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Validación de un modelo *in situ* de predicción de absorción en colon. Aplicación al desarrollo de formas de liberación controlada

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Que el trabajo presentado por la licenciada Dña. Isabel Lozoya Agulló para optar al grado de Doctor, titulado: **"Validación de un modelo *in situ* de predicción de absorción en colon. Aplicación al desarrollo de formas de liberación controlada"**, ha sido realizado bajo nuestra dirección y asesoramiento.

Concluido el trabajo experimental y bibliográfico, autorizamos la presentación de esta Tesis Doctoral para que sea juzgada por el tribunal correspondiente.

Y para que así conste, firmamos el presente certificado en Valencia, a dieciséis de enero de dos mil diecisiete.

M. Merino Sanjuán M.V. Bermejo Sanz M. González Álvarez

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"La ciencia se compone de errores,
que a su vez, son los pasos hacia la verdad"
Julio Verne

Abreviaturas

- ABL: Aqueous Boundary Layer
- AE%: Association Efficiency
- ANOVA: Analysis of Variance
- ATP: Adenosín trifosfato
- AUC: Area under the Curve
- BBB: Blood-Brain Barrier
- BCRP: Breast Cancer Resistance Protein
- BCS: Sistema de Clasificación Biofarmacéutica / Biopharmaceutical Classification System
- Caco-2: human cancer of the colon epithelial cell line
- CLD: Closed-Loop Doluisio
- CM: Carrier Mediated
- COX: Cyclo-oxigenase
- CR: controlled release
- CV%: Coefficient of Variation
- CYP3A4: citrocromo P450
- Da: Dalton
- DDI: Drug Delivery Index
- DMEM: Dulbecco's Modified Eagle's Medium
- DMSO: Dimethyl sulfoxide
- DRW: Dynamic Range Window
- EMA: European Medicine Agency
- F: biodisponibilidad
- F_a: fracción oral absorbida/oral fraction absorbed
- F_{abs}: fracción oral absorbida/oral fraction absorbed
- FDA: Food and Drug Administration
- GIT: Gastrointestinal Tract
- GOF: Goodness of Fit
- GRAS: Generally Regarded as Safe
- H: High permeability

- HBSS: Hank's Balanced Salt Solution
- HPLC: High Performance Liquid Chromatography
- HT29-MTX: mucus-secreting cell line
- IR: Immediate Release
- IVIVC: Correlación *in vitro-in vivo*
- IVIVR: Relación *in vitro-in vivo*
- K_a: constante de velocidad de absorción intínseca
- L: Low permeability
- Log P: Coeficiente de reparto
- MDCK: Madin-Darby Canine Kidney epithelial cell line
- MMM: Magnetic Marker Monitoring
- MRP: Multidrug Resistance-associated Protein
- MW: Molecular Weight
- N/A: Not Available data
- n: tamaño muestral/sample size
- NPs: Nanoparticles
- NS: Not Statistically significant differences
- NSAID: Nonsteroidal Anti-Inflammatory Drug
- OATP: Organic Anion-Transporting Polypeptide
- OCT: Organic Cation Transporter
- OMS: Organización Mundial de la Salud
- PAMPA: Parallel Artificial Membrane Permeation Assay
- PAMPA-DS: parallel artificial membrane permeability assay, Double-Sink™ intestinal model (Pion Inc.)
- P_{app}: Permeability
- PBPK: Physiologically-based Pharmacokinetic
- PBS: Phosphate Buffered Saline
- PDI: Polydispersity Index
- P_{eff}: Permeability
- PEPT: Peptide Transporter Protein
- P-gp: glicoproteína P
- pKa: constante de disociación

- PLGA: Poly(Lactic-co-Glycolic) Acid
- PM: Peso Molecular
- PVA: Poly(Vinyl Alcohol)
- QSAR: Quantitative Structure-Activity Relationship
- r.p.m.: revolutions per minute
- R^2/r^2: Coeficiente de determinación
- Raji B: cell line from human Burkitt's linfoma
- RSS: Residual Sum of Squares
- S.D.: Standard Deviation
- S: Statistically significant differences
- SEM: Scanning Electron Microscope
- SPIP: Single-Pass Intestinal Perfusion
- TEER: Transepithelial Electrical Resistance
- TFA: Trifluoroacetic Acid
- UPLC: Ultra Performance Liquid Chromatography
- USP: United States Pharmacopeia
- UV: ultraviolet
- ZP: Zeta Potential
- λ: wavelength

Resumen

El intestino delgado presenta las características idóneas (anatómicas, fisiológicas y fisicoquímicas) para llevar a cabo la absorción de xenobióticos, por lo que es considerado el órgano por excelencia para realizar dicho proceso. El colon, por el contrario, no se ha tenido en cuenta tradicionalmente como órgano destinado a la absorción. Por este motivo, existe una escasez de estudios científicos relacionados con la absorción de moléculas en colon.

Sin embargo, en las últimas décadas, el auge de las fórmulas de liberación controlada ha provocado que los estudios de absorción de moléculas en el colon ganen importancia, ya que para este tipo de formulaciones farmacéuticas el tramo distal del tracto gastrointestinal es el lugar principal de absorción. Por tanto, resulta necesario conocer la permeabilidad de los fármacos en este segmento intestinal antes de abordar la formulación de estas formas de administración.

Por todo ello, en esta Tesis Doctoral se ha realizado la validación de un modelo de perfusión *in situ* que permita predecir la absorción de compuestos en colon y para tal fin se han establecido y analizado diferentes correlaciones. Adicionalmente, se han estudiado dos posibles estrategias para modificar la absorción en colon de fármacos de alta y baja permeabilidad.

La perfusión *in situ* sin recirculación basada en el método de Doluisio fue la técnica escogida para determinar la permeabilidad en colon de rata. Por lo que, en primer lugar, se procedió a validar esta técnica en intestino delgado de rata comparando los resultados que proporciona con los obtenidos utilizando el método de paso simple, ampliamente empleado para predecir la absorción de compuestos en intestino delgado. Las correlaciones establecidas demostraron que ambos métodos son igual de válidos para determinar la absorción en intestino delgado de rata, predecir la absorción

intestinal en humanos y clasificar los compuestos según los criterios del Sistema de Clasificación Biofarmacéutica (BCS).

A continuación, se procedió a validar la técnica de perfusión *in situ* utilizada en colon de rata. Para ello se establecieron correlaciones entre la permeabilidad de compuestos obtenida en colon de rata y en intestino delgado de la misma especie animal. Asimismo, se correlacionaron los valores de permeabilidad en colon de rata con aquellos obtenidos utilizando modelos *in vitro* de diferentes características (cultivos celulares y membranas artificiales). Por último, para completar la validación se establecieron correlaciones con parámetros de absorción determinados en colon humano.

Las correlaciones obtenidas pusieron de manifiesto que a partir de la permeabilidad determinada en intestino delgado de rata es posible predecir la permeabilidad en colon de esta misma especie. Asimismo, los monocultivos celulares de Caco-2 también demostraron ser una técnica *in vitro* con la que se puede predecir la absorción en colon de rata. Además, el método de perfusión *in situ* sin recirculación es válido para predecir la absorción colónica en humanos y, por tanto, es útil para realizar el cribado de moléculas candidatas a ser formuladas en formas farmacéuticas de liberación controlada.

Una vez validado el método de perfusión, se estudió el desarrollo de un par iónico y de nanopartículas poliméricas como estrategias para modificar la absorción en colon de fármacos modelo de baja y alta permeabilidad. La formación de un par iónico con atenolol permitió aumentar la permeabilidad en colon de este compuesto, clasificado de acuerdo con los criterios que se establecen en el BCS como de baja permeabilidad, por lo que esta estrategia sería útil para conseguir una adecuada absorción en colon de moléculas con carga y poco permeables tras ser administradas en formas farmacéuticas de liberación controlada. Por otro lado, con la formulación de nanopartículas poliméricas de ibuprofeno se

consiguió obtener una liberación sostenida de este principio activo, clasificado en el BCS como fármaco de alta permeabilidad. Los estudios de liberación mostraron que la liberación de este principio activo desde las nanopartículas formuladas es mayor a partir de pH 6,8. Esta característica las convierte en una opción apropiada para el desarrollo de formas farmacéuticas orales dirigidas a liberar la molécula activa en el colon.

La presente Tesis Doctoral se estructura en ocho capítulos. En el Capítulo 1 se realiza una revisión exhaustiva de las propiedades y características del colon y de las herramientas existentes para predecir la absorción en el tracto gastrointestinal, así como de las formas de liberación controlada para administración por vía oral y evaluación de las mismas. En el Capítulo 2 se plantean esquemáticamente el objetivo general y los objetivos específicos de la Tesis. El Capítulo 3 contiene una descripción detallada de la técnica de perfusión *in situ* sin recirculación, objeto de validación en esta Memoria de Tesis, además, se incluyen los artículos científicos 1 y 2 dedicados a la validación de la misma. El Capítulo 4 abarca la validación de la técnica de perfusión *in situ* sin recirculación en colon, para lo que se han establecido correlaciones con datos obtenidos mediante técnicas de distinta naturaleza tal y como se muestra en los artículos científicos 3, 4, 5 y 6. En el Capítulo 5 se estudian dos estrategias para modificar la absorción en colon de fármacos modelo, en concreto, el artículo científico 7 profundiza en el desarrollo de un par iónico para el atenolol y el artículo científico 8 se centra en el análisis de nanopartículas poliméricas elaboradas con el ibuprofeno. En el Capítulo 6 se resumen y discuten los resultados más destacables obtenidos en los artículos científicos que se aportan. Además, el Capítulo 7 finaliza con las conclusiones generales alcanzadas en los trabajos realizados. Por último, en el Capítulo 8 se adjuntan los cuatro artículos científicos que se han publicado hasta la fecha.

Índice

1. INTRODUCCIÓN

1.1. Generalidades

1.1.1. Absorción gastrointestinal

El proceso de absorción de nutrientes, xenobióticos y fármacos administrados por vía oral tiene lugar a lo largo del tracto gastrointestinal. El tracto gastrointestinal se compone de tres tramos principales bien diferenciados morfológicamente: estómago, intestino delgado y colon (Figura 1.1).

1.1.1.1. Lugares de absorción

La absorción puede realizarse en cualquiera de los tres tramos del tracto gastrointestinal que se han mencionado pero el lugar especializado para este proceso es el intestino delgado [1]. Sin embargo, en los últimos años, debido al creciente desarrollo de fórmulas de liberación controlada, la absorción en el colon está cobrando importancia y los estudios centrados en este tramo distal del tracto gastrointestinal han aumentado de forma notable [2, 3].

Intestino delgado

El intestino delgado posee una longitud aproximada de 7 metros [4], conecta el estómago y el colon y está dividido en tres segmentos: duodeno, yeyuno e íleon (Figura 1.1.1). Este órgano es el lugar de absorción por excelencia tras una administración por vía oral debido a su localización anatómica, justo después del estómago, a su entorno fisicoquímico y a sus características fisiológicas y biofarmacéuticas [2].

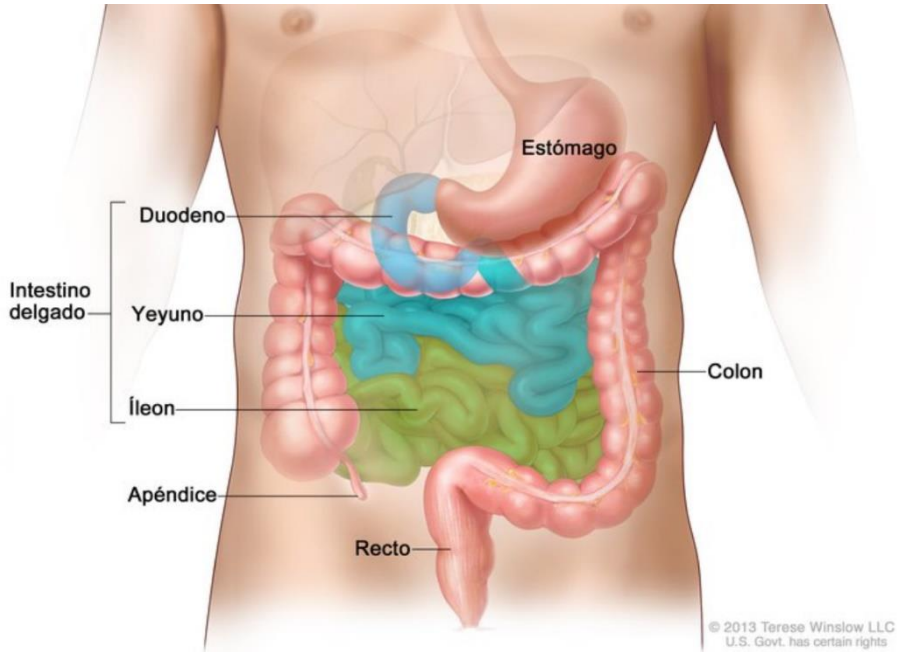

Figura 1.1.1 Partes del tracto gastrointestinal y del intestino delgado [5]. Imagen tomada con permiso de Terese Winslow LLC.

El intestino delgado posee numerosas estructuras morfológicas encaminadas a aumentar su superficie, de hecho, la gran ventaja que presenta este tramo del tracto gastrointestinal frente al resto de tramos es su elevada superficie útil de absorción [1], gracias al desarrollo de tres subestructuras a partir de la estructura cilíndrica como se muestra en la Figura 1.1.2 [6, 7]:

- Pliegues circulares o pliegues de Kerkring.
- Vellosidades intestinales o villi.
- Microvellosidades o microvilli, presentes en las células columnares epiteliales, absorbentes o enterocitos, que conforman el ribete en cepillo.

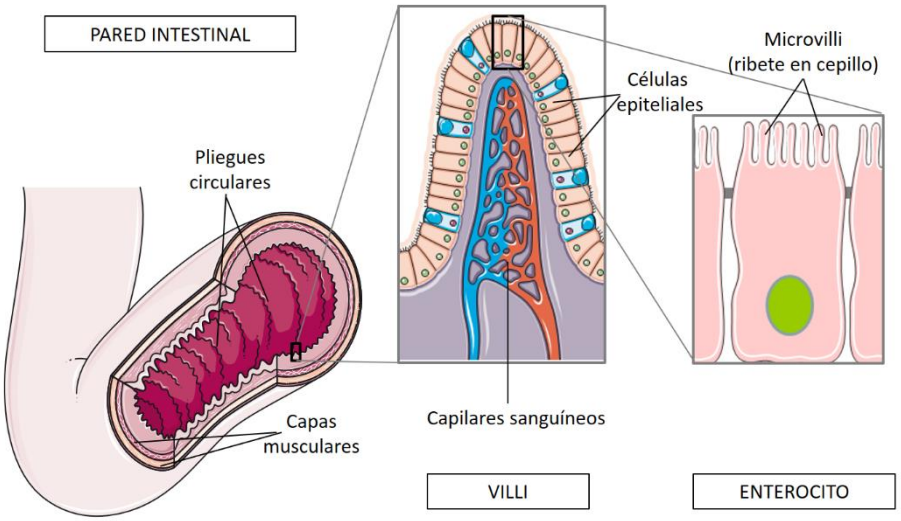

Figura 1.1.2. Esquema de las distintas modificaciones del intestino delgado. Imagen adaptada de la base de datos de imágenes cedidas por Servier [8].

Todas estas estructuras confieren un incremento en la superficie útil de absorción disponible (Tabla 1.1.1), lo que implica que sólo el intestino delgado está estructurado de manera que permite la máxima absorción de nutrientes, xenobióticos y fármacos, por lo que es considerado el lugar más eficiente para este proceso.

Tabla 1.1.1. Factor de amplificación de la superficie del intestino delgado respecto al cilindro fundamental [9].

	Cilindro	**Pliegues**	**Villi**	**Microvilli**
Factor de amplificación	x 1	x 3	x 30	x 600

Colon

El colon constituye la parte principal del intestino grueso. En los humanos se diferencian cuatro partes: colon ascendente, colon transverso,

colon descendente y colon sigmoide (Figura 1.1.3). Comparado con el intestino delgado, el colon es más corto y su lumen es más amplio. Este tramo gastrointestinal presenta una longitud aproximada de 1,5 m [10, 11].

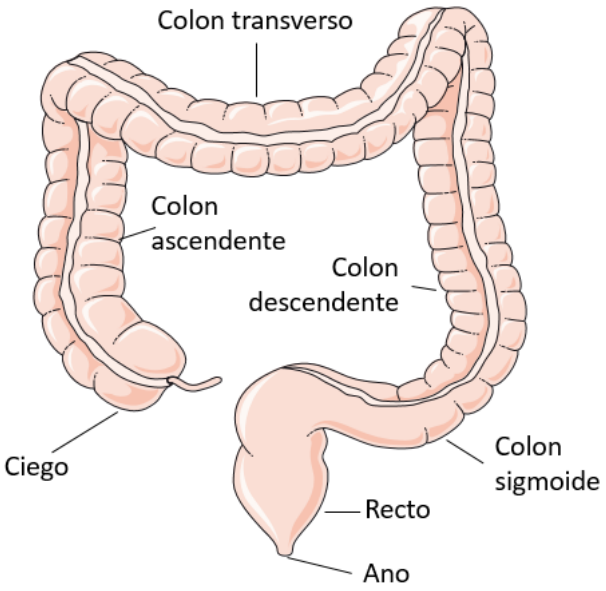

Figura 1.1.3. Partes del intestino grueso. Imagen adaptada de la base de datos de imágenes cedidas por Servier [8].

A diferencia del intestino delgado, la mucosa del colon está formada por una superficie plana de epitelio que presenta criptas invaginadas. La característica más destacable es la ausencia de villi (Figura 1.1.4) [12]. La composición celular es similar a la del intestino delgado aunque con mayor proporción de células caliciformes (secretoras de moco); además, las células absorbentes del colon o colonocitos, al igual que los enterocitos del intestino delgado, también poseen microvellosidades [13]. El colon está especializado en mantener el balance de electrolitos y agua, es el principal responsable de la absorción de Na^+, Cl^- y H_2O, y de la secreción de HCO_3^- y K^+ [14].

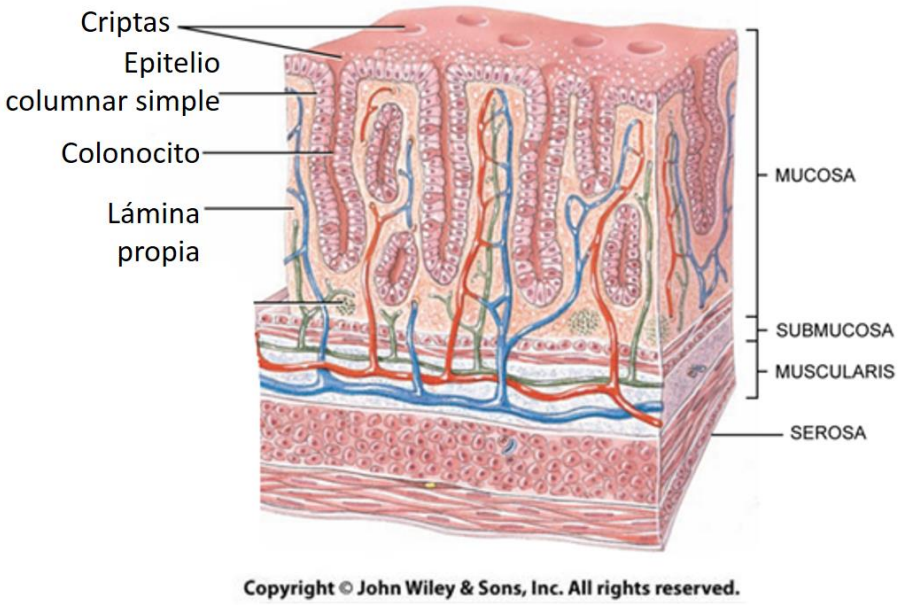

Figura 1.1.4. Estructura de la pared del colon [15]. Imagen tomada con permiso de John Wiley & Sons.

Las características comentadas en el párrafo anterior contribuyen a que la capacidad de absorción del colon de los distintos compuestos y nutrientes sea marcadamente reducida en comparación con la del intestino delgado. En la Figura 1.1.5 se pueden observar los cambios y diferencias existentes entre la pared de los dos tramos gastrointestinales. La diferencia más importante es la falta de villi en el colon. Por todo ello, los compuestos se absorben principalmente durante su paso a través del intestino delgado, con lo que el colon juega un papel secundario en la absorción sistémica.

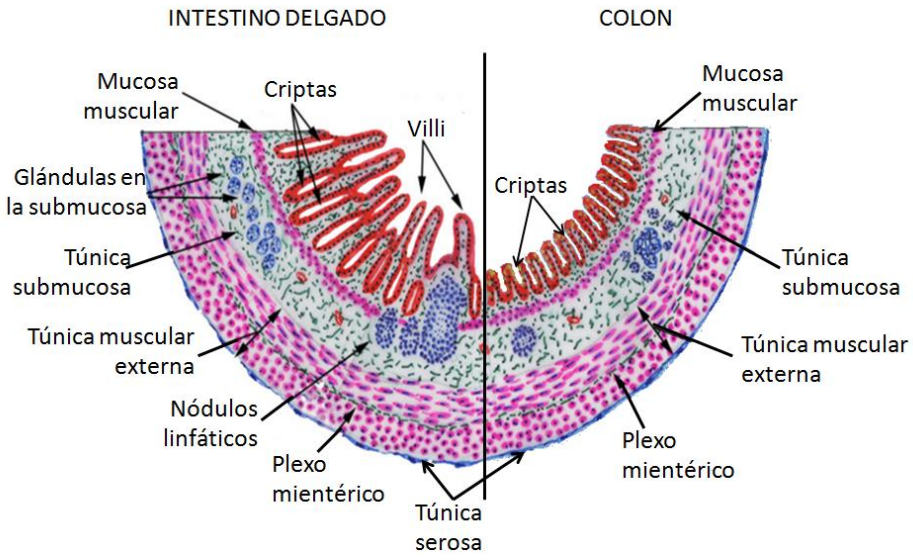

Figura 1.1.5. Esquema comparativo de la pared del intestino delgado y la pared del colon [16].

1.1.1.2. Mecanismos de absorción

Los mecanismos de absorción a través de la membrana de las células del tracto gastrointestinal son de dos tipos:

1. Difusión pasiva.
2. Mecanismos especializados de transporte:
   - Difusión facilitada
   - Transporte activo

Cada compuesto se puede absorber por uno o varios de los mecanismos mencionados anteriormente. En la Figura 1.1.6 se muestra una representación esquemática de los distintos mecanismos de absorción.

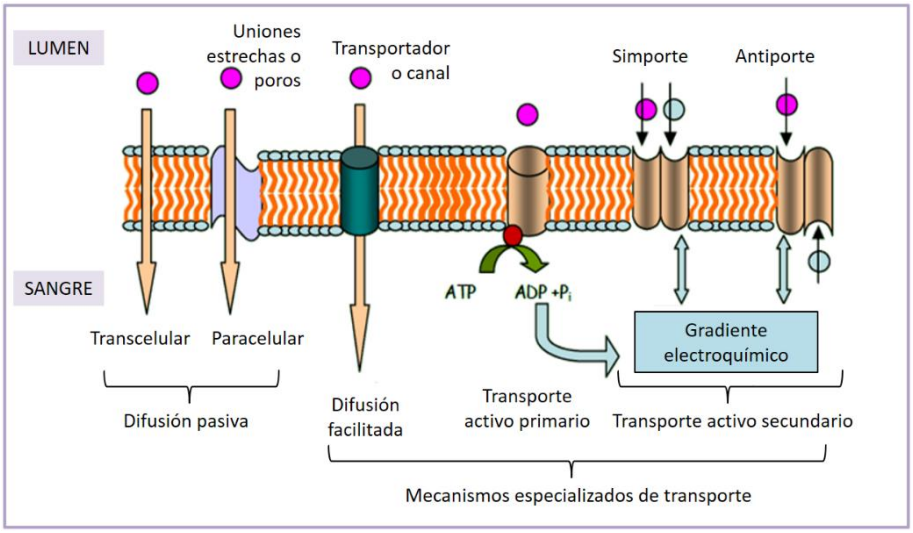

Figura 1.1.6. Mecanismos de absorción. Imagen modificada de Oltra-Noguera, D. [17].

Difusión pasiva

La absorción de sustancias está caracterizada principalmente por el proceso de absorción por difusión pasiva. La difusión pasiva no consume energía y ocurre siempre a favor de gradiente de concentración [18]. Es un proceso complejo, en el que se distinguen factores extracelulares (zona luminal) e intracelulares o tisulares (zona serosa), y destacan las características de la membrana lipoidea fundamental, localizada en el extremo superior de las células columnares, paralela a la superficie de las microvellosidades [19].

Se diferencian dos procesos:

1. Ruta paracelular: paso a través de las uniones intercelulares.
2. Ruta transcelular: difusión a través de membranas lipoideas.

- *Ruta paracelular*

Dentro de la difusión pasiva se puede considerar la entrada de xenobióticos que ocurre a través de los espacios intercelulares y/o poros acuosos de la membrana, es decir, a través de las aperturas que dejan las uniones entre las células columnares adyacentes, también conocidas como uniones estrechas (*tight junctions*). En este caso, la difusión es inversamente proporcional al tamaño molecular del fármaco, pues, en general esta vía solo es accesible para los solutos de bajo peso molecular (< 200–250 Daltons) e hidrofilia elevada [20] y no se incluiría la membrana lipídica en la ruta global de difusión del xenobiótico.

- *Ruta transcelular*

El paso de moléculas a través de la membrana celular intestinal es la ruta principal. Las moléculas de soluto se distribuyen a favor de gradiente, desde la zona donde se encuentra una mayor concentración hasta aquélla en la que están menos concentradas, para ello se reparten entre el fluido del lumen intestinal y la bicapa lipídica de la membrana celular.

Las sustancias liposolubles poseen una mayor facilidad para atravesar las membranas biológicas. El reparto tendrá lugar hasta que el soluto se equilibre a ambos lados de la bicapa lipídica.

El transporte a través de la ruta transcelular depende de las características fisicoquímicas de la molécula transportada, principalmente del grado de ionización y de la lipofilia, ya que normalmente sólo se absorbe la fracción no ionizada de la molécula, por ser ésta la más lipófila.

En el proceso que siguen los solutos para alcanzar el plasma a través de la ruta transcelular se pueden diferenciar tres zonas:

1) Interfase lumen-membrana (zona luminal)

Está compuesta por tres áreas continuas de distintas características fisicoquímicas. En la Figura 1.1.7 se puede observar su disposición:

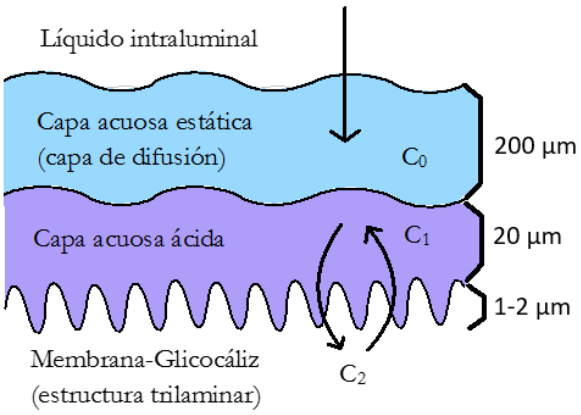

Figura 1.1.7. Disposición de la interfase lumen-membrana. Imagen tomada con permiso de Ruiz-García, A. [21].

La primera zona que se aprecia engloba el lumen intestinal y está compuesta por el *líquido luminal*. El pH de este líquido varía a lo largo de todo el intestino. De hecho, el pH aumenta de 6 a 8 desde la zona proximal al tramo final del intestino [22].

En contacto directo con el líquido luminal se localiza la *capa acuosa estática de difusión o capa límite*, con un espesor de unos 200 µm aproximadamente. Presenta la misma composición que el líquido luminal, aunque, en la capa acuosa de difusión, las moléculas de agua ejercen una resistencia al paso de sustancias. Esta resistencia actúa como factor limitante en el proceso de absorción de moléculas altamente lipófilas. Adyacente a la capa acuosa de difusión se observa una capa mucosa compuesta por más del 90% de agua y entre un 0,5% y 5% de mucina [23].

A continuación, y en contacto con la membrana, se ubica la *capa acuosa ácida* o *microclima* cuyo pH es ligeramente más ácido que el del

líquido intraluminal, ya que sus valores se sitúan entre 5,3 y 6,1 [24]. Las características de la capa acuosa ácida no se conocen en profundidad, parece que su espesor es del orden de 20 µm y, hasta la fecha, no se ha demostrado que interfiera de forma importante en el proceso de absorción de sustancias.

2) Membrana lipoidea

Se trata de una membrana trilaminar con características diferenciales del resto de membranas celulares del organismo. Según el modelo de mosaico fluido, propuesto por Singer y Nicholson (Figura 1.1.8), está formada por una zona central compuesta por dos capas fosfolipídicas con cadenas hidrocarbonadas unidas entre sí mediante fuerzas de Van der Waals, de tal manera que los grupos polares se encuentran recubriendo la cara interna y la cara externa de la bicapa lipídica. Asimismo, existen proteínas globulares que revisten algunas zonas o aparecen interpuestas entre los lípidos [25].

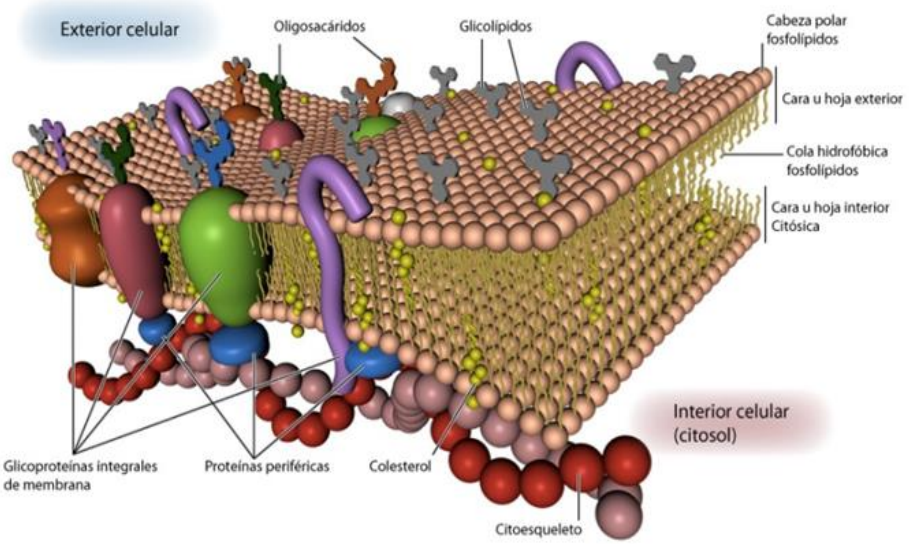

Figura 1.1.8. Estructura de la membrana lipoidea [26].

La membrana lipoidea tiene función de "contenedor" para el citosol y los diferentes compartimentos celulares, además de otorgar protección mecánica. En su composición se encuentran mayoritariamente fosfolípidos (fosfatidiletanolamina y fosfatidilcolina), así como colesterol, glúcidos y proteínas (integrales y periféricas).

Esta membrana presenta características que la diferencian del resto de membranas biológicas. Destacan su espesor, un micrómetro superior al resto de membranas celulares [27] y su composición química, ya que posee un elevado contenido de proteínas (con una relación proteína/lípido tres veces superior a la normal), lo que indica la existencia de enzimas y proteínas transportadoras.

Otra característica diferencial respecto al resto de membranas es su proporción de lípidos. Presenta porcentajes esfingomielina/lecitina del 30% y colesterol/fosfolípidos de un 20%, siendo estos superiores a los del resto de membranas biológicas. Además, tiene una elevada proporción de glicolípidos (54%) resultante de la abundancia de mucopolisacáridos presentes en el glicocáliz [28].

3) Interfase membrana-plasma

Para desplazarse desde el interior de la célula columnar hasta el plasma sanguíneo, las moléculas deben atravesar los distintos elementos que constituyen esta interfase.

Existe una parte de estas moléculas que se retiene en las estructuras subcelulares, pero esta retención no afecta a la difusión del xenobiótico al interior celular. En esta interfase pueden tener lugar interacciones con enzimas biotransformadoras, por tanto, existe la posibilidad de que se modifique o inactive el soluto. La resistencia que presenta la membrana basal a la penetración de las moléculas es mucho menor que la resistencia mostrada por la membrana apical [29]. En la membrana basal también se

localizan transportadores, pero de características diferentes y en una cantidad inferior a la existente en la membrana apical.

Una vez que los solutos consiguen atravesar la membrana basal, el camino de difusión restante hacia el plasma sanguíneo ya no presenta dificultades, puesto que los capilares están muy próximos y sus endotelios son altamente porosos, por lo que no ofrecen resistencia a la difusión de moléculas.

Mecanismos especializados de transporte

La existencia de mecanismos especializados de transporte está ligada de la presencia de estructuras, normalmente proteicas, capacitadas para transportar el soluto de un lado a otro de la membrana. Tiene lugar una unión específica entre la sustancia y la proteína, cuya translocación causa el paso del compuesto a través de la membrana. Las proteínas transportadoras tienen un papel esencial en varios contextos como la absorción oral, la biodisponibilidad de los compuestos [30] y la resistencia a fármacos [31, 32]. Además, también son importantes en los procesos de excreción y metabolismo en hígado y riñón, en la toxicidad relacionada con ciertos medicamentos [33], y en determinadas características farmacocinéticas y farmacodinámicas de otros [34-36].

Se distinguen dos tipos de mecanismos especializados de transporte:

1. Difusión facilitada: no necesita energía.
2. Transporte activo: se consume energía en el proceso.

- *Difusión facilitada*

Se denomina también difusión mediada por transportadores, ya que, aunque se realiza mediante proteínas específicas de membrana, no requiere consumo de energía metabólica. Junto con la difusión pasiva (transcelular y

paracelular) conforma el transporte pasivo, pues ambos son procesos que no consumen energía y ocurren a favor de gradiente [37].

Los solutos que no pueden atravesar la membrana por difusión libre, tienen la opción de hacerlo mediante proteínas transmembrana específicas, que presentan la función de proteínas transportadoras. Se diferencian dos tipos de proteínas transportadoras: las proteínas canales, que forman pequeños poros mediante los cuales los iones de tamaño apropiado y carga determinada pueden atravesar la membrana por difusión libre; y las proteínas *carriers* que sufren cambios conformacionales al unirse a moléculas específicas dando lugar a la apertura de los canales por donde las moléculas que van a ser transportadas pueden pasar a través de la membrana y ser liberadas al otro lado.

A diferencia de la difusión pasiva, la difusión facilitada es un proceso específico y saturable [37].

- *Transporte activo*

El transporte activo ocurre en contra de gradiente electroquímico y de concentración. Se requiere de una fuente de energía para transportar el soluto a través de la membrana celular desde un compartimento en el que se encuentre baja concentración de dicho soluto a otro de alta concentración. Por tanto, no puede producirse de forma espontánea. La unión entre sustrato y transportador específica y la existencia de un número determinado de transportadores, lo convierten en un proceso saturable [38].

Según la energía requerida y el sentido en el que se vaya a transportar el soluto, se distinguen distintos subtipos dentro del transporte activo:

a) Según la fuente de energía utilizada para producirlo:

- *Transporte activo directo o primario*: la energía deriva directamente de la hidrólisis del adenosín trifosfato (ATP).

- *Transporte activo indirecto o secundario*: se considera un transporte acoplado, ya que una molécula es cotransportada gracias a la energía que procede del gradiente generado por el transporte activo primario de otra molécula distinta. Es decir, la transferencia de un soluto depende de la transferencia simultánea o secuencial de otro soluto.

  Según la dirección de los solutos, el transporte secundario se puede clasificar a su vez en:

  - *Simporte o unidireccional*: transporte de ambos solutos en la misma dirección.

  - *Antiporte o de intercambio*: transporte de los diferentes solutos en dirección opuesta.

b) Según el sentido de transporte en el lumen intestinal:

- *Transporte de absorción o influx*: el transporte del fármaco a través de la membrana se produce desde el lumen intestinal hasta la sangre. Las proteínas de la familia MRP son un ejemplo de influx, excepto la MRP2.

- *Transporte de secreción o efflux:* las proteínas transportadoras aquí implicadas interrumpen el paso del fármaco, devolviendo al lumen las moléculas que atraviesan la membrana del enterocito. Como resultado, la absorción es menor de la esperada. Destacan la Glicoproteína-P y MRP2.

- *Transportadores*

Los transportadores de membrana se encuentran en numerosos epitelios, entre ellos en la membrana del tracto intestinal que se caracteriza por poseer una gran riqueza y diversidad de los mismos. Las proteínas

portadoras se encuentran en las membranas luminales y/o basales de los enterocitos y colonocitos y pueden facilitar el paso de sustancias a la zona citosólica o serosa. Por tanto, pueden condicionar la absorción o la secreción de los sustratos.

Los transportadores intestinales son cruciales en la absorción de muchos fármacos por vía oral y su interés es creciente dada la importancia que tienen en la farmacocinética de los compuestos. Existe una gran cantidad de transportadores identificados, pero de todos estos sólo unos pocos han demostrado estar involucrados en la absorción intestinal. En la Figura 1.1.9 se muestra un esquema de las proteínas transportadoras más relevantes existentes en las células epiteliales intestinales y su posición, apical o basolateral.

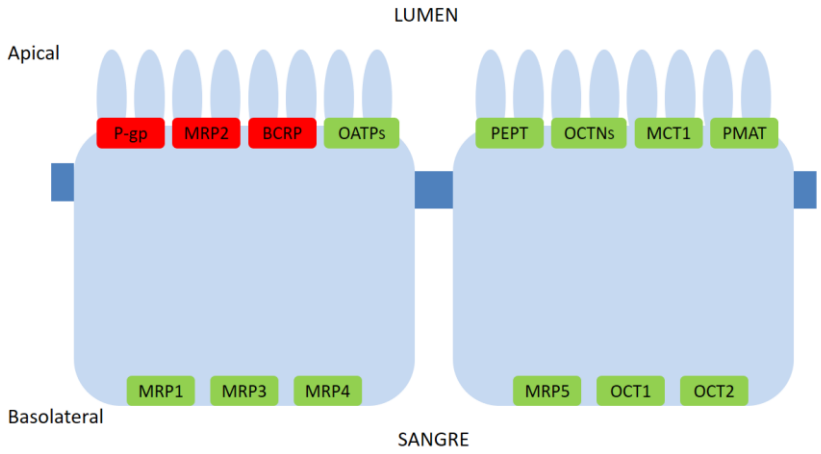

Figura 1.1.9. Diagrama de los transportadores más importantes expresados en el epitelio intestinal [39]. Rojo: secreción; verde: absorción.

Las proteínas transportadoras más investigadas en el intestino son los transportadores de *efflux* pertenecientes a la familia ABC *(ATP binding cassette transporters)*: glicoproteína-P (P-gp), MRP2 y BCRP, siendo altamente abundantes, todos ellos, en la membrana apical de los enterocitos y, además, limitan la absorción de muchos fármacos importantes

clínicamente y frecuentemente prescritos. La glicoproteína-P es la más relevante y la más estudiada hasta la fecha [39-42].

Mecanismos de absorción a lo largo del tracto gastrointestinal

El paso de compuestos y nutrientes a través de la pared intestinal varía a lo largo del intestino delgado y del colon y está influenciado por los mecanismos de transporte involucrados.

- *Difusión pasiva*

La difusión pasiva es el proceso predominante en la absorción de moléculas. La ruta transcelular presenta las mismas características en el intestino delgado completo y en colon. En cuanto a la ruta paracelular, se encuentra descrito en la literatura que las uniones existentes entre una célula y otra (*tight junctions)* son más estrechas en la capa de células epiteliales colónicas que en la del intestino delgado, además no existen poros acuosos en este segmento [43]. Por todo ello en colon sólo se considera relevante la vía de absorción transcelular. Estas características unidas a la menor superficie útil de absorción del colon sugieren que el componente pasivo es mayor en el intestino delgado [2, 3].

- *Mecanismos especializados de transporte*

Los transportadores intestinales están expresados en mayor cantidad en yeyuno e íleon, por lo que en estos tramos la absorción estará más influenciada por mecanismos especializados de transporte [39, 44]. Según el estudio llevado a cabo por Drozdzik y colaboradores [45] en 2014, la cantidad y tipo de proteínas transportadoras, considerando los transportadores presentes en el tracto intestinal (Figura 1.1.9), es similar en los distintos tramos del intestino delgado, pero en colon este porcentaje varía notablemente al compararlo con el intestino delgado.

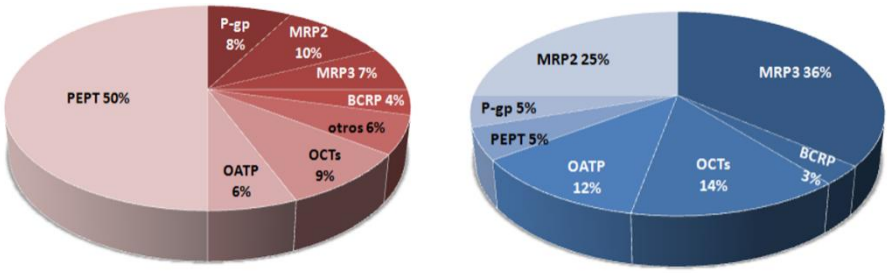

Figura 1.1.10. Porcentaje que representan los principales transportadores en intestino delgado (izquierda) y colon (derecha). Datos obtenidos de Drozdzik y colaboradores [45].

En la Figura 1.1.10 se observa el porcentaje que representa cada transportador en los dos tramos gastrointestinales principales: intestino delgado y colon. Llama la atención que en el intestino delgado el 50% corresponde al transportador con función de absorción PEPT; sin embargo, está mínimamente expresado en colon (5%) [46]. En este último tramo el mayor porcentaje (36%) pertenece al transportador MRP3 que contribuye al paso de sustancias a la sangre.

Se muestra una representación más detallada de los transportadores presentes en el tracto gastrointestinal en la Figura 1.1.11, donde aparecen los transportadores expresados en estómago y en cada segmento del intestino delgado.

En cuanto a la glicoproteína P (MDR1) los datos existentes no son concluyentes. La expresión de P-gp aumenta de las regiones proximales a las distales del intestino delgado [47], pero no está clara su expresión en colon. Muchos estudios sugieren que su presencia es mayor en colon que en intestino delgado mientras que otros afirman que hay una expresión menor, por ejemplo, los datos del estudio presentado por Tannergren y col. en 2009 no muestran un claro efecto limitante en la absorción colónica mediada por la glicoproteína P [2, 48-51].

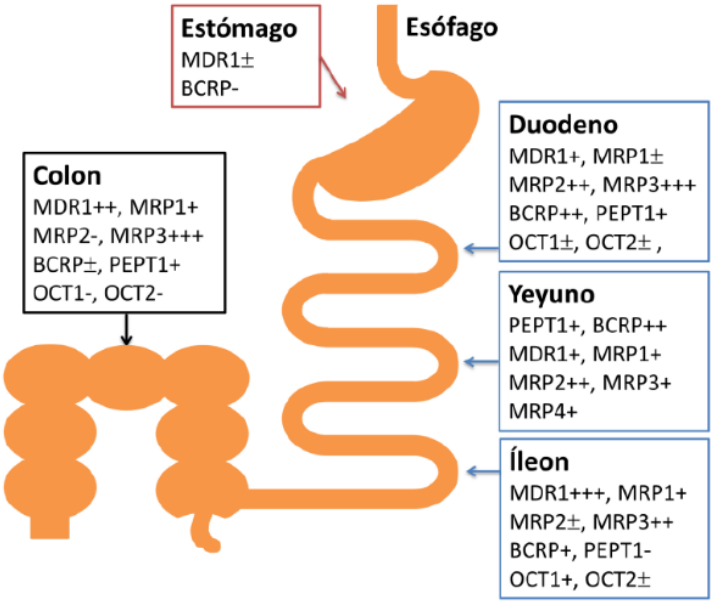

Figura 1.1.11. Localización y expresión de los transportadores a lo largo del tracto gastrointestinal (de mayor a menor nivel de expresión: +++, ++, +, ±, -). Imagen tomada con permiso de los editores [52].

1.1.1.3. Parámetros de absorción

La mayoría de los modelos experimentales evalúan la absorción intestinal, tanto en magnitud como en velocidad, a partir de datos de concentración remanente en lumen a distintos tiempos. Según el diseño, resulta más conveniente obtener un tipo u otro de parámetro representativo del fenómeno de desaparición del soluto en estudio. Los parámetros más utilizados son la constante de velocidad de absorción de primer orden y el coeficiente de permeabilidad.

Existe además la opción de cuantificar la aparición del soluto objeto de estudio en sangre portal para determinar la fracción oral absorbida. Es una estimación más precisa del fenómeno de absorción, pero presenta inconvenientes de tipo experimental.

Constante de velocidad de absorción

La constante de absorción o constante de velocidad de absorción intrínseca, k_a, es el parámetro que rige la velocidad a la que se absorbe un compuesto en condiciones estandarizadas y reproducibles, generalmente en animales de experimentación como sustrato biológico, tras aislar convenientemente el lugar de absorción (algún tramo o todo el tracto intestinal). Al caracterizar este parámetro, se debe evitar que la disolución del soluto a ensayar sea un factor limitativo de la absorción, para ello éste debe estar totalmente disuelto.

El valor de la constante de absorción intrínseca está condicionado por los siguientes factores:

- *Factores característicos de la técnica experimental:* área superficial de la membrana absorbente, pH de la disolución ensayada, grado de agitación, temperatura de la zona de estudio y viscosidad del medio. Si la técnica experimental está convenientemente estandarizada, estos factores pueden considerarse constantes.

- *Factores característicos del fármaco:* lipofilia intrínseca (afinidad de la molécula hacia la membrana absorbente), peso, volumen y área molecular, así como, la configuración espacial y electrónica; que determinan el coeficiente de difusión del compuesto tanto en el medio acuoso como en el lipídico. Además, esta última, condiciona su interacción con transportadores.

El valor de k_a se calcula por ajuste no lineal de las concentraciones remanentes en el lugar de absorción estudiado frente al tiempo, asumiendo una cinética de primer orden.

Coeficiente de permeabilidad

El coeficiente de permeabilidad (P_{eff}) es el parámetro utilizado para describir la permeabilidad intestinal. Esta última se entiende como la velocidad de paso de cualquier compuesto a través de las membranas del tracto intestinal, lo que contribuye en gran medida al rendimiento del proceso de la absorción oral de fármacos [53].

El coeficiente de permeabilidad se relaciona con la constante de velocidad de absorción como se muestra en la expresión (1):

$$P_{eff} = k_a \cdot R/2 \quad (1)$$

donde R es el radio del segmento intestinal donde se realiza el ensayo.

Fracción oral absorbida

La fracción oral absorbida, F_a *o* F_{abs}, es la fracción de la dosis administrada por vía oral que atraviesa la membrana apical del enterocito, que coincidirá con la biodisponibilidad en caso de que el fármaco no sufra efecto de primer paso, intestinal o hepático. Ya que la vía de administración oral es la más utilizada por sus numerosas ventajas, la predicción de la absorción oral de un fármaco es uno de los aspectos más relevantes en su etapa de desarrollo, por ello el cálculo de la F_a es un proceso de gran importancia. Dada la dificultad de determinar la fracción oral absorbida de manera directa en humanos, se han desarrollado métodos indirectos para predecirla [54]:

- Modelos de predicción de la fracción oral absorbida basados en descriptores moleculares y parámetros fisicoquímicos.

- Modelos de predicción de la fracción oral absorbida tiempo-independientes o en estado estacionario, basados en establecer un balance de masas.

- Modelos de predicción de la fracción oral absorbida dinámicos que también permiten predecir la velocidad de absorción.

1.1.1.4. Propiedades fisicoquímicas de las moléculas que afectan a su absorción

La absorción y permeabilidad de un principio activo se verán influenciadas por sus propiedades fisicoquímicas. Éstas deben de tenerse en cuenta en el momento de preparar una fórmula farmacéutica, ya que la biodisponibilidad del fármaco dependerá de dichas propiedades. A continuación, se comentan las propiedades fisicoquímicas más relevantes.

Lipofilia

Se entiende por lipofilia el grado de afinidad que presenta una molécula por los lípidos. Esta propiedad se mide con el coeficiente de reparto (LogP), parámetro que describe el reparto de un soluto entre agua y un solvente orgánico inmiscible, generalmente n-octanol [55].

La absorción de fármacos administrados por vía oral se realiza principalmente por difusión pasiva a través de la membrana lipídica. Este mecanismo de absorción depende de las propiedades fisicoquímicas del soluto, en especial del grado de ionización y de su lipofilia, ya que, normalmente, solo atraviesa la membrana la fracción no disociada de la molécula, que es la más lipófila. Por este motivo, muchos principios activos ven limitada su permeabilidad, sobre todo los compuestos ionizados y las moléculas hidrófilas [14]. Por tanto, la lipofilia intrínseca de una molécula condiciona la absorción de los fármacos administrados por vía oral, pues las sustancias más liposolubles pueden atravesar más fácilmente las membranas

hasta que el soluto se equilibre a ambos lados de la bicapa. Conviene tener presente que la capa acuosa estática de difusión, localizada en la interfase lumen-membrana, genera una resistencia que actúa como factor limitativo para la absorción de moléculas altamente lipófilas.

Tamaño y forma molecular

La permeabilidad de la membrana parece ser inversamente proporcional al tamaño o peso molecular, ya que las moléculas de un peso molecular igual o inferior a 250 Daltons pueden atravesar la membrana intestinal por las uniones intercelulares y/o poros acuosos por lo que, en estas moléculas, además de la absorción atribuida a la ruta transcelular, también hay que tener en cuenta la absorción de la ruta paracelular.

En cuanto a la forma, las moléculas esféricas presentan una mayor facilidad para atravesar las membranas biológicas que las moléculas no esféricas.

pK_a

El pK_a, también conocido como constante de disociación, indica la fuerza que tienen las moléculas para disociarse. Un ácido será más fuerte cuanto menor es su pK_a; sin embargo, con las bases ocurre lo contrario, son más fuertes cuanto más alto es su pK_a.

La mayor parte de fármacos son ácidos o bases débiles con grupos funcionales capaces de ionizarse. En estos casos la relación existente entre el pK_a del fármaco y el pH del medio condiciona su grado de ionización, por lo que sus formas ionizadas y no ionizadas coexisten y la proporción de ellas dependerá del pH. La fracción no ionizada de un compuesto (*f*) puede calcularse con la ecuación de Hendersson-Hasselbach, como se muestra en la ecuación (2) para ácidos y en la ecuación (3) para bases. La fracción no ionizada aporta información sobre la absorción de un fármaco, ya que, como

se ha mencionado anteriormente, sólo las formas no ionizadas son susceptibles de absorción a través de la membrana lipídica.

$$f = \frac{1}{1 + 10^{(pH - pK_a)}} \quad (2)$$

$$f = \frac{1}{1 + 10^{(pK_a - pH)}} \quad (3)$$

Teniendo en cuenta las diferencias de pH existentes a lo largo del tracto gastrointestinal, una misma molécula puede presentar variaciones en su absorción dependiendo del tramo en el que se halle. En el estómago encontramos un pH ácido que puede oscilar entre 1,0 y 3,5 dependiendo de la ausencia o presencia de alimentos. En el intestino delgado el pH va aumentando desde 5,0, en la zona proximal, hasta 8,0, en la zona distal [56]. Y en el intestino grueso el pH luminal se sitúa entre 7,5-8,0 [57].

Así, un compuesto ácido presentará una mayor fracción no ionizada en estómago y zonas proximales del intestino delgado por lo que su absorción será mayor en estas localizaciones. Pero, un compuesto básico verá favorecida su absorción en las zonas distales del tracto gastrointestinal. Sin embargo, el lugar de absorción prioritario de un fármaco no solo depende de su pH, la solubilidad de la fracción ionizada y no ionizada también afecta a este proceso. En el apartado siguiente se comenta la relación existente entre las fracciones ionizada y no ionizada y la solubilidad y sus repercusiones en la absorción.

Solubilidad

La solubilidad es la capacidad que presenta una sustancia (soluto) para disolverse cuando se mezcla con un líquido (disolvente).

Para que los medicamentos puedan ser absorbidos por el organismo, es necesario que la forma farmacéutica en la cual está contenido el fármaco,

libere el principio activo y que éste se solubilice y disuelva a cierta velocidad, para luego poder difundir a través de la membrana epitelial intestinal y ser distribuido hacia el lugar donde ejercerá su acción farmacológica [58].

Dado que los fluidos intestinales son de naturaleza acuosa, los fármacos que presentan baja solubilidad en agua a menudo presentan una absorción escasa o errática.

Para determinar la solubilidad de un compuesto hay que tener en cuenta el pH del medio de disolución. Como se ha comentado en el punto anterior, el pH de los fluidos gastrointestinales cambia en los diferentes tramos del tracto gastrointestinal. Por tanto, los ácidos débiles presentarán mayor solubilidad a pH básico, ya que su grado de ionización será mayor. Sin embargo, las bases débiles serán más solubles en los medios acuosos cuanto más ácido sea el pH de dicho medio [56, 59]. Por tanto, aunque sea la fracción no ionizada de un fármaco la que se absorbe, si ésta no es soluble en medio acuoso su absorción será difícil.

1.1.2. Sistema de Clasificación Biofarmacéutica

El Sistema de Clasificación Biofarmacéutica o BCS por sus siglas en inglés (Biopharmaceutic Classification System), es un sistema para clasificar los fármacos basado en la solubilidad acuosa de los mismos y en su permeabilidad intestinal. Fue desarrollado por el Dr. Gordon Amidon y está ampliamente aceptado actualmente en el mundo farmacéutico, concretamente en el ámbito académico, industrial y reglamentario del medicamento.

El BCS traslada el punto de atención al lugar de absorción, puesto que la liberación en el medio intestinal, la concentración a saturación de dicho medio y la permeabilidad en la membrana intestinal son los determinantes de la velocidad y magnitud de la absorción oral.

1.1.2.1. Orígenes del BCS

Las bases del BCS [60] se remontan a la investigación llevada a cabo por Gordon Amidon sobre absorción y farmacocinética de fármacos durante los años 70 y 80. Pero no fue hasta 1990-1991, durante una estancia del Dr. Amidon en la FDA (Food and Drug Administration), cuando la necesidad de establecer una clasificación de los fármacos se volvió aparente. El objetivo inicial fue desarrollar un sistema para simplificar los requerimientos normativos y reguladores relacionados con la disolución de fármacos administrados por vía oral.

Durante 1990, Gordon Amidon desarrolló varios métodos para predecir la absorción en humanos utilizando la permeabilidad intestinal obtenida en yeyuno de animales. Estos métodos predictivos establecieron que la permeabilidad de la membrana intestinal, medida con el coeficiente de permeabilidad (P_{eff}), era una variable clave en la predicción de la absorción humana.

Paralelamente, el Dr. Hans Lennernas adaptó y desarrolló una técnica (Loc-I-Gut®) para determinar la permeabilidad intestinal en humanos. La disponibilidad de datos en humanos fue crucial para obtener avances en el ámbito reglamentario y regulador que apoyaran el uso del BCS, además de un factor fundamental para predecir la absorción.

Con todo esto el enfoque se fue perfilando y, finalmente, fue en 1995 cuando se publicó el artículo científico del BCS que tuvo un gran impacto en la comunidad científica y que ha cambiado la perspectiva del desarrollo de medicamentos [61].

1.1.2.2. Bases del BCS

La primera ley de Fick (4) fue el concepto científico usado como punto de partida para esta clasificación [61]:

$$J = \frac{1}{A} \cdot \frac{\partial M}{\partial t} = P_{eff} \cdot C_s \quad (4)$$

donde, *J* representa el transporte intestinal del fármaco, A es la superficie del intestino, M es la masa absorbida por unidad de tiempo (*t*), P_{eff} es la permeabilidad efectiva del compuesto y C_s es concentración del fármaco en el lumen intestinal. De acuerdo con esta ecuación, la permeabilidad y la solubilidad son los factores que determinan el transporte del fármaco a través de la membrana intestinal [62]. Si se considera la Ley de Fick para definir los factores que determinan la biodisponibilidad de un fármaco administrado por vía oral, se puede establecer que la velocidad de absorción de un fármaco depende de su permeabilidad a través de la membrana y de su concentración en el lumen. La cantidad total de fármaco que se absorbe depende de estos mismos factores y del tiempo que permanece el fármaco disponible para su absorción. A su vez, la concentración del fármaco en el lumen depende de su solubilidad y de la velocidad de disolución *in vivo*.

Por lo anteriormente expuesto se puede afirmar que dos formas farmacéuticas orales de liberación inmediata, con el mismo principio activo y los mismos perfiles de concentración en función del tiempo en el lumen intestinal, tendrán la misma velocidad y grado de absorción, por tanto, podrán considerarse bioequivalentes. Para poder aplicar esta premisa ninguno de los componentes de las formulaciones puede afectar la permeabilidad de la membrana o el tiempo de tránsito intestinal y ambas formulaciones deben tener el mismo perfil de disolución en todas las condiciones luminales.

Amidon determina en su publicación de 1995 tres números adimensionales para caracterizar un fármaco: número de absorción (*An*), número de dosis (*Do*) y número de disolución (*Dn*).

El número de absorción es la relación entre la permeabilidad (P_{eff}), el radio del intestino (*R*) y el tiempo de residencia (T_{res}). Este número también

puede expresarse como la razón entre el tiempo de residencia y el tiempo de absorción (T_{abs}), como se muestra en la ecuación (5).

$$An = \frac{P_{eff}}{R} \cdot \langle T_{res} \rangle = \frac{\langle T_{res} \rangle}{\langle T_{abs} \rangle} \quad (5)$$

Como se puede deducir de la ecuación anterior, los compuestos con altos valores de permeabilidad se absorben en un corto periodo de tiempo y, por tanto, se absorberán completamente durante su tránsito intestinal, siempre que no existan factores que modifiquen su liberación desde la forma farmacéutica o su solubilidad.

La fracción absorbida (F_a) de una solución sigue una función exponencial y se puede calcular según la ecuación (6) que establece la relación entre fracción absorbida y número de absorción.

$$F_a = 1 - e^{-2An} \quad (6)$$

El número de dosis es la relación entre la concentración que tiene la dosis en el lumen intestinal y la solubilidad del fármaco (ecuación (7)).

$$Do = \frac{D/250}{C_s} \quad (7)$$

donde, *D* es la dosis del fármaco, 250 es el volumen de agua con que se administra la dosis al paciente, que usualmente es un vaso de agua, y C_s es la solubilidad del fármaco.

Cuando un fármaco posee una alta solubilidad, la disolución no está limitada por la solubilidad y, por tanto, la absorción no depende de estos factores.

En fármacos cuya solubilidad y permeabilidad es alta, la velocidad de absorción está controlada por el vaciado gástrico.

El número de disolución es la relación entre el tiempo de residencia y el tiempo de disolución (T_{diss}). El tiempo de disolución depende de la solubilidad (C_s), la difusividad (D), densidad (ρ) y radio inicial de las partículas (r) (ecuación (8)).

$$Dn = \left(\frac{3D}{r^2}\right) \cdot \left(\frac{C_s}{\rho}\right) \cdot \langle T_{res} \rangle = \frac{T_{res}}{T_{diss}} \tag{8}$$

Una disolución rápida asegura que la disolución *in vivo* no es el factor limitante del proceso de absorción [62].

Teniendo en cuenta las definiciones dadas para los tres números adimensionales, queda claro que la permeabilidad y la solubilidad son los parámetros fundamentales que controlan la absorción de un fármaco. Por lo que los principios activos pueden ser divididos en cuatro clases, según su grado de solubilidad y de permeabilidad, como se muestra en la Figura 1.1.12.

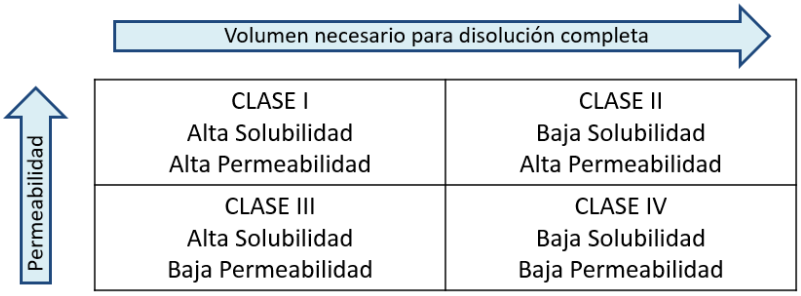

Figura 1.1.12. Clasificación de los fármacos según el BCS.

Clase I: fármacos de alta solubilidad-alta permeabilidad: los compuestos pertenecientes a este grupo se absorben bien y el paso limitante de la velocidad de absorción es la disolución del fármaco o el vaciado gástrico si la disolución es muy rápida.

Clase II: fármacos de baja solubilidad-alta permeabilidad: el paso que controla la absorción del fármaco es su disolución *in vivo*, la absorción suele ser más lenta que en la clase I.

Clase III: fármacos de alta solubilidad-baja permeabilidad: la permeabilidad es el factor limitante en la absorción de los fármacos pertenecientes a este grupo.

Clase IV: fármacos de baja solubilidad-baja permeabilidad: este tipo de compuestos presentan problemas significativos en su absorción oral.

Sin embargo, la disolución de los fármacos pertenecientes a las clases II y IV depende en gran medida de la naturaleza ácida o básica del fármaco y de las características del lumen intestinal. Por este motivo, Tsume y colaboradores [59] propusieron en 2014 una subclasificación para las clases II y IV del BCS (Tabla 1.1.2) teniendo en cuenta si el fármaco es ácido, básico o neutro y el rango de pH fisiológico (pH < 7,5).

Tabla 1.1.2. Subclasificación biofarmacéutica tomada de Tsume y colaboradores [59].

Subclasificación BCS	Solubilidad a pH 2 (estómago)	Solubilidad a pH 6.5 (intestino)	Permeabilidad
I	Alta	Alta	Alta
IIa	Baja	Alta	Alta
IIb	Alta	Baja	Alta
IIc	Baja	Baja	Alta
III	Alta	Alta	Baja
IVa	Baja	Alta	Baja
IVb	Alta	Baja	Baja
IVc	Baja	Baja	Baja

En la clase IIa se engloban los ácidos débiles, con un pK_a inferior a 5. Este tipo de compuestos presenta mayor solubilidad a pH básico, por tanto,

su porcentaje de absorción será mayor en los tramos distales del tracto gastrointestinal como el íleon y el colon. Dado el elevado tiempo de residencia que muestra cualquier molécula en el intestino grueso, los fármacos pertenecientes a la clase IIa tienen tiempo más que suficiente para una disolución y absorción completas, por lo que su comportamiento es bastante similar a aquéllos clasificados en la clase I del BCS.

Dentro de la clase IIb se encuentran las bases débiles, con un pK_a mayor a 6. Su solubilidad se ve incrementada a pH ácido, lo que les confiere mayor absorción en los tramos proximales, como el duodeno. El pH más básico de las zonas distales del intestino delgado y del colon podría provocar su precipitación, por lo que el tiempo de residencia del que disponen estos fármacos para una absorción óptima es relativamente corto.

La clase IIc son compuestos neutros y su solubilidad no está afectada por los cambios de pH que se dan a lo largo del tracto gastrointestinal. Se absorben más lentamente, por lo tanto, presentan un alto grado de absorción en colon ya que más cantidad de fármaco alcanza esta zona distal. Esta característica los convierte en compuestos ideales para fórmulas de liberación controlada.

Con estas premisas, se puede hacer una extrapolación similar para los fármacos de clase IV. Aunque, no hay que perder de vista que este grupo presenta más problemas debido a su baja permeabilidad.

1.1.2.3. Aplicaciones del BCS

Si combinamos los conceptos de solubilidad y permeabilidad del BCS con las características de disolución *in vitro* de un fármaco, tendremos los tres factores más importantes que gobiernan la absorción de un compuesto administrado en una forma farmacéutica oral sólida de liberación inmediata: solubilidad, permeabilidad intestinal y grado de disolución.

Una sustancia es altamente soluble cuando la dosis más alta administrada se disuelve en 250 mL de agua, lo que equivale a un vaso de agua; y en un rango de pH de 1,0-7,5. Será de alta permeabilidad cuando el grado de absorción intestinal, determinado experimentalmente, sea igual o superior al 90% [63].

Una aplicación muy importante del BCS es el uso de bioexenciones. Se entiende por bioexención la posibilidad de sustituir el ensayo de bioequivalencia *in vivo* por datos obtenidos en ensayos de disolución *in vitro.* Se aplica a fórmulas farmacéuticas orales sólidas de liberación inmediata con acción sistémica y consiste en el estudio comparativo de los perfiles de disolución *in vitro* entre la formulación que se ensaya y el producto de referencia.

Actualmente se utilizan en la aprobación de productos genéricos que contienen principios activos con ciertas características de solubilidad y permeabilidad, lo que supone una reducción de los estudios realizados *in vivo* y, como consecuencia, una disminución tanto en los gastos de investigación de los genéricos como en la repercusión ética que tienen los estudios en humanos.

El BCS ha proporcionado una sólida base científica para decidir cuándo los estudios de bioequivalencia *in vitro* son suficientes o incluso más discriminativos que los estudios *in vivo*. Amidon y colaboradores [64] defienden que para los fármacos clase I y III, los ensayos de disolución pueden aportar inclusive mayor evidencia de bioequivalencia que los estudios *in vivo*, ya que en estos últimos se pueden producir variaciones en los niveles plasmáticos del fármaco que pueden deberse a modificaciones en el tránsito intestinal y gástrico, impidiendo observar las diferencias entre las formulaciones a comparar.

Según el BCS en aquellas formulaciones sólidas de liberación inmediata que contengan fármacos de alta solubilidad (clase I y III), que

disuelvan al menos el 85% de la sustancia activa en 15 minutos o menos, se puede asegurar que la biodisponibilidad del fármaco no está limitada por la disolución; en esos casos, el paso limitante de la velocidad de absorción es el vaciado gástrico [65].

1.1.3. Modelos experimentales para determinar la permeabilidad intestinal

Según la FDA, un fármaco es considerado de alta permeabilidad cuando el grado de absorción en humanos es igual o mayor al 90%. Hay diversos métodos que pueden ser usados para la determinación de la permeabilidad de un compuesto; los cuales deben ser desarrollados y validados y demostrar que son adecuados para establecer la permeabilidad de fármacos, con el uso de varios fármacos modelo y el de un control negativo. El metoprolol ha sido aceptado como fármaco de referencia para establecer el punto de corte para clasificar los fármacos según su permeabilidad. Todos los compuestos con una permeabilidad igual o superior a la del metoprolol serán considerados de alta permeabilidad y, por el contrario, todos aquellos con una permeabilidad inferior serán de baja permeabilidad [66].

1.1.3.1. Métodos in silico

Los métodos computacionales (*in silico*) son útiles para realizar un cribado inicial en la fase de descubrimiento de nuevas moléculas con actividad terapéutica. Estos métodos presentan una ventaja frente a los métodos experimentales, ya que no requieren que el fármaco esté sintetizado para llevar a cabo los estudios.

Los métodos *in silico* pueden predecir propiedades importantes de un compuesto relacionadas con su absorción, como la lipofilia, solubilidad, permeabilidad o metabolismo. Por lo que con estas estimaciones se puede reducir los posibles fallos en fases más avanzadas de la investigación.

Destacan dentro de los métodos *in silico* aquellos basados en las relaciones cuantitativas estructura-actividad (QSAR) que permiten relacionar los cambios en la estructura química de una molécula con los datos biológicos y farmacológicos, por lo que se puede saber de qué manera las modificaciones estructurales pueden afectar a las propiedades biofarmacéuticas. También existe la posibilidad, en etapas tempranas del descubrimiento y desarrollo de fármacos, de realizar una clasificación simple y rápida en los cuatro grupos del BCS a través de la obtención de los parámetros de interés mediante el uso de modelos *in silico*, utilizando propiedades físico-químicas derivadas solamente de descriptores moleculares [67].

1.1.3.2. Métodos in vitro

Membranas artificiales

Las membranas artificiales (PAMPA: parallel artificial membrane permeation assay) son utilizadas para determinar la permeabilidad, además de clasificar, los compuestos que presentan una absorción de forma pasiva, concretamente, aquellos que atraviesan las membranas permeables por transporte transcelular [68]. En esta técnica se usa una membrana de permeación formada por lecitina y un disolvente orgánico inerte. Además, se utiliza una placa de microvaloración compuesta por numerosos insertos hidrofóbicos que actúan como soporte de la membrana [69].

La ventaja más importante que presenta este sistema es la posibilidad de investigar numerosos compuestos en un solo día, ya que permite realizar ensayos múltiples. Sin embargo, solamente es útil para clasificar de forma sencilla los compuestos estudiados en baja, media y alta probabilidad de absorción intestinal.

En ensayos posteriores se han realizado variaciones en el método para incluir la difusión paracelular [70] y se han correlacionado los datos de

permeabilidad de numerosos compuestos obtenidos en Caco-2 con los obtenidos mediante el uso de membranas artificiales [71].

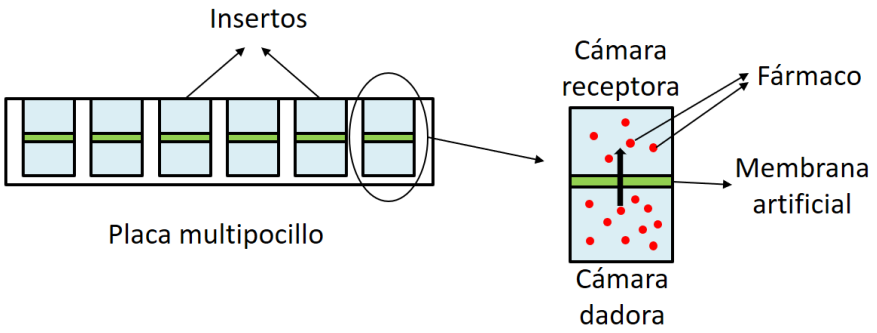

Figura 1.1.13. Representación esquemática de un modelo PAMPA.

Cultivos celulares: monocapas Caco-2

Actualmente, las monocapas de células Caco-2 cultivadas en membranas permeables (membranas de policarbonato) se utilizan para la realización de estudios de transporte transepitelial de numerosos fármacos y nutrientes [72]. Los ensayos en cultivos celulares se llevan a cabo en placas con un determinado número de pocillos. Cada uno de estos pocillos consta de un inserto donde se siembran las células que formarán la monocapa, además se distingue una cámara apical y otra basolateral (Figura 1.1.14). En la mayoría de los casos existe la posibilidad de analizar si un fármaco se transporta de forma activa o pasiva a través de la membrana intestinal; además, si el transporte es activo, permite identificar el transportador más importante [73, 74].

La línea celular Caco-2 fue aislada en 1977 por Fogh y colaboradores [75] a partir de carcinoma de colon humano. Es la única línea celular capaz de diferenciarse en células con gran similitud a los enterocitos de manera espontánea [76, 77]. Estas células, al igual que los enterocitos, presentan dos

lados bien diferenciados (cara apical y basal) y uniones estrechas (*tight junctions*).

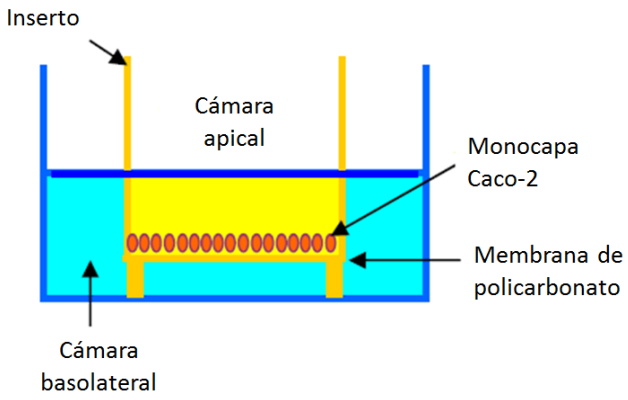

Figura 1.1.14. Representación esquemática de un pocillo donde se realizan los ensayos de cultivos celulares.

La existencia de una gran parte de las enzimas que se encuentran en el borde en cepillo del enterocito, además de otros sistemas de transporte especializado para aminoácidos, dipéptidos, vitaminas y citostáticos, es otra característica importante que le confiere a la línea celular Caco-2 gran parecido con la membrana intestinal [78]. La membrana basolateral también se relaciona con actividad ATP-asa dependiente de sodio-potasio y con receptores hormonales. Asimismo, aparecen enzimas intracelulares, de fase I (oxidación) y de fase II (conjugación) [79, 80].

Las métodos *in vitro* con monocapas Caco-2 han demostrado ser un modelo igual de válido que otros modelos más complejos para los estudios de absorción intestinal de fármacos [81].

Las ventajas que presentan los cultivos celulares frente a los modelos de absorción convencionales son:

- La rápida determinación de la permeabilidad y del metabolismo de los fármacos.

- Posibilidad de estudiar los mecanismos de absorción bajo condiciones controladas.
- La rápida evaluación de métodos que mejoren la absorción de fármacos.
- La posibilidad de realizar estudios con líneas celulares humanas.
- La reducción de la experimentación animal.

Es importante resaltar la existencia de diferencias entre los valores de permeabilidad de un mismo fármaco obtenidos en diferentes laboratorios. Este hecho se debe a la variabilidad en las condiciones experimentales y en las propias líneas celulares usadas en cada laboratorio [20].

Modelos basados en tejidos

En estos modelos se emplean segmentos intestinales (generalmente de animales, aunque en ocasiones se usan tejidos humanos) para el estudio de la absorción de fármacos. En general el ensayo consiste en colocar una solución con el fármaco a estudiar en la zona mucosa del tejido y se determina la velocidad de absorción por la desaparición del fármaco de la solución o por la aparición de fármaco en la zona serosa. En estos modelos se conservan las características de las membranas biológicas y además son útiles en la determinación de la absorción gastrointestinal de diferentes segmentos del intestino [68].

Existen dos grandes tipos de sistemas experimentales que se basan en estos modelos:

1) Cámaras de difusión:

Estos modelos de absorción determinan el flujo transepitelial de los compuestos a través del tejido epitelial intacto. La técnica consiste en aislar y seccionar de forma correcta el segmento intestinal objeto de estudio para obtener capas planas de tejido. Las porciones de tejido se disponen

posteriormente sobre las células de difusión estándar que se rellenan con un tampón adecuado para simular los fluidos extracelulares [82].

Una de las técnicas más conocidas dentro de las cámaras de difusión es la cámara Ussing (Figura 1.1.15). Esta técnica fue desarrollada en 1951 [83] y ha sido utilizada para determinar la permeabilidad con tejido intestinal animal. Pero en estudios más recientes también se ha utilizado con tejido aislado del tracto intestinal humano [84, 85]. Se puede evaluar tanto el transporte activo como el pasivo y ofrece la posibilidad de estudiar el transporte en distintas zonas del tracto gastrointestinal.

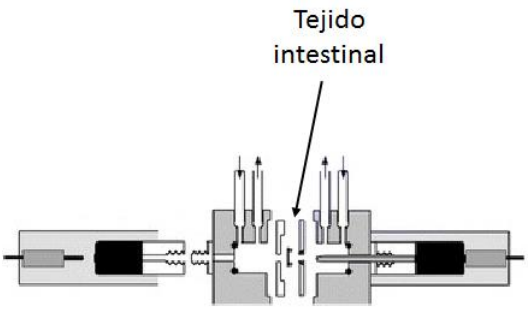

Figura 1.1.15. Esquema del emplazamiento de una cámara Ussing [86].

2) Sacos evertidos:

Los sacos evertidos se utilizan en estudios de acumulación de fármacos en segmentos intestinales completos. Los segmentos intestinales deben ser invertidos y divididos en pequeñas porciones en forma de anillos. Para invertir el segmento se debe introducir un aplicador a través del mismo, anudar el extremo y tirar con suavidad hasta que el intestino se vuelva del revés como se muestra en la Figura 1.1.16. A continuación, se incuban en tampones oxigenados donde se encuentra el fármaco objeto de estudio, bajo condiciones controladas de agitación y temperatura [87].

El sistema de sacos evertidos permite el estudio de la absorción intestinal la evaluación de la conversión de un profármaco en fármaco por la

acción del tejido intestinal, y la discriminación de los mecanismos de absorción de fármacos en transporte pasivo o activo [87, 88].

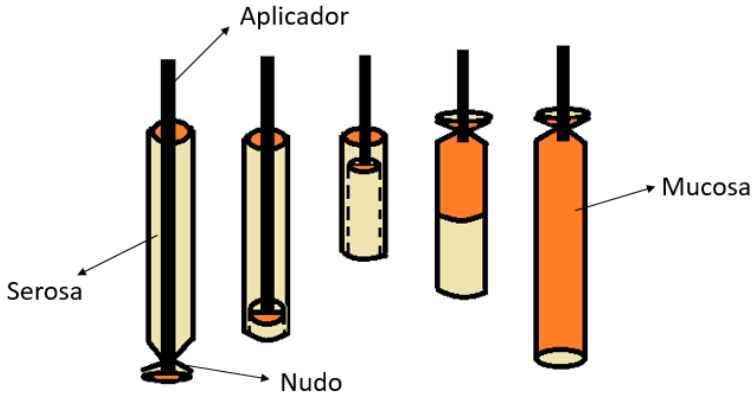

Figura 1.1.16. Procedimiento a seguir para obtener un saco evertido.

1.1.3.3. Métodos in situ

En los modelos *in situ* el aporte neural, endocrino, sanguíneo y linfático de la zona en estudio permanece intacto y, por tanto, son sensibles a influencias farmacológicas y fisiológicas. Entre las técnicas *in situ* de perfusión intestinal destaca el método de paso simple (single pass) y el método de Doluisio (closed loop).

Método de paso simple (single pass)

En este método el segmento del intestino que se desea estudiar, se perfunde a velocidad controlada con la solución del fármaco (Figura 1.1.17). Con esta técnica se puede controlar la concentración del fármaco, el pH, la osmolaridad, y la velocidad de entrada del mismo. El cálculo de la permeabilidad se realiza por medida de la desaparición del fármaco del lumen intestinal en estado estacionario [89-91].

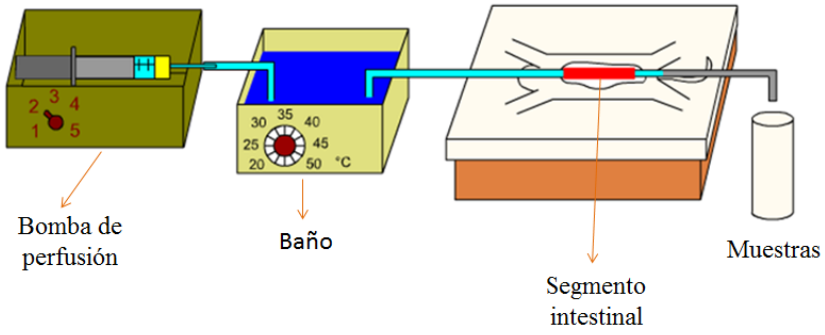

Figura 1.1.17. Esquema de la técnica de perfusión *in situ* de paso único. Tomado con permiso de "Biofarmacia Moderna V.6.0".

Método de Doluisio (closed loop)

Este método está basado en la técnica desarrollada por Doluisio y colaboradores [92] en 1969. Al contrario de lo que ocurre en el paso simple, en este caso, la solución a ensayar se mantiene en el segmento intestinal durante todo el experimento. El segmento de interés queda aislado (Figura 1.1.18) dando lugar a un compartimento estanco de absorción del cual se toman muestras para observar la desaparición del fármaco del lumen.

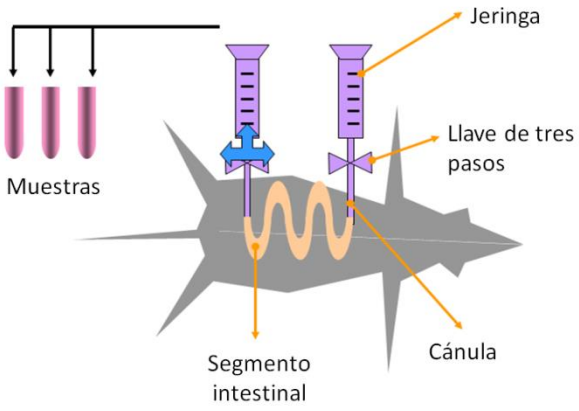

Figura 1.1.18. Representación esquemática de la técnica de perfusión *in situ* basada en el método de Doluisio. Imagen tomada con permiso de Bermejo, M. [93].

1.1.3.4. Métodos in vivo

Los métodos *in vivo* en rata se utilizan para realizar los estudios de farmacocinética y de biodisponibilidad. Se entiende por biodisponibilidad la fracción o porcentaje de la dosis del fármaco administrado que accede de forma inalterada a la circulación sistémica y la velocidad a la que se produce dicho proceso.

Para llevar a cabo estos estudios se implanta un catéter de silicona en la vena yugular de la rata de forma permanente, bajo condiciones de anestesia y analgesia adecuadas. Una vez el animal esté recuperado de la intervención, se pueden tomar muestras sanguíneas a través del catéter sin necesidad de anestesia y sin limitar su movilidad (Figura 1.1.19), lo que evita el estrés adicional para el animal [94]. Esta técnica permite administrar un compuesto por cualquier vía de administración, en general por vía parenteral y por vía oral, y medir su aparición en sangre, de esta manera se puede realizar una predicción indirecta de la permeabilidad, ya que si el compuesto aparece en sangre implica que ha atravesado la membrana absorbente.

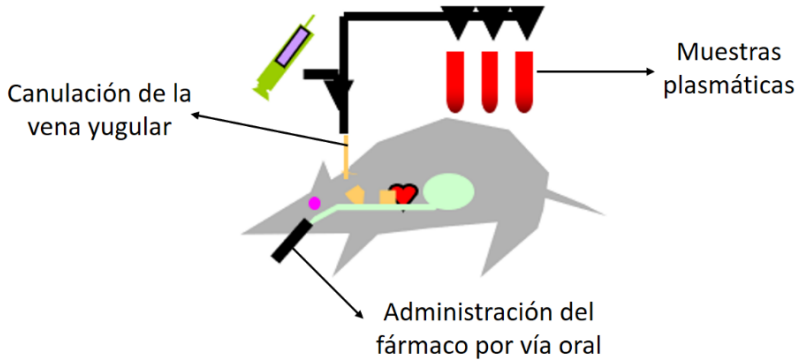

Figura 1.1.19. Representación esquemática del método de canulación de la vena yugular en rata. Imagen tomada con permiso de Bermejo, M. [95].

1.1.3.5. Determinación de la permeabilidad en humanos

Métodos indirectos

1) *Estudio farmacocinético de balance de masas:* es un estudio farmacocinético completo que se realiza con el fármaco radiomarcado. Se lleva a cabo en voluntarios sanos a los que se les administra por vía oral el fármaco y luego se recolectan muestras de sangre, orina y heces a fin de cuantificar el fármaco y sus metabolitos en cada muestra biológica obtenida [96].

2) *Estudio de biodisponibilidad absoluta:* en este tipo de estudios se comparan los datos disponibles tras la administración de un fármaco administrado por vía oral con los datos obtenidos tras la administración del mismo por vía intravenosa, obteniéndose información útil sobre la distribución y eliminación del fármaco e indirectamente se determina la permeabilidad oral y la influencia de enzimas y transportadores presistémicos. El fármaco puede estudiarse en plasma sanguíneo o en orina y debe conocerse con exactitud los metabolitos que se pueden encontrar en la muestra biológica estudiada. El método analítico ha de ser adecuado para cuantificar tanto el fármaco como los metabolitos. La razón entre el área bajo la curva de concentración plasmática-tiempo obtenida tras la administración de la dosis oral y la dosis intravenosa, permite el cálculo de la biodisponibilidad absoluta del fármaco administrado por vía oral [97].

Métodos directos

Las medidas directas de permeabilidad intestinal y secreción de fármacos en humanos son posibles gracias a técnicas de perfusión intestinal de la región de interés [98, 99]. La determinación de la permeabilidad se basa en el cálculo de la absorción intestinal como función de la desaparición de un

fármaco en el segmento estudiado, por lo que reflejará la velocidad de transporte a través de la barrera epitelial del intestino, expresada en centímetros por segundo [100].

En general se utilizan cuatro sistemas de perfusión diferentes [101]. Dos de ellos son sistemas abiertos: doble lumen y triple lumen (Figura 1.1.20-A), el tercero es un sistema semiabierto de tubo multi-lumen con un balón de oclusión proximal (Figura 1.1.20-B) y el último es un método de doble balón con tubo multi-lumen donde se delimita un segmento intestinal de 10 cm (Figura 1.1.20-C): Loc-I-Gut y Loc-I-Col dependiendo de si el segmento es de intestino delgado o de colon [102].

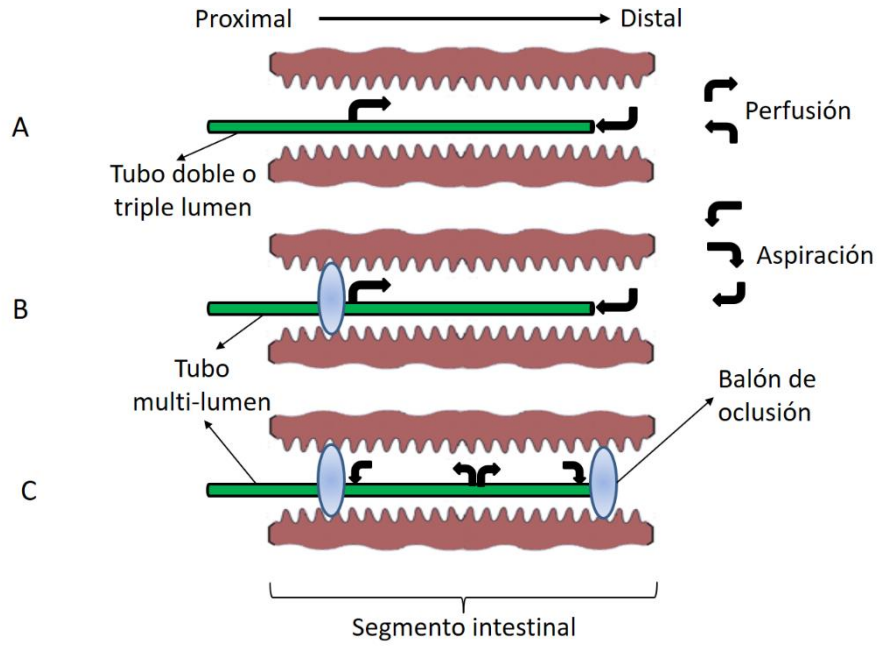

Figura 1.1.20. Esquema de los modelos de perfusión intestinal en humanos.

1.1.4. Estrategias para aumentar la permeabilidad intestinal

Existen numerosos fármacos que presentan baja biodisponibilidad tras una administración por vía oral debido a diferentes problemas en lumen gastrointestinal (degradación enzimática, degradación por microflora bacteriana, etc.) y/o problemas de absorción o permeabilidad a través de las membranas absorbentes del tracto intestinal.

Hay numerosas estrategias para mejorar la absorción de un compuesto. Muchas de ellas se centran en aumentar la solubilidad en medio acuoso, ya que para que un fármaco se absorba en primer lugar debe disolverse. Esto es de especial importancia para los compuestos pertenecientes a la clase II del BCS, en los que su escasa solubilidad acuosa es el factor limitante de su absorción. Sin embargo, para aquellos principios activos que pertenecen a la clase III se deben valorar estrategias para aumentar su permeabilidad, puesto que no presentan problemas de solubilidad. Para los de clase IV, que presentan problemas tanto de solubilidad como de permeabilidad, es más complejo conseguir una mejor absorción, se necesitan estrategias que mejoren ambos parámetros.

A continuación, se comentan algunas de las estrategias más relevantes para mejorar la absorción de un fármaco. Aunque también pueden ser útiles para aumentar la solubilidad, el enfoque dado en este apartado se centra en el incremento de la permeabilidad para conseguir una mejora en la absorción intestinal. Por lo tanto, estas estrategias serían aplicables a fármacos pertenecientes a las clases III y IV del BCS. No hay que perder de vista que existen opciones diferentes a las explicadas en este apartado que también pueden ser utilizadas para mejorar la permeabilidad de un principio activo.

1.1.4.1. Excipientes

Un excipiente es una sustancia inactiva usada para incorporar el principio activo, se utilizan para conseguir la forma farmacéutica deseada y facilitan la preparación, conservación y administración del medicamento.

Diversos estudios muestran que los excipientes, habitualmente considerados sustancias inertes, pueden modificar parámetros fundamentales que condicionan la biodisponibilidad de un fármaco, como la permeabilidad. De hecho, la absorción total de un compuesto puede verse afectada por la combinación de excipientes incluidos en una formulación.

Varios autores han estudiado el aumento de absorción modulando el transporte a través de la membrana intestinal con el uso de excipientes. Por ejemplo, se ha demostrado que los tensionactivos, como el lauril sulfato sódico, clásicamente utilizados con el objetivo de aumentar la solubilidad acuosa, también incrementan considerablemente la permeabilidad, ya que afectan a las uniones estrechas aumentando la distancia intercelular y son capaces de arrastrar la capa de difusión acuosa estática [103]. La difusión pasiva también puede verse afectada por el uso de ciertos excipientes que producen cambios en la membrana apical y/o basolateral de los enterocitos [104]. Además, existen excipientes que provocan un incremento de la permeabilidad debido a la inhibición de transportadores de secreción [105].

1.1.4.2. Profármacos

Un profármaco es un derivado químico del fármaco sin actividad farmacológica que posteriormente, en el organismo, se convierte en el fármaco activo. Los profármacos se obtienen mediante modificaciones químicas biorreversibles del compuesto activo y su objetivo es mejorar las propiedades farmacocinéticas, farmacodinámicas o farmacéuticas de la molécula, haciendo hincapié en características como la permeabilidad, solubilidad, estabilidad o toxicidad. Con el uso de un profármaco se

consiguen mejoras, principalmente, en la absorción y biodisponibilidad del principio activo. El paso a sustancia activa ocurre a través de una reacción química o enzimática *in vivo*, con esta bioconversión se libera el fármaco activo y la parte restante del profármaco, la cual debería ser fisiológicamente inerte y rápidamente eliminada [106].

El uso de profármacos se ha vuelto muy popular, es una estrategia que se ha venido utilizando cada vez más en las últimas décadas [107]. Pero no hay que perder de vista que las modificaciones químicas utilizadas dan lugar a una nueva entidad molecular con características distintas a las del fármaco de origen, por lo que la función del principio activo podría verse afectada [108].

1.1.4.3. Par iónico

Un par iónico es un complejo formado por dos iones de carga opuesta unidos por atracción electrostática sin presencia de enlaces covalentes, de tal forma que el complejo obtenido presenta una carga global neutra.

Esta estrategia se utiliza para compuestos que presentan uno o varios grupos ionizados en el rango de pH fisiológico, ya que son las moléculas sin carga las que atraviesan las membranas biológicas. Por tanto, se puede incrementar la lipofilia de compuestos que presenten grupos ionizables eligiendo el contraión adecuado; de manera que el complejo obtenido será más lipófilo que el principio activo de interés, por lo que aumentará la difusión pasiva a través de la membrana intestinal lo que traerá consigo un incremento de la permeabilidad del fármaco. Un par iónico se comporta como una única entidad y se disocia después de su absorción, una vez que ya ha pasado a sangre [109].

La formación de un par iónico presenta varias ventajas. Además de aumentar la permeabilidad a través de las membranas biológicas de principios activos ionizables y poco permeables, también se pueden utilizar

para mejorar la solubilidad y estabilidad. Cabe destacar que la formación de un par iónico no implica ninguna alteración en la estructura y función del fármaco y elimina la necesidad de reacciones enzimáticas específicas para su activación [110].

1.1.4.4. Microencapsulación y nanoencapsulación

La microencapsulación y nanoencapsulación se definen como aquel proceso tecnológico por el que se forma una cubierta sobre pequeñas partículas de principio activo, sólidas o líquidas, utilizando materiales de distinta naturaleza. El producto obtenido tras la aplicación de este proceso se denomina micropartícula o nanopartícula, dependiendo de su tamaño. Las micropartículas se sitúan dentro de la escala micrométrica y poseen un diámetro menor a 1 mm. Sin embargo, las nanopartículas se engloban dentro de la escala nanométrica, con un diámetro inferior a 1 µm. Tanto las nanopartículas como las micropartículas pueden constituir una forma farmacéutica por sí solas y administrarse en forma de suspensión, o pueden acondicionarse en otras formas farmacéuticas, como por ejemplo en cápsulas de gelatina rígida [111].

La formulación de micro y nanopartículas es especialmente útil para fármacos que presentan una degradación temprana y/o baja solubilidad en el tracto gastrointestinal, ya que mejorando estos dos aspectos se conseguirá aumentar la permeabilidad de una molécula poco permeable [112]. Además, la micro y nanoencapsulación también pueden ser beneficiosas para reducir ciertos efectos adversos locales, porque se limita el contacto directo del principio activo con las mucosas del tracto digestivo. Asimismo, se utilizan para enmascarar características organolépticas indeseables, lo que puede llegar a ser de vital importancia en pacientes pediátricos.

Por último, otra ventaja destacable de este tipo de encapsulación es la posibilidad de obtener una liberación controlada del fármaco. Pues, en el caso de la micro y nanoencapsulación, el compuesto encapsulado se libera

gradualmente a una velocidad que puede ser controlada bajo unas condiciones específicas. Esta ventaja también puede ser interesante para fármacos que presentan alta permeabilidad para conseguir una liberación prolongada y sostenida en el tiempo aumentando, de este modo, la duración del efecto terapéutico [113, 114].

1.1.5. Correlaciones entre parámetros utilizadas como modelos de predicción

Desde un punto de vista matemático, existe una correlación cuando hay una interdependencia entre unos datos cuantitativos y cualitativos o una relación entre una variable medible y una categoría [115]. Los modelos matemáticos que se derivan de estas correlaciones pueden llegar a ser una herramienta crucial en la predicción de la absorción gastrointestinal. En el contexto biofarmacéutico, las correlaciones entre distintos parámetros se han venido utilizando para varios objetivos. Algunos de ellos se describen a continuación.

1.1.5.1. Obtención de modelos biofísicos de absorción

El método que muchos grupos de investigación han aplicado en sus estudios para obtener los modelos biofísicos de absorción, consiste en establecer correlaciones sencillas entre parámetros de absorción y parámetros fisicoquímicos. Los valores de la constante de velocidad de absorción o de permeabilidad intestinal son tomados como parámetros de referencia del proceso de absorción; por otro lado, la lipofilia y el peso molecular se toman como parámetros fisicoquímicos. Tanto los parámetros de absorción como los fisicoquímicos condicionan la velocidad de absorción de los fármacos que se absorben por difusión pasiva, ya sea por vía transcelular o por vía paracelular.

Teniendo en cuenta este planteamiento, se han obtenido los modelos biofísicos de absorción con los que se puede predecir la absorción de un

determinado compuesto a partir de la determinación del parámetro fisicoquímico seleccionado, por ejemplo, su lipofilia. Las relaciones obtenidas son características del lugar del tracto gastrointestinal en el que se hayan obtenido los parámetros de absorción y han servido para explicar la influencia de las características fisiológicas particulares de cada segmento del tracto gastrointestinal sobre el proceso de absorción de los fármacos [43].

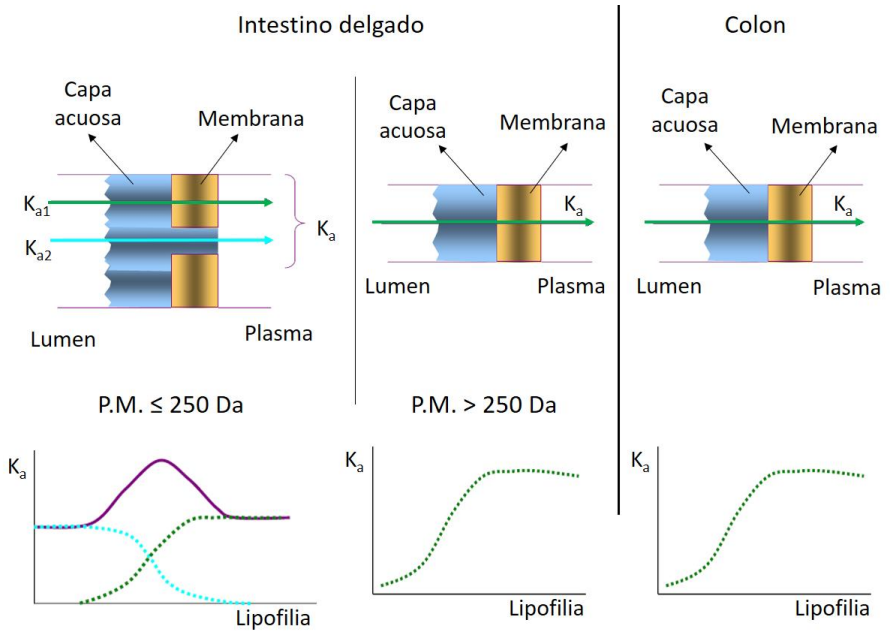

Figura 1.1.21. Correlaciones absorción-lipofilia obtenidas tras la obtención de los parámetros de absorción en intestino delgado y colon utilizando un método de perfusión *in situ*. K_a: constante de velocidad de absorción, P.M.: peso molecular, Da: Daltons [54].

En la Figura 1.1.21 se observan las correlaciones resultantes entre la constante de absorción obtenida en ensayos de absorción *in situ* desarrollados en intestino delgado y colon y la lipofilia de los compuestos. Se aprecia que en el intestino delgado los compuestos con un peso molecular de 250 Daltons o inferior, que son capaces de atravesar las membranas

absorbentes por vía transcelular y por vía paracelular, presentan una absorción global igual a la suma de la absorción por ambas vías. Sin embargo, los compuestos con un peso molecular superior a 250 Daltons solo se absorben por la ruta transcelular. En colon sólo la absorción transcelular tiene importancia, independientemente del peso molecular del compuesto.

1.1.5.2. Predicción de la absorción oral

La predicción del rendimiento de la absorción de fármacos administrados por vía oral frecuentemente se realiza a partir de las correlaciones entre parámetros de disolución y parámetros de absorción. Estas correlaciones se conocen con el nombre de correlaciones *in vitro-in vivo* (IVIVCs) y en el ámbito biofarmacéutico, durante los últimos años, han ganado importancia ya que entre sus aplicaciones cabe destacar su utilización para predecir la absorción *in vivo* de una molécula a partir de la obtención de parámetros de disolución obtenidos *in vitro,* así como la capacidad de determinar la bioequivalencia *in vitro.*

Se puede distinguir dos definiciones para el término IVIVCs [116]:

- La FDA define las IVIVCs como un modelo matemático predictivo que describe la relación entre una propiedad de una formulación obtenida utilizando métodos *in vitro* y una respuesta relevante obtenida utilizando métodos *in vivo*. Normalmente como propiedad *in vitro* se emplea la velocidad de disolución del fármaco y como respuesta *in vivo,* la concentración plasmática o la cantidad de fármaco absorbida a un determinado tiempo.

- La USP define las IVIVCs como el establecimiento de una relación racional entre una propiedad biológica, o un parámetro derivado de una propiedad biológica, y una propiedad fisicoquímica o característica de la forma de dosificación. La propiedad biológica debe estar inducida por la forma de dosificación administrada.

Entre las diferentes IVIVCs descritas en el contexto de la biofarmacia y farmacocinética cabe resaltar las IVIVCs encaminadas a apoyar bioexenciones, evitando la realización de estudios de biodisponibilidad *in vivo* en humanos, ya que conociendo los parámetros de disolución obtenidos *in vitro* se podrían predecir a partir de ellas los parámetros de biodisponibilidad *in vivo*. Además, existen motivos éticos que promueven el desarrollo de estas IVIVCs, porque con ellas se puede reducir el número de ensayos en voluntarios humanos, lo que además reduce el coste y tiempo necesario para que un fármaco sea comercializado [117].

En este contexto se puede resaltar el proyecto OrBiTo (Oral Biopharmaceutic Tools) que tiene como principal objetivo aumentar la comprensión del proceso de absorción desde el tracto gastrointestinal de los fármacos administrados por vía oral, así como desarrollar nuevas pruebas *in vitro* y modelos *in silico* que permitan mejorar la predicción de la acción de un compuesto administrado por vía oral en humanos. Dado que las IVIVCs son una herramienta que está ganando importancia en el campo biofarmacéutico, tanto en las formulaciones de liberación inmediata como en las de liberación controlada o modificada, los integrantes del proyecto OrBiTo han realizado una extensa revisión del tema señalando las lagunas existentes actualmente en el conocimiento relativo al ámbito de las IVIVCs [118]. En los siguientes párrafos se resumen los aspectos más relevantes de dicha revisión.

Los autores diferencian entre correlaciones *in vitro-in vivo* (IVIVC) y relaciones *in vitro-in vivo* (IVIVR). Ambos términos se pueden usar indistintamente; aunque algunos autores, como por ejemplo los integrantes del proyecto OrBiTo, usan IVIVC cuando existe una ecuación matemática e IVIVR cuando se observa una tendencia determinada, pero no se ha establecido una ecuación matemática que la describa. Ambas son clave en el desarrollo y en la comercialización de un fármaco, ya que son las bases para comprender la relación entre los datos *in vitro* y los parámetros *in vivo.* Sin

embargo, desde la perspectiva industrial y normativa se cuestiona su utilidad debido a los problemas que presenta el desarrollo de una IVIVC y/o IVIVR para formas farmacéuticas de administración oral.

Como ya se comentó anteriormente, una IVIVC se define como una relación matemática predictiva que conecta, por ejemplo, los datos de disolución obtenidos *in vitro* con los datos farmacocinéticos obtenidos *in vivo*. Pero en ciertas ocasiones el término IVIVC también se emplea para describir una amplia variedad de enfoques que unen varios aspectos del comportamiento de una formulación *in vitro* con el rendimiento, medido o predicho clínicamente, de una forma de dosificación.

Por otro lado, una IVIVR hace referencia a aquellas ocasiones en las que cambios en los parámetros de disolución obtenidos *in vitro* no tienen ningún impacto en la bioequivalencia, para un determinado compuesto y en unas condiciones concretas del ensayo. De hecho, esta idea es la base de las bioxenciones para los compuestos pertenecientes a la clase I del BCS. El término IVIVR va ligado al concepto "espacio seguro", entendido como el rango en el que variaciones en la disolución no alteran la biodisponibilidad de un principio activo.

Por tanto, existen tres posibles relaciones entre la disolución *in vitro* y el comportamiento *in vivo*:

1. Una correlación matemática entre los parámetros de disolución *in vitro* y el rendimiento en la absorción *in vivo,* de tal manera que una modificación dada en la disolución puede ser utilizada para predecir un cambio correspondiente a un parámetro *in vivo.*

2. Cambios en los parámetros de disolución *in vitro* pueden ser tolerados sin ningún tipo de impacto sobre el rendimiento *in vivo,* resultando una disolución dentro del "espacio seguro".

3. Pequeños cambios de los parámetros de *in vitro* no tienen impacto en los parámetros de absorción *in vivo,* pero cambios mayores sí pueden afectar.

Los tres escenarios pueden ser útiles para entender la relevancia que presenta un resultado de una prueba *in vitro* sobre el rendimiento de absorción *in vivo.* El caso 1 se refiere a una IVIVC clásica y los casos 2 y 3 están relacionados con el término IVIVR.

Las IVIVCs han sido recomendadas e incluidas en las guías de la EMA y la FDA para demostrar la predictibilidad de los métodos *in vitro*. Según estas guías, cuando un método *in vitro* demuestra una predictibilidad robusta y precisa, junto con una IVIVC validada, para aprobar la comercialización de un medicamento se pueden sustituir los estudios de permeabilidad en humanos por una prueba de disolución. Sin embargo, el concepto IVIVR no está incluido actualmente en ningún documento normativo, lo que significa que no está aceptado por las instituciones gubernamentales (FDA o EMA) como las IVIVCs, que se establecen cuando ligeros cambios en el parámetro determinado *in vitro* conllevan cambios en la variable respuesta seleccionada (parámetro *in vivo*).

Pero las IVIVCs suelen estar limitadas y a veces fallan debido a razones desconocidas, probablemente relacionadas con las condiciones no fisiológicas que presentan los ensayos *in vitro.* Además, en el proceso de tratamiento matemático de datos hay que prestar atención a varios aspectos para maximizar la probabilidad de éxito de la IVIVC. Asimismo, establecer una IVIVC con fármacos altamente variables es problemático, porque, o bien la variabilidad intraindividual puede enmascarar diferencias en la formulación, o la variabilidad en los factores que regulan el proceso de disolución *in vivo* puede afectar negativamente al proceso.

Normalmente, se asume que una correlación IVIVC entre parámetros de disolución y parámetros de absorción debe ser lineal; sin embargo, una correlación no lineal también es aplicable y útil [118].

Por todas las razones que se acaban de señalar, y dada la importancia que puede tener cualquier cambio en una formulación sobre la biodisponibilidad de un principio activo, en la revisión realizada por el grupo de trabajo del proyecto OrBiTo, se concluye que se necesitan más estudios para aumentar la probabilidad de desarrollar una IVIVC/IVIVR fiable que pueda utilizarse con seguridad en el desarrollo de medicamentos y en el ámbito normativo. Además, se requiere más conocimiento sobre los conceptos IVIVR y "espacio seguro", así como, resultados de este tipo de relaciones desarrollados en voluntarios sanos y pacientes para, de este modo, poder utilizar las IVIVRs en el contexto normativo.

1.1.5.3. Validación de modelos experimentales utilizados para estimar parámetros de absorción

Las correlaciones entre parámetros de absorción estimados mediante modelos *in silico, in vitro, in situ e in vivo* se utilizan para validar los diferentes métodos empleados para obtener los parámetros de absorción con el objetivo final de seleccionar el método que mejor prediga la absorción intestinal en humanos de un determinado compuesto y, a su vez, sea lo más sencillo posible. Se pueden construir a partir de los parámetros de absorción obtenidos en modelos *in silico*, en modelos *in vitro*, en modelos *in situ* y/o en modelos in *vivo*.

Todos los métodos requieren que los parámetros de absorción se determinen en condiciones que aseguren que el proceso de absorción transcurre de acuerdo con una cinética de primer orden, es decir que el mecanismo predominante es la difusión pasiva, porque se ha podido demostrar que el grado de expresión de los transportadores en los modelos de absorción habitualmente utilizados, *in vitro, in situ e in vivo*, no es el

mismo que en humanos. En consecuencia, para fármacos que se absorben mediante procesos especializados de transporte las correlaciones entre los parámetros de absorción obtenidos por métodos diferentes no es adecuada y tiene escaso valor predictivo [119].

A continuación, se comentan algunos ejemplos de las correlaciones disponibles en la literatura científica utilizando datos de absorción proporcionados por métodos *in silico, in vitro, in situ* e *in vivo*:

Correlaciones entre parámetros de absorción *in silico-in vitro*

Las correlaciones obtenidas a partir de parámetros de absorción determinados utilizando métodos *in silico* e *in vitro* pueden resultar útiles para predecir parámetros de absorción en estadíos tempranos del proceso de desarrollo de un fármaco. De hecho, existen varios modelos *in silico* para predecir la absorción *in vitro* de compuestos, entre ellos el modelo computacional propuesto por Paixão y colaboradores [120], en el cual correlacionan la permeabilidad de 296 fármacos calculada *in silico* de acuerdo con el modelo propuesto y la permeabilidad obtenida en ensayos en monocapas celulares Caco-2, por ser un modelo *in vitro* que ha demostrado ser útil para estudios de absorción intestinal, para estos mismos fármacos. La buena correlación obtenida demuestra que el modelo que proponen los autores puede ser una herramienta útil de predicción para el cribado inicial de compuestos.

Correlaciones entre parámetros de absorción *in vitro-in vitro*

También es posible correlacionar parámetros de absorción obtenidos utilizando distintas técnicas *in vitro* como hacen Rastogi y colaboradores [121] en su estudio sobre la absorción intestinal de seis polifenoles. En este caso se establece una correlación entre la permeabilidad obtenida utilizando membranas artificiales (PAMPA), usado como modelo de transporte pasivo, y monocapas celulares Caco-2, escogida por ser una técnica que permite

estimar el transporte pasivo y activo. En el estudio se obtiene una buena correlación lineal con una R^2 de 0,82, por lo que los autores concluyen que el transporte pasivo juega un papel principal en la absorción de este tipo de compuestos, por tanto, la conclusión que alcanzan los autores de este estudio es que las membranas artificiales son un modelo efectivo para predecir la absorción intestinal de los polifenoles.

Correlaciones entre parámetros de absorción *in situ-in vitro*

Estas correlaciones son relaciones lineales que ofrecen la posibilidad de predecir la permeabilidad *in situ*, en general en rata ya que es el modelo animal utilizado en este tipo de estudios, usando los datos de permeabilidad obtenidos con técnicas *in vitro.*

Rodríguez-Ibáñez y colaboradores [122] correlacionaron la permeabilidad *in situ* en rata con la permeabilidad *in vitro* en células Caco-2 para una serie de quinolonas. Esta correlación puso de manifiesto que la principal diferencia entre ambas técnicas es el área efectiva real para el transporte. Este es uno de los motivos principales por los que la permeabilidad determinada *in situ* en rata es más alta que los valores obtenidos en monocapas celulares Caco-2, ya que las monocapas solo presentan microvilli, sin embargo, los modelos *in situ* cuentan con una mayor superficie de absorción proporcionada además por los pliegues y los villi [43].

Correlaciones fracción absorbida-parámetros de absorción *in vitro*

Existen varios estudios que demuestran la utilidad de los modelos *in vitro* basados en monocapas celulares Caco-2 para predecir la absorción en humanos [20, 123, 124].

Shiyin Yee [125] observó las limitaciones encontradas en la literatura para los cultivos celulares de Caco-2, y se propuso evaluar la utilidad de este modelo *in vitro* como herramienta para predecir la absorción en humanos.

Para cumplir tal objetivo estableció una correlación entre la permeabilidad *in vitro* y la fracción absorbida en humanos de 35 fármacos donde obtuvo un coeficiente de correlación de 0,90. Pero este coeficiente de correlación mejoró a un 0,95 al suprimir, con la ayuda de un inhibidor, el mecanismo de secreción debido a la glicoproteína-P en las células Caco-2. Este autor concluyó que las células Caco-2 son una buena herramienta, tanto predictiva como de cribado, siempre que las moléculas atraviesen las membranas absorbentes por transporte pasivo [125]. Además, esta conclusión está de acuerdo con las guías de la FDA donde solo se consideran apropiados los métodos *in vitro* y los modelos animales para fármacos transportados de forma pasiva [119].

Correlaciones fracción absorbida-parámetros de absorción *in situ*

Mediante los parámetros de absorción obtenidos con métodos *in situ* es posible predecir la fracción oral absorbida. Se muestra un ejemplo de este tipo de correlación en la Figura 1.1.22, donde se aprecia la relación existente entre las permeabilidades obtenidas *in situ* en intestino delgado de rata y las fracciones orales absorbidas (F_a) en la misma especie. Los puntos corresponden a la permeabilidad obtenida con el método de perfusión *in situ* de Doluisio [126] y los triángulos, a los valores obtenidos con la técnica del paso simple [127]. En este tipo de correlaciones (Figura 1.1.22) se alcanza una asíntota tanto si se absorbe completamente la dosis administrada como si no, ya que la fracción absorbida se calcula en relación a la cantidad máxima que se puede absorber. Los valores de la fracción absorbida más próximos a la unidad se asocian con los valores más altos de permeabilidad.

De este mismo modo también se puede correlacionar la fracción oral absorbida en humanos con la permeabilidad intestinal en rata obtenida mediante técnicas *in situ*. Por ejemplo, Zakeri-Milani y colaboradores [128] realizaron un estudio cuyo objetivo era obtener correlaciones fiables para predecir la permeabilidad intestinal y la fracción oral absorbida en humanos usando datos de permeabilidad intestinal en ratas. Seleccionaron 13

compuestos y calcularon el coeficiente de permeabilidad en rata mediante la técnica *in situ* de paso simple, los datos en humanos los obtuvieron de la literatura. Las correlaciones resultantes fueron altamente satisfactorias (R^2 > 0,90) y corroboraron de nuevo la condición establecida por la FDA de correlacionar únicamente datos de absorción pasiva para poder considerar que el modelo utilizado es útil para predecir la absorción intestinal. Estos resultados les permitieron concluir que la permeabilidad en rata puede usarse para predecir la absorción oral en humanos [128].

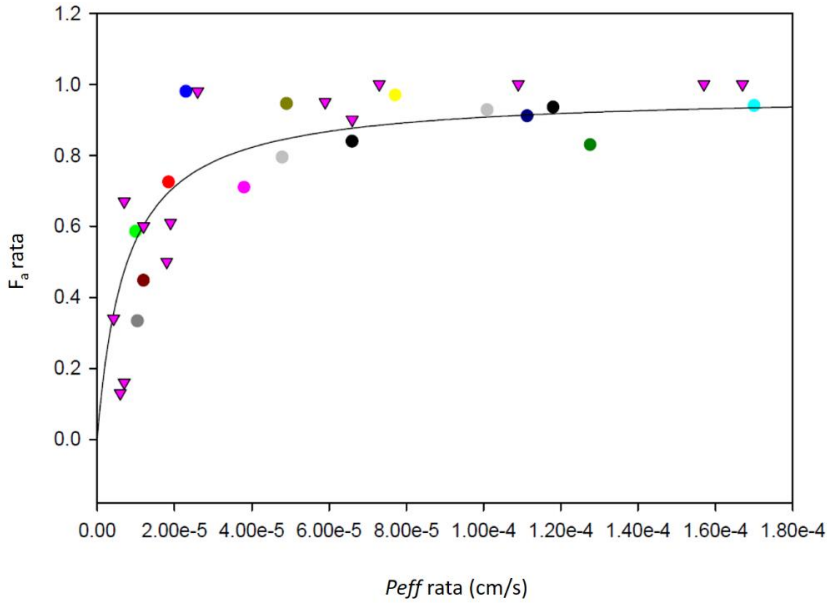

Figura 1.1.22. Correlación fracción absorbida- coeficiente de permeabilidad obtenido en rata mediante un método de perfusión *in situ* [126, 127].

Correlaciones biodisponibilidad-parámetros de absorción *in situ*

Estas correlaciones se utilizan con frecuencia para validar los métodos de absorción in situ ya que con ellas existe la posibilidad de estimar la biodisponibilidad *in vivo* en rata a partir de parámetros de absorción obtenidos con técnicas *in situ* en la misma especie animal.

Sánchez-Castaño y colaboradores [126] establecieron una correlación entre la constante de velocidad de absorción en rata, obtenida mediante el método *in situ* de Doluisio, y la biodisponibiliad en esta misma especie animal de una serie de siete quinolonas, calculada con una técnica *in vivo* basada en canular la vena yugular de rata, (Figura 1.1.23).

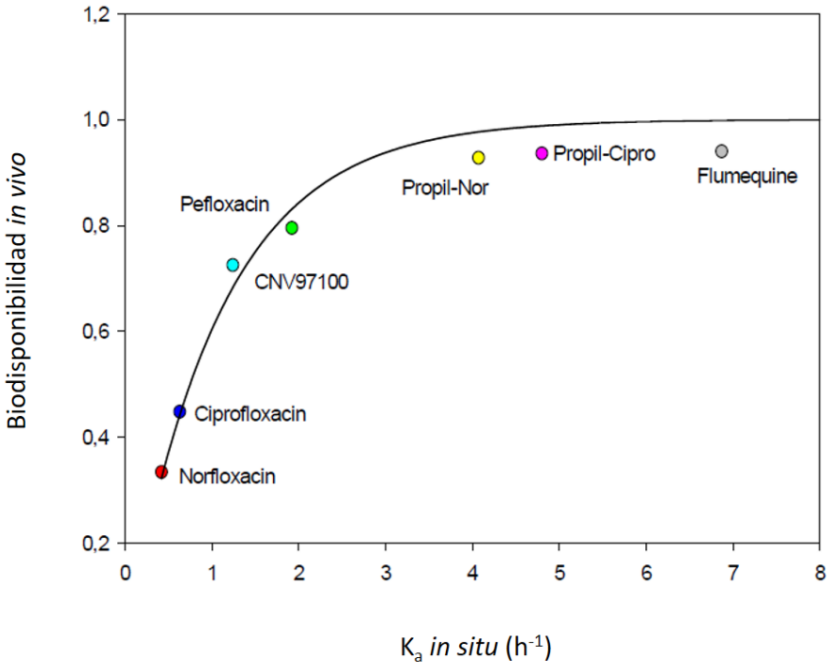

Figura 1.1.23. Correlación biodisponibilidad oral-*in situ* [126].

Con ayuda de esta correlación se estableció la siguiente ecuación para predecir la biodisponibilidad:

$$F = 1 - e^{-k_a T} \qquad (9)$$

donde F hace referencia a la biodisponibilidad, k_a es la constante de velocidad de absorción, y T representa el tiempo medio de absorción. La biodisponibilidad predicha con la ecuación anterior, usando la k_a intestinal obtenida en rata, resultó ser fiable cuando las moléculas se absorben por

difusión pasiva siempre que el efecto de primer paso sufrido por los compuestos, al igual que ocurre con las quinolonas, no sea significativo [126].

1.1.6. Formas farmacéuticas de liberación controlada de administración oral

Uno de los inconvenientes que pueden presentar las fórmulas convencionales, o de liberación inmediata, es la corta duración de la acción terapéutica. En un tratamiento crónico esta característica obliga a realizar una toma repetida de dosis a intervalos de tiempo reducidos; lo que puede provocar la falta de efecto farmacológico o la presencia de efectos adversos si las fluctuaciones en la concentración plasmática del principio activo no se mantienen dentro del margen terapéutico (Figura 1.1.24).

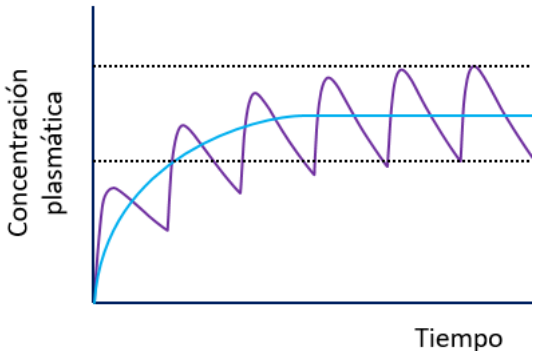

Figura 1.1.24. Perfil concentración plasmática-tiempo de una formulación de liberación inmediata (línea púrpura) y una formulación de liberación controlada (línea azul). La franja comprendida entre las dos líneas punteadas corresponde al margen terapéutico.

Con el objetivo de evitar, dentro de lo posible, los inconvenientes de las formulaciones convencionales, se desarrollaron las formas farmacéuticas de liberación modificada. Estos productos hacen referencia a aquellos sistemas de liberación de fármacos en los que se modifica y controla su velocidad de liberación y/o el lugar donde se liberan, de tal forma que se pueden alcanzar objetivos terapéuticos que no son posibles para las fórmulas

convencionales. La FDA adopta la terminología "sistemas de liberación controlada" (controlled release) para este tipo de formulaciones, mientras que la USP opta por nombrarlos "sistemas de liberación modificada" (modified release). En ambos casos se diferencian, en términos generales, las formulaciones o sistemas de liberación prolongada o sostenida los cuales permiten reducir la frecuencia de dosificación, porque están liberando el principio activo durante un largo periodo de tiempo; y las formulaciones de liberación retardada que no liberan el fármaco inmediatamente después de su administración [129]. En esta tesis doctoral se ha utilizado principalmente la terminología de la FDA.

El objetivo fundamental de las formas de liberación controlada es optimizar la seguridad y eficacia de los tratamientos, simplificando su posología. Para ello se debe alcanzar y mantener niveles plasmáticos eficaces en estado de equilibrio estacionario, con las mínimas fluctuaciones posibles e intervalos de dosificación de 12 o 24 horas.

Este tipo de formulaciones presenta una serie de ventajas que se resumen a continuación [129, 130]:

1. Para los fármacos con una semivida biológica corta se incrementa el intervalo de dosificación, lo que reduce la frecuencia de administración de la forma farmacéutica.

2. Se previenen los efectos adversos indeseables y los períodos de infradosificación asociados a las fluctuaciones de la concentración plasmática del fármaco, propias de las fórmulas convencionales y, particularmente importante en aquellos compuestos con un margen terapéutico estrecho.

3. Se reducen los efectos secundarios relacionados con las dosis elevadas. El concepto de liberación controlada implica que las formulaciones tengan un contenido elevado de principio activo, pero éste se liberará

lentamente, por lo que se evitan los efectos adversos de una liberación masiva.

4. Se consigue una respuesta farmacológica más uniforme al alcanzar una concentración plasmática estable dentro del margen terapéutico (Figura 1.1.24).

5. Todas las ventajas comentadas inciden directamente en un incremento de la adherencia terapéutica al tratamiento por parte del paciente. Este hecho es especialmente importante en aquellos pacientes con tratamiento crónico y polimedicados.

Para desarrollar una forma farmacéutica de liberación modificada es necesario no perder de vista tres aspectos fundamentales:

1. Las características físico-químicas, farmacocinéticas y farmacodinámicas del principio activo con el que se desea desarrollar la formulación de liberación controlada.

2. Las características de la forma farmacéutica diseñada y el modo de liberación del fármaco a partir de ella.

3. Las características anatómicas y fisiológicas del tracto gastrointestinal, principalmente las zonas distales, donde se producirá la mayor parte de la absorción. En este punto cobra especial importancia la permeabilidad que presente el principio activo en colon.

La formulación de un principio activo en forma de liberación controlada es un proceso para el que existen numerosos métodos y estrategias que dan lugar a una gran variedad de sistemas con características y mecanismos de liberación diferentes. Sin embargo, todos ellos presentan como objetivo común que la molécula de interés terapéutico alcance el colon tras su administración por vía oral, y así poder beneficiarse de las ventajas que suponen las fórmulas de liberación controlada.

No existe una clasificación única para los distintos tipos de sistemas de liberación controlada. Una de las maneras más esclarecedoras es establecer una clasificación según la estrategia en la que se basa el proceso de liberación del fármaco [57]. Las diferentes estrategias se fundamentan en el correcto aprovechamiento de las características fisiológicas que presenta el tracto gastrointestinal. Las características que más se explotan son las siguientes:

- Los cambios de pH existentes a lo largo del tracto gastrointestinal.

- El diferente tiempo de tránsito de las moléculas en cada tramo del sistema digestivo.

- La microflora característica del colon que confiere propiedades enzimáticas y metabólicas exclusivas a este segmento intestinal.

- La mayor presión intraluminal existente en el colon debido a los intensos movimientos peristálticos y a la mayor viscosidad del contenido de este tramo distal del tracto gastrointestinal.

- El fenómeno físico de la presión osmótica que ocurre cuando el agua atraviesa una membrana semipermeable. En este caso se debe evitar que el agua penetre en la fórmula farmacéutica durante su tránsito a lo largo de los segmentos superiores del tracto gastrointestinal, para conseguir que el principio activo alcance el colon sin ser liberado.

Hay ciertas ocasiones en las que el desarrollo de un sistema de liberación controlada se basa en varias estrategias. De esta manera, se consigue evitar los inconvenientes que puede presentar el uso de una estrategia de forma aislada, ya que las características fisiológicas pueden presentar variaciones entre individuos o verse modificadas en determinadas patologías [131].

Los métodos existentes para desarrollar un sistema de liberación controlada se abordan de una forma más exhaustiva en el review titulado

"Preclinical models for colonic absorption, application to controlled release formulation development". Este trabajo de revisión se muestra a continuación, ya que forma parte del desarrollo de la presente tesis doctoral. Asimismo, en dicho artículo de revisión, se incluyen otros aspectos relacionados con las formulaciones de cesión controlada como los métodos usados para evaluar la eficacia de estas formas farmacéuticas o los modelos disponibles para analizar la permeabilidad de un principio activo en colon.

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1.2. Artículo de revisión

Preclinical models for colonic absorption, application to controlled release formulation development

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ABSTRACT

The oral controlled release (CR) formulations have many benefits and have become a valuable resource for local and systemic administration of drugs. The most important characteristic of these pharmaceutical products is that the main drug absorption occurs in the colon. Therefore, this review analyses the physiological and physicochemical features that can affect a CR product administered orally, as well as the different strategies to develop a CR dosage form and the methods used to evaluate the formulation efficacy. The available models to study the intestinal permeability and their applicability to colonic permeability determinations are also discussed.

Keywords: controlled release formulations, colon, colonic permeability, drug absorption, oral route.

INTRODUCTION

The oral route is the most physiological to administer drugs and it has several advantages for the patients. Controlled Release (CR) formulations for oral route have gained importance during the last decades. A CR system is able to provide a therapeutic control, because it delivers the drug at a determined velocity and/or in a determined site, according to the needs that it wants to cover. This review is focused in oral CR formulations targeted to colon and in the drug colonic absorption, because the absorption in this segment of the gastrointestinal tract has a great relevance for oral CR formulations.

Colon as targeted absorption site

The colon is one of the sites where local and systemic delivery of drugs can take place. Both, local treatment and systemic, when targeted to the colonic region, can offer considerable therapeutic benefits to patients. Colonic drug delivery is interesting in the treatment of colonic diseases like

ulcerative colitis, Crohn's disease, colon cancer and local infections. Thereby, a direct treatment at the affected area will be ensured, so the patients need a lower dose which causes less systemic side effects [1, 2].

In addition to local therapy, the colon can also be used as a targeted window for introducing drugs into the systemic circulation in order to reduce gastric irritation or first pass metabolism of orally ingested drugs [3]. There are evidences that suggest a lower activity of cytochrome P450 3A (CYP3A) in colon than in small intestine, so a delivery of a CYP3A substrate in colon may be reflected in higher plasma levels and enhanced oral bioavailability [2, 4, 5]. Other drugs such as peptides and proteins are degraded in stomach and small intestine, therefore, oral delivery of this type of compounds is possible because colon provides a more suitable environment than the upper gastrointestinal tract [6, 7]. This approach is very interesting for drugs like insulin [8, 9].

Colon CR formulations can be employed when a delay or a modification in the absorption is required, despite being a drug with high permeability and good absorption through the small intestine. The CR products can be beneficial for drugs with short elimination half-life, side effects associated to peak plasma concentration and multiple daily doses[10, 11]. The objective of developing a CR formulation with this type of drugs is to achieve constant concentrations for prolonged periods of time; thus, it may be feasible to increase the effect duration, reach a once daily dose, avoid fluctuations of plasmatic concentration and reduce the adverse effects. Independently if the CR is for local or systemic therapy, the main goal of CR products is to improve the patient compliance and, therefore, the treatment efficiency [10-12].

Moreover, recently it has been demonstrated that the Biopharmaceutic Classification System (BCS) employed to classify the drugs according to their solubility and permeability in small intestine [13], can be applied to colon absorption [14]. In addition, several studies show that many

drugs have a significant absorption in colon, which is important in the development of CR formulations with systemic effects [14-18].

Anatomical and physiological characteristics of the colon

Traditionally the Immediate Release (IR) forms have been designed to release the drug in upper regions of gastrointestinal tract, because only the small intestine is structured to provide a maximum absorption, due to its higher available absorption surface and its higher transporter protein abundance [19]. An orally administered dosage form takes about 3 h to travel through the small intestine to the beginning of colon [3], and a CR formulation is designed to release the drug during 12-24 h [20]. Therefore, to achieve a successful CR product, it is necessary to take into account the specific characteristics of the colon, which will affect the design and development of the CR formulation; Table 1.2.1 shows a comparison of features between human colon and human small intestine [3, 12, 19, 21-30]. Moreover, the total time for transit along the colon tends to be highly variable and influenced by several factors such as diet, mobility, stress, diseases and drugs[3]; this fact can also affect the CR products final effect.

The principal role of the colon is to confer a mechanism for the orderly disposal of waste products of digestion [31]. The colon is specialized in keeping the electrolytes and water balance, absorbing Na^+, Cl^- ions and H_2O, and secreting of HCO_3^- and K^+ [32]. The most important characteristic of colon is its high transit time, longer than 24 h; whereas the small intestine transit time is shorter, about 2-5 h [24, 25]. This high transit time allows the drugs to be in contact with colon mucosa for a longer period than in small intestine which compensates the lower surface of colon for absorption [2].

Table 1.2.1. Human small intestine and human colon characteristics [3, 12, 19, 21-30].

		Small intestine	Colon
Length (m)		7	1.5
Absorption surface area (m^2)		120	0.3
Absorption surface provided by:		Folds, villi and microvilli	Folds and microvilli
Transit time (h)		2-5	~ 24
pH		6.0-7.0 (duodenum), 6.0-7.7 (jejunum), 6.5-8.0 (ileum)	5.5-7.5 (ascending colon), 7.0-8.0 (descending colon)
Water volume (mL) (fasting conditions)		105	13 (higher water absorption: contents more viscous)
Microorganism (Organism/g)		10^2 (duodenum), 10^5 (jejunum), 10^7 (ileum)	$10^{11} – 10^{12}$ (important role in the gut immune system)
Enzymes[a]		CYP3A family	Enzymes secreted by colonic microflora
Absorption pathway	**Passive**	Transcellular, paracellular	Transcellular
	Active[b]	PEPT, MRP2, P-gp	MRP3, MRP2, OCTs

[a] The most important enzymes in small intestine and colon.

[b] The most important transporter protein in small intestine and colon according to Drozdzik et al.[19]

CYP3A, cytochrome P450; PEPT, peptide transporter protein, MRP, multidrug-resistance-associated protein; P-gp, P-glycoprotein; OCT, organic cation transporter.

Factors affecting drug absorption

As it has been mentioned, drug absorption through the colon membrane is very important for CR formulations. All the anatomical and

physiological characteristics of the colon can influence the drug absorption and they should be taken into account during the CR formulation development. However, there are more factors to consider than biological features: the physicochemical properties of drug.

1. Drug lipophilicity (distribution coefficient):

The lipophilicity can be measured with the distribution coefficient: with a higher distribution coefficient, the drug will be more lipophilic. The passive diffusion is the main pathway for absorption of drugs administered by oral route, the compounds go through the lipid membrane. Therefore, the more lipophilic drugs have higher permeability. For example, ibuprofen and carbamazepine, with a distribution coefficient of 3.97 and 2.45 respectively, are more lipophilicity drugs than ranitidine and amoxicillin, with a distribution coefficient of 0.27 and 0.87 respectively.

2. Aqueous drug solubility:

The dissolution of a hydrophobic drug is the limiting factor for its absorption through the intestinal membrane, because the intestinal fluids are aqueous. However, for hydrophilic drugs, the rate of drug permeation across the biological membrane is the determining factor for the absorption.

3. pKa (dissociation constant):

The relationship between the drug pKa and the intestinal pH determines the ionization degree of a molecule, only the unionized forms of a drug can be absorbed. The unionized fraction *(f)* can be calculated according to the Henderson-Hasselbach equation: $f=1/1+10^{(pKa-pH)}$ for basic drugs, and $f=1/1+10^{(pH-pKa)}$ for acid drugs. For example, atenolol (pKa = 9.6), a low permeability drug, has a low unionized fraction at pH 7 (f=0.25%). However, cimetidine (pKa = 6.8) has a bigger unionized fraction at pH 7 (f=61%), so its permeability is higher than atenolol permeability at pH 7. The

f is not the only parameter that determines the drug absorption, but it provides useful information.

4. Particle size and effective surface area:

Particle size and surface area are inversely proportional; a smaller particle size provides a greater surface area. So, the solid particles which have a big surface area, have higher dissolution rate.

5. Polymorphism:

Polymorphism is the ability of a solid material to exist in more than one form or crystal structure. They differ from each other in their physical properties like solubility, melting point, density, etc. Meta stable forms have higher aqueous solubility and better bioavailability than stable forms.

6. Drug stability:

If the drug suffers degradation into inactive form the bioavailability will be lower. Moreover, interactions between excipients or gastrointestinal contents can be other reasons for poor absorption.

METHODS FOR CR FORMULATIONS DEVELOPMENT

There are different techniques to develop a CR formulation. All of them are included into one of the five basic strategies described below:

pH-dependent strategy

One of the more used approaches to achieve colon release is to take advantage of the differences in the pH values throughout the gastrointestinal tract. Coating the drug with polymers with different solubility at different pH environment is the method employed to develop these systems. Nevertheless, to use this strategy is important to consider that the pH in the

colon can vary and depends on many factors such as diet, food intake, motility and disease state [3].

Eudragit is one of the most employed polymers to develop pH dependent CR formulations [33]. Bussemer et al. [34] demonstrated that Eudragit FS is more appropriated for drug delivery into the ileocolonic part. pH-dependent systems are commercially available for the treatment of ulcerative colitis, as Asacol® and Salofalk®, and for Crohn's disease treatment, as Entrocort® [3, 35].

Time-dependent strategy

The site of drug release is decided by the transit time of a formulation in the gastrointestinal tract. The aim of this strategy is to avoid the release to 3-5 hours after the CR product reaches the small intestine. However, the gastric emptying can be different between patients and some colon diseases can affect the transit time in colon [3, 36, 37].

For example, Hoffman-La Roche Inc. patented a tablet formulation according to a time-dependent strategy. This tablet releases the drug in the colonic region by a time-dependent explosion mechanism, the release happens after a consistent period of 4-6 h [38]. Another example of time-dependent strategy is the hydrophilic sandwich capsule. It consists of a capsule inside another capsule. In the inter-capsular space between them, there is a layer with a hydrophilic polymer [35].

Bacteria-dependent strategy

The basis of this strategy is the exploitation of the specific enzymatic activity of the microflora only present in the colon, or the change in the redox potential due to this microflora. The method most used in this strategy is the formation of prodrugs which becomes active with the metabolic processes of the colonic bacteria [3, 33].

The earliest example of bacteria-dependent strategy CR formulation is the drug sulfasalazine (Salazopyrin®) employed for inflammatory bowel disease and rheumatoid arthritis treatment. The diazoreductase bacteria from colon cleaves the drug into 5-amino salicylic acid and sulfapyridine [2].

Pressure-dependent strategy

This strategy takes advantage of the higher luminal pressure in colon resulting from peristaltic motion. This feature is attributed to the higher viscosity of luminal contents [39].

Takaya et al. [40] developed some CR products based on the pressure-dependent strategy. The system employed in these products is the same: a capsule coated with ethylcellulose (water-insoluble). The release of the active pharmaceutical ingredient happens after the disintegration of the ethylcellulose capsule due to the pressure in the colonic lumen [41].

Osmotically-dependent strategy

This method uses the physical phenomena of osmotic pressure. The osmotic pressure controlled systems, or osmotic pumps, reach the colon intact and there the drug release takes place due to the osmotic pressure generated by the entry of the water throughout a semi-permeable membrane [3, 33, 42]. The osmotic pumps are usually coated with a polymer layer to avoid the pass of water in the upper parts of the gastrointestinal tract.

Alza corporation developed OROS®-CT, based on the osmotically-dependent strategy. The main characteristic of this system is that it has about 5 push-pull units in a hard gelatine capsule. Alza corporation, also developed a miniature osmotic pump (Osmet™) with the same mechanism that OROS®-CT system [35]. A schematic representation of a push-pull unit can be observed in the osmotically-dependent strategy section of Table 1.2.2.

Table 1.2.2 shows a summary classification of some methods and different strategies and technologies employed to develop the CR products, as well as, some schematic figures.

Examples shown in this table are only the simplest methods for each strategy, but there are more options. For example, a multiparticulate system can be performed with different particles coated with polymers sensitive to different pHs [37, 43-45]. Thus, release at different gastrointestinal segments can be achieved with the oral administration of a unique formulation. It deserves to be mentioned that the time-dependent strategy is employed to develop delayed dosage forms. In addition, with the aid of an enteric coating, it is possible to obtain gastro-resistance products that avoid the drug degradation by the acid gastric pH.

Moreover, more recently have been designed systems that combine different strategies to develop a CR product. Thereby, the disadvantages of one strategy can be avoided and more secured systems will be obtained. For example, the Pulsincap® system is a pulsatile drug delivery system which mix pH and time-dependent strategies; it is composed of an impermeable capsule and a hydrogel polymer plug [46-48]. The Port® system combines osmotic and time dependent strategies, it consists of a hard gelatine capsule, a semipermeable membrane and an osmotic agent [35, 49, 50]. CODES™ is another combined system with pH and microflora- dependent strategies, it has polysaccharides that are only degraded by colonic bacteria and a layer of pH-sensitive polymer [3, 33, 35]. Regardless of the method or strategy used, the lag time can be controlled with the thickness of the layer employed to coat the drug.

Table 1.2.2. Summary of methods and systems employed to CR development according to the different strategies.

Strategy	Methods/Systems employed	References
pH-dependent strategy	- Coating with pH dependent polymers (single layered or multilayer products). - Embedded in pH sensitive matrices. - Based on pH sensitive hydrogel.	[3, 33, 37, 51, 52]
Time-dependent strategy	- Time-dependent mechanism by solubilization/ erosion of a polymeric membrane (i.e. Time Clock® system). - Drug embedded in a matrix. It can be a hydrophilic matrix that progressively dissolves forming a gel-layer, through which the drug diffuses. Or, it can be a non-erodible matrix where the drug dissolves and leaves the matrix slowly. - Repetabs: the dose in the coating is immediately release, but the core release the drug several hour later.	[3, 7, 33, 53-55]

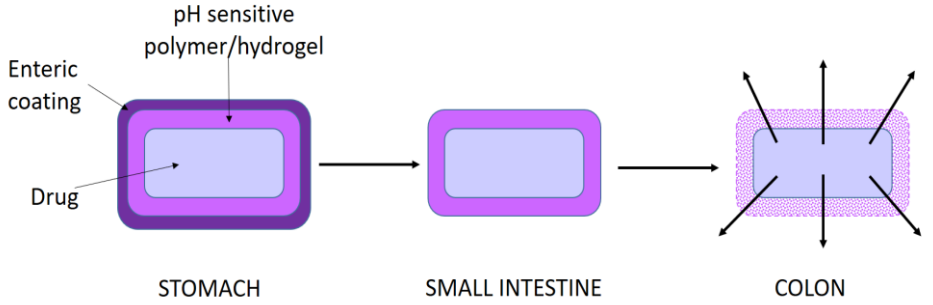


Table 1.2.2. Summary of methods and systems employed to CR development according to the different strategies (continued).

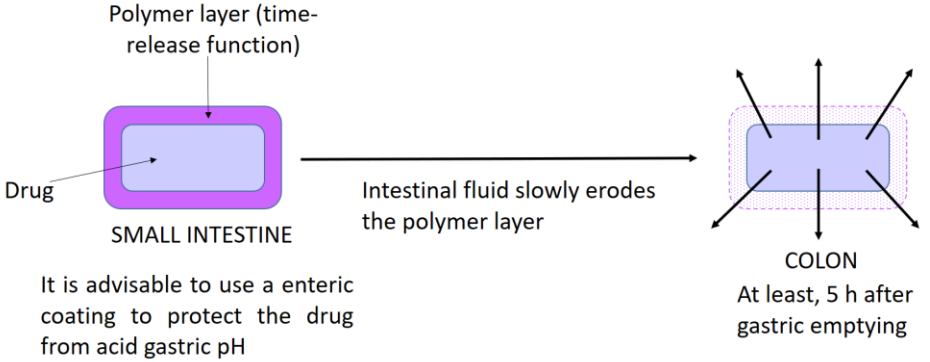
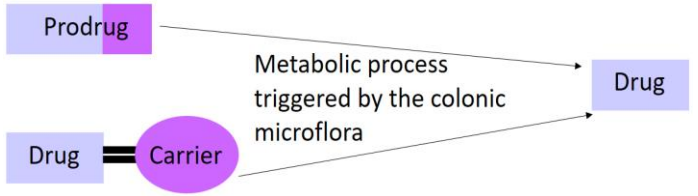
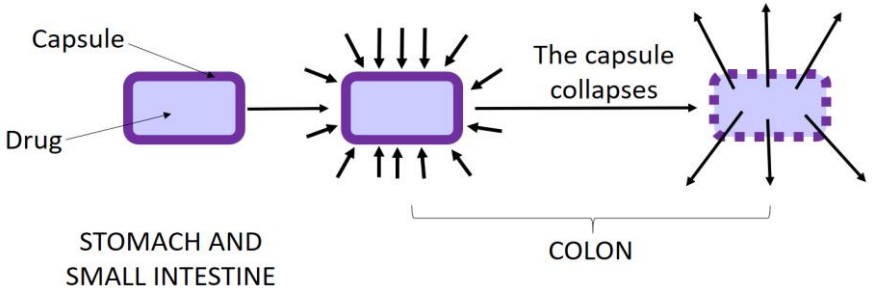
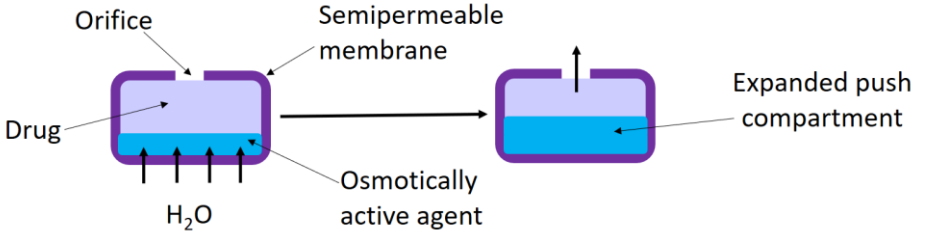
Time-dependent strategy		
	- Biodegradable polymer/matrix. - Prodrugs (such as azo conjugates or polymeric prodrugs). - Redox sensitive polymer coating. - Covalent linkage with a carrier (such as cyclodextrin or amino acids).	[3, 33, 37, 52, 56]
Microflora-dependent strategy		

Table 1.2.2. Summary of methods and systems employed to CR development according to the different strategies (continued).

Pressure-dependent strategy	- Capsule rupture by colonic luminal pressure. - Capsule with shell of ethyl cellulosa.	[3, 37, 39-41]
		
Osmotically-dependent strategy	- Osmotic controlled delivery: OROS-CT (it can be a single unit or 5-6 push pull units) - Osmotic pump with non-expanding second chamber: controlled porosity osmotic pump, osmoting bursting osmotic pump, liquid OROS	[3, 37, 42]
		

METHODS USED TO EVALUATE THE FORMULATION TARGETING EFFICACY

The methods employed to evaluate the release efficacy of a CR formulation cannot be the same employed with IR formulations because their different designs give them different characteristics but in most cases, they have adapted from them since they are the most known and studied. Next, the methods used to evaluate the CR formulations efficacy are described.

In vitro methods

For *in vitro* methods, there is not a standardized evaluation technique available to evaluate a controlled release product. The ideal *in vitro* technique should be able to distinguish the effects of the upper gastrointestinal tract on the CR formulation and, to mimic the *in vivo* colonic characteristics.

1. Conventional dissolution methods

The conventional basket method can be employed at different pHs and different periods of time to reproduce the gastrointestinal tract. Three different media are used with pH 1.2, 6.8 and 7.4, respectively, to simulate the gastric, intestinal and colonic fluids. The experimental time in the different media is modified according to the transit time through the stomach and the intestine. The colonic media usually has enzymes depending on the method or technique employed to develop the delivery system [2, 49, 57-60]. Biorelevant media to reproduce gastric and small intestinal conditions already exists. Moreover, nowadays, there are companies developing new biorelevant media in order to reproduce the colonic conditions under fasted or fed situations, with the aid of tris/maleate buffer [61].

However, in the available conventional dissolution methods, the volume, the composition of the dissolution media and the viscosity are not completely representative of the colon physiological characteristics.

2. Animal caecal contents

Animal caecal contents can be utilized as alternative dissolution media in order to avoid the limitations of the conventional dissolution methods. According to existent studies in the literature, the animal most employed is the rat due to the similarity between the human and rat colonic microflora and, its easy handle and availability [62, 63].

The rat caecal contents are diluted in phosphate buffer at pH 7.0. The complete experimental procedure is carried out under anaerobic conditions, with CO_2 or nitrogen atmosphere, in sealed glass vessels, at 37 °C during at defined period of time [57].

3. Human faecal slurries

The dissolution using human faecal slurries is another alternative dissolution test. It is more useful for CR formulations activated with colon microflora. In this case, the faecal contents are obtained from healthy human volunteers, and the dissolution media is prepared by homogenizing the fresh faeces in sodium phosphate buffer under anaerobic conditions [57, 64, 65].

4. Dynamic multi-compartment systems

These systems have been developed to simulate the *in vivo* drug dissolution as similar as possible to the physiological processes involved during the gastrointestinal passage [66-68]. For example, the research organisation TNO patented the *in vitro* gastrointestinal model TIM-1 with four compartments: stomach, duodenum, jejunum and ileum [69]. Kobayashi et al., Sugawara et al. and Gu et al., also developed systems with similar

characteristics, the three devices have three different compartments: for stomach, for small intestine and to simulate the absorption process [70-72].

But, the dynamic systems mentioned above, do not include the colon. Four systems with compartments to simulate the colon are described below.

4.1. Three-stage compound culture system [57, 73, 74]

This system was developed in 1988 to solve the inaccessibility of the ascending colon. It is a modular fermenter and it was designed to reproduce the spatial, nutritional, temporal and microflora characteristics in the different colon segments. The three-stage compound system has three glass fermentation vessels. The working volume for the vessel 1 is 200 mL, the pH is 5.5 and it simulates the proximal colon; the volume for the vessel 2 is also 200 mL, the pH is 6.2 and it reproduces the transverse colon; and, the volume for the third vessel is 280 mL, the pH is 6.8 and it represents the distal colon. All the vessels are under magnetic stirring and kept at 37 °C and in an anaerobic atmosphere. Moreover, each fermentation vessel is inoculated with a preparation of freshly faecal slurries from healthy human volunteers.

4.2. SHIME [57, 75]

With a similar objective to the three-stage compound culture system, a 5-step multi-chamber reactor was developed in 1993 by Molly et al. [75]. The purpose of this model is to simulate the gastrointestinal microbial ecosystem of humans (small and large intestine). This model has five different vessels: the vessel 1 is to simulate duodenum and jejunum, the vessel 2, for ileum; the vessel 3 simulates caecum and the ascending colon; the vessel 4 correspond to the transverse colon; and, the vessel 5 is for the descending colon. Vessels 1 and 2, corresponding to the small intestine, are inoculated with the supernatant of a human diet suspension. On the other hand, vessels 3, 4 and 5, corresponding to the large intestine, are inoculated with a faecal suspension. Figure 1.2.1 shows the scheme of the SHIME model.

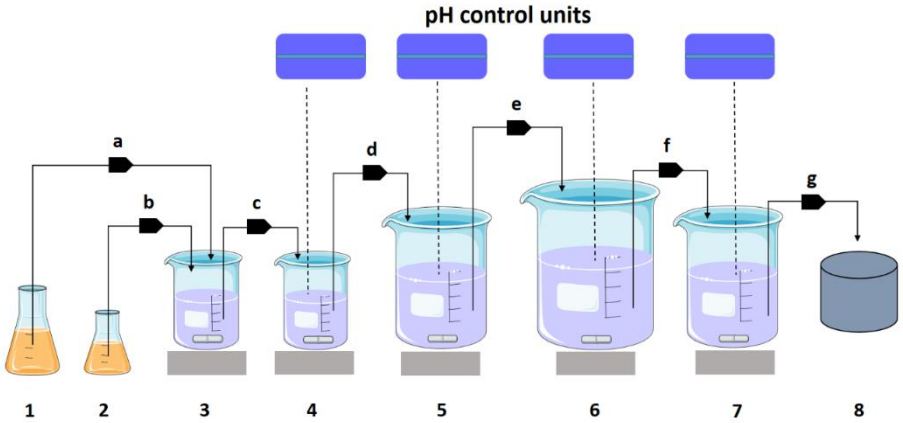


Figure 1.2.1. Schematic representation of the SHIME model. 1, food; 2, pancreatic solution; 3, duodenum and jejunum vessel; 4, ileum vessel; 5, caecum and ascending colon vessel; 6, transverse colon vessel; 7, descending colon vessel; 8, waste collection; a-g, pumps to transfer the contents.

4.3. TIM-2 [76, 77]

After patenting TIM-1, TNO patented the *in vitro* model of the colon (TIM-2) developed in 1999 by Minekus et al. [76]. TIM-2 has four interconnected glass compartments with a flexible membrane inside. This membrane simulates the peristaltic waves of the large intestine which produces the mixing and the movement of the luminal contents; therefore, TIM-2 mimics better than previous models the physiological conditions in the large intestine. The system is incubated with faecal slurries from healthy volunteers and it is kept under anaerobic conditions. Similar to TIM-1, TIM-2 is also a computer-controlled model, so it is highly reproducible and it is able to predict results in the proximal colon, segment that cannot be measured in a clinical trial. Moreover, TIM-2 can be use isolated or in combination with TIM-1.

4.4. Multi-reactor gastrointestinal model [78]

Recently, a multi-reactor gastrointestinal model, consisting of five consecutive reactors, has been developed by Sadeghi-Ekbatan et al. [78]. The five reactors simulate the stomach, small intestine, ascending colon, transverse colon and descending colon. These reactors are interconnected by plastic tubing and peristaltic pumps. The whole system is connected to a computer that automatically controls the food added to the stomach reactor, the pH, the temperature and the flow of intestinal content between reactors. As the systems described before, the anaerobic atmosphere is maintained with nitrogen, so colonic microflora can be added to study their effect on the CR formulation. Figure 1.2.2 shows a schema of the model developed by Sadeghi-Ekbatan et al. [78].

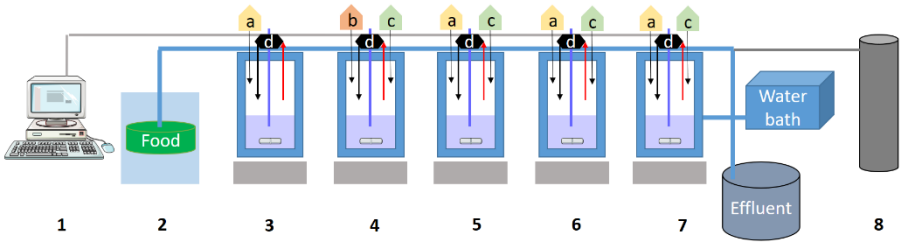


Figure 1.2.2. Representation of the multi-reactor gastrointestinal model. 1, data acquisition and control software; 2, refrigerator; 3, stomach reactor; 4, small intestine reactor; 5, ascending colon reactor; 6, transverse colon reactor; 7, descending colon reactor; 8, nitrogen cylinder; a, acid; b, pancreatic solution; c, base; d, peristaltic pump.

***In vivo* methods**

When the results obtained with *in vitro* test are positive, the next step to evaluate a CR product is to study its pharmacokinetic profile. It can be studied with *in vivo* methods in animals and/or humans, both options are explained next.

1. Animal studies

Different animals can be used to evaluate the drug release of a CR formulation, such as rats, guinea pigs, dogs or pigs because of their similarity with the physiology of the human gastrointestinal tract [49].

The choice of the correct animal model will depend on the system or technique employed to develop the CR product. For example, the distribution of some enzymes is similar in rat, rabbit and guinea pig to those in human. In the case of the rat, its colonic microflora is similar to human colonic microflora [79]. Dogs can be used as well, but the results cannot be extrapolated to humans due to the differences between the gastrointestinal anatomy [80].

The experimental procedure consists in collecting blood samples from the jugular vein after an oral administration of the studied formulation. The samples are taken during a period of time long enough to cover the complete release of the drug from the pharmaceutical form [81, 82]. It is important not to forget that the data obtained from animal models should be interpreted with caution and that it cannot always be extrapolated to humans.

2. Human studies

2.1. Pharmacokinetic studies

In vivo experiments similar to those explained for animals can be performed in humans. Blood samples are collected over a period at least of 24 h, after an oral CR drug administration. The data can be used to calculate pharmacokinetic parameters [7, 60, 82]. A pharmacokinetic parameter calculated after administering a colonic product is the Drug Delivery Index (DDI), which relates the relative colonic tissue exposure to the drug with the relative amount of drug in blood. The colon drug delivery will be better with a higher DDI [7].

2.2. Imaging techniques

Sometimes, the pharmacokinetic studies do not generate enough information of a colon drug delivery system. In these cases, the combination of the pharmacokinetic studies with the imaging techniques can generate more useful data. The imaging techniques allow to follow the CR formulation through the gastrointestinal human tract under physiological conditions in a non-invasive manner, as gamma-scitingraphy and roentgenography thecniques or the Magnetic Marker Monitoring.

2.2.1. Gamma-scintigraphy [49, 83, 84]

This technique was first employed in 1976 by Alpsten et al. [85] and Casey et al. [83] and became a technique extensively used to monitor new drugs. A dosage form is labelled with a gamma-emitting radioisotope, usually technetium-99m-DTPA, which allows monitoring the intraluminal location as a function of time, the colon arrival time and the integrity of the labelled dosage form through the gastrointestinal tract. The gastrointestinal transit is recorded by a gamma-camera and the data are collected in a computer for the subsequent analysis.

2.2.2. Roentgenography [49]

This strategy was applied to *in vivo* evaluation of drugs, the first time in 1965 by Steinberg et al. [86]. The roentgenography technique implies that a radio opaque material, like barium sulfate, was incorporated to the dosage form. The CR formulation can be visualized taking X-rays of abdomen after oral administration. With the roentgenography technique, the location, movement and the integrity of the dosage form can be observed.

2.2.3. Magnetic Marker Monitoring (MMM) [87, 88]

MMM was developed in 1990 by Weitschies et al. [89] as an alternative technique to avoid the disadvantages of the gamma scintigraphy and the

roentgenography for the volunteers and the investigators, because both are techniques that require essential precautions and regulatory issues. The MMM is based on the detection of magnetically labelled dosage forms. Ferromagnetic particles are incorporated to the CR formulation and then, the formulation is marked as a permanent magnetic dipole. The MMM allows obtaining a more detailed insight than gamma-scintigraphy and roentgenography of the dosage form into the gastrointestinal tract.

PERMEATION MODELS

Before developing a CR formulation, it is essential to have information about the permeability of the active pharmaceutical ingredient in colon, because this segment will be the main site of absorption for a CR product. It is advisable that the drug has a high permeability to achieve a correct controlled release. Therefore, after knowing the permeability data, should be assessed if the drug has a permeability high enough to administer it as a CR formulation or, on the contrary, it is necessary to improve the permeability value of the compound [90]. When the permeability is suitable, the system employed to develop the CR formulation has to be chosen according to the drug features, the type of release sought (i.e. extended, delayed, repeated) and the desired release site: colon or small intestine and colon.

The models available to analyse the permeability of an active pharmaceutical ingredient in colon are described below.

In silico models

The experimental models need a certain amount of synthetized drug to perform the study. However, to save time and money, during the stage of development of a new drug it is necessary a screening of the promising molecules. This screening is done taking into account the prediction of some characteristics of the compounds before their synthesis. The prediction is

carried out with computationally models, also known as *in silico* models. Two main approaches can be differentiated in *in silico* models: QSAR and PBPK models.

1. QSAR (Quantitative Structure-Activity Relationship)

QSARs are mathematical models that relate the chemical structure of a molecule with its physicochemical properties or biological activity. Therefore, they are useful to predict the properties and/or activity of new molecules when their chemical structure is known. Moreover, these quantitative relationships also can be used to predict pharmacokinetics properties in the early stages of drug development [91, 92]. Several QSAR models have been proposed to predict oral absorption, they base on the *in vivo* human intestinal absorption, *in vivo* absorption rate constants and *in vivo* jejunal permeability [93-95].

Until now, there are not a useful QSAR model to predict absorption in colon, so this mathematical approach cannot be use with certainty to develop CR formulations.

2. PBPK (Physiologically-Based Pharmacokinetic) models

PBPK are mechanistic models that are built based on the anatomical structure of the organism and they take *in vitro* dissolution and *in silico* data as inputs to simulate the *in vivo* behaviour of a drug administered orally [96-98]. These models are useful in the early part of the discovery and development of drugs. Their focus is to predict the amount of compound absorbed as a function of time [96]. In a PBPK model the organs are represented by compartments, interconnected by the blood circulation. There are several *in silico* software to build PBPK models, for example: GastroPlus®, the Simcyp simulator, PK-Sim®, MATLAB® and STELLA® [96, 98].

Despite that the software packages have one or several compartments for the colon, the colonic absorption and physiology are less defined than those in small intestine; thus, there are important gaps for colonic appropriate descriptors. An improved colon model would increase the accuracy in the predictions of the oral fraction absorbed and plasma profile. Moreover, a better human permeability database for drugs with different absorption properties would be useful to achieve more rational development of CR dosage forms [23, 99].

***In vitro* models**

The *in vitro* assays are experiments performed in a controlled environment outside a living organism. The conditions of an *in vitro* model not always correspond with *in vivo* conditions, but the *in vitro* methods have replaced some *in vivo* assays during the screening of compounds. The most important and accepted *in vitro* models employed to characterize the drug permeability are the artificial membranes, the cell models and the tissue based models.

1. Parallel Artificial Membrane Permeation Assay (PAMPA)

The PAMPA method, developed by Pion-Inc company [100], has two aqueous compartments separated by a filter coated with phospholipids to mimic the passive transport of small molecules [101]. But, this method is only useful to predict the transcellular transport and not for the paracellular one. The main advantages are the low cost and the speed of the experiment procedure, so it is possible to test multiple compounds in a short period of time [102].

PAMPA permeability values have been correlated with permeability obtained with the cell model Caco-2 and, also, with the rat small intestine permeability data. The results obtained with these correlations indicated that PAMPA technique is useful to predict the small intestine absorption

[102]. The permeability in colon might be estimated with PAMPA adjusting the pH of the solution tested to the colonic pH (7.5) [103]; however, there are not published studies to show if PAMPA is a good method to predict absorption in colon.

2. Cell models

The *in vitro* methods using Caco-2 monolayers have demonstrated to be a valid model to predict the intestinal absorption of drugs [104]. The Caco-2 line was obtained from human epithelial colorectal adenocarcinoma and its cells show to be able to differentiate themselves in cells very similar to the enterocytes [105]. Caco-2 cells have apical and basolateral sides, in addition to the typical enzymes from the enterocytes, tight junctions, ATPase activity and specialized mechanism of transport. Therefore, they are useful to study the passive and specialized mechanism of drug absorption and the drug metabolism [106, 107]. Moreover, with the use of Caco-2 it is possible to reduce the animals employed in the early stages of a drug development.

Hence, the Caco-2 monolayers is an *in vitro* model widely accepted to predict the small intestine absorption in animals and humans [104, 108-111]. However, the utility of Caco-2 cells to predict the colon absorption is not as studied as the small intestine absorption, therefore, there is few information in the scientific literature to predict colon absorption with Caco-2. Rubas et al. [112] compared the Caco-2 permeability with permeability values from colonic human tissue of seven compounds, the relationship between both sets of data was good, but the number of drugs employed was small to obtain a robust conclusion. Recently, Lozoya-Agullo et al. [14] correlated the permeability value of rat colon with Caco-2 permeability data of fifteen drugs with different properties, the correlation obtained indicates a good relationship between rat colon permeability and Caco-2 permeability. These two commented studies suggest that Caco-2 monolayers can be employed as an *in vitro* model to predict the permeability in colon of a molecule. Therefore, the Caco-2 cells could be useful to the early stages of a CR

development; but more research is needed to considerer Caco-2 monolayer as a valid model to predict colonic absorption.

3. Tissue based models

These models use intestinal segments or intestinal tissue fragments to study the intestinal drug absorption, they also can be named *ex vivo* models. The *ex vivo* models have an important advantage: the characteristics of the *in vivo* epithelial membrane are maintained during the experimental procedure. Moreover, the permeability can be study in different intestinal segments, including the colon. There are two different techniques to study the intestinal absorption inside the tissue based models: everted gut sacs and diffusion chambers.

3.1. Everted gut sacs

This technique employs animal intestinal segments to perform the absorption studies. The intestinal segment has to be inverted and incubated in an oxygenated buffer with the drug dissolved, at temperature of 37 °C [113]. This system is useful to study the intestinal absorption and to discriminate between passive or carrier-mediated transport [114].

The everted sacs can be employed with different intestinal segments: duodenum, jejunum, ileum and colon. In fact, there are several studies published where different drugs with different mechanism of absorption are analysed using the *in vitro* everted gut sac technique. These studies are performed in different segments of the gastrointestinal tract, colon included, and the results show that everted gut sac is a useful technique to predict the permeability in colon [115-120].

3.2. Diffusion chambers

The most used and recognized diffusion chamber to study the intestinal permeability is the Ussing chamber [121]. To use this method, the

intestinal segment has to be isolated and divided in fragments to achieve plain layers, which are mounted in the Ussing chamber with a buffer continuously oxygenated and maintained at 37 °C for the whole experimental process. This method allows to evaluate the passive transport and the carrier-mediated mechanism.

The Ussing chamber has been widely used to measure the permeability of drugs in animal intestinal tissue from different gastrointestinal segments: duodenum, jejunum, ileum and colon [18, 122, 123]. Moreover, the permeability values obtained with the Ussing chamber are useful to predict the human absorption in small intestine [123, 124].

In addition, this technique can be employed with human intestinal tissue. Human intestinal sections are collected immediately after surgical resection from different patients [125, 126]. Besides, in the last years the Ussing chamber has been used with human colon tissue, obtaining promising results [127, 128]. This fact may suppose an advancement to analyse the permeability of a candidate drug to CR development.

***In situ* models**

The *in situ* studies, or perfusion studies, are employed mainly in animals, although similar techniques used in humans will be described later. The organ perfused needs to be isolated from the nearby tissues, but it must not be removed from the living organism. These studies are performed with animals under anesthetized conditions. The *in situ* techniques keep the neural, endocrine, blood and lymphatic contributions intact, which confers characteristics similar to those *in vivo*. Therefore, these models are sensitive to pharmacological and physiological modifications; hence, they can detect more variability in the absorption parameters than *in vitro* models [129].

In order to study the intestinal absorption of drugs, the Single Pass Intestinal Perfusion (SPIP) and the closed-loop technique, based on the

Doluisio's method, are the most employed *in situ* methods to study the intestinal permeability in rats. Both techniques measure the disappearance of the drug from the intestinal lumen.

1. Single Pass Intestinal Perfusion (SPIP)

The studied intestinal segment is perfused at a constant flow rate during all the experiment and the sample collection is done under steady state conditions. The SPIP is established as a valid model to predict the human permeability, several studies show good correlations between rat jejunum permeability obtained with SPIP and human jejunum permeability, also, good correlations between rat permeability determined with SPIP and human oral fraction absorbed have been published [23, 124, 130-133].

However, the studies to measure the rat colon permeability using the SPIP method are limited as few drugs have been studied with this technique [134-138]. The results of these studies suggest that SPIP might be a good technique to determine rat colon permeability but, until now, a systematic assay with drugs of different properties does not exist.

2. Closed-loop (Doluisio's method)

The closed-loop technique employed for intestinal absorption studies is based on the method developed by Doluisio et al. [139]. In contrast to the SPIP technique, in the Doluisio's method, the drug solution remains inside the intestinal segment throughout the absorption experiment, and the sample collection starts at five minutes after introducing the solution in the intestinal segment. Recently, it has been shown that permeability obtained with Doluisio's method correlated perfectly with permeability obtained with SPIP, so Doluisio's method is as valid as SPIP to predict human intestinal absorption [140, 141].

Moreover, Lozoya-Agullo et al. [14] have validated an *in situ* perfusion model, based on Doluisio's method, to study the colonic absorption in rat. Therefore, the closed-loop method can be used to analyse the colon permeability of a possible candidate for CR formulation.

***In vivo* models**

The *in vivo* assays are performed with a complete alive organism, so the physiological conditions are guaranteed for these studies.

1. Animal models

The *in vivo* methods in animals, mainly in rat, allow to study the bioavailability and the pharmacokinetics of a pharmaceutical dosage after oral administration. Plasmatic samples are taken by a catheter implanted in the jugular vein at pre-established times; therefore, the drug that goes through the intestinal permeable membrane and that does not have first-pass metabolism, will appear in systemic circulation [142]. Thus, these assays can be useful to analyse the pharmacokinetic profile of an oral CR formulation [143-145].

2. Human studies

The human studies give more information than the other models explained; but, obviously, they are more complicated and have several ethical problems. Therefore, the studies in human are suitable only for the final stages of the drug development. Next, the different studies in human are classifies into two groups (indirect and direct method) and they are briefly described.

2.1. Indirect methods

It is necessary to consider that for the indirect methods the analytical procedure of the samples has to differentiate between the drug and its metabolites.

2.1.1. Mass balance studies

It is a pharmacokinetic study performed with a radiolabelled drug. The assay is done in healthy volunteers, the drug is administered orally and blood, urine and faeces samples are taken to measure the drug in all the sample recollected [146]. This technique can be useful to study the pharmacokinetic profile of a CR formulation.

2.1.2. Absolute bioavailability studies

Blood samples or urine samples are taken after drug administration by oral route and by intravenous (IV) route. These data are compared to obtain drug distribution and elimination information. Moreover, the intestinal permeability can be estimated indirectly. In addition, the absolute bioavailability of the drug administrated orally can be determinate as AUC_{oral} / AUC_{IV} [147]. As the mass balance studies, the absolute bioavailability studies can be used to analyse the pharmacokinetic profile of a CR product.

2.1.3 Deconvolution studies

Recently, a deconvolution method to study the regional permeability has been validated. The studied drug can be administered as an intraluminal bolus dose at different intestinal regions. The deconvolution method is applied to the plasma concentration-time data obtained after the intraluminal administrations and the human intestinal or colonic permeability is indirectly estimated [148, 149]. Therefore, the deconvolution studies can be used to estimate the permeability of drugs formulated into CR dosage forms.

2.1.4. Estimated fraction absorbed after colonic administration

The oral fraction absorbed is a valuable absorption parameter for drugs administered by oral route. By definition, it is the fraction of the administrated drug that goes through the intestinal membrane. However, knowing the fraction of drug that passes the colonic membrane is very useful to evaluate an active pharmaceutical ingredient candidate to CR formulation. Tannegren et al. [10] developed an indirect method to estimate colonic fraction absorbed (Fa_{colon}). The compound of interest is administered to the colon as solution with colonoscopy techniques. The relative bioavailability in the colon ($Frel_{colon}$) is calculated by $AUC_{colon}/AUC_{reference}$, and the Fa_{colon} is estimated with the oral fraction absorbed (*Fa*) of the drug as $Fa \times Frel_{colon}$ [10]. Thus, this indirect method to estimate the colonic fraction absorbed is very useful to study *in vivo* human absorption data of a compound that will be included in a CR formulation.

Consequently, the indirect methods to estimate human absorption parameters are appropriate to predict the gastrointestinal absorption.

2.2. Direct methods

The direct determination of the human permeability is based on single-pass perfusion techniques in different regions of the human gastrointestinal tract. The permeability is calculated by measuring the disappearance rate of substances from the intestinal lumen during the intestinal perfusion [150].

Lennernas developed a double-balloon and multilumen tube system for single-pass perfusion in human (Loc-I-Gut) to determine the jejunal human permeability. The Loc-I-Gut is a validated and accepted method to study the oral drug absorption in human after the administration of an immediate release formulation [151, 152]. A similar system has been

developed for colorectal regions (Loc-I-Col) [153, 154], but the data of human colon permeability of drugs is very limited.

REGULATORY CONSIDERATIONS

The CR products are regulated as any other pharmaceutical product by the different government agencies in order to obtain the marketing authorization. In this section, the considerations of the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) about the candidate drugs to obtain controlled release formulations are briefly explained.

The EMA published in 2014 the final document of the "Guideline on quality of oral modified release products" [155]. This guideline is focused on the delayed release oral formulations (gastro-resistant products) and the prolonged release oral dosage forms. The document explains the aspects related with the development and quality evaluation, mainly using *in vitro* assays, of both controlled release formulations.

The guideline establishes that the prolonged release products need to be analysed under different dissolution conditions to ensure the representation of the whole gastrointestinal tract. Moreover, the dissolution test needs to demonstrate the batches consistency and the stability of the drug release during the expected life time of the product under the correct storage conditions. Besides, information about the drug pharmacokinetic and physicochemical characteristics and information about the release system characteristics is required. In addition, the release system needs to show a correct kinetic release, independently of the physiological variability [155].

Furthermore, regarding the bioavailability assays of the CR products, the EMA decrees the characteristics that the tested batches should have. The

results of the bioavailability test must show the pharmacokinetic data and a 90% confidence interval for the generic products [155].

On the other hand, the FDA has, since 1997, the document "Guidance for industry. Extended release oral dosage forms: development, evaluation, and application of *in vitro/in vivo* correlations" [156]. The main objective of this guideline is to provide advice to document the development of an *in vitro-in vivo* correlation (IVIVC) for oral CR formulations. The FDA describes the different IVIVCs categories, how to develop and evaluate these correlations, and the applications of the IVIVCs to the regulatory field of the CR formulations. The dissolution tests recommended by the FDA to perform an IVIVC are described in the United States Pharmacopeia (USP) [156].

CONCLUSION

The controlled release formulations have gained importance during the last years, because they have several advantages over immediate release formulations and, also, can be beneficial for many active pharmaceutical ingredients and for many pathologies. The CR oral products have meant a huge progress in the pharmacy field.

The permeability in colon of the candidate drugs to develop a CR formulation should be known, due to the drug absorption from a CR product takes place mainly in the colon. However, the amount of permeability studies performed in this distal segment of the gastrointestinal tract are scarce. It is necessary to validate more models to study the colon absorption and to predict the human colonic permeability.

To study the human permeability in distal parts of the intestinal tract involves difficulties and several ethical problems. Nevertheless, achieving more colonic human permeability values would be helpful to build more robust data bases with human data. Only when a good and wide data base about human colonic will be established, the simpler models may be

validated and may be reliable to predict the colonic absorption in humans. Therefore, *in silico* and *in vitro* models may be used with confidence to do a screening of candidate molecules to CR formulations development without need of animal or human assays. Nevertheless, given the lack of direct experimental data in humans, nowadays, the most approximated and reliable option to study the colonic human permeability would be to use animal studies. This is due to more simple models, like the cell cultures, although useful to do an initial screening of a new active molecule, do not reproduce all the complexity of the intestinal and colonic membrane. Therefore, the rat is a particularly useful species due to its easy handling and its similarity with the human gastrointestinal tract. Then to obtain more accurate permeability values, *in vivo* or *in situ* studies with rats would be a better option.

The recent advances in our knowledge of human colon physiology regarding water volumes and fluid composition, the variability and distribution of bacterial population and the transit times should lead to the improvement of the current in vitro method for evaluating the efficacy of the controlled release mechanism or the proposal of new ones adapted to each release mechanism. On the other hand, more information is needed about the physiological changes in colon environment in patients as this can affect to the release conditions or release triggering mechanisms.

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2. OBJETIVOS

El objetivo global de esta Tesis Doctoral es validar un modelo de perfusión intestinal *in situ* en rata que permita predecir la absorción humana en intestino y colon. Para dar respuesta a este objetivo se establecen los siguientes objetivos específicos:

- Analizar las correlaciones entre los valores de permeabilidad obtenidos para un conjunto de compuestos en diferentes tramos del tracto gastrointestinal utilizando dos métodos de perfusión *in situ,* el propuesto por Doluisio y colaboradores (compartimento estanco y sin recirculación) y el de paso simple.

- Analizar las correlaciones entre los valores de permeabilidad obtenidos para un conjunto de compuestos de referencia en colon de rata mediante la técnica de perfusión *in situ* sin recirculación y los obtenidos mediante diferentes métodos *in vitro* y en tejido intestinal de colon de procedencia humana.

- Desarrollar estrategias de formulación para favorecer la absorción de fármacos de baja y alta permeabilidad en colon.

2. OBJECTIVES

The main objective of this work is to validate an *in situ* rat perfusion model to predict human intestinal and colonic absorption. To achieve this objective, some specific objectives were established:

- To analyse the correlation between the permeability values of a set of model drugs in different gastrointestinal segments obtained by two *in situ* perfusion models, the closed-loop perfusion method and the single-pass intestinal perfusion technique.

- To analyse the correlations between the permeability values of a set of model drugs obtained in rat colon by the *in situ* closed-loop perfusion technique and those obtained by different *in vitro* methods and human colonic absorption data.

- To develop formulation strategies to favour the colonic absorption of high-permeability and low-permeability drugs.

3. VALIDACIÓN DE LA TÉCNICA DE PERFUSIÓN *IN SITU*

Según el Sistema de Clasificación Biofarmacéutica (BCS), los dos parámetros fundamentales que controlan la absorción de un fármaco son la solubilidad acuosa y la permeabilidad a través de la membrana gastrointestinal [1]. Determinar la solubilidad, a pesar de su complejidad y dificultades técnicas, no suele ser un obstáculo si se usa el protocolo adecuado, sin embargo, no ocurre lo mismo con la permeabilidad. De hecho, dada la dificultad existente para determinar la permeabilidad de compuestos en humanos [2], se han desarrollado numerosos modelos animales *in vivo* e *in situ* y modelos *in vitro* usando tejido intestinal o cultivos celulares.

Las técnicas de perfusión *in situ* en intestino de rata es una de las opciones más fiables y efectivas ya que el flujo sanguíneo y la inervación nerviosa se mantiene intacta, lo que confiere unas condiciones experimentales cercanas a la situación *in vivo* [3]. Existen dos técnicas de perfusión *in situ* principales: el paso simple o SPIP, por sus siglas en inglés (Single Pass Intestinal Perfusion), y el método de Doluisio. Ambas técnicas presentan las siguientes diferencias en su procedimiento [4, 5]:

- En el método de Doluisio la disolución se perfunde a través del intestino delgado completo, sin embargo, en el SPIP se utiliza únicamente un segmento de 10 cm, normalmente en yeyuno.

- En el método de Doluisio la disolución del fármaco permanece en el lumen intestinal durante el tiempo en el que se desarrolla el experimento, pero en el SPIP la solución pasa a través del segmento perfundido sin permanecer en el mismo.

- En el SPIP se perfunde la disolución y la toma de muestras se inicia después de una hora con el objetivo de alcanzar el estado estacionario. Transcurrido este tiempo se inicia el periodo de toma de muestra, que se prolonga durante una hora tomando muestras cada 10 minutos. En el desarrollo experimental del método de Doluisio la disolución se perfunde

durante 30 minutos y se comienza a tomar muestra transcurridos los 5 primeros minutos.

A pesar de las diferencias descritas, ambas técnicas presentan un objetivo común: determinar la permeabilidad de un fármaco midiendo la desaparición de dicho fármaco del lumen intestinal. Sin embargo, se ha establecido el SPIP como modelo de referencia para determinar la permeabilidad intestinal de compuestos en animales de experimentación, ya que la literatura científica le confiere un alto valor predictivo de la permeabilidad en humanos. Por el contrario, el método de Doluisio queda relegado para los casos en los que se dispone de pocas cantidades de líquido de perfusión y/o fármaco, ya que este último método, en relación con el SPIP, requiere volúmenes reducidos para llevar a cabo el experimento [3, 6-11].

En este capítulo se describe detalladamente el método de Doluisio, así como sus ventajas, inconvenientes y aplicaciones. Además, en los artículos científicos 1 y 2, este método se compara con el método de SPIP con el fin de demostrar que ambos son modelos útiles para estudiar la permeabilidad intestinal en rata y predecir la permeabilidad en intestino delgado de humanos.

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3.1. Capítulo de libro

Perfusión *in situ* sin recirculación

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INTRODUCCIÓN

La técnica de perfusión *in situ* sin recirculación permite calcular la constante de absorción de fármacos a través del tracto gastrointestinal del animal anestesiado o tramos del mismo. Esta técnica fue descrita por Doluisio y colaboradores en 1969 [1], y supuso una mejora, respecto a las técnicas *in situ* existentes para calcular la constante de absorción de los fármacos a través del tracto gastrointestinal en ratas.

En el año 1969 existían diversas técnicas para estudiar la absorción gastrointestinal:

- Brodie y colaboradores empleaban una técnica *in situ* en ratas para determinar el equilibrio entre las concentraciones de fármaco en sangre y en el lumen intestinal, mediante inyección simultánea del fármaco en el torrente sanguíneo y la perfusión de disoluciones de fármaco a través del intestino [2].

- Otros autores como Schanker o Kakemi [2], estudiaron la dinámica del proceso de absorción midiendo la desaparición a partir de disoluciones de perfusión gástrica o intestinal mediante experimentos de un solo paso o experimentos de circulación.

- Perfusión de un solo paso: la disolución de fármaco se perfunde a través de un tramo intestinal y se determina la concentración entrante y saliente. La diferencia entre ambas concentraciones se asume como concentración absorbida en ese tramo intestinal. Se supone que la absorción sigue una cinética de primer orden. Por tanto, son experimentos de circulación en los que se determina la disminución de concentración de fármaco en la disolución circulante. Este método tiene el inconveniente que el volumen de perfusión debe de ser mucho mayor que el volumen de la luz intestinal.

Otros investigadores intentaron estudiar la absorción gastrointestinal con técnicas *in vitro* usando segmentos aislados de intestino. Pero estos segmentos carecen de suministro sanguíneo, lo cual es un inconveniente importante.

A pesar de que se aprendió mucho sobre el proceso de absorción de fármacos mediante experimentos que utilizaban las técnicas mencionadas anteriormente, ninguna técnica o combinación de ellas permitía al investigador hacer algo más que comparar el valor de permeabilidad entre los compuestos respecto a las tasas de absorción orales. Además, en la mayoría de preparaciones *in situ* las constantes aparentes de velocidad de absorción eran relativamente lentas; mientras que en humanos y animales intactos la absorción de fármacos calculada con datos plasmáticos era muy rápida.

VENTAJAS E INCONVENIENTES

La principal ventaja del método de Doluisio es que permite calcular constantes de velocidad de absorción comparables con las obtenidas a partir de datos de concentración plasmática tras la administración oral de un fármaco. Además, la técnica experimental es sencilla, el material necesario es barato y se dispone de él fácilmente, un solo animal produce datos experimentales suficientes para el análisis de la cinética de absorción, la variación de los resultados cinéticos entre animales es mínima y el tiempo requerido para llevar a cabo el experimento no es mucho (de 45 a 60 minutos, cirugía y toma de muestras) [1].

Con este método se mantienen las características propias de la realidad fisiológica en el animal completo, como puede ser la capa de mucus y la presencia de los movimientos peristálticos o de las secreciones intestinales. Pero como todos los modelos animales, conlleva problemas éticos, logísticos y económicos. Las condiciones hidrodinámicas son distintas

de los ensayos *in vivo*, por lo que las estimaciones de los valores de permeabilidad para los compuestos de baja lipofilia son menos precisos [3].

Otra limitación es que la permeabilidad intestinal se calcula a partir de las concentraciones remanentes en el lumen intestinal, por ello es necesario comprobar que la desaparición se produce únicamente por absorción y no por degradación o metabolismo.

En el tracto gastrointestinal, tiene lugar un proceso de reabsorción de agua paralelo al de absorción del fármaco. En algunos casos supone la reducción de más del 10% del volumen al finalizar el ensayo de absorción, sigue una cinética de orden cero y es necesario tenerlo en cuenta para el cálculo adecuado de la permeabilidad intestinal. Como parámetro representativo del proceso de absorción se calcula la constante aparente de velocidad de absorción, k_a, que lleva implícita la superficie intestinal. Para poder comparar los resultados obtenidos "*in situ*" en intestino delgado con los obtenidos "*in vitro*" en líneas celulares deben transformarse los valores de k_a en permeabilidades.

ACONDICIONAMIENTO

Instalaciones

Una instalación para animales de laboratorio (bioterio, animalario o estabulario) debe facilitar la investigación mediante la disminución de variables experimentales imprevistas, mientras provee todos los requerimientos fisiológicos, sociales y de comportamiento del animal.

Las funciones de estos centros van, desde la producción de reactivos biológicos de alta calidad, hasta el mantenimiento de especies o cepas no producidas en sus instalaciones. En ellos se debe evitar la intromisión de animales y personas incontroladas y llevar una permanente vigilancia de las poblaciones, garantizando la constitución genética y el perfecto estado

sanitario. Sirven de suministrador a los investigadores y/o mantienen los animales en fase experimental, asesorando a otros centros productores o a los usuarios. En ellos se puede realizar la centralización de otros elementos relacionados con el animal (material y equipamiento) y el desarrollo de investigación relacionada con el animal de experimentación, además de la formación y/o especialización de profesionales. Muchos de estos centros actúan como empresas o forman parte de los servicios técnicos de apoyo a la investigación o centros de instrumentación científica de universidades, hospitales, centros de investigación o laboratorios [4].

Equipamiento y material

Además de rata Wistar o Sprague-Dawgle de peso comprendido entre 250-300 g se necesita el siguiente material:

- Baño a 37 ± 0,5 ºC.

- Solución de anestesia: Pentobarbital sódico en suero fisiológico.

- Suero fisiológico.

- Solución de lavado A y solución de lavado B.

    - Solución A:

        NaCl.....9 g

        KCl.....0.34 g

        $CaCl_2 \cdot 2H_2O$ .....3.9 mL

        $NaH_2PO_4 \cdot 2H_2O$ .....6.1 mL

        H_2O bidestilada.....c.s.p. 1L

- Solución B:

NaCl.....9 g

$NaH_2PO_4 \cdot 2H_2O$ 1/15M.....3.9 mL

Na_2HPO_4 1/15M.....6.1 mL

Ajustar pH a 7,00 con NaOH o HCl

H_2O bidestilada.....c.s.p. 1L

- Disolución del fármaco en "solución B". Ajustar el pH a 7,00 antes de enrasar.

- Tubos de vidrio neutro de 25 mL de capacidad.

- Tubos para la recogida de muestras.

- Pipetas de diferentes volúmenes.

- 2 cánulas de vidrio acodadas de unos 3 mm de diámetro interno y 4 mm de diámetro externo.

- 2 tubos de polietileno de unos 3 cm de longitud y 4 mm de diámetro interno.

- 2 llaves de 3 pasos.

- 2 jeringuillas de vidrio con luer-lock de 10 mL de capacidad.

- Algodón hidrófilo.

- Hilo de seda.

- Pinzas de diente de ratón.

- Pinzas de punta fina.

- Tijera quirúrgica.

- 2 pinzas de sujeción unidas a soportes verticales.

PERSONAL

El personal investigador que diseñe y/o realice la técnica quirúrgica deberá entrar acreditado según la legislación vigente en el país donde se realice el ensayo.

BUENAS PRÁCTICAS DE TRABAJO

Siempre que se usen animales en investigación se debe tener en cuenta que su bienestar es muy importante. Además, existen razones éticas, científicas, jurídicas y económicas para asegurar que los animales sean cuidados adecuadamente [5]:

- Si el animal sufre estrés y/o dolor podrían verse afectados los resultados del experimento. (Razón científica).

- A través de normas bioéticas han sido adoptados los principios de las 3 Rs [6]:

Reemplazar: sustituir los animales de laboratorio por equivalentes que no empleen animales, es decir, usar métodos alternativos siempre que sea posible. (Razón ética).

Reducir el número de animales empleados en la investigación. Usar un número menor de animales resulta más barato y el trabajo se reduce al mínimo. (Razón económica).

Refinar: usar procedimientos que minimicen el sufrimiento o la ansiedad de los animales empleados en la experimentación. (Razón ética).

- La legislación europea (razón jurídica) en su Directiva 86/609/CEE del Consejo de Europa, de 24 de noviembre de 1986, que se complementa con la Resolución 86/C 331/02, dicta:

Los experimentos deben tener como objetivo el bien del hombre o de la naturaleza.

Se debe evitar todo sufrimiento y ansiedad y paliar el que obligadamente se deba producir.

Los experimentadores deben estar adecuadamente capacitados.

No deben existir alternativas al empleo de los animales.

Es obligatorio el uso de métodos indoloros de eutanasia.

Debe fomentarse la búsqueda de técnicas alternativas.

- Además, existen las Buenas Prácticas de Laboratorio que son un conjunto de Normativas sometidas a revisiones constantes que tienden a afinar progresivamente en los principios éticos: minimizar el sufrimiento de los animales, disminuir el número empleado y usar sistemas alternativos igualmente fiables. (Razón ética).

- Por otro lado, se encuentra la organización CIOMS (Council for International Organizations of Medical Sciences). Es una organización científica establecida por la Unesco y la OMS en 1949 que establece, entre otros, los siguientes principios éticos universales:

El avance del conocimiento, la protección de la salud y/o el bienestar de los hombres y los animales requiere de la experimentación con animales vivos.

Siempre que sea apropiado usar métodos alternativos (3 R's).

Realizar experimentación en animales después de estudiar su importancia para la salud humana y animal y para el avance del conocimiento biológico.

Seleccionar animales de especie y calidad apropiadas.

Evitar o minimizar las molestias, la angustia y el dolor.

Los animales que puedan sufrir dolor crónico o severo, angustia, molestia o invalidez, que no puedan ser aliviados, deben ser sacrificados sin dolor.

Los animales deben tener las mejores condiciones de vida posibles.

TIPOS DE ANIMALES

Los conceptos básicos sobre absorción gastrointestinal de fármacos están basados, en gran medida, en experimentos realizados en ratas[1]. Las razas más empleadas en estudios de absorción son la Wistar y la Sprague-Dawley [7, 8].

Wistar

El investigador Donaldson adquirió cuatro parejas de ratas albinas para el Instituto Wistar de Filadelfia, en EEUU, las cuales dieron origen a la bien conocida estirpe que lleva el nombre de dicha institución.

Actualmente es una de las cepas de ratas más populares utilizadas para la investigación de laboratorio. Se caracteriza por su cabeza ancha,

orejas largas, y con una longitud de la cola que es siempre inferior a la longitud de su cuerpo. Es una rata con buena tasa de crecimiento, dócil y fácil de manipular.

Sprague Dawley

El profesor Robert W. Dawley, de Wisconsin, fue quien crió y tipificó la cepa que lleva su apellido al que antepuso el de su esposa Sprague. Esta cepa de rata fue la primera producida por la compañía de animales Sprague Dawley en Madison.

Es una rata albina que procede de una serie de cruces iniciados con un macho de cabeza pigmentada y seis hembras albinas de origen desconocido. La rata Sprague Dawley se utiliza ampliamente en investigación médica, su principal ventaja es su tranquilidad y facilidad de manejo.

TÉCNICA EXPERIMENTAL

Acondicionamiento del animal

El animal se mantiene en ayuno durante un periodo de unas 4 horas, con objeto de que la cantidad de detritus y heces formes sea mínima en lumen intestinal. Durante ese periodo de ayuno al animal se le permite libre acceso al agua para evitar su deshidratación.

Anestesia del animal

Se procede a pesar al animal y a continuación se calcula el volumen de solución de anestesia que debe administrarse. La dosis necesaria de pentobarbital sódico es 40 mg/kg. Ésta se administra por inyección intraperitoneal. A continuación, se espera entre 5 y 10 min, hasta comprobar la pérdida del reflejo parpebral.

Técnica quirúrgica

A partir de este momento el animal debe mantenerse a una temperatura ambiental de 25 °C para evitar que se enfríe ya que, de lo contrario, se podría modificar la perfusión sanguínea y falsear los resultados del ensayo de absorción.

Se coloca al animal en posición de decúbito supino sobre un tablero quirúrgico y se sujeta las extremidades mediante cinta adhesiva. Con ayuda de unas pinzas de diente de ratón, se hace una incisión longitudinal en la línea alba de la capa muscular abdominal del animal. Para descubrir el paquete abdominal se corta a lo largo de esta línea desde 1 cm por encima del poro genital hasta 1 cm por debajo del apéndice xifoides, evitando lesionar los músculos abdominales y tratando de producir la mínima hemorragia posible.

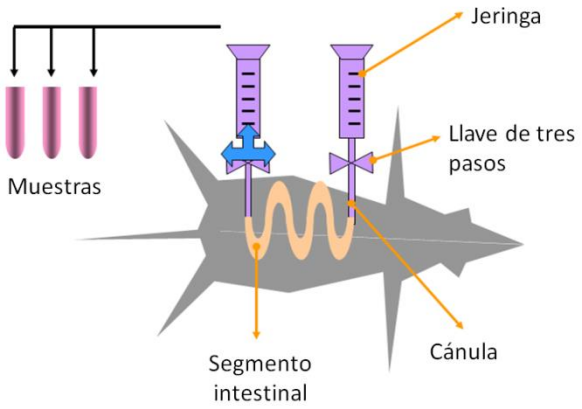

Figura 3.1.1. Esquema de la técnica experimental.

Se localiza el estómago, a partir del cual empieza el duodeno que es el primer tramo del intestino delgado. En la zona proximal del duodeno se practica un pequeño corte en bisel. A continuación, se introduce una cánula acodada. Esta cánula se sujeta con hilo de seda a la pared intestinal.

Se localiza el conducto biliar y se practica una ligadura con hilo de seda para impedir el paso de la bilis al lumen intestinal.

La cánula se une a una llave de tres pasos mediante un tubo de polietileno. Ésta última se conecta a una jeringa de 10 mL de capacidad, que se sujeta mediante una pinza a un soporte vertical. Debe de procurarse que la posición de la cánula respete la disposición natural del intestino en la medida de lo posible. La llave de tres pasos permite la correcta realización de los ensayos.

A continuación, para aislar el intestino delgado, se localiza el ciego, tramo donde acaba el intestino delgado y empieza el intestino grueso. Se practica, como en el caso anterior, un corte en bisel. Aunque el animal ha sido sometido a 4 h de ayuno, siempre quedan restos de quimo en el lumen intestinal, por lo que es necesario proceder al lavado de la zona, haciendo pasar solución A (unos 25 mL aproximadamente, atemperado a 37ºC). Debe evitarse el lavado enérgico ya que se podría lesionar la mucosa intestinal. Finalmente se hacen pasar unos 25 mL de la solución B para restaurar el pH intestinal.

Se coloca en esta segunda incisión una cánula de forma similar a la primera. Una vez ligada la cánula con hilo de seda se introduce varias veces aire en el intestino para provocar la salida de los restos de la solución de lavado. Así se evita, en lo posible, la dilución de la solución de perfusión. La cánula se une a una llave de tres pasos y ésta, a su vez, a una jeringa de 10 mL que se sujeta por una pinza a un soporte vertical.

Dado que la cavidad abdominal permanece al descubierto, para evitar la desecación del intestino, se introduce en ella un pequeño volumen de líquido de solución B, atemperado a 37ºC, y se cubre la incisión con una torunda de algodón empapado en la misma. Conviene controlar que no se enfríe la cavidad abdominal durante el ensayo, ya que podría disminuir el riego mesentérico. Esto se consigue reponiendo la torunda varias veces.

Perfusión y toma de muestras

Se introducen 10 mL de la solución de perfusión en la jeringa proximal con la llave de tres pasos en posición jeringa-intestino, mientras la llave distal conecta el intestino con el exterior. Así el aire que ocupa el interior del intestino es desplazado por la solución a medida que esta se introduce. Se pone en marcha el cronómetro y se colocan ambas llaves en posición jeringa-exterior con lo que el intestino se convierte en un compartimento estanco ocupado por la solución de perfusión.

A los tiempos de toma de muestra, cada 5 minutos hasta un total de 6 muestras, se toma aire en la jeringa contraria a la de muestreo y seguidamente se colocan las dos llaves en posición jeringa-intestino. Se bombea el aire de la jeringa contraria a la de muestreo mientras en la jeringa que se toma la muestra, se succiona la solución de perfusión. Una vez que se ha extraído todo el volumen posible de la solución se toman 0.2 mL con ayuda de una micropipeta. A continuación, se devuelve la solución remanente al interior del intestino; para ello, se coloca la llave en la que se ha cogido la muestra en posición jeringa-intestino y la otra llave en posición intestino-exterior para facilitar la salida de aire. Tras introducir la solución, ambas llaves se colocan en posición jeringa-exterior, convirtiendo de nuevo el intestino en un compartimento estanco.

Las muestras se toman alternativamente por cada una de las jeringas. La primera muestra se toma en la jeringa distal y la última en la jeringa proximal. Las muestras se recogen en tubos adecuados, se centrifugan a 5000 rpm durante 5 minutos para separar la muestra de restos de mucosa. A continuación, se conservan de forma adecuada hasta la valoración cuantitativa.

Una vez tomadas todas las muestras, se extrae todo el líquido remanente del intestino. Para ello, se desconecta la cánula de la jeringa distal y se coloca en un tubo de vidrio de 25 mL. La otra cánula se mantiene

conectada a la jeringa proximal, con la cual se toma aire y se ejerce presión para forzar la salida del líquido remanente a través de la cánula. Seguidamente, con ayuda de unas tijeras se corta el mesenterio y se separa lentamente el asa intestinal del resto del animal. Una vez aislado el intestino mediante una torunda de algodón húmedo, se presiona ligeramente, de principio a fin y se vacía completamente su contenido en el tubo.

El volumen de líquido recogido se centrifuga durante 5 minutos a 5000 rpm para separar los restos de mucosa que se han podido arrastrar en el vaciado. Tras retirarlos se mide el volumen de líquido recuperado. El volumen remanente al final del ensayo (V_f) corresponde a la suma de este volumen más el volumen de las muestras extraídas para la valoración. Para los cálculos posteriores se emplea como volumen inicial (volumen a tiempo cero, V_o) 10,7 mL. Por último, se comprueba al final del ensayo el pH de la solución recogida.

APLICACIONES DE LOS ENSAYOS *IN SITU*

Determinación de la permeabilidad intestinal y mecanismos de transporte [9]

Gracias a esta metodología podemos conocer parámetros fundamentales en el desarrollo de cualquier fármaco como la constante aparente de velocidad de absorción, la permeabilidad y los mecanismos implicados en el transporte de la molécula.

Las muestras obtenidas tras aplicar la técnica *in situ* sin recirculación se someten a un procedimiento analítico, normalmente cromatografía líquida de alta resolución, para poder determinar la concentración de compuesto estudiado que existe en cada muestra. De este modo se puede calcular la constante aparente de velocidad de absorción (K_{ap}) que se obtiene mediante ajuste por regresión no lineal a la siguiente ecuación:

$$C = C_0 \cdot e^{-k_{ap} \cdot t} \quad (1)$$

donde C es la concentración de fármaco a tiempo t y C_0 corresponde a la concentración inicial de fármaco disponible para la absorción (t=0).

Para obtener un valor representativo de la constante de velocidad de absorción, el ensayo se realiza en un mínimo de seis animales para cada concentración ensayada. Con esto se obtiene un valor medio que es característico de las condiciones del ensayo.

Es posible determinar los valores de permeabilidad intestinal efectiva (P_{eff}) por transformación de la constante aparente de velocidad de absorción según la ecuación:

$$P_{eff} = \frac{k_{ap} \cdot R}{2} \quad (2)$$

donde R es el radio del segmento intestinal donde se ha perfundido la disolución a ensayar.

Para poner en evidencia la existencia o no de transportadores implicados en el mecanismo de absorción de un compuesto, hay que diseñar un estudio en el que se determine la permeabilidad a diferentes concentraciones de compuesto. La comparación estadística de las constantes aparentes de velocidad de absorción obtenidas con las distintas concentraciones ensayadas permite poner de manifiesto la presencia o no de un transporte activo y saturable, pues la presencia de diferencias estadísticamente significativas nos indica que existen transportadores implicados en la absorción.

Estudios de mecanismos de inhibición [10]

Se puede evaluar la capacidad que tienen ciertos inhibidores de provocar una menor absorción, y por tanto disminuir la permeabilidad del fármaco, a través de fenómenos inhibitorios.

Para ello se debe adicionar una cantidad adecuada de inhibidor a una concentración de compuesto que no provoque la saturación de los transportadores. Una vez finalizado el ensayo se comparan estadísticamente los resultados obtenidos entre el fármaco en solución libre y el fármaco adicionado de inhibidor.

Detección de ventanas de absorción [10]

Una vez obtenida la evidencia experimental de la presencia de un proceso no lineal, es decir, presencia de transportadores, se podrían realizar ensayos de absorción en los diferentes tramos fisiológicos del intestino delgado de la rata.

El objetivo de estos ensayos es caracterizar la zona de absorción con niveles mayores de expresión del transportador, lo que se conoce como determinar la presencia de ventanas de absorción.

Para ello se efectuarían ensayos en duodeno, considerando como tal la zona comprendida entre el píloro y el ligamento de Treitz (longitud aproximada 10 cm), en yeyuno tomando 45 cm desde el ligamento de Treitz y en íleon, caracterizado como los 45 cm previos a la válvula íleo-cecal. Por último, se realizan también ensayos en colon que se aísla desde el ciego a la zona rectal (aproximadamente 10 cm).

Correlaciones [11, 12]

Esta técnica experimental también permite establecer correlaciones a partir de datos previamente publicados.

Para poder establecer correlaciones se debe aislar el componente pasivo del proceso de absorción, siempre y cuando sea posible ya que hay compuestos en los que no se consigue aislar.

- Correlaciones *in situ-in vitro:* calcular la permeabilidad en rata usando la permeabilidad *in vitro*.

- Correlaciones fracción absorbida-*in situ*: estimar la fracción absorbida en humanos basándose en la permeabilidad intestinal en rata.

- Correlaciones biodisponibilidad-K_a *in situ*: permiten calcular la biodisponibilidad *in vivo* usando las constantes de absorción calculadas *in situ*.

- Correlaciones *in situ*-lipofilia: permiten calcular la absorción (permeabilidad) a partir de la lipofilia de los compuestos.

FACTORES QUE AFECTAN A LA TÉCNICA EXPERIMENTAL

Factores pre-experimentales

Entre los factores pre-experimentales en rata que conducen a la obtención de medidas de permeabilidad diferentes para un mismo fármaco están la raza y el sexo. Otro aspecto a tener en cuenta es el estado nutricional del animal, ya que en ciertos compuestos se puede modificar la absorción y la permeabilidad debido a la desnutrición [13].

Además, existen otros factores que pueden alterar al animal y afectar a los resultados. Éstos pueden ser biológicos y no biológicos [4].

Factores biológicos: aire, agua, alimento, contaminación ambiental, personal trabajador, presencia de insectos y parásitos en el estabulario.

Factores no biológicos: diseño del estabulario, clima, temperatura, humedad, luz, ruido, jaulas.

Factores experimentales

Con las técnicas *in situ*, el aporte neural, endocrino, sanguíneo y linfático de la zona en estudio permanece intacto. Estos modelos son, por tanto, sensibles a influencias farmacológicas y fisiológicas, por lo que se observa una gran variabilidad en comparación con los sistemas *in vitro*.

Esta técnica *in situ* sin recirculación, basada en el método de Doluisio, puede llevarse a cabo en intestino completo o en tramos intestinales, creando un compartimento estanco de absorción, donde se observa cómo va desapareciendo el fármaco del lumen intestinal. Dependiendo de la zona elegida para realizar el experimento, puede haber diferencias en los datos de permeabilidad obtenidos para un mismo compuesto.

Las muestras se toman cada 5 minutos alternando las jeringas del compartimento estanco, durante 30 minutos. Por lo que cualquier error en el montaje experimental o en la toma de muestras, también puede afectar a los valores de permeabilidad.

Factores post-experimentales

Existe un proceso de reabsorción de agua simultáneo al proceso de absorción de la sustancia ensayada de modo que la solución remanente en lumen se concentra [14]. Este proceso puede falsear por exceso el valor de la concentración remanente en lumen, sobre todo en las últimas muestras (25 y 30 min).

Con el fin de estimar las concentraciones reales de compuesto se calcula el volumen remanente a cada tiempo de toma de muestra. Dado que el volumen varía según una cinética de orden cero, la ecuación diferencial representativa del proceso es [15]:

$$\frac{dV}{dt} = -k_0 \quad (3)$$

Su forma integrada es:

$$V = V_0 - k_0 \cdot t \quad (4)$$

en la que V es el volumen remanente en el intestino a cada tiempo, V_0 es el volumen remanente a tiempo inicial y k_0 representa la constante de velocidad de reabsorción de agua (mL/min).

Mediante regresión lineal por mínimos cuadrados de los volúmenes obtenidos, se obtienen los parámetros V_0 como ordenada en el origen y k_0 como pendiente. Estos parámetros permiten determinar los volúmenes teóricos para cada tiempo de toma de muestra (V_t), y con ellos se corrigen los valores experimentales de las concentraciones de soluto en las muestras, utilizando la ecuación:

$$C = E \cdot \frac{V_t}{V_0} \quad (5)$$

en la que C corresponde a la concentración de soluto corregida y E es la concentración obtenida experimentalmente.

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3.2. Artículo científico 1

In situ intestinal rat perfusions for human F_{abs} prediction and BCS permeability class determination: investigation of the single-pass vs the Doluisio experimental approaches

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ABSTRACT

Intestinal drug permeability has been recognized as a critical determinant of the fraction dose absorbed, with direct influence on bioavailability, bioequivalence and biowaiver. The purpose of this research was to compare intestinal permeability values obtained by two different intestinal rat perfusion methods: the single-pass intestinal perfusion (SPIP) model and the Doluisio (closed-loop) rat perfusion method. A list of 15 model drugs with different permeability characteristics (low, moderate, and high, as well as passively and actively absorbed), was constructed. We assessed the rat intestinal permeability of these 15 model drugs in both SPIP and the Doluisio methods, and evaluated the correlation between them. We then evaluated the ability of each of these methods to predict the fraction dose absorbed (F_{abs}) in humans, and to assign the correct BCS permeability class membership. Excellent correlation was obtained between the two experimental methods (r^2=0.93). An excellent correlation was also shown between literature F_{abs} values and the predictions made by both rat perfusion techniques. Similar BCS permeability class membership was designated by literature data and by both SPIP and Doluisio methods for all compounds. In conclusion, the SPIP model and the Doluisio (closed-loop) rat perfusion method are both equally useful for obtaining intestinal permeability values that can be used for F_{abs} prediction and BCS classification.

Keywords: biopharmaceutics classification system, intestinal permeability, bioequivalence, biowaiver, oral drug absorption, perfusion study.

INTRODUCTION

Amidon et al. [1] revealed that the two fundamental parameters controlling the rate and extent of drug absorption following oral administration are the permeability of the drug through the gastrointestinal (GI) membrane and the solubility/dissolution of the drug dose in the GI

milieu, and developed the Biopharmaceutics Classification System (BCS). Based on this work, the FDA initiated the replacement of *in vivo* bioequivalence (BE) studies with *in vitro* dissolution tests for immediate-release (IR) solid oral dosage forms when formulated as rapidly dissolved drug product [2]. To date, only BCS class I, high-solubility high-permeability drugs, may be eligible as candidates for this biowaiver according to the American FDA. Under the EMA jurisdiction, class III drugs may also be eligible for biowaivers under certain conditions [3].

While the solubility parameter is fairly straightforward when assigning a BCS classification, intestinal permeability is not routinely measured, and difficulties to prove high-permeability classification may limit the broad regulatory application of the BCS-based biowaiver concept [4-8]. The permeability class of a drug can be determined in human subjects using mass balance, systemic bioavailability, or intestinal perfusion approaches. Recommended methods not involving human subjects include *in vivo* or *in situ* intestinal perfusion in a suitable animal model, and/or *in vitro* permeability methods using excised intestinal tissues, or monolayers of suitable epithelial cells [2].

While human data is more convincing than pre-clinical or *in vitro* experiments, the difficulty in obtaining human results is tremendous and cannot be ignored [4, 9, 10]. In fact, in many cases it is probably less difficult to run the traditional human bioequivalence study than to obtain human intestinal permeability data, so the whole purpose of the biowaiver concept is missed. Among all non-human FDA approved methods for BCS permeability classification, intestinal perfusion studies in rats emerge as the most reliable and cost effective option. A high correlation between human and rat small intestine permeability (r^2=0.8-0.95) was reported for drug intestinal permeability with both carrier-mediated absorption and passive diffusion mechanisms [11-14]. Perfusion studies in the rat intestine appear to correlate best with human data, even though the type of transporters and

their expression levels may vary between species [15-17]. Rat perfusion studies even allow to assess segmental-dependent permeability throughout the entire small intestine, a factor that to date cannot be measure in humans [6, 7, 11, 18-21].

The purpose of this research was to compare two different intestinal rat perfusion methods: the single-pass intestinal perfusion (SPIP) model and the Doluisio rat perfusion method. While both of these experimental methods measure disappearance from the intestinal lumen as a measure for drug absorption, they have substantial differences between them; the SPIP model focuses on a 10 cm intestinal segment (typically the jejunum) [22, 23], whereas the Doluisio method typically measures the absorption throughout the entire small intestine [24, 25], although with appropriate adjustments segmental-dependent permeability can be measured by both techniques [7, 11, 18, 26-29]. Moreover, while in the SPIP model each drug molecule gets only one passage through the investigated intestinal segment, in the Doluisio method the tested drug solution remains within the intestinal lumen throughout the entire experiment. We constructed a list of 15 model drugs with different permeability characteristics: low, moderate, and high permeability, as well as passively and actively absorbed compounds. We assessed the rat intestinal permeability of these 15 model drugs in both SPIP and the Doluisio experimental methods, and evaluated the correlation between them. We then evaluated the ability of each of these methods to predict the fraction dose absorbed (F_{abs}) in humans, and to assign the correct BCS permeability class membership. Overall, this research can aid and guide the correct assignment of BCS permeability classification using FDA approves animal models.

MATERIALS AND METHODS

Materials

Antipyrine, atenolol, caffeine, carbamazepine, chloramphenicol, cimetidine, colchicine, codeine, ibuprofen, digoxin, furosemide, labetalol, metoprolol, paracetamol and valsartan were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, methanol and water (Merck KGaA, Darmstadt, Germany) were UPLC grade. All other chemicals were of analytical reagent grade.

The model drugs

A list of 15 model drugs was constructed: antipyrine, atenolol, caffeine, carbamazepine, chloramphenicol, cimetidine, colchicine, codeine, ibuprofen, digoxin, furosemide, labetalol, metoprolol, paracetamol and valsartan. These model drugs were chosen while making sure to include drugs with different permeability characteristics: low, moderate, and high permeability, as well as passively and actively absorbed compounds, as will be discussed hereinafter.

Single-pass rat intestinal perfusion

The effective permeability coefficient (P_{eff}) of the 15 model drugs using the single-pass rat intestinal perfusion model were conducted using protocols approved by the Ben-Gurion University of the Negev Animal Use and Care Committee (Protocol IL-60-11-2010). Male Wistar rats (250-300 g, Harlan, Israel) were housed and handled according to the Ben-Gurion University of the Negev Unit for Laboratory Animal Medicine Guidelines.

Rats were anesthetized with an intra-muscular injection of 1 mL/kg of ketamine-xylazine solution (9%:1%, respectively), placed on a heated surface maintained at 37 °C (Harvard Apparatus Inc., Holliston, MA), and a midline abdominal 3 cm incision was made. A proximal 10 cm jejunal

segment, starting 2 cm below the ligament of Treitz, was cannulated on two ends, and was rinsed with blank perfusion buffer. All solutions were incubated in a 37 °C water bath [30-32].

At the starting point of each experiment, perfusion solution containing the investigated drug, 10 mM MES buffer, pH 6.5, 135 mM NaCl, 5 mM KCl, and 0.01 mg/mL phenol red, with an osmolarity of 290 mOsm/L, was perfused through the intestinal segment (Watson Marlow 205S, Watson-Marlow Bredel Inc, Wilmington, MA), at a flow rate of 0.2 mL/min. The perfusion buffer was perfused for 1 hour without sampling, to ensure steady state conditions, followed by additional 1 h of perfusion with samples taken every 10 min. The pH of the collected samples was measured at the outlet, to verify that there was no pH change throughout the perfusion (pH 6.5). The samples were immediately assayed by UPLC. The length the perfused intestinal segment was measured at the endpoint of the experiment.

The effective permeability (P_{eff}; cm/sec) through the rat gut wall was determined according to the following equation:

$$P_{eff} = \frac{-Qln(C'_{out}/C'_{in})}{2\pi RL} \quad (1)$$

where Q is the perfusion buffer flow rate (0.2 mL/min), C'_{out}/C'_{in} is the ratio of the outlet and the inlet concentration of drug that has been adjusted for water transport via the non-absorbable marker phenol red [33-36], R is the radius of the intestinal segment (set to 0.2 cm), and L is the length of the perfused intestinal segment.

Doluisio's (closed-loop) rat intestinal perfusion

The studies were approved by the Scientific Committee of the Faculty of Pharmacy, Miguel Hernandez University, and followed the guidelines

described in the EC Directive 86/609, the Council of the Europe Convention ETS 123 and Spanish national laws governing the use of animals in research.

Fasted male Wistar rats weighing ~250 g with free access to water were used in these studies. Rats were anesthetized using a mixture of diazepam (1.67 mg/kg, Valium, Roche), ketamine (50 mg/kg, Ketolar, Parke-Davis), and atropine (1 mg/kg, Atropina sulfato, Braun), and placed on heated surface maintained at 37 °C. A midline abdominal incision was made and the small intestine was exposed. The bile duct was ligated in order to avoid drug enterohepatic circulation and the presence of bile salts in lumen. The method consists of creating a small intestinal compartment with the aid of two syringes and two three-way stopcock valves. Two incisions were made in the intestine, the first at the beginning of the duodenal segment, and the second at the end of the ileum segment, just before the cecum, and the entire small intestine was cannulated through these incisions [37, 38]. Care was taken to avoid disturbance of the intestinal blood supply. In order to remove all intestinal contents, the small intestine was thoroughly flushed with a warm physiologic solution. The catheters were then connected to a glass syringe using a stopcock three way valve. The intestine was carefully placed back into the peritoneal cavity and the abdomen was covered with cotton wool pads to prevent peritoneal liquid evaporation and heat loss. This set up ensures the isolation of the small intestine, and drug solution can be introduced and sampled with the aid of the syringes and stopcock valves. To take a sample, the luminal content is pushed out from one syringe to the other. This procedure is done alternatively from the proximal syringe to the distal one, assuring the mixing of the solution in the intestinal lumen. The samples were collected every 5 minutes up to a period of 30 minutes[39, 40].

Water flux throughout the experiment may be significant, and hence must be accounted for [35]. A method based on direct measurement of the remaining solution volume was employed to calculate the water reabsorption zero order constant (k_0). For each tested compound, the initial

volume (V_0) was determined on groups of three animals, while the the endpoint volume (V_t) was measured on every animal used. The drug concentration in the samples was corrected as: $C_t=C_e(V_t/V_0)$, where C_t represents the concentration in the absence of water reabsorption at time t, and C_e the experimental value. The corrected concentration, C_t, was then used the actual absorption rate coefficient calculations [41].

The absorption rate coefficients (k_a) of the compounds were determined by nonlinear regression analysis of the remaining concentrations in the intestinal lumen (C_t) versus time:

$$C_t = C_0 \cdot e^{-k_a t} \quad (2)$$

These absorption rate coefficients were then transformed into permeability values using the relationship: $P_{app} = k_a \cdot R/2$, where R is the effective radius of the intestinal segment, calculated from the area/volume relationship considering a 10 mL volume and an intestinal length of 100 cm.

Analytical methods

Analytical analyses of the SPIP and the Doluisio samples were performed on a Waters (Milford, MA) Acquity UPLC H-Class system, and a Waters 2695 HPLC Separation Module, respectively. Both systems were equipped with photodiode array UV detector and Empower software. The simultaneous determination of the investigated drug and the non-absorbable marker phenol red in the SPIP samples was achieved using a Waters (Milford, MA) Acquity UPLC BEH C_{18} 1.7 µm 2.1 × 100 mm column. The determination of the investigated drug in the Doluisio samples was carried out using a Waters C_{18} 3.5 µm 4.6×250 mm XTerra column. A general gradient mobile phase consisted of 90:10 going to 20:80 (v/v) water:acetonitrile (both with 0.1% TFA) over 7 minutes was suitable for all analyses. Injection volumes ranged from 2 to 50 µL.

Statistical analysis

All animal experiments were replicated with n = 4-6. Values are expressed as mean ± standard deviation (SD). To determine statistically significant differences among the experimental groups, the nonparametric Kruskal-Wallis test was used for multiple comparisons, and the two-tailed nonparametric Mann–Whitney *U*-test for two-group comparison where appropriate. A p value of less than 0.05 was termed significant.

RESULTS

The permeability values (cm/s) obtained for the different drugs in both the SPIP and the Doluisio experimental methods are presented in Figure 3.2.1, and summarized in Table 3.2.1. The correlation between the two sets of data, the SPIP vs the Doluisio P_{eff} values, is presented in Figure 3.2.2. Excellent correlation was obtained between the two experimental methods, as evident by a coefficient of determination (r^2) of 0.93.

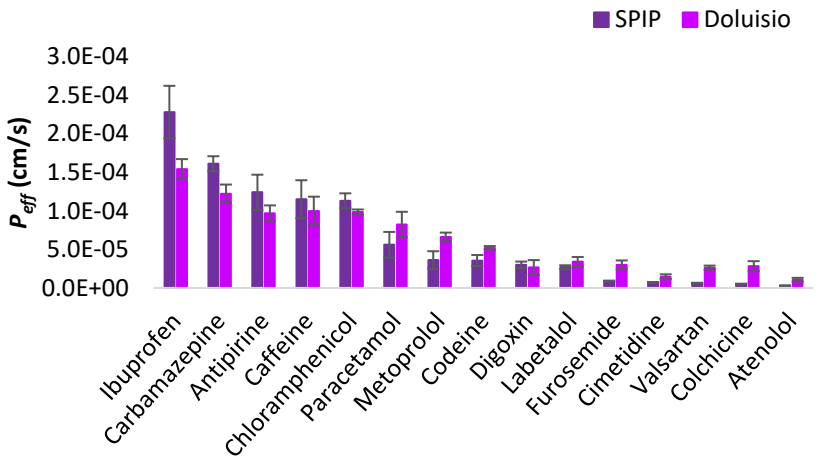

Figure 3.2.1. The permeability values (cm/s) obtained for the different drugs in both the SPIP and the Doluisio experimental methods.

Table 3.2.1. The concentrations (μM) and permeability values (cm/s) of the different drugs in both experimental methods. Data presented as means (SD).

The Drug	Single-pass rat jejunal perfusion		Doluisio's rat perfusion	
	Conc. (μM)	P_{eff} (cm/sec)	Conc. (μM)	P_{eff} (cm/sec)
Ibuprofen	250	$2.2 \cdot 10^{-4}$ ($3.4 \cdot 10^{-5}$)	500	$1.5 \cdot 10^{-4}$ ($1.3 \cdot 10^{-5}$)
Carbamazepine	500	$1.6 \cdot 10^{-4}$ ($9.7 \cdot 10^{-6}$)	500	$1.2 \cdot 10^{-4}$ ($1.2 \cdot 10^{-5}$)
Antipirine	200	$1.2 \cdot 10^{-4}$ ($2.2 \cdot 10^{-5}$)	500	$9.7 \cdot 10^{-5}$ ($1.0 \cdot 10^{-5}$)
Caffeine	500	$1.1 \cdot 10^{-4}$ ($2.4 \cdot 10^{-5}$)	500	$9.9 \cdot 10^{-5}$ ($1.8 \cdot 10^{-5}$)
Chloramphenicol	1000	$1.1 \cdot 10^{-4}$ ($9.6 \cdot 10^{-6}$)	500	$9.8 \cdot 10^{-5}$ ($3.4 \cdot 10^{-6}$)
Paracetamol	330	$5.6 \cdot 10^{-5}$ ($1.7 \cdot 10^{-5}$)	500	$8.2 \cdot 10^{-5}$ ($1.6 \cdot 10^{-5}$)
Metoprolol	500	$3.6 \cdot 10^{-5}$ ($4.8 \cdot 10^{-6}$)	500	$6.6 \cdot 10^{-5}$ ($5.6 \cdot 10^{-6}$)
Codeine	750	$3.5 \cdot 10^{-5}$ ($7.1 \cdot 10^{-6}$)	750	$5.2 \cdot 10^{-5}$ ($2.3 \cdot 10^{-6}$)
Digoxin	150	$3.0 \cdot 10^{-5}$ ($4.2 \cdot 10^{-6}$)	500	$2.6 \cdot 10^{-5}$ ($9.6 \cdot 10^{-6}$)
Labetalol	500	$2.7 \cdot 10^{-5}$ ($2.3 \cdot 10^{-6}$)	500	$3.4 \cdot 10^{-5}$ ($6.1 \cdot 10^{-6}$)
Furosemide	500	$9.0 \cdot 10^{-6}$ ($5.0 \cdot 10^{-7}$)	500	$3.0 \cdot 10^{-5}$ ($5.7 \cdot 10^{-6}$)
Cimetidine	500	$7.2 \cdot 10^{-6}$ ($4.7 \cdot 10^{-7}$)	500	$1.4 \cdot 10^{-5}$ ($3.0 \cdot 10^{-6}$)
Valsartan	250	$6.0 \cdot 10^{-6}$ ($6.7 \cdot 10^{-7}$)	500	$2.6 \cdot 10^{-5}$ ($2.7 \cdot 10^{-6}$)
Colchicine	15	$5.0 \cdot 10^{-6}$ ($4.0 \cdot 10^{-7}$)	500	$2.8 \cdot 10^{-5}$ ($6.5 \cdot 10^{-6}$)
Atenolol	200	$2.8 \cdot 10^{-6}$ ($3.6 \cdot 10^{-7}$)	500	$1.0 \cdot 10^{-5}$ ($2.3 \cdot 10^{-6}$)

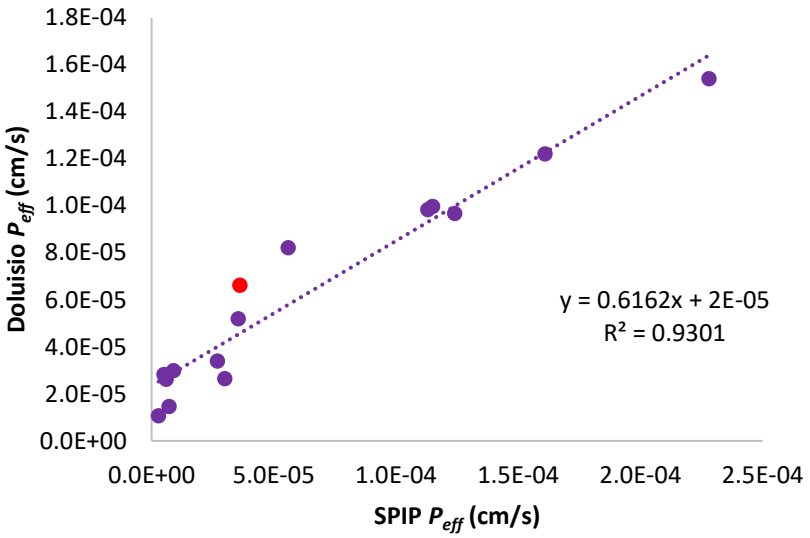

Figure 3.2.2. The correlation between the two sets of data, the SPIP vs. the Doluisio P_{eff} values. Metoprolol, the traditional low/high permeability class boundary marker, is highlighted in red.

The correlation between the permeability values obtained in this study and literature oral fraction dose absorbed, F_{abs}, in humans, of the different drugs, was evaluated, using the relationship:

$$F_{abs} = 1 - e^{\left(-P_{app}\frac{2}{R}T\right)} \quad (3)$$

where R represents the intestinal radius, and T the effective absorption time [42, 43]. Figure 3.2.3, Figure 3.2.4 and Figure 3.2.5 illustrate the correlations between F_{abs} and SPIP P_{eff}, Doluisio P_{eff}, and all P_{eff} data, respectively. It can be seen that in all cases a good fit was obtained between the predicted and the literature F_{abs} values, indicating the high capabilities of both rat perfusion models to predict human fraction dose absorbed.

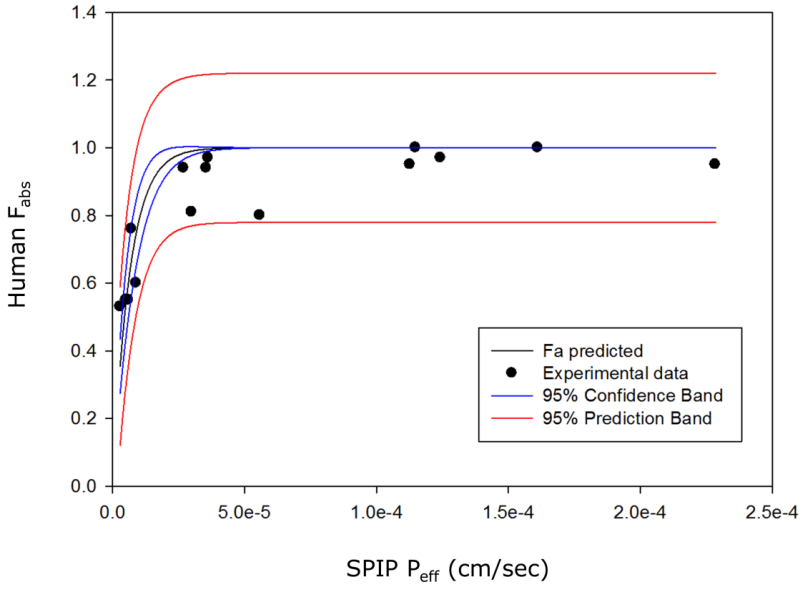

Figure 3.2.3. Correlation between the SPIP permeability values obtained in this study and the literature oral fraction dose absorbed, F_{abs}, in humans, of the different drugs.

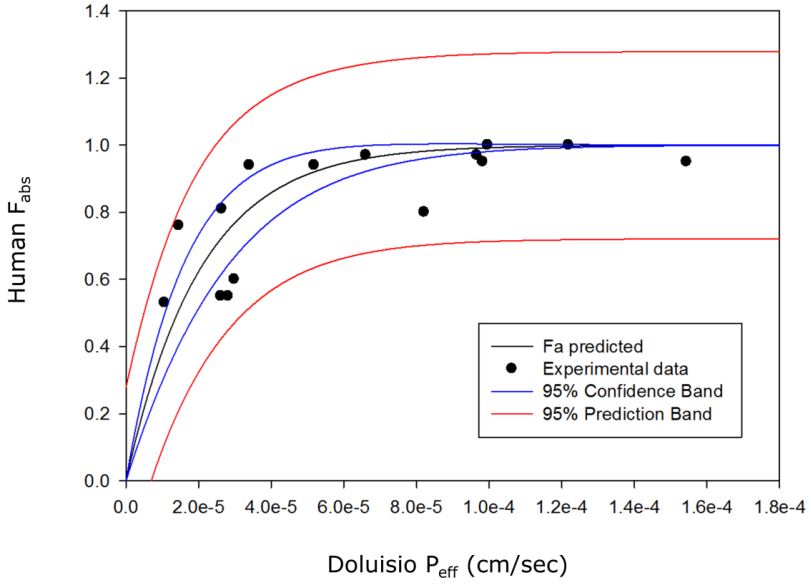

Figure 3.2.4. Correlation between the Doluisio permeability values obtained in this study and literature oral fraction dose absorbed, F_{abs}, in humans, of the different drugs.

Table 3.2.2 summarizes the literature human F_{abs} data, and the corresponding SPIP and Doluisio predicted F_{abs} and BCS permeability class. With the exception of one drug, digoxin, for which definite permeability classification could not be found, similar BCS permeability class membership was designated by literature data and by both SPIP and Doluisio methods. The predicted F_{abs} obtained by both SPIP and Doluisio methods were also in very good agreement to literature human F_{abs} values, and for both the SPIP and the Doluisio methods, all experimental values were within the 95% confidence band of the predicted fit.

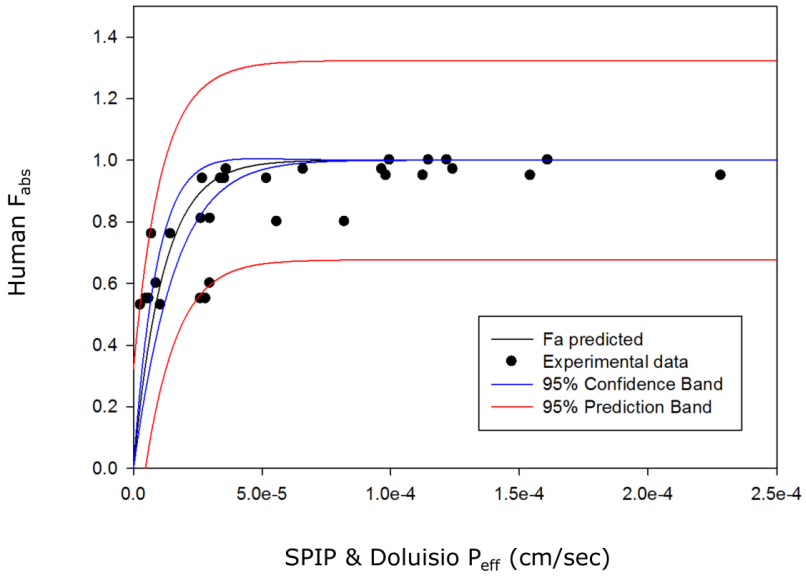

Figure 3.2.5. The correlation between the SPIP and Doluisio permeability values obtained in this study and literature oral fraction dose absorbed, F_{abs}, in humans, of the different drugs.

Table 3.2.2. Literature human F_{abs} data [44-48], and the corresponding SPIP and Doluisio predicted F_{abs} and BCS permeability class. H, high; L, low; N/A, not available.

The Drug	Literature human data		SPIP		Doluisio's rat perfusion	
	F_{abs} (%)	Permeability Class	Predicted F_{abs}	Permeability Class	Predicted F_{abs}	Permeability Class
Ibuprofen	95	H	100	H	99.9	H
Carbamazepine	100	H	100	H	99.7	H
Antipirine	97	H	100	H	99.1	H
Caffeine	100	H	100	H	99.2	H
Chloramphenicol	100	H	100	H	100	H
Paracetamol	80	H	99.9	H	98.2	H
Metoprolol	98	H	100	H	100	H
Codeine	94	H	99.5	H	92.0	H
Digoxin	81	N/A	99	H	72.5	L
Labetalol	94	H	98.4	H	88.0	H
Furosemide	60	L	74.8	L	76.7	L
Cimetidine	76	L	66.9	L	50.9	L
Valsartan	55	L	60.4	L	72.2	L
Colchicine	55	L	54.3	L	74.6	L
Atenolol	50	L	35.4	L	40.6	L

DISCUSSION

Rat intestinal perfusions represent an efficient and reliable experimental tool in human F_{abs} predictions, BCS classification, regional-dependent absorption, permeability mechanisms, and absorption related drug-drug interactions. The purpose of this study was to compare two different intestinal rat perfusion methods: the single-pass intestinal perfusion (SPIP) model and the Doluisio (closed-loop) rat perfusion method. A list of 15 model drugs was constructed to allow the comparison between the two experimental methods.

A coefficient of determination (R^2) of 0.93 was obtained when correlating the P_{eff} data from both experimental methods (Figure 3.2.2). This excellent correlation was achieved in spite of very different experimental settings. First and foremost, with the SPIP model, only a 10-cm long jejunal segment was perfused and used to determine the drugs' permeability. On the other hand, the entire small intestine was used to generate the P_{eff} values in the Doluisio method. Since permeability is position dependent and pertains to a specific point along the intestinal membrane [5, 7, 11, 20, 21], this difference could potentially lead to very different results between the two models. However, the SPIP and the Doluisio methods actually agreed with each other very well, and were able to correctly classify 14 of the 15 investigated drugs. This result is in corroboration with the good correlation that was found between human jejunal P_{eff} and the overall human fraction of dose absorbed [14, 19, 49, 50], indicating that in general the jejunum can be predictive for the overall absorption. Of course, there are cases in which looking solely at the jejunum cannot predict the overall absorption, as will be discussed hereinafter.

For 14 out of the 15 investigated drugs, similar BCS permeability class membership was designated by literature data and by both SPIP and Doluisio methods (Table 3.2.2.). The predicted fraction dose absorbed (F_{abs}) obtained by both SPIP and Doluisio methods (Figure 3.2.3, Figure 3.2.4 and Figure

3.2.5) were also in very good agreement to literature human F_{abs} values (Table 3.2.2). It should be noted that very frequently, literature human F_{abs} values do not include any indication for the margin of error, and only the average value is reported. Taking into account the expected variability in the human F_{abs} data, the good correlation between the experimental and the SPIP/Doluisio predicted F_{abs} can be even more appreciated. The only difficult case to analyze was that of digoxin; reports of its F_{abs} had large variability, no clear BCS permeability classification could be found in the literature for this drug, and different predictions were obtained by the two experimental models. While almost complete absorption was predicted by the SPIP model, F_{abs} value of only 72% was indicated by the Doluisio method. The transport of digoxin is very well known to be mediated by P-gp, and together with the drug's low dose, and intermediate physicochemical properties, inconsistent and unpredictable absorption can be expected [51-54]. In this case, the different settings of the two experimental methods may become significant; the expression of P-gp throughout the rat small intestine was shown to follow a gradient, increasing from the proximal to the distal segments [11, 34, 55]. Similar trend, with higher variability, was show in humans too [56-58]. By considering only the upper jejunum, an intestinal region with lower P-gp expression, the SPIP model may give a higher P_{eff} value for digoxin than the Doluisio method that takes into account the entire small intestine, including segments with high P-gp expression. This explains the single disagreement between the two experimental methods in the case of digoxin. This case also highlights that the correlation between the two techniques could be improved with standardization of the perfused intestinal segment, the pH/composition of the perfusion solution, and the drug concentration used.

Both experimental techniques follow the disappearance of drug from the intestinal lumen as a measure for drug absorption. Nevertheless, the experimental settings lead to significantly different kinetic conditions in each method. In the SPIP model the drug solution is perfused through the

intestinal segment until steady-state is reached in the segment, i.e., the overall input rate equals to the overall output rate. In this case the drug output from the perfused intestinal segment corresponds to the absorption process and to the drug leaving the intestine without being absorbed. In the closed-loop technique the drug solution is kept in the segment, and the change in drug concentration with time corresponds to the rate of drug permeation. As a consequence, a different mathematical treatment is necessary in order to estimate the drug permeability. Nevertheless, an excellent correlation was obtained between the two methods.

A noticeable difference in the obtained data was related to the range of the P_{eff} values; while in the SPIP model the permeability values ranged ~100-fold between $2.8 \cdot 10^{-6}$ and $2.2 \cdot 10^{-4}$ cm/sec, in the Doluisio method only ~10-fold range was obtained, from $1.0 \cdot 10^{-5}$ and $1.5 \cdot 10^{-4}$ cm/sec (Table 3.2.1). As a consequence, it may be easier to distinguish between drugs with close (but not similar) intestinal permeability using the SPIP model. This trend may be attributable to the use of short segment (SPIP) vs. the entire small intestine (Doluisio), as well as to the steady-state (SPIP) vs. non-steady-state (Doluisio) conditions. In any case, the drugs' P_{eff} rank was similar with both experimental models, and both were able to correctly classify all of the investigated drugs.

CONCLUSION

The single-pass intestinal perfusion (SPIP) model and the Doluisio (closed-loop) rat perfusion method are both equally useful for obtaining intestinal permeability values that can be used for F_{abs} prediction and BCS classification. A very good correlation was shown between the two experimental techniques, as well as between literature F_{abs} values and the predictions made by the rat perfusions. Similar BCS permeability class membership was designated by literature data and by both SPIP and Doluisio methods for all compounds.

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3.3. Artículo científico 2

Segmental-dependent permeability throughout the small intestine following oral drug administration: Single-pass vs. Doluisio approach to *in situ* rat perfusion

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ABSTRACT

Intestinal drug permeability is position dependent and pertains to a specific point along the intestinal membrane, and the resulted segmental-dependent permeability phenomenon has been recognized as a critical factor in the overall absorption of drug following oral administration. The aim of this research was to compare segmental-dependent permeability data obtained from two different rat intestinal perfusion approaches: the single-pass intestinal perfusion (SPIP) model and the closed-loop (Doluisio) rat perfusion method. The rat intestinal permeability of 12 model drugs with different permeability characteristics (low, moderate, and high, as well as passively and actively absorbed) was assessed in three small intestinal regions: the upper jejunum, mid-small intestine, and the terminal ileum, using both the SPIP and the Doluisio experimental methods. Excellent correlation was evident between the two approaches, especially in the upper jejunum (R^2=0.95). Significant regional-dependent permeability was found in half of drugs studied, illustrating the importance and relevance of segmental-dependent intestinal permeability. Despite the differences between the two methods, highly comparable results were obtained by both methods, especially in the medium-high P_{eff} range. In conclusion, the SPIP and the Doluisio method are both equally useful in obtaining crucial segmental-dependent intestinal permeability data.

Keywords: biopharmaceutics classification system, intestinal permeability, segmental-dependent permeability, oral drug absorption.

INTRODUCTION

Although oral drug absorption is a complex cascade of processes influenced by many physicochemical, physiological and pharmaceutical factors, the work of Amidon et al revealed that the solubility of the drug dose in the intestinal milieu, and the drug permeability through the intestinal membrane are the two key factors governing the fraction of dose absorbed

[1-3]. However, these two parameters are not constant throughout the gastrointestinal tract (GIT) [4, 5]. It is well recognized that the solubility of some drugs may differ dramatically as a function of the location throughout the GIT, for instance due to pH changes, or bile salts content. The regulatory requirement to supply solubility data in all luminal conditions before assigning a BCS (biopharmaceutics classification system) solubility classification is a reflection of this fact [6-9]. On the other hand, no parallel thinking currently exists for the BCS permeability classification, which is based merely on the jejunal permeability value. This policy may miss the complexity behind the permeability measure, considering the whole of the intestine; permeability is location dependent, and pertaining to each point throughout the GIT [7, 10-13]. The mechanisms behind segmental-dependent intestinal drug permeability may include ionization variations due to pH changes [4, 14-16], different expression levels of various influx/efflux transporters [17, 18], local water absorption, membrane structure/ composition variations, *etc.* [10, 19-21].

Human data on site-specific intestinal permeability would be highly appreciated and convincing. Recently, Sjögren et al indirectly estimated such human information from clinical trials that administered drug solutions to different intestinal regions [11, 22], but still, human regional-dependent intestinal permeability data is unfortunately very rare and hard to get [5, 23]. Cell culture based experimental approaches (Caco-2, MDCK, etc.) are also very limited in representing the intestinal membrane permeability process in all of its complexity. Currently, the main source for segmental-dependent permeability data is animal studies, typically in rats, which allow a fairly straightforward investigation of the different GIT regions. Given the lack of direct experimental data in humans, this experimental approach represents the best we can do today, providing a good indication of the expected situation in humans [24-26]. For instance, we have shown that the permeability of the H_2 blockers and P-gp substrates cimetidine varies along the rat intestine, and is inversely correlated with the P-gp expression levels

in the different segments [17, 27], while the same segmental-dependent permeability, at the same magnitude (approximately 2-fold), was reported for cimetidine intestinal absorption in humans [28].

The aim of this research was to compare segmental-dependent permeability data obtained from two different rat intestinal perfusion methods: the single-pass intestinal perfusion (SPIP) model and the Doluisio rat perfusion method. While both of these experimental methods measure disappearance from the intestinal lumen as an indication for drug absorption [29], they have substantial differences between them [30]. For instance, while in the SPIP model each drug molecule gets only one passage through the investigated intestinal segment, in the Doluisio method the tested drug solution remains within the intestinal lumen throughout the entire experiment. We have constructed a list of 12 model drugs with different permeability characteristics: low, moderate, and high permeability, as well as passively and actively absorbed compounds. We assessed the rat intestinal permeability of these 12 model drugs in three small intestinal regions: the upper jejunum, mid-small intestine, and the terminal ileum, using both SPIP and the Doluisio experimental methods, and evaluated the correlation between them. While we have recently presented analysis concerning the upper small intestine [30], this is the first time to analyse segmental-dependent permeability using this two experimental approaches. Overall, this research provides a deeper insight into segmental-dependent permeability following oral drug administration, with emphasize on the two experimental approaches investigated.

MATERIAL AND METHODS

Materials

Acetaminophen, antipyrine, caffeine, codeine, digoxin, furosemide, glipizide, ibuprofen, labetalol, metoprolol, pseudoephedrine and sotalol

were purchased from Sigma-Aldrich. Methanol, acetonitrile and water were HPLC grade. All other chemicals were of analytical reagent grade.

Single-pass rat intestinal perfusion

The single-pass intestinal perfusion studies were performed using protocols approved by the Ben-Gurion University of the Negev Animal Use and Care Committee (Protocol IL-08-01-2015). Male Wistar rats (280–320 g, Harlan, Israel) were housed and handled according to the Ben-Gurion University of the Negev Unit for Laboratory Animal Medicine Guidelines.

The technique used for the *in situ* single-pass perfusion experiments followed previous reports [27]. Rats were anesthetized with an intra-muscular injection of 1 mL/kg of ketamine–xylazine solution (9%:1%, respectively), placed on a heated surface (37 °C; Harvard Apparatus Inc., Holliston, MA), and a 3 cm midline abdominal incision was made. Three individual 10 cm segments of the small intestine were simultaneously perfused in each rat: (1) the proximal jejunum, starting 2 cm distal to the ligament of Treitz; (2) mid-small intestine, between the end of the upper and the start of the lower segments; and (3) the distal ileum, ending 2 cm proximal to the caecum. The three segments were cannulated on two ends, and were rinsed witch blank perfusion buffer. All solutions were incubated in a 37° C water bath.

To closely mimic the physiological intestinal conditions, each segment was perfused with a phosphate buffer containing the investigated drug and the physiological pH relevant to each segment: 6.5 for the upper jejunal segment, 7.0 for the mid-small intestine, and pH 7.5 for the ileal segment. These perfusion buffers (freshly prepared 30 min prior to starting the experiments) were prepared by adding different ratios of potassium phosphate monobasic and sodium phosphate dibasic to give the pH values of 6.5, 7.0 and 7.5, while osmolality (290 mOsm/L) and ionic strength (50 mM) were similar in all buffers. The perfusion buffer was perfused through

the intestinal segment (Watson Marlow 205S, Watson-Marlow Bredel Inc., Wilmington, MA) at a flow rate of 0.2 mL/min, first for 1 h without sampling, to ensure steady state conditions, followed by additional 1 h of perfusion with samples (2 mL) taken every 10 min. The pH of the collected samples was measured at the outlet, to verify that the pH did not change throughout the perfusion. The samples were immediately assayed for drug content by UPLC. The exact length of each perfused intestinal segment was measured at the endpoint of the experiment.

The effective permeability (P_{eff}; cm/sec) through the rat gut wall was determined according to the following equation:

$$P_{eff} = \frac{-Qln(C'_{out}/C'_{in})}{2\pi RL} \quad (1)$$

where *Q* is the perfusion buffer flow rate (0.2 mL/min), C'_{out}/C'_{in} is the ratio of the outlet and the inlet concentration of drug that has been adjusted for water transport via the non-absorbable marker phenol red [18, 31-33], *R* is the radius of the intestinal segment (set to 0.2 cm), and *L* is the length of the perfused intestinal segment.

Doluisio's (closed-loop) rat intestinal perfusion

The Doluisio studies were approved by the Scientific Committee of the Faculty of Pharmacy, Miguel Hernandez University, and followed the guidelines described in the EC Directive 86/609, the Council of the Europe Convention ETS 123 and Spanish national laws governing the use of animals in research. The experimental protocols were also approved by the Ethics Committee for Animal Experimentation of the University of Valencia (Spain, code A1330354541263).

The absorption rate coefficients and the permeability values of each model drug were evaluated by the *in-situ* close-loop perfusion method based on Doluisio's technique [34], in the same three 10 cm segments appointed in

the single-pass intestinal perfusion method: the upper jejunum, mid-small intestine, and the distal ileum. Male Wistar rats (body weight, 260-300 g), fasted 4 hours prior to the experiment with free access to water, were used in these studies. Rats were anesthetized using a mixture of diazepam (1.67 mg/kg, Valium, Roche), ketamine (50 mg/kg, Ketolar, Parke-Davis), and atropine (1 mg/kg, Atropina sulfato, Braun), and were placed on heated surface maintained at 37 °C. A midline abdominal incision was made, and the small intestine was exposed. The perfusion technique consists of creating three isolated compartments in the three intestinal segments of interest with the aid of six syringes and six three-way stopcock valves. Care was taken to avoid disturbance of the intestinal blood supply. In order to remove all intestinal contents, the three segments were thoroughly flushed with a warm physiologic solution at the physiologic pH of each segment. The catheters were then connected to a glass syringe using a stopcock three-way valve. This set up ensures the isolation of the three compartments, and drug solution (pH 6.5 for the upper segment, 7.0 for the middle segment, and pH 7.5 for the distal segment, similar to the SPIP buffers) were then introduced into each segment and sampled with the aid of the syringes and stopcock valves. The samples were collected every 5 min up to a period of 30 min [35, 36]. The length of the perfused intestinal segment was accurately measured at the endpoint of the experiment, as well as the pH.

Water flux during the experiment may be significant, and hence must be accounted for [33]. A method based on direct measurement of the remaining solution volume was employed to calculate the water reabsorption zero order constant (k_0). For each segment, the initial volume (V_0) was determined, and the endpoint volume (V_t) was measured. The drug concentration in the samples was corrected as: $C_t=C_e(V_t/V_0)$, where C_t represents the concentration in the absence of water reabsorption at time t, and C_e the experimental value. The corrected concentration, C_t, was then used as the actual absorption rate coefficient calculations [37].

The absorption rate coefficients (k_a) of the compounds were determined by nonlinear regression analysis of the remaining concentrations in the intestinal lumen (C_t) vs. time:

$$C_t = C_0 \cdot e^{-k_a t} \quad (2)$$

These absorption rate coefficients were then transformed into permeability values using the relationship: $P_{app} = k_a \cdot R/2$, where R is the effective radius of the intestinal segment, calculated from the area/volume relationship considering a 2 mL volume and the measure of each segment length in each animal.

Analytical methods

Analytical analyses of the SPIP and the Doluisio samples were performed on a Waters (Milford, MA) Acquity UPLC H-Class system, and a Waters 2695 HPLC Separation Module, respectively. Both systems were equipped with photodiode array (PDA) detector and Empower software.

The simultaneous determination of the investigated drug and the non-absorbable marker phenol red in the SPIP samples was achieved using a Waters (Milford, MA) Acquity UPLC BEH C_{18} 1.7 µm 2.1x100 mm column. The determination of the investigated drugs in the Doluisio samples were carried out using a Waters C_{18} 3.5 µm 4.6x250 mm XTerra column. A general gradient mobile phase consisted of 90:10 going to 20:80 (v/ v) water:acetonitrile (both with 0.1% TFA) over 7 min was suitable for all analyses. Injection volumes ranged 5-20 µL in all analyses.

Statistical analysis

All animal experiments were replicated with n= 4-7. Values are expressed as mean ± standard deviation (SD). Permeability values determined in each intestinal segment and perfusion method were compared using Student's t-test to detect the existence of significant

differences at the 0.05 probability level. All statistical analyses were made using the statistical package SPSS V.20, as well as STATA software.

RESULTS

The correlation between the permeability values (cm/sec) obtained for the 12 model drugs using the SPIP vs. the Doluisio experimental methods in the jejunum (upper panel), mid small intestine (middle panel) and in the ileum (lower panel) are presented in Figure 3.3.1. The shadowed area around the trendline represents the standard error (SE). These results illustrate that excellent correlation exists between the SPIP and the Doluisio approaches in the jejunum (R^2=0.95). A good correlation between the two experimental methods was also obtained in the mid-SI (R^2=0.9), as well as in the lower ileum (R^2=0.85), yet not as good as the upper segment.

A broader look on the correlation between the SPIP and the Doluisio approaches is illustrated in Figure 3.3.2, where all of the 36 permeability values gathered in these experiments (12 drugs x 3 small intestinal segments) are shown altogether. A very high correlation between the two sets of data can be seen, as evident by a coefficient of determination (R^2) of 0.9. This good correlation highlights that in spite of the significant differences between the two methods, e.g. single-pass vs. closed-loop, and equilibrium vs. non-equilibrium, both approaches track the same fundamental process, and use disappearance of drug from the gut lumen as a measurement for absorption, which eventually governs the final result.

In Figure 3.3.3 we have examined the Doluisio/SPIP ratio of the 36 permeability values obtained in this work. This Figure reveals that in the medium-high P_{eff} range, the permeability values obtained by the two methods resembled a lot (as evident by Doluisio/SPIP ratio close to 1). On the other hand, in the low-permeability range the Doluisio method produces higher values than the SPIP approach, as evident by Doluisio/SPIP ratio significantly higher than 1. This trend was irrespective of the small intestinal

segment being examined, and was shown to be stronger as the permeability value gets lower.

The permeability values (cm/sec) obtained for the 12 model drugs in the three small-intestinal segments (jejunum, upper panel; mid-SI, middle panel; ileum, lower panel) using both the SPIP approach (blue bars) and the Doluisio experimental method (red bar) are shown in Figure 3.3.4. The 12 drugs were organized from the lowest to the highest permeability, according to the proximal jejunum (upper panel), and this order was maintained in the other panels; it can be seen that already at the mid-SI, and more profoundly at the lower ileum, this order failed to represent the lowest to the highest P_{eff} order. This illustrates the importance of segmental-dependent permeability throughout the gastrointestinal tract; it shows that low-permeability in the jejunum does not necessarily indicate that this drug will show low-permeability in other intestinal regions, as will be further discussed hereinafter.

DISCUSSION

In this work, a comparison of two different rat intestinal perfusion methods, the single-pass intestinal perfusion (SPIP) approach and the Doluisio rat perfusion model, is presented. Specifically, we aimed to study the ability of these experimental approaches to investigate segmental-dependent permeability throughout the small intestine.

Traditionally, the Doluisio (closed-loop) method is performed on the entire small intestine, and we have previously evaluated permeability results of 15 model drugs obtained in this manner [30]. An excellent correlation (R^2=0.93) was obtained between the jejunal SPIP and the Doluisio results, in spite of the fact that the entire SI was used in the Doluisio method.

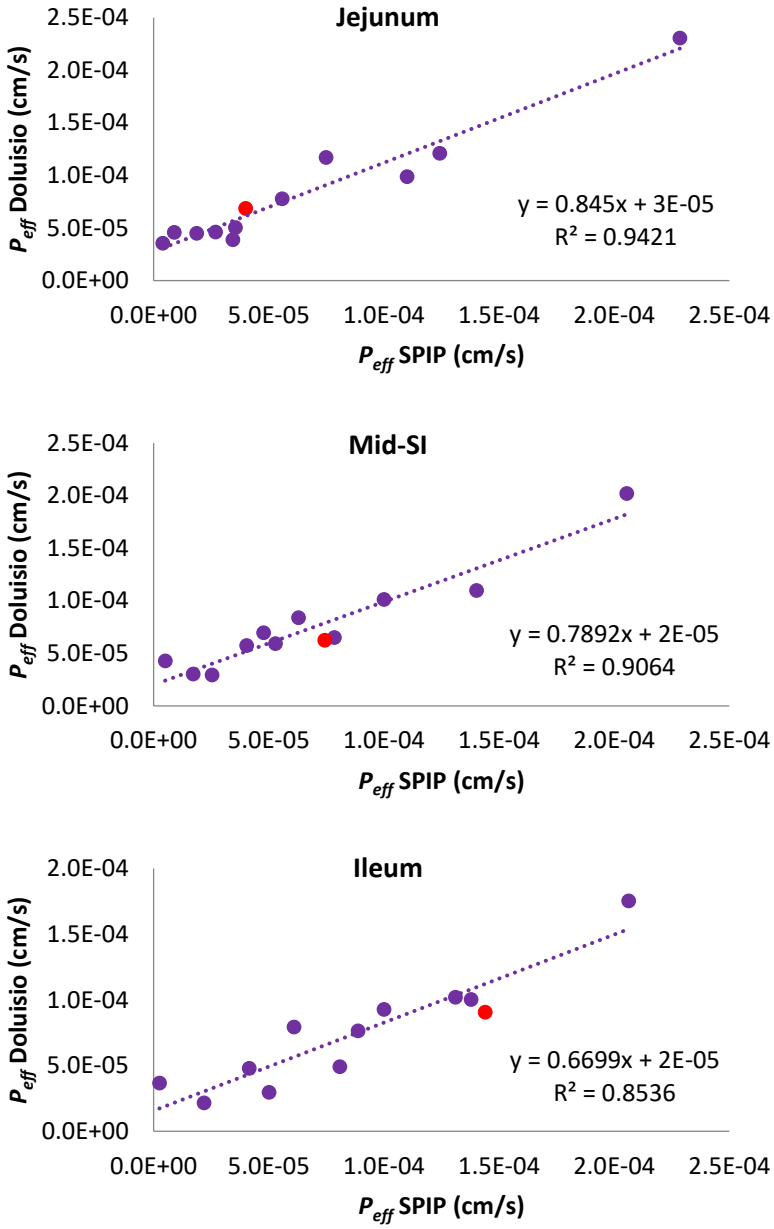

Figure 3.3.1. The correlation between the permeability values (cm/sec) obtained for the 12 model drugs using the SPIP vs. the Doluisio experimental methods in the jejunum (upper panel), mid-small intestine (middle panel) and in the ileum (lower panel). The red symbol corresponds to metoprolol.

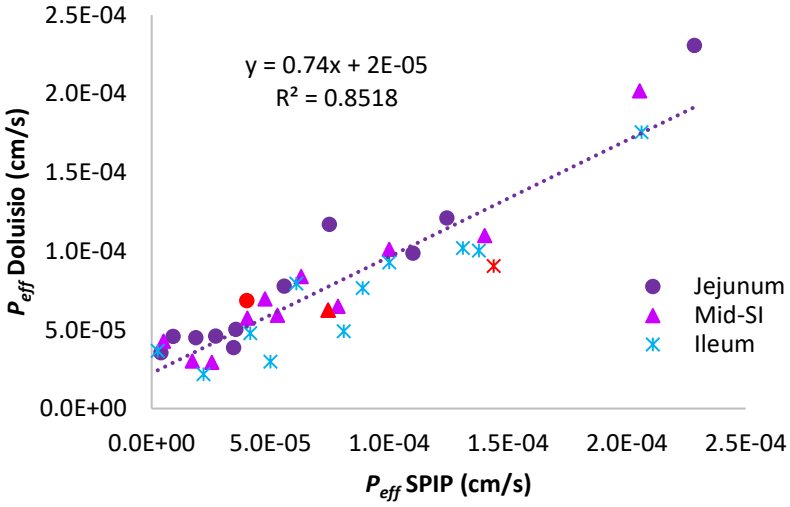

Figure 3.3.2. The correlation between all of the 36 permeability values obtained in this study using the SPIP vs. the Doluisio experimental methods. Red symbols correspond to metoprolol.

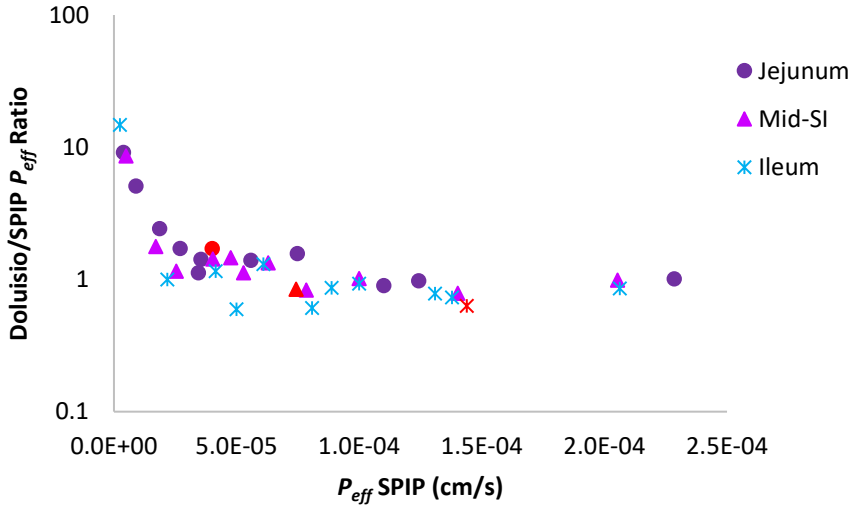

Figure 3.3.3. Examination of the Doluision/SPIP ratio of the 36 permeability values obtained in this work. Red symbols correspond to metoprolol.

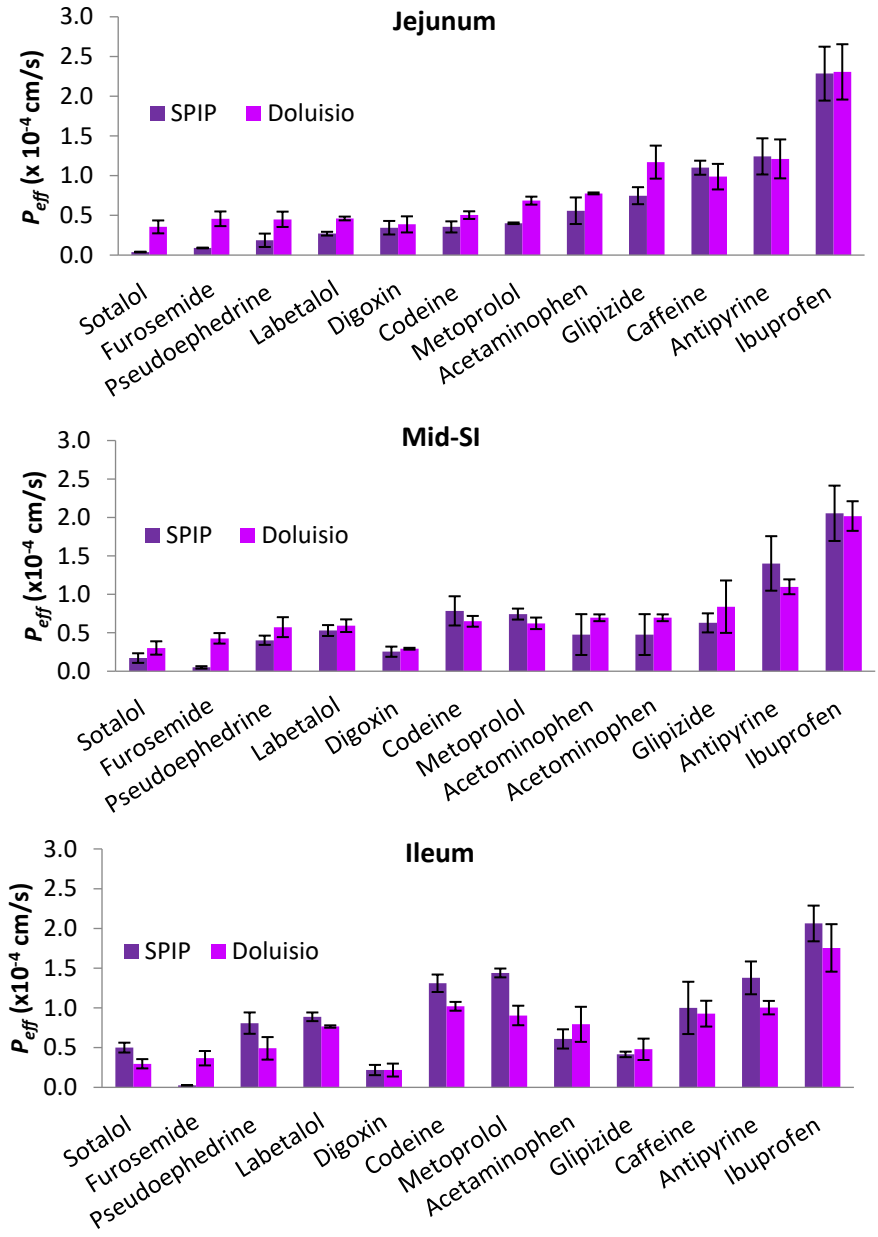

Figure 3.3.4. The permeability values (cm/sec) obtained for the 12 model drugs in the three small-intestinal segments (jejunum, upper panel; mid-SI, middle panel; ileum, lower panel) using both the SPIP approach (dark purple bars) and the Doluisio experimental method (light purple bar).

In the current work, even better correlation was evident for the upper intestinal segment (R^2=0.95) between the SPIP and the Doluisio results, since this time the Doluisio method was focused solely on a 10-cm jejunal segment, similarly to the SPIP approach. Yet, the excellent correlation obtained previously reveals that when the entire SI was used in the Doluisio method, the jejunal segment was the dominant one, dictating the overall result. This may be attributable to the fact that a jejunal buffer (pH 6.5) was used in the entire SI Doluisio experiments, and hence those studies closely represented the upper SI and were highly correlated to the jejunal SPIP results. This analysis highlights that using too long intestinal segment in permeability studies may miss the complexity of the small intestine, as a complicated organ with a diverse environment throughout.

Permeability is position dependent and pertains to a specific point along the intestinal membrane [5, 10, 38]. A variety of mechanisms may result in the phenomenon of segmental-dependent permeability, in which a certain drug may move from low- to high-permeability, and *vice versa*, during the travel throughout the gastrointestinal tract. As demonstrated in this work, this may happen not only due to differences between the small and the large intestine, but even between the different segments of the small intestine itself. Table 3.3.1 summarizes the permeability classification of the 12 model drugs in the three small intestinal regions investigated, using the SPIP vs. the Doluisio method. The classification was determined compared to the jejunal P_{eff} value of metoprolol (for each experimental method); values lower than metoprolol's jejunal P_{eff} indicated lowpermeability, while values similar/higher than metoprolol's jejunal P_{eff} indicated high permeability. It can be seen that for five drugs (over 40%) a permeability classification switch was detected throughout the small intestine, highlighting the relevance and significance of accounting for regional variations throughout the GIT in drug permeability classification. It should be noted that significant different permeability values in the different small intestinal regions were very likely to occur in two situations: (1) when active transporters are known to play a

significant role in the overall absorption process, e.g. digoxin, furosemide, and glipizide; and (2) when the pK_a and Log *P* of the drug molecule support a significant ionization state variations in the small intestinal pH range, e.g labetalol, sotalol, and pseudoephedrine. Occasionally, both mechanisms may be relevant for the same drug molecule, e.g. glipizide and furosemide. An interesting phenomenon was observed for the cases where segmental-dependent passive permeability was significant due to pHdependent lipophilicity: while the segmental differences obtained with the SPIP approach were in accordance with the theoretical distribution coefficients (Log D), the Doluisio's method showed much lower differences than expected. This may be attributable to the critical difference between the two models: single-pass of the solution through the intestinal segment vs. closed loop. With the SPIP approach, fresh buffered solution is continuously perfused through the intestinal segment, and thus, the pH is kept at the initial value. On the other hand, with the Doluisio technique it is possible that during the experiment, since the same buffer remains constantly within the investigated intestinal segment, the relatively long contact time of the perfusion solution with the intestinal compartment, the water reabsorption and the intestinal secretions interchange, alter the initial buffer pH. This may increase and decrease the buffer pH in the proximal and distal segments, respectively, thus masking the regional-dependent permeability in these cases. For this reason, the segmental P_{eff} differences in the Doluisio method were attenuated, and even when permeability trend similar to that of the SPIP approach was observed, no statistical significant difference could be detected. This analysis reveals another technical difference between the two methods: with the SPIP approach a fresh drug solution is continuously perfused through the investigated intestinal segment, and hence, a large volume of drug solution is required for these experiments. On the other hand, since with the Doluisio technique the same drug solution remains constantly within the investigated intestinal segment, a small volume of drug solution is required for this method. This may be critical when the

investigated drug is very expensive, or during initial stages of drug development, when only small quantity of the drug is available (Table 3.3.2).

A close look at Figure 3.3.3 reveals that in the medium-high P_{eff} range, the permeability values obtained by the 2 methods resembled a lot (as evident by Doluisio/SPIP ratio close to 1), while in the low-permeability range the Doluisio method produces higher values than the SPIP approach, as evident by Doluisio/SPIP ratio significantly higher than 1. This trend was irrespective of the small intestinal segment being examined, and was shown to be stronger as the permeability value gets lower. This phenomenon may be related to the equilibrium/non-equilibrium state of the drug transport; while with the SPIP approach steady-state is assured before starting the permeability measurements, the Doluisio method measures the initial, non-equilibrium phase of the drug transport. It should be noted that in our previous SPIP-Doluisio comparison when the entire SI was used in the Doluisio method, this phenomenon was even more profound; in that work both the low-permeability and the high-permeability range were narrowed in the Doluisio technique. As a consequence, while in the SPIP model the permeability values ranged 100-fold between the lowest to the highest P_{eff}, in the Doluisio method only 10-fold range was obtained [30]. In this current work, where the Doluisio method was focused solely on a 10-cm intestinal segment, the high-permeability range was similar to that obtained with the SPIP method, highlighting the advantage of measuring the permeability in individual restricted intestinal regions rather than in the entire SI. In spite of all of the discussed differences between the two approaches, the correlation between both methods was found to be very good in the three small intestinal segments, with minimal variations. Moreover, the BCS permeability classification relative to metoprolol's jejunal permeability was highly comparable with both methods, supporting our previous analysis that states that if a compound has high fraction of dose absorbed, it will have high-permeability, not necessarily in the jejunum, but somewhere along the GIT.

Table 3.3.1. The permeability classification of the 12 model drugs in the 3 small intestinal segments, using the SPIP approach vs. the Doluisio method. The classification was determined compared to the jejunal P_{eff} value of metoprolol (for each experimental method). H, high; L, low; N/A, not available.

Drug	*F*abs	Literature P_{eff} Classification	SPIP			Doluisio		
			Jejunum	Mid-SI	Ileum	Jejunum	Mid-SI	Ileum
Glipizide	100	H	H	H	H	H	H	L
Caffeine	100	H	H	H	H	H	H	H
Metoprolol	98	H	H	H	H	H	H	H
Antipyrine	97	H	H	H	H	H	H	H
Ibuprofen	95	H	H	H	H	H	H	H
Sotalol	95	H	L	L	H	L	L	L
Pseudoephedrine	95	H	L	H	H	L	L	L
Labetalol	94	H	L	H	H	L	L	H
Codeine	94	H	H	H	H	L	L	H
Digoxin	81	N/A	H	L	L	L	L	L
Paracetamol	80	N/A	H	H	H	H	H	H
Furosemide	60	L	L	L	L	L	L	L

Table 3.3.2. Relevant literature physicochemical properties of the 12 model drugs, the solubility classification, and the concentrations (mM) used in all of the permeability experiments. H, high; L, low; N/A, not available.

Drug	Concentration (μM)	pK_a	Log *P*	Literature Solubility Classification
Glipizide	100	5.9	1.9	L
Caffeine	500	14; 0.6	-0.07	L
Metoprolol	500	9.7	1.9	H
Antipyrine	200	2.2; 1.4	-1.1	H
Ibuprofen	250	5.2	4	L
Sotalol	500	9.8, 8.3	0.2	H
Pseudoephedrine	100	9.9	1.1	H
Labetalol	500	7.3; 8.8	1.3	H
Codeine	750	7.9	1.1	H
Digoxin	100	7.1	1.2	L
Paracetamol	330	9.9	0.2	N/A
Furosemide	500	4.7; 3.9	2	L

CONCLUSIONS

In conclusion, the SPIP and the Doluisio methods presented a very good correlation in the three studied small intestinal segments, and were demonstrated to be valuable approaches to elucidate segmental permeability differences. Both methods were able to correctly classify drugs according to the BCS. Irrespective of the experimental method, it is advisable to measure the permeability in individual restricted intestinal segments, rather than using wider regions.

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4. PREDICCIÓN DE LA ABSORCIÓN EN COLON

Trabajos científicos realizados durante más de 40 años han descrito y demostrado que existe una buena correlación entre la permeabilidad obtenida en intestino delgado de rata e intestino delgado de humanos, así como con la fracción absorbida en intestino humano, lo que ha permitido considerar el intestino delgado de rata como un modelo válido para predecir la absorción intestinal en humanos [1-4].

Con la aparición y difusión comercial de las formas de liberación controlada el colon cobra un papel protagonista como órgano de absorción. Además de ello, la mayor prevalencia de enfermedades de colon (cáncer de colon o enfermedad inflamatoria intestinal) ha hecho necesario obtener formulaciones que liberen el principio activo en colon para aumentar la eficacia terapéutica minimizando los efectos adversos. Sin embargo, la cantidad de estudios publicados sobre la absorción en colon es considerablemente menor comparada con aquellos relacionados con el intestino delgado. Tal y como postulan Sjögren y colaboradores en su publicación titulada "*In vivo* methods for drug absorption – Comparative physiologies, model selection, correlations with *in vitro* methods (IVIVC), and applications for formulation/API/excipient characterization including food effects" del año 2014, parece que el colon de rata puede ser útil para predecir la absorción de fármacos en colon de humanos y, de este modo, obtener datos que puedan aplicarse a la formulación de productos de liberación controlada [5]. Sin embargo, aunque la absorción en colon es un campo de investigación en auge en la actualidad y se están realizando muchos estudios respecto a modelos *in vitro* e *in situ* de permeabilidad y mecanismos de absorción y correlaciones, se requiere un análisis sistemático para validar las técnicas y poder determinar la fiabilidad de los modelos [5]. Además, los datos publicados de absorción en colon [6-8] no son suficientes para generar un modelo animal para predecir la permeabilidad en colon de humanos.

Asimismo, para conseguir una validación completa del modelo animal empleado y de esta manera considerarlo útil para predecir la absorción en

colon se requieren datos precisos, obtenidos en humanos, de distintos parámetros de absorción de este tramo del tracto gastrointestinal [9]. Pero, la dificultad y los problemas éticos que conllevan los ensayos *in vivo* para obtener datos de absorción en colon de humanos justifican la escasez de datos existente. Sin embargo, en los últimos años se han comenzado a publicar estudios en los que se ha determinado la permeabilidad en colon humano usando diferentes técnicas menos invasivas y poco problemáticas. Una de ellas emplea la técnica de la cámara Ussing para determinar la permeabilidad en colon utilizando tejido de colon humano escindido en cirugías [10, 11]. Otra técnica, desarrollada recientemente, consiste en utilizar un método de deconvolución para estimar de manera indirecta la permeabilidad en distintas regiones del tracto gastrointestinal humano. Este método de deconvolución estima la permeabilidad usando los perfiles plasmáticos concentración-tiempo después de administrar la disolución del compuesto de interés en una región específica del tracto intestinal [12, 13].

En el presente capítulo se muestra la validación de una técnica de perfusión *in situ* en colon de rata. Puesto que en el capítulo anterior se ha demostrado que el método de Doluiso es un método de perfusión igual de fiable que la técnica de SPIP, la técnica de perfusión *in situ* empleada en los ensayos en colon de rata que se describe en este capítulo está basada en el método de Doluisio. Además, para obtener una validación lo más completa posible, se han establecido correlaciones entre los datos de permeabilidad en colon de rata y los obtenidos a partir de otros modelos diferentes (PAMPA, cultivos celulares y cámara Ussing), así como con datos humanos obtenidos de los estudios publicados hasta la fecha.

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4.1. Artículo científico 3

In situ perfusion model in rat colon for drug absorption studies: comparison with small intestine and Caco-2 cell model

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ABSTRACT

Our aim is to develop and to validate the *in situ* closed loop perfusion method in rat colon and to compare with small intestine and Caco-2 cell models. Correlations with human oral fraction absorbed (Fa) and human colon fraction absorbed (Fa_colon) were developed to check the applicability of the rat colon model for controlled release (CR) drug screening. Sixteen model drugs were selected and their permeabilities assessed in rat small intestine and colon, and in Caco-2 monolayers. Correlations between colon/intestine/Caco-2 permeabilities versus human Fa and human Fa_colon have been explored to check model predictability and to apply a BCS approach in order to propose a cut off value for CR screening. Rat intestine perfusion with Doluisio's method and single-pass technique provided a similar range of permeabilities demonstrating the possibility of combining data from different laboratories. Rat colon permeability was well correlated with Caco-2 cell-4 days model reflecting a higher paracellular permeability. Rat colon permeabilities were also higher than human colon ones. In spite of the magnitude differences, a good sigmoidal relationship has been shown between rat colon permeabilities and human colon fractions absorbed, indicating that rat colon perfusion can be used for compound classification and screening of CR candidates.

Keywords: colon absorption, fraction absorbed, permeability, absorption, colonic drug delivery, *in vitro* models, Caco-2 cells, site-specific absorption.

INTRODUCTION

The small intestine is the main site of absorption, because of its anatomical, physiological, physicochemical environment and biopharmaceutical features [1]. Traditionally, the colon has been considered less important than the small intestine for drug absorption. Anatomically, colon is shorter and wider than small intestine; colonic lumen has not extra

surface area provided by villi; moreover, epithelial cell layer junctions are tighter in colon than in small intestine. So, the available surface for drug absorption in colon is smaller and morphologically less well equipped than that of the small intestine [1-4]. Differences also affect the expression of efflux and uptake transporters. Intestinal drug transporters are predominant in the jejunum and ileum [5, 6] and, according to Drozdzik et al. [7] the transporter protein abundance was similar in the jejunum and ileum but markedly different in the colon. In fact, the major transporter protein in the small intestine (around 50%) was the uptake carrier PEPT1, and in the colon, the major transporter was the basolateral efflux carrier ABCC3 [8]. In addition, the gastrointestinal tract is populated with a large number of bacteria and most of them reside in the large intestine; the stability of a drug to the microbiota is clinically relevant; the metabolism can transform a drug in pharmacologically active, inactive or toxic. Many drugs are substrates for colonic bacteria, which could result in degradation and influence their bioavailability [9, 10]. These arguments also support that the permeability and the absorptive capacity for drugs of the colonic membrane are considered lower than the small intestine. However, this is not always so simple. Distal regions of intestine, including the colon, can significantly contribute to the overall absorption as well. Several studies have demonstrated that some drugs show fairly high permeability in colon [11-14].

Colon has one main advantage for absorption: the transit time of drugs. The colonic transit time is longer than 24 h, whereas the small intestinal transit time is shorter about 2-5 h [15, 16]. Thus, colonic absorption can be beneficial to ensure the performance of oral controlled released (CR) products. As CR formulations are designed to release the drug during 12-24 h, it is obvious that drug absorption in colon is a prerequisite for oral CR development [3, 13]. Despite the fact that there are various *in vitro* and *in situ* models to predict the absorption and permeability in small intestine, there is less knowledge regarding the large intestine [1, 17]. There is a need

for exploratory *in vivo* studies to clarify regional drug absorption along the intestine, and especially from the colon in order to validate animal or *in vitro* biopharmaceutical colon models as these models would have a clear impact for the study of oral CR formulations [4].

It is well known that oral drug absorption is defined mainly by two parameters: solubility and permeability. These parameters are the basis for the Biopharmaceutics Classification System (BCS) for immediate release (IR) products developed by Amidon et al. [18]. The premise of BCS is that the rate and extent of drug absorption depends on the solubility and permeability of the drug. The applicability of BCS for CR formulations and colonic absorption has been discussed [19-21]. Some authors have remarked that to extend the BCS model to the CR formulations with a simple measure of permeability is not adequate [21], but on the contrary, Tannegren et al. [1] demonstrated that compounds with high permeability had good absorption in colon, whereas low permeability drugs presented unfavorable colon absorption, that is, permeability data could be used for early assessment of the colonic absorption potential during CR development.

The first purpose of this work is to develop and to validate the technique of *in situ* perfusion in rat colon based on Doluisio's method and to correlate the colon permeability values with permeability data obtained in small intestine to characterize the segmental differences in the rat model. Moreover, in order to obtain a simpler model for screening drug absorbability in colon, the second objective is to compare the animal model with an *in vitro* model based on Caco-2 monolayers. For those purposes, 16 model drugs with different permeability characteristics have been selected and the permeability has been assessed in both segments of the rat gastrointestinal tract: small intestine and colon, and in Caco-2 monolayers. Small intestine/colon and *in situ/in vitro* correlations and the comparison with literature *in vivo* data will be used to apply a BCS approach to colon

absorption in order to propose a cut off value for screening during CR product development.

MATERIALS AND METHODS

Antipyrine, caffeine, carbamazepine, metoprolol, naproxen, theophylline, verapamil, amoxicillin, atenolol, cimetidine, codeine, colchicine, furosemide, ranitidine, terbutaline and valsartan were purchased from Sigma-Aldrich. Methanol, acetonitrile and water were HPLC grade. All other chemicals were of analytical reagent grade.

Table 4.1.1 summarizes the contribution of transporters that can affect the permeability of the test compounds and some physicochemical properties [1, 6, 22-42]. In this work a high concentration of drugs (500 μM) was used to ensure saturating conditions in order to get the diffusional component of the transport and a negligible contribution of the transporters.

Absorption Studies

Male Wistar rats were used in accordance with 2010/63/EU directive of September 22, 2010 regarding the protection of animals used for scientific experimentation. The Ethics Committee for Animal Experimentation of the University of Valencia approved the experimental protocols (Spain, code A1330354541263).

The absorption rate coefficient and the permeability value in the whole small intestine of each compound was evaluated by *in situ* "close loop" perfusion method based on Doluisio's Technique [43]. Male Wistar rats (body weight, 250-300 g) fasted 4 hours and with free access to water were used for these studies. Rats were anesthetized using a mixture of pentobarbital (40 mg/kg) and butorphanol (0.5 mg/kg). A midline abdominal incision was made. The intestinal segment was manipulated in order to minimize any intestinal blood supply disturbances. The bile duct was always tied up in

order to avoid drug enterohepatic circulation and the presence of bile salts in lumen. The perfusion technique consists of creating an isolated compartment in the intestinal segment of interest, with the aid of two syringes and two three-way stopcock valves. Two incisions were performed in the intestine, the first one at the beginning of the duodenal portion and the second one at the end of the ileum portion just before the cecum sac. Surgical ligature to a catheter was placed at the duodenal incision. In order to remove all intestinal contents, the small intestine was copiously flushed with a physiologic solution: isotonic saline (pH 6.9) with 1% Sörensen phosphate buffer (v/v), 37 °C. After that, the end of the segment was tied up and connected to a second catheter. Both catheters were connected to a glass syringe using a stopcock three-way valve. The intestinal segment was carefully placed back into the peritoneal cavity and the abdomen was covered with a cotton wool pad avoiding peritoneal liquid evaporation and heat losses. Once this system is set up, the intestinal segment is an isolated compartment where the drug solution (10 mL) can be introduced and sampled with the aid of the syringes and stopcock valves. The samples were collected every 5 minutes up to a period of 30 minutes.

The absorption rate coefficient and the permeability value in the colon were evaluated with a similar technique to that used for the whole small intestine [3]. The colon was flushed with a physiologic solution: isotonic saline (pH 7.5) with 1% Sörensen phosphate buffer (v/v), 37 °C. The drug solution (5 mL) was introduced in the isolated compartment and it was sampled at fixed times.

Drug solutions for experiments in colon and intestine were prepared in isotonic saline buffered with Sörensen phosphate buffer (pH 7.0). For furosemide and valsartan, 1% DMSO (dimethyl sulfoxide) was used to achieve complete dissolution of these compounds.

Table 4.1.1. Transporters involved on the intestinal permeation of each drug and physicochemical properties.

Drug	*CYP 3A4*	Efflux transporters			Uptake transporters			Physicochemical properties		
		P-gp	**MRP**	**BCRP**	**OATP**	**PEPT1**	**OCT**	**MW**	**LogP**	**pKa**
Antipyrine [22]	x							188.2	0.38	1.50[a]
Caffeine [23, 24]		x						212.2	-0.07	10.4[b]
Carbamazepine [25, 26]								236.3	2.45	13.9[b]
Metoprolol [27]								267.4	1.88	9.70[b]
Naproxen [28]					x			230.3	3.18	4.15[a]
Theophylline [1]								180.2	-0.02	8.81[b]
Verapamil [6, 28-30]	x	x	x	x	x	x	x	491.1	3.79	8.92[b]
Amoxicillin [1, 6]		x				x		365.4	0.87	2.40[a]
Atenolol [1, 6, 31]			x		x			266.3	0.16	9.60[b]
Cimetidine [1, 32-34]		x		x			x	252.3	0.40	6.80[b]
Codeine [35, 36]	x						x	299.4	1.19	8.21[b]
Colchicine [6, 37]	x	x	x					399.4	1.30	1.85[a]
Furosemide [6, 38, 39]			x	x			x	330.7	2.03	3.80[a]
Ranitidine [1, 6]		x					x	314.4	0.27	8.20[b]
Terbutaline [40]							x	274.3	0.90	8.80[b]
Valsartan [18, 41, 42]		x			x			435.5	3.65	4.90[a]

CYP 3A4: cytochrome P450; P-gp: P-glycoprotein; MRP: multidrug resistance associated protein; BCRP: breast cancer resistance protein; OATP: organic anion transporting polypeptide; PEPT1: peptide transporter protein; OCT: organic cation transporter. MW: molecular weight (g/mol), LogP and pKa. [a] acid drugs, [b] basic drugs.

At the end of the experiments the animals were euthanized. In order to separate solid components (mucus, intestinal contents) from the samples, they were centrifuged 5 minutes at 2991 g. All samples were analyzed by HPLC.

The absorption rate coefficient (K_a) and permeability values (P_{app}) for 16 drugs were determinate in two segments of gastrointestinal tract: entire small intestine and colon (n = 5-7). The perfusion solution of each drug was prepared at 500 µM. After dissolving the drugs, the pH of the solution was readjusted to 7.0.

The reduction in the volume of the perfused solutions at the end of the experiments was significant (up to 20%), and a correction became necessary in order to calculate the absorption rate constants accurately. Water reabsorption was characterized as an apparent zero order process. A method based on direct measurement of the remaining volume of the test solution was employed to calculate the water reabsorption zero order constant (k_o). The volume at the beginning of the experiment (V_0) was determined on groups of three animals, whereas the volume at the end (V_t) was measured on every animal used. The concentration in the samples was corrected as

$$C_t = C_e(V_t/V_0) \quad (1)$$

where C_t represents the concentration in the gut that would exist in the absence of the water reabsorption process at time *t,* and C_e represents the experimental value. The C_t values (corrected concentrations) were used to calculate the actual absorption rate coefficient [44].

The absorption rate coefficients (k_a) of compounds were determined by nonlinear regression analysis of the remaining concentrations in lumen C_t versus time.

$$C_t = C_0 e^{-k_a t} \quad (2)$$

The absorption rate coefficients (k_a) were transformed in to permeability values with the following relationship:

$$P_{app} = k_a R/2 \quad (3)$$

where *R* is the effective radius of the intestinal segment. *R* value was calculated considering the intestinal segment as a cylinder with the relationship:

$$Volume = \pi R^2 L \quad (4)$$

Estimation was carried out using a 10 mL perfusion volume and an intestinal length of 100 cm for the whole small intestine, and a 5 mL perfusion volume and a length of 10 cm for the colon. The effective radius of the intestinal segment was fixed at a value of 0.1784 cm for small intestine and 0.3989 cm for colon.

The relationship between absorption rate coefficient and the permeability value is described in detail next:

- First order disappearance from lumen:

$$\frac{dC}{dt} = -k_a \cdot C \quad (5)$$

where *C* is luminal concentration and k_a the absorption rate coefficient.

- Diffusion flux trough the membrane:

$$\frac{dQ}{dt \cdot A} = -P_{app} \cdot C \quad (6)$$

where Q represents amount, C luminal concentration and P_{app} the apparent permeability coefficient.

- Dividing both terms by the luminal volume V:

$$\frac{dQ}{dt \cdot V} = -\frac{A}{V} \cdot P_{app} \cdot C \rightarrow \frac{dC}{dt} = -\frac{A}{V} \cdot P_{app} \cdot C$$

where:

$$\frac{A}{V} \cdot P_{app} = k_a$$

- Taking the area and the volume of a cylinder:

$$\frac{2\pi RL}{\pi R^2 L} \cdot P_{app} = k_a \rightarrow \frac{2}{R} \cdot P_{app} = k_a$$

Cell culture and transport studies

Caco-2 cells were grown in Dubelcco's Modified Eagle's Media containing L-glutamine, fetal bovine serum and penicillin–streptomycin. Cell monolayers were prepared by seeding 250,000 cells/cm^2 on the polycarbonate membrane whose surface of the insert placed in each well. They were maintained at 37 °C temperature, 90% humidity and 5% CO2 for 4 days until confluence. Afterwards, the integrity of each cell monolayer was evaluated by measuring the transepithelial electrical resistance (TEER). Hank's balanced salt solution supplemented with HEPES was used to fill the receiver chamber and to prepare the drug solution that was placed in the donor chamber.

Transport studies were conducted in an orbital environmental shaker at constant temperature (37 °C) and agitation rate (50 rpm). Four samples were taken from the receiver side at prefixed sample times 15, 30, 45 and 90 min. Samples of the donor side were taken at the beginning and at the end of the experiment. The amount of compound in cell membranes and inside

the cells was determined at the end of experiments in order to check the mass balance and the percentage of compound retained in the cell compartment as always less than 5%.

Drug transport studies were performed from apical-to-basal (A-to-B) direction using the same concentration that in the *in situ* assays (500 µM). The TEER values were measured before and after the transport experiments and were always between 800 and 1100 ohms/cm^2, which ensure the integrity of the cell monolayer at assayed concentrations (500µM).

The apparent permeability coefficient was calculated according the following equation:

$$C_{receiver,t} = \frac{Q_{total}}{V_{receiver}+V_{donor}} + \left(\left(C_{receiver,t-1} \cdot f \right) - \frac{Q_{total}}{V_{receiver}+V_{donor}} \right) \cdot e^{-P_{app\ 0,1} \cdot S \cdot \left(\frac{1}{V_{receiver}} + \frac{1}{V_{donor}} \right) \cdot \Delta t} \quad (7)$$

where $C_{receiver,t}$ is the drug concentration in the receiver chamber at time *t*, Q_{total} is the total amount of drug in both chambers, $V_{receiver}$ and V_{donor} are the volumes of each chamber, $C_{receiver,t-1}$ is the drug concentration in receiver chamber at previous time, f is the sample replacement dilution factor, *S* is the surface area of the monolayer, Δt is the time interval and P_{app} is the permeability coefficient that might be $P_{app\ 0}$ or $P_{app\ 1}$. This equation considers a continuous change of the donor and receiver concentrations, and it is valid in either sink or non-sink conditions. The new feature is the option to estimate two permeability coefficients ($P_{app\ 0}$ and $P_{app\ 1}$) to account for atypical profiles in which the initial rate is different [45].

The non-linear regression to fit the equation to data was performed in Excel® using Solver tool for minimization of the Sum of Squared Residuals.

HPLC Analysis

All the samples were analyzed using a Perkin-Elmer HPLC with an UV detector or with a fluorescence detector depending on the characteristics of each drug. The standard calibration curves were prepared by dilution from the drug solution assayed. Sample concentrations were within the linear range of quantitation for all the assays. Analytical methods were validated for each compound in terms of specificity, selectivity, linearity, precision and accuracy. All the compounds were analyzed with a flow rate of 1 mL/min and at room temperature. µBondapak 150 mm x 4.6 mm C-18 column (5 µm particle size) was used for antipyrine, caffeine, carbamazepine, naproxen, verapamil, amoxicillin, furosemide, ranitidine and terbutaline; and a µBondapak 250 mm x 4.6 mm C-18 column (5 µm particle size) was required for metoprolol, colchicine, valsartan, atenolol, theophylline, codeine and cimetidine. An injection volume of 10 µL was used for the compounds analyzed with fluorescence detection, and 20 µL for the drugs analyzed with UV detection. Analytical details are presented in Table 4.1.2 [46].

Data Analysis

Permeabilities determined in each intestinal region were compared using Student's t-test to detect the existence of significant differences at the 0.05 probability level. The statistical comparison was made using the statistical package SPSS, V.20.

In order to characterize the relationship between permeability values (P_{app}) and oral fraction absorbed (F_a) the following equations were used for small intestine and colon (8), and Caco-2 respectively (9):

$$F_a = 1 - e^{\left(-P_{app}\frac{2}{R}T\right)} \quad (8)$$

$$F_a = 1 - e^{\left(-P_{app}\frac{A}{V}T\right)} \quad (9)$$

Table 4.1.2. Summary of HPLC conditions for the 16 tested compounds [46]**.**

Drug	Detection[a]	Mobile phase (aqueous:organic)	λ (nm)	Retention time (min)
Amoxicillin	UV	50 mM KH_2PO_4 (pH 5): MeCN (95:5)	230	3.6
Antipyrine	UV	Water (pH 3): MeCN (65:35)[b]	235	2.3
Atenolol	F	Water (pH 3): MeOH: MeCN (90:5:5)[b]	231/307[c]	7.0
Caffeine	UV	Water(pH 3): MeOH (65:35)[b]	273	3.6
Carbamazepine	UV	50 mM KH_2PO_4 (pH 7): MeCN (50:50)	280	2.9
Cimetidine	UV	Water pH 3: MeCN (87:13)[b]	220	4.9
Codeine	UV	0.05 mM acid phosphoric (pH 3): MeCN (75:25)	212	6.5
Colchicine	UV	10 mM NaH_2PO_4 (pH 3): MeCN (65:35)	245	4.8
Furosemide	UV	25 mM Na_2HPO_4 (pH 3): MeCN (45:55)	254	2.5
Metoprolol	F	Water (pH 3): MeOH: MeCN (60:20:20)[b]	231/307[c]	4.3
Naproxen	UV	Water (pH 3): MeCN: glacial acetic acid (34:65:1)[b]	244	2.8
Ranitidine	UV	10 mM Na_2HPO_4 (pH 7): MeOH (30:70)	320	2.0
Terbutaline	UV	10 mM KH_2PO_4 (pH 3.1): MeCN (92:8)	212	4.2
Theophylline	UV	Water (pH 3): MeCN (85:15)[b]	280	6.0
Valsartan	F	100 mM $(NH_4)_2HPO_4$ (pH 3): MeCN (40:60)	265/370[c]	6.2
Verapamil	F	Water (pH 3): MeCN (50:50)[b]	275/320[c]	2.7

MeCN: acetonitrile, MeOH: methanol; [a] UV: drug analyzed with an ultraviolet detector, F: drug analyzed with a fluorescence detector. [b] Water (pH 3) was 0.01% trifluoroacetic acid in water. [c] λ for excitation/λ for emission.

Where *R* represents the effective radius of the intestinal segment that was fixed at a value of 0.1784 cm for small intestine and 0.3989 cm for colon, *A* and *V* represent the surface area of the well and the volume in the donor chamber in cell culture experiments and *T* is the effective absorption time.

Additionally, a simultaneous fit of both equations to data from Caco-2 cells (21 days model) and rat colon and intestine permeability was performed, keeping a shared *T* parameter and including a surface factor correction factor, *S*, in the exponent of equation (9), to account for the surface difference between the monolayer model and the intestine and colon (because of villi and folds).

RESULTS

Table 4.1.3 summarizes the apparent first-order absorption rate coefficients (k_a, h^{-1}) and apparent permeability values (P_{app}, 10^{-4} cm/s) of the 16 drugs in whole small intestine and colon.

Figure 4.1.1 shows a comparative graphic of P_{app} values in small intestine and colon of each assayed drug. A good linear correlation was established between rat colon and intestinal permeability with R^2=0.94 (see Figure 4.1.2).

Figure 4.1.3 shows a comparative graphic of P_{app} values in colon and Caco-2 cells; 4 days (top panel) and 21 days (bottom panel) respectively of each assayed drug. P_{app} Caco-2 data of theophylline, metoprolol, furosemide and atenolol (4 days) and codeine and valsartan (21 days) were obtained from literature [32, 47]. Data are summarized in Table 4.1.4.

Table 4.1.3. Apparent first order absorption rate coefficients (*ka*, h^{-1}) and apparent permeability values (P_{app}, 10^{-4} cm/s) of the 16 drugs in whole small intestine and colon.

Drugs	Small intestine				Colon			
	k_a	SD	P_{app}	SD	k_a	SD	P_{app}	SD
Naproxen	7.434	0.492	1.841	0.116	3.016	0.227	1.673	0.123
Carbamazepine	5.164	0.510	1.279	0.120	2.575	0.425	1.424	0.230
Verapamil	4.304	0.470	1.056	0.101	2.221	0.556	1.230	0.308
Caffeine	4.221	0.790	1.046	0.186	1.565	0.363	0.870	0.196
Antipyrine	4.094	0.435	1.014	0.108	1.527	0.155	0.848	0.086
Theophylline	3.983	0.254	0.986	0.067	1.907	0.150	1.058	0.081
Metoprolol	2.518	0.239	0.624	0.273	1.505	0.344	0.814	0.187
Codeine	2.200	0.090	0.545	0.023	1.053	0.165	0.583	0.091
Furosemide	1.265	0.242	0.313	0.057	0.401	0.127	0.222	0.069
Cimetidine	1.211	0.229	0.299	0.057	0.639	0.146	0.346	0.078
Valsartan	1.050	0.087	0.262	0.022	0.606	0.115	0.328	0.062
Colchicine	1.010	0.158	0.250	0.039	0.591	0.157	0.320	0.085
Amoxicillin	0.708	0.172	0.167	0.041	0.553	0.095	0.299	0.051
Terbutaline	0.672	0.114	0.159	0.027	0.650	0.132	0.352	0.071
Ranitidine	0.573	0.136	0.135	0.032	0.546	0.135	0.295	0.073
Atenolol	0.430	0.096	0.107	0.024	0.390	0.079	0.211	0.043

Values represent mean ± SD (standard deviation) of five to seven rats for each drug and region.

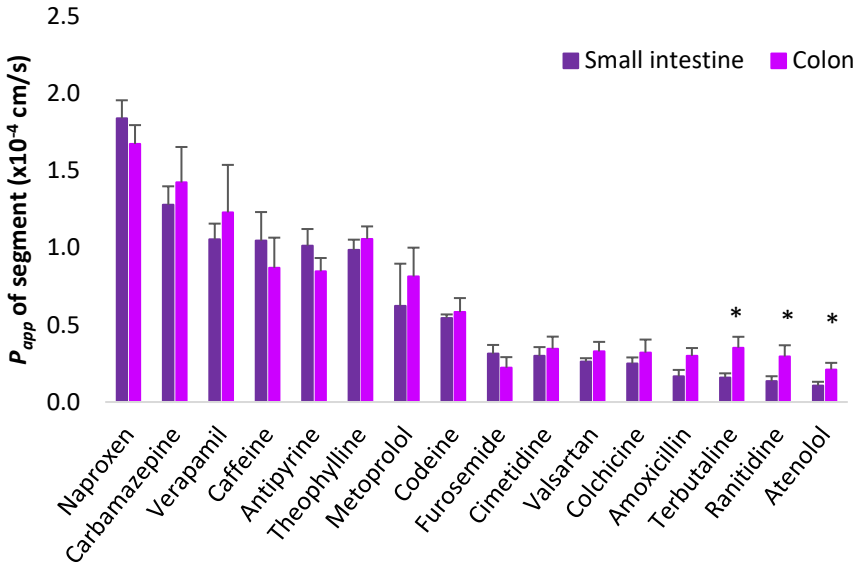

Figure 4.1.1. Permeability values of the drugs in small intestine and colon. *Significant differences between both segments ($p < 0.05$).

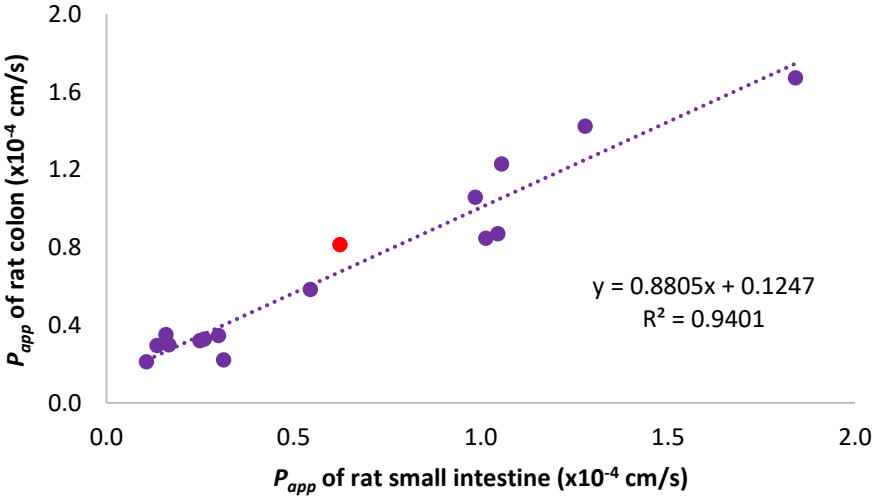

Figure 4.1.2. Correlation between apparent permeability coefficients in rat small intestine and rat colon (red symbol corresponds to metoprolol).

Table 4.1.4. Permeability values in colon and in caco-2 monolayers (4 and 21 days).

Drug	P_{app} colon ($x10^{-4}$ cm/s)	SD	P_{app} Caco-2 ($x\ 10^{-4}$ cm/s)					
			4 days model			21 days model		
			Papp	SD	Ratio	Papp	SD	Ratio
Naproxen	1.673	0.123	1.058	0.063	0.632	0.311	0.043	0.186
Carbamazepine	1.424	0.230	0.466	0.067	0.328	0.418	0.021	0.294
Theophylline	1.058	0.081	0.610	0.009	0.576	0.249	0.018	0.236
Caffeine	0.870	0.196	0.537	0.096	0.618	0.292	0.066	0.336
Antipiryne	0.848	0.086	0.347	0.047	0.409	0.282	0.019	0.333
Metoprolol	0.814	0.187	0.400	0.014	0.491	0.230	0.067	0.283
Codeine	0.583	0.091	0.084	0.003	0.143	0.229		0.393
Terbutaline	0.352	0.071				0.047	0.010	0.132
Cimetidine	0.346	0.078	0.015	0.002	0.045	0.037	0.003	0.107
Valsartan	0.328	0.062	0.012	0.002	0.036	0.007		0.021
Colchicine	0.320	0.085	0.005	0.001	0.016	0.009	0.002	0.029
Amoxicillin	0.299	0.051				0.020	0.003	0.067
Ranitidine	0.295	0.073	0.008	0.001	0.027	0.020	0.001	0.067
Furosemide	0.222	0.069	0.033	0.001	0.150	0.031	0.002	0.140
Atenolol	0.216	0.043	0.026	0.002	0.121	0.016	0.003	0.076

P_{app} Caco-2 data of theophylline, metoprolol, furosemide and atenolol (4 days) and codeine and valsartan (21 days) were obtained from literature [32, 47]. Values represent mean ± SD. Ratio between P_{app} obtained in Caco-2 and P_{app} obtained in colon.

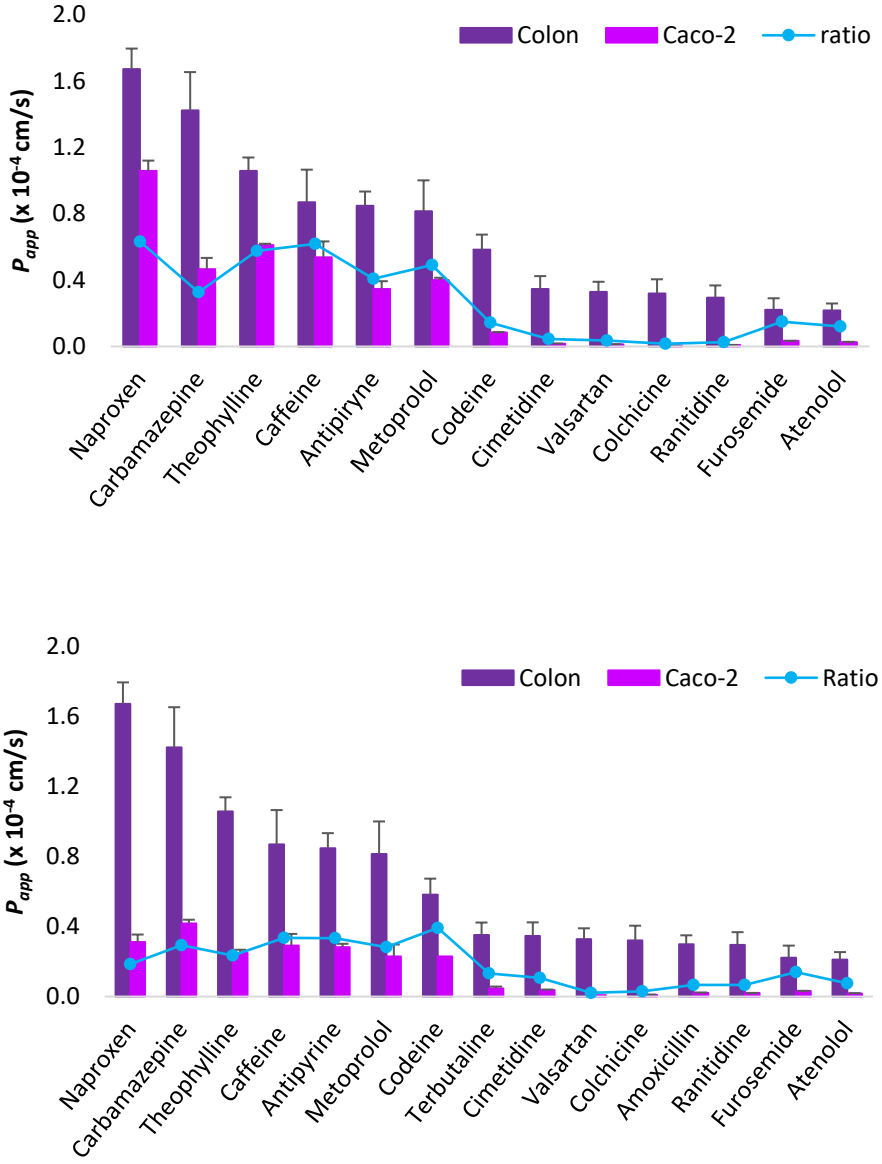

Figure 4.1.3. Top panel: permeability values of the drugs in colon and Caco-2, 4 days model. Bottom panel: permeability values of the drugs in colon and Caco-2, 21 days model.

Figure 4.1.4 shows in the top panel the correlation obtained between *in situ* (colon of the rat) and *in vitro* (Caco-2) models, whereas the bottom panel displays the correlation among the permebility values of antipyrine, metoprolol, atenolol and ranitidine obtained in rat colon and permeabiliy values obatined in human colonic tissue with Ussing chamber [48, 49].

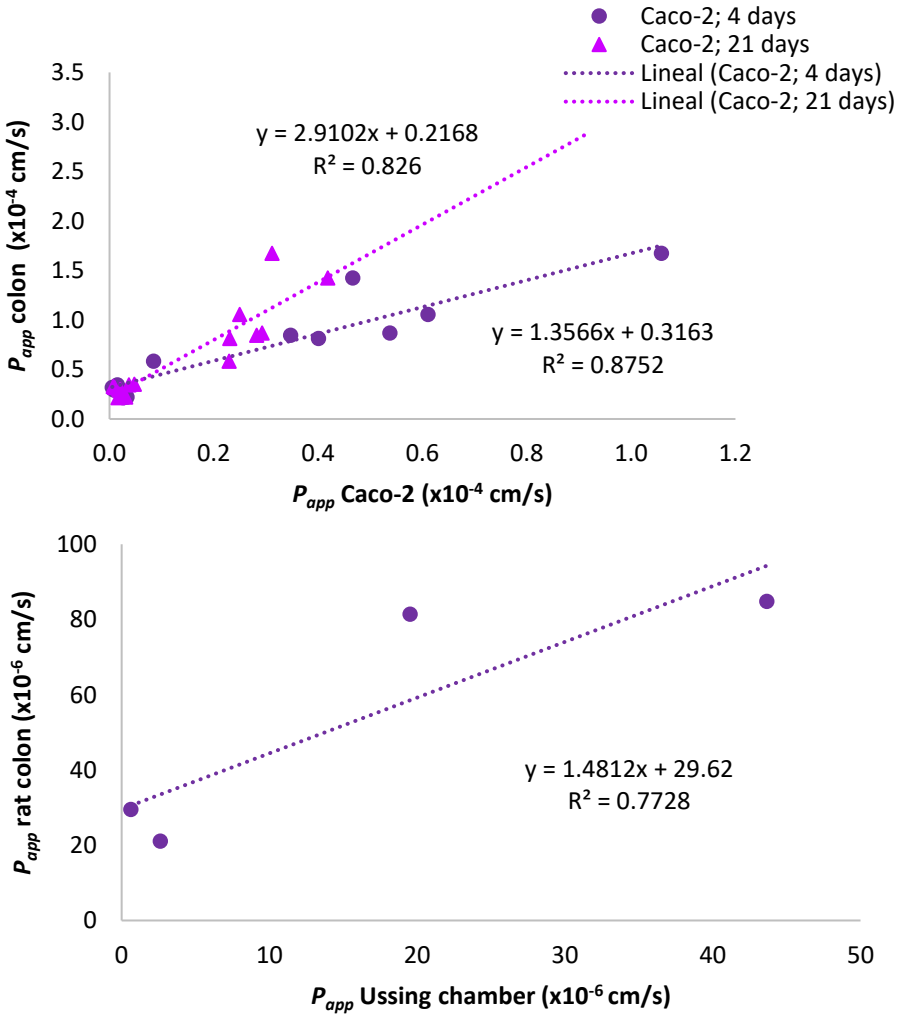

Figure 4.1.4. Top panel: correlation between apparent permeability coefficients in rat colon and Caco-2, 21 days model and Caco-2, 4 days model. Bottom panel: Correlation between rat colon permeability obtained in this study and human colon tissue in Ussing chambers (average values from references [48, 49]).

Figure 4.1.5 displays the rat intestinal permeability obtained with our closed loop method versus human oral fraction absorbed. Data obtained in rat small intestine with single-pass perfusion method by Salphati et al. [50] and in rat intestine with Ussing chambers [51] are also represented in Figure 4.1.5 to explore the similarity of data from different laboratories and experimental techniques. The human fractions absorbed were obtained from literature [32, 52-54].

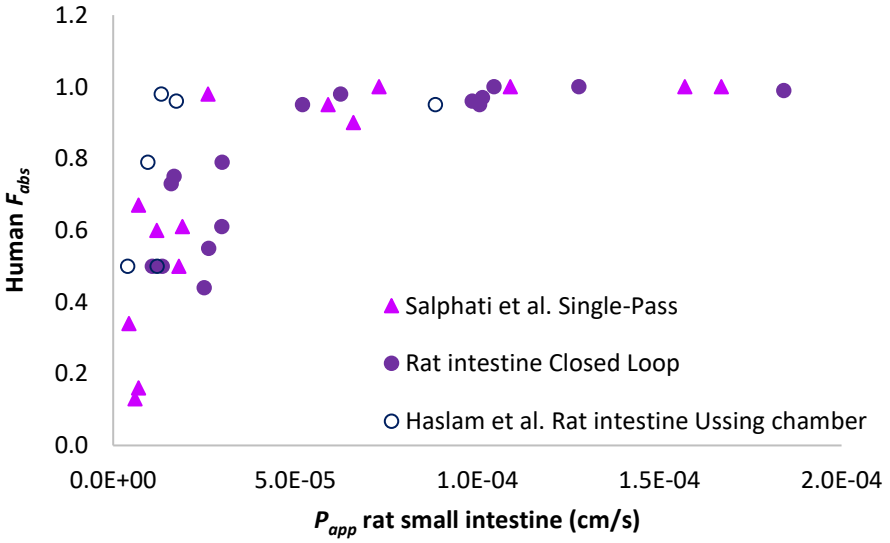

Figure 4.1.5. Correlation between permeability values obtained in rat small intestine with closed loop method and oral fraction absorbed of the different drugs. Asterisk represents permeability data obtained with single-pass method in rat small intestine by Salphati et al. [50]. Hollow circles correspond to Ussing chamber data with rat intestine obtained by Haslam et al. [51].

In Figure 4.1.6 a simultaneous fit of fraction absorbed versus permeability in Caco-2 model (21 days and 4 days) rat small intestine and colon is represented to show the shift of the curves because of the differences in surface area for transport in cell system versus the *in situ* models.

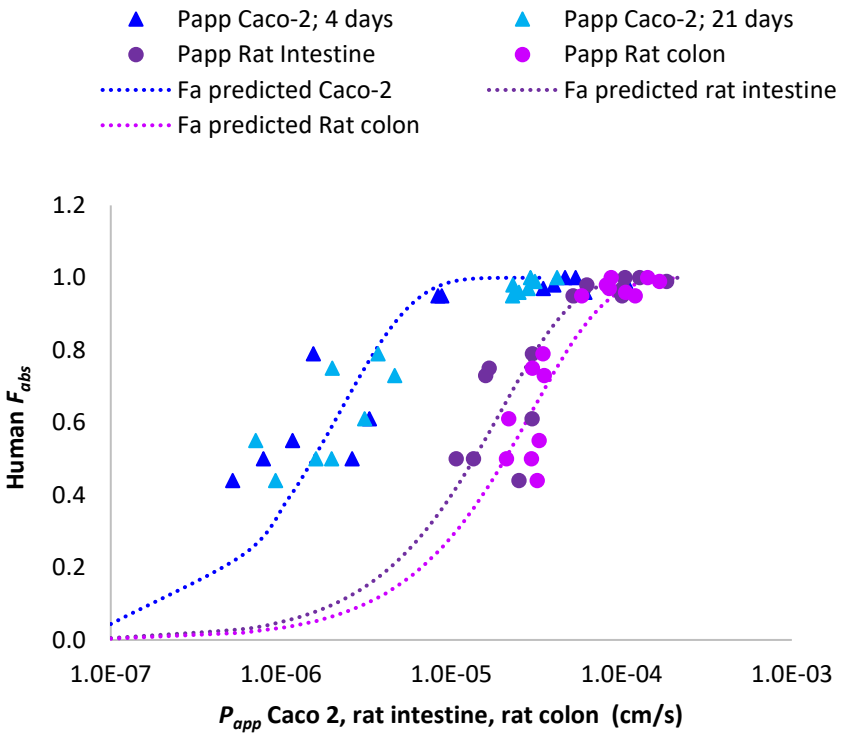

Figure 4.1.6. Simultaneous fit of permeability values obtained in different models (rat intestine, rat colon and Caco-2; 21 and 4 days) to estimate the difference in surface area correction factor (47.0 for small intestine and 32.0 for rat colon).

Fraction absorbed in human colon [48] were included in Figure 4.1.7 to explore the predictability of rat colon permeability to classify compounds in high/low colon absorbability. A previous correlation of human colon P_{app} and human colon fractions absorbed is overlapped for comparison [47].

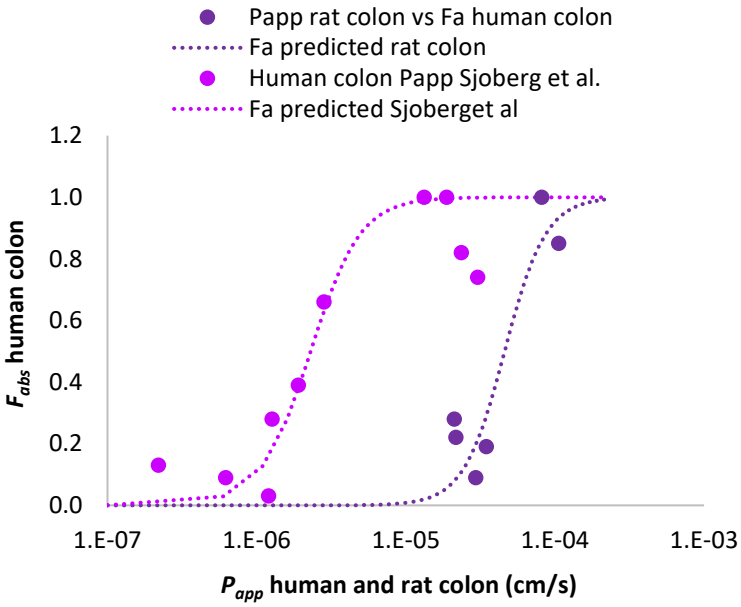

Figure 4.1.7. Correlation between permeability values obtained in rat colon and oral fraction absorbed in colon taken from Tannergren et al. and Sjoberg et al. [1, 48]. Fitted lines were obtained with a four parameter logistic model y=B/[1+(x/C)^D] where B is 1, C is P_{app} at Fa=0.5 ($4.6 \cdot 10^{-5}$ cm/s); D is the slope factor=-3.15 (R^2=0.87, $p < 0.05$). The sigmoidal correlation between human colonic P_{app} and human Fa in colon obtained by Sjoberg et al. is superimposed for comparison.

Table 4.1.5 shows the permeability class of the different compounds assayed according to the BCS classification (literature data by comparison with metoprolol) [32, 52, 54, 55] and the permeability class according to the results of this research in small intestine and colon (*in situ* method) and Caco-2 monolayers (*in vitro* method) obtained by comparison with metoprolol as high permeability model drug. A BCS-based classification derived from Figure 4.1.7 and considering a cut-off value of fraction absorbed in colon of 50% is also shown.

Table 4.1.5. Literature BCS permeability class data [32, 52, 54, 55] and permeability class according to the results of this research.

Drugs	Fraction absorbed *in vivo*	Permeability class				
		BCS	Small intestine	Colon	Caco-2	Colon*
Antipyrine	97	H	H	H	H	H
Caffeine	100	H	H	H	H	H
Carbamazepine	100	H	H	H	H	H
Metoprolol	98	H	H	H	H	H
Naproxen	99	H	H	H	H	H
Theophylline	96	H	H	H	H	H
Verapamil	95	H	H	H	N/A	H
Codeine	95	H	H	L	L	H
Amoxicillin	75	L	L	L	L	L
Atenolol	50	L	L	L	L	L
Cimetidine	79	L	L	L	L	L
Colchicine	44	L	L	L	L	L
Furosemide	61	L	L	L	L	L
Ranitidine	50	L	L	L	L	L
Terbutaline	73	L	L	L	L	L
Valsartan	55	L	L	L	L	L

H: high permeability; L: low permeability. (Classification taking metoprolol as high permeability model). * Classification taking as boundary value $4.6 \cdot 10^{-5}$ cm/s for CR developability corresponding to a F_a in colon of 50%. N/A: Not available.

DISCUSSION

The main purpose of this study is to validate the technique of *in situ* perfusion in colon and intestine of Wistar rat based on Doluisio's method and to compare the permeability values in both segments with permeability values in two Caco-2 cells-based models. A second objective is to explore whether the BCS system could be applicable for colonic absorption, at least for permeability classification and screening for CRS development. To reach these objectives, several drugs with different physicochemical characteristics (acids, bases, and lipophilicity) and transport mechanism have been chosen (See Table 4.1.1). Compounds have been assayed at a high concentration (500 µM) to ensure saturating conditions in the case that the tested compound is absorbed through a specialized, carrier-mediated mechanism. In these conditions (high concentration), the contribution of the transporters in both intestinal segments (small intestine and colon) would be negligible and absorption process could be described as a passive diffusion mechanism.

Our research shows that the correlation obtained between the permeability values in small intestine and colon is excellent ($R^2 > 0.9$). There are not statically significant differences in the permeability of both segments assayed for most of the drugs but for three low permeability compounds, terbutaline, ranitidine and atenolol. Interestingly, for these basic drugs, colonic permeability was higher in our rat model. These results are not because of differences in ionization degree in intestine versus colon as the perfusion solution were prepared and buffered at the same pH (7.00) for both segments.

Other authors have shown that polar drugs are less permeable in colon than in small intestine, whereas high permeability drugs present similar or higher permeability in colon in a rat model and in human tissue in Ussing chambers [48, 56]; thus, our results present a different trend. On contrary, Rozehnal et al. [49] did not found significant differences between human small intestine and human colon in Ussing chambers. From our results in rat

colon and the comparison with the two models of Caco-2 cells monolayers it seems that paracellular pathway in rat colon is less restrictive than in human intestine or at least there are less regional differences in the rat. Rat colon showed better correlation with Caco-2, 4 days model in which the paracellular pathway is less tight. On the contrary, the correlation between human colon permeability obtained in Ussing chambers and rat colon permeability is good (see Figure 4.1.4), but demonstrated higher permeability values in rat colon compared with human tissue (slope close to 1.5). This linear correlation also reflects the difference in surface area for transport that is higher in human tissue (represented by the intersection with Y axis ~30). The higher permeability values in rat colon versus human tissue in spite of the higher surface area available in the human tissue could reflect higher paracellular permeability in the rat model.

As it can be seen in Figure 4.1.5, the relationship between permeability values obtained in rat small intestine with Doluisio's technique and human fraction absorbed can be combined with permeability data obtained in rat small intestine with single pass-perfusion method as well as with rat tissue in Ussing chambers [50, 51]. In accordance with these results, recently a good correlation between permeability values obtained in rat small intestine using these two experimental techniques (single-pass and Doluisio's method) in different laboratories has been demonstrated [57]. This indicates the robustness of the rat model for predicting human Fa and the possibility of using this curve of combined data to predict oral fraction absorbed without the need of developing a full correlation with 20 model compounds but instead, running one or two model drugs of low, intermediate and high permeability and checking wheter the data fall in the same line. A similar approach has been proposed for the human permeability versus human Fa correlation [48].

Figure 4.1.6 shows that a good sigmoidal relationship can be obtained with all the experimental systems used in this work between permeability

values and human fractions absorbed but with a shift to higher permeability values in the *in situ* models (rat intestine or colon). This difference in the permeability ranges in cell culture versus whole tissue models reflects the difference in surface area available for transport as in the cell model only microvilli are present, whereas in the tissue or in the animal model villi and folds contribute to a higher area availability.

Figure 4.1.6 also makes evident the higher permeability values obtained in rat colon versus small intestine in particular for low permeability drugs. Even if the differences in permeability between intestine and rat colon were statistically different only for three compounds, the fitted curve permits to point out the difference between both regions. These results could be further explored by performing experiments with markers of paracellular permeability with a wider range of molecular weights.

Finally, to validate the rat colon perfusion method to predict human colon absorption it was necessary to have data availability from permeability values in human colon and/or human fraction absorbed in this region. Only recently, these data have been published [48, 49] making this comparison possible. In Figure 4.1.4, a good correlation between human colon tissue and rat is shown. In Figure 4.1.7, a sigmoidal correlation between rat colon permeability and human fraction absorbed in colon is shown. In the same figure the correlation between human colon tissue and human colon Fa have been represented to show the shift to higher permeability values in rat colon, which nevertheless show a similar relationship.

Eventually, that means that rat colon permeability values can be used for classification purposes in high-low colon permeability compounds. In that case, the boundary value for classification has to be selected by considering the objective of the classification. That is, if the objective is to explore CR developability, a permeability value ensuring Fa > 50% could be reasonable, (in our rat model that corresponds to a permeability value of $4.6 \cdot 10^{-5}$ cm/s). Table 4.1.5 shows the permeability class assigned to the different drugs

assayed in this work based on the different experimental models and considering metoprolol as high permeability model, and the classification for CR developability based in our rat colon permeability values and the correlation with human colon Fa values (from Figure 4.1.7).

CONCLUSION

In situ rat perfusion technique in colon and the same technique in small intestine are both equally useful to predict human oral fraction absorbed. Rat perfusion with Doluisio's method and single-pass provided a similar range of values demonstrating the robustness of the animal model and the possibility of combining data from different laboratories.

Rat colon permeability is well correlated with Caco-2 cell 4 days model reflecting higher paracellular permeability. Rat colon permeability values are also higher than human colon permeability values. In spite of the magnitude differences between rat colon and human colon, a good sigmoidal relationship has been shown between rat colon values and human fraction absorbed in colon indicating that rat colon perfusion can be used for compound classification and screening of candidates for CR development.

ACKOWLEDGMENTS

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4.2. Artículo científico 4

Usefulness of Caco-2/HT29-MTX and Caco-2/HT29-MTX/Raji B co-culture models to predict intestinal and colonic permeability compared to Caco-2 monocultures

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ABSTRACT

The Caco-2 cellular monolayer is a widely accepted *in vitro* model to predict human permeability, but suffering from several and critical limitations. Therefore, some alternative cell cultures to mimic the human intestinal ephitelium, as closely as possible, have been developed to achieve more physiological conditions, as the Caco-2/HT29-MTX co-culture and the triple Caco-2/HT29-MTX/Raji B models. In this work the permeability of 12 model drugs of different Biopharmaceutical Classification System (BCS) characteristics, in the co-culture and triple co-culture models was assessed. Additionally, the utility of both models to classify compounds according to the BCS criteria was scrutinized. The obtained results suggested that the co-culture of Caco-2/HT29-MTX and the triple co-culture of Caco-2/HT29-MTX/Raji B were useful models to predict intestinal permeability and to classify the drugs in high or low permeability according to BCS. Moreover, to study thoroughly the transport mechanism of a specific drug, to use a more complex model than Caco-2 monocultures is more suitable because, co-culture and triple co-culture are more physiological models, so the results obtained with them will be closer to those obtained in the human intestine.

Keywords: *in vitro* models, Caco-2, co-culture, triple co-culture, permeability.

INTRODUCTION

The oral route is the first choice for drug administration as it is the physiological mechanism for incorporating nutrients and other exogenous molecules. The main place of absorption after administering a drug by oral route is the small intestine; therefore, the colon plays a secondary role in systemic absorption for immediate release formulations. However, for modified release products its absorptive function becomes more important [1-3].

During the development stage of any drug product it is necessary to test its permeability. Nevertheless, obtaining data of human intestinal permeability has ethical implications and can be extremely complex [4]. Thus, there are alternative options to study a drug permeability in the early stages of its development, like *in vivo* and *in situ* techniques with different animal models [5-7], *ex vivo* models using excised intestinal tissues from human or animals [8-10] or *in vitro* cell culture models [11-13]. The *in vitro* cell models consist in simulating the intestinal barrier with cells from human colon carcinoma. They avoid the difficulties implicit in collecting human data, as referred above. Also, avoid the animal models that, despite showing functional cells, are more expensive and laborious than *in vitro* techniques. Moreover, the most important objective of the 3R's policy is to reduce the use of animals in research [14].

The *in vitro* model more widely accepted to study the human oral drug absorption is the Caco-2 monolayers [13, 15, 16]. Caco-2 cells are able to get morphologic and functional characteristics very similar to enterocytes, for example, they show tight junctions, apical and basolateral side and a brush border with microvilli on the apical surface [17]. However, these Caco-2 monolayers have several limitations. On one hand, the tight junctions are tighter than those present in the small intestine, they are more similar to colon epithelium cells, which causes less permeability of drugs through the paracellular route. On the other hand, these cell cultures only have absorptive cells, which do not mimic the human intestinal epithelium as it is an integration of different cell types: absorptive cells or enterocytes, goblet cells (mucus producer cells), endocrine cells and M cells. The most abundant cells are the enterocytes and the second most frequent cell type is the goblet cells [18]. Others limitations of the Caco-2 cell model are the different permeability of compounds transported via carried-mediated absorption and the overexpression of P-glycoprotein [18, 19].

Therefore, to improve the cellular models for drug intestinal absorption, more studies are still needed. Hence, many research groups have proposed to use the co-culture of Caco-2/HT29-MTX as a model that mimics the human intestinal epithelium better than the simple Caco-2 monolayers [18-22]. The mucus producing HT29-MTX cell line is used as a model to study the mucus role in the transport of drugs through the intestinal tract. Mucus secreting goblet cells are usually obtained from adenocarcinoma cell line HT29. HT29 cells are treated with methotrexate to get mature goblet cells so-called HT29-MTX. This cell line shows sparse and shorter microvilli than Caco-2 cells but a similar size and cellular complexity [23].

The amount of goblet cells changes along the intestinal tract, from 10% in small intestine to 24% in distal colon [18]. Different studies confirm that the closest ratio to physiologic conditions is 9:1 for Caco-2 to HT29-MTX cells, respectively [18, 21]. Several works prove the validity of these co-cultures; in fact, with them, the permeability increases for compounds with paracellular pathway and P-glycoprotein substrates [24-26].

A more recent strategy to develop an *in vitro* model able to reproduce the intestinal epithelium more approximately is the triple co-culture Caco-2/HT29-MTX/Raji B [27]. Caco-2 cells cultured with Raji B lymphocytes have the ability to acquire M cell phenotype. These Caco-2 cells lose the brush border organization, the microvilli and the typical digestive function from enterocytes [28]. They play an important role in the immune system and have the capacity to transport particulate matters by endocytosis, such as bacteria, viruses, nanoparticles and microparticles [26, 29]. The triple co-culture was first developed to test the permeability of insulin in solution and mediated by nanoparticles. Afterwards, Araújo *et al.* characterized better the triple co-culture and proved that the three cell types present the features of the human intestinal epithelium cells when they were cultured together [30].

The objective of this work was to study the permeability in the Caco-2/HT29-MTX co-culture and triple co-culture Caco-2/HT29-MTX/Raji B

models of 12 model drugs with different characteristics (metoprolol, atenolol, verapamil, furosemide, valsartan, caffeine, theophylline, cimetidine, naproxen, codeine, colchicine and carbamazepine) and classify them according to the Biopharmaceutical Classification System (BCS) criteria. For a better data understanding, correlations with rat small intestine permeability, rat colon permeability, Caco-2 monocultures permeability and human Fabs have been established. The final aim was to study whether the co-culture and the triple co-culture are better tools to predict the oral absorption of drugs than the reference *in vitro* model used until now, the Caco-2 monocultures. In addition, the utility of both models to classify compounds in high or low BCS permeability classes was assessed.

MATERIALS AND METHODS

Material and cell lines

Metoprolol, atenolol, verapamil, furosemide, valsartan, caffeine, theophylline, cimetidine, naproxen, codeine, colchicine and carbamazepine were purchased from Sigma-Aldrich (Spain). Methanol, acetonitrile and water were HPLC grade. All other chemicals were of analytical reagent grade.

Caco-2 and Raji B cell lines were from American Type Culture Collection (ATCC, USA); HT29-MTX cell line was kindly provided by Dr. T. Lesuffleur (INSERM U178, Villejuif, France).

Cell culture

Caco-2, HT29-MTX and Raji B cells were grown separately in Dulbecco's Modified Eagle's medium (DNEM) containing L-glutamine supplemented with 10% (v/v) inactivated Fetal Bovine Serum, 1% (v/v) Non-essential amino acids and 1% (v/v) antibiotic-antimitotic mixture (100 U/mL penicillin and 100 U/mL streptomycin). Cells were subcultured once a week and seeded at a density of 5 x 10^5 cells per 75 cm^2 flask and the culture

medium was replaced every other day. They were maintained in an incubator at 37 °C temperature and 5% CO_2 in a water saturated atmosphere.

***In vitro* cell models**

The techniques employed to obtain the models used for the *in vitro* experiments were described in Araújo et al. paper [30]. Briefly, in the Caco-2/HT29-MTX co-cultures, were seeded on the apical chamber of 6-well Transwell plate in a proportion 90:10, respectively, to reach a monolayer with a final density of 1 x 10^5 cells/cm^2 in each insert and they were maintained for 21 days until the experiment. To get the Caco-2/HT29-MTX/Raji B triple co-culture model, after the co-culture grew for 14 days, 1 x 10^5 Raji B cells were added to the basolateral chamber and the three cell types were maintained for 7 days more before the experiment.

Cell monolayer integrity

The transepithelial electrical resistance (TEER) of co-cultures and triple co-cultures was measured every time that the medium was replaced to check the confluence evolution and their integrity during the 21 days before the permeability studies. Moreover, the TEER values were measured before and after the transport experiments to ensure the integrity of the monolayer. It should be noted that only the monolayers with TEER values between 150-250 Ω·cm^2 were selected for permeability experiments [19, 30].

Permeability studies

To prepare the experimental solutions a high concentration of drugs (0.5 mM) was used to ensure saturating conditions in order to get the diffusional component of the transport and a negligible contribution of the transporters. Before starting permeability assays, the culture medium was removed and the cell monolayers were washed. Hank's balanced salt solution (HBSS) was used to do the washes and to prepare the drug solutions.

The basolateral chamber was filled with 2.5 mL of HBSS and the apical chamber was filled with 1.5 mL of drug solution. The permeability studies were carried out from apical-to-basolateral (A-to-B) direction in an orbital shaker at 37 °C and 100 rpm. At different time points, 200 µL samples were collected from the basolateral compartment and the drug concentrations were analysed by HPLC (High Performance Liquid Chromatography).

The apparent permeability coefficient (P_{app}, cm/s) was calculated according to the following equation:

$$C_{receiver,t} = \frac{Q_{total}}{V_{receiver}+V_{donor}} + \left(\left(C_{receiver,t-1} \cdot f \right) - \frac{Q_{total}}{V_{receiver}+V_{donor}} \right) \cdot e^{-P_{app\ 0,1} \cdot S \cdot \left(\frac{1}{V_{receiver}} + \frac{1}{V_{donor}} \right) \cdot \Delta t} \quad (1)$$

where $C_{receiver,t}$ is the drug concentration in the receiver chamber at time t, Q_{total} is the total amount of drug in both chambers, $V_{receiver}$ and V_{donor} are the volumes of each chamber, $C_{receiver,t-1}$ is the drug concentration in receiver chamber at previous time, f is the sample replacement dilution factor, S is the surface area of the monolayer, Δt is the time interval and P_{app} is the permeability coefficient which might be $P_{app\ 0}$ or $P_{app\ 1}$. This equation considers a continuous change of the donor and receiver concentrations, and it is valid in either sink or non-sink conditions. Its main feature is the option to estimate two permeability coefficients ($P_{app\ 0}$ and $P_{app\ 1}$) to account for atypical profiles in which the initial rate is different [31], but the drugs employed in this work don't show an atypical profile.

HPLC analysis

All the experimental samples were analysed using a Perkin-Elmer HPLC with an UV or fluorescence detector depending on the drug characteristics. The standard calibration curves were prepared by dilution from the drug solution assayed. Sample concentrations were within the linear range of quantitation for all the assays. Analytical methods were validated for each

compound calculating specificity, selectivity, linearity, precision and accuracy. The details of the analytical method of each drug were obtained from Lozoya-Agullo et al. work [2].

Rat small intestine, rat colon, Caco-2 monolayers and Fabs data

The Papp from *in situ* animal techniques, the Papp from *in vitro* Caco-2 monolayers and human fraction absorbed data used to stablish the correlations shown in this paper, have been taken from the previous work developed by Lozoya-Agullo et al. [2].

Moreover, in order to characterize the relationship between permeability values (P_{app}) and oral fraction absorbed in humans (Fabs) the following equation was used to calculate the predicted Fabs:

$$F_a = 1 - e^{\left(-P_{app}\frac{S \cdot A}{V}T\right)} \quad (2)$$

where *A* and *V* represent the surface area of the well and the volume in the donor chamber in cell culture experiments and *T* is the effective absorption time. *S* corresponds to a factor to account for the increment in area of transport due to microvilli presence. This model was fit simultaneously to the three data sets keeping in common the value of *T* (fixed to 2 hours) and estimating a different *S* factor for each cell culture.

Data analysis

To determine the goodness of fit of the linear regressions employed to obtain the different correlations, R^2 was taken into consideration. A value of R^2 closer to 1 means a better adjustment. Moreover, to know if the correlations are adequate from a statistical point of view, the residual sum of squares (RSS) and the significance of F ($p < 0.05$) were calculated using the "data analysis" tool from Excel®. Smaller RSS values indicate a better

adjustment, and a significance of F smaller than 0.05 implied that the correlation was appropriate from a statistical point of view.

The permeability determined for each drug with the different techniques contrasted (Caco-2/HT29-MTX co-culture, Caco-2/HT29-MTX/Raji B triple co-culture, rat small intestine, rat colon and monoculture Caco-2) were compared using ANOVA test. Levene's statistic was calculated to study the homogeneity of variances and Scheffé or Dunnett's T3 *Post Hoc* test were applied accordingly to determine the differences between groups. Moreover, the co-culture and triple co-culture were compared between them using the t-Student test ($p < 0.05$). The statistical comparison was made using the statistical package SPSS, V.22.

RESULTS

The results of the experimental design using co-culture and triple co-culture cell models in order to be accepted as *in vitro* methods to predict human oral fraction absorbed are shown in this section. The correlations between Caco-2 cells, rat small intestine and colon permeability were established and discussed in this work.

Table 4.2.1 summarizes the results of the permeability assays with the different selected drugs and the two *in vitro* models employed in this work (Caco-2/HT29-MTX co-culture and Caco-2/HT29-MTX/Raji B triple co-culture). The correlation stablished between the two models is shown in Figure 4.2.1, the R^2 obtained ($R^2 > 0.90$) indicates a good correlation between both techniques.

Table 4.2.1. Apparent permeability coefficient (Papp, $x10^{-4}$ cm/s) of the 12 drugs with the two *in vitro* models studied (Caco-2/HT29-MTX co-culture and Caco-2/HT29-MTX/Raji B triple co-culture). S.D.: Standard Deviation. C.V.%: Coefficient of variation. * Denotes significant differences between both models ($p<0.05$).

DRUG	CO-CULTURE			TRIPLE CO-CULTURE		
	P_{app}	S.D.	C.V.%	P_{app}	S.D.	C.V.%
Atenolol	0.142	0.006	3.87	0.160	0.016	9.90
Caffeine	0.603	0.084	13.97	0.557	0.027	4.86
Carbamazepine	0.429	0.068	15.74	0.364	0.057	15.74
Cimetidine*	0.102	0.009	8.94	0.243	0.048	19.65
Codeine	0.391	0.076	19.34	0.408	0.085	20.82
Colchicine	0.247	0.048	19.62	0.238	0.033	14.00
Furosemide	0.083	0.020	23.65	0.124	0.018	14.26
Metoprolol	0.361	0.028	7.69	0.323	0.015	4.70
Naproxen	0.468	0.043	9.23	0.457	0.041	8.91
Theophylline	0.388	0.024	6.18	0.399	0.015	3.67
Valsartan*	0.083	0.015	18.10	0.196	0.034	17.37
Verapamil	0.417	0.047	11.17	0.427	0.056	13.09

Permeability data from rat small intestine and rat colon obtained with an *in situ* perfusion technique and the Caco-2 monolayer *in vitro* model [2] were correlated with the two *in vitro* models assayed in this work, the correlations obtained are shown in Figure 4.2.2 and Figure 4.2.3. R^2 values for each correlation appears in the plots, but a more exhaustive analysis of goodness and validity can be seen in Table 4.2.2.

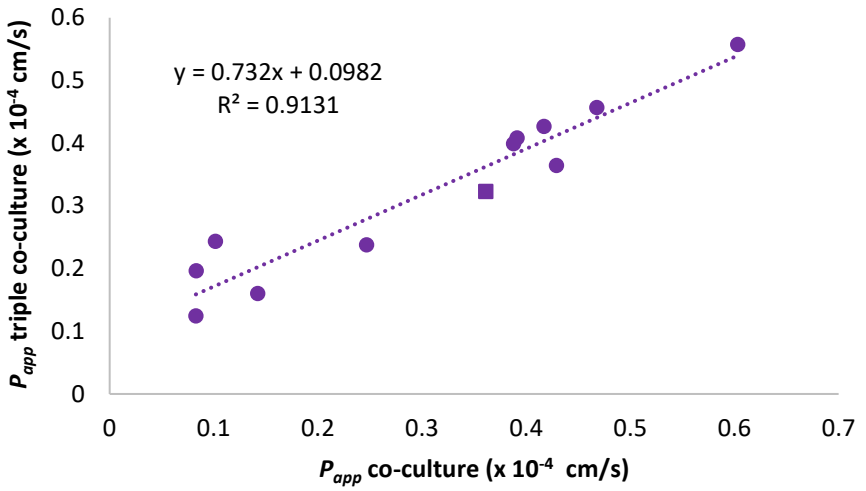

Figure 4.2.1. Correlation between apparent permeability coefficients obtained using the Caco-2/HT29-MTX co-culture and the Caco-2/HT29-MTX/Raji B triple co-culture. Square symbol corresponds to metoprolol.

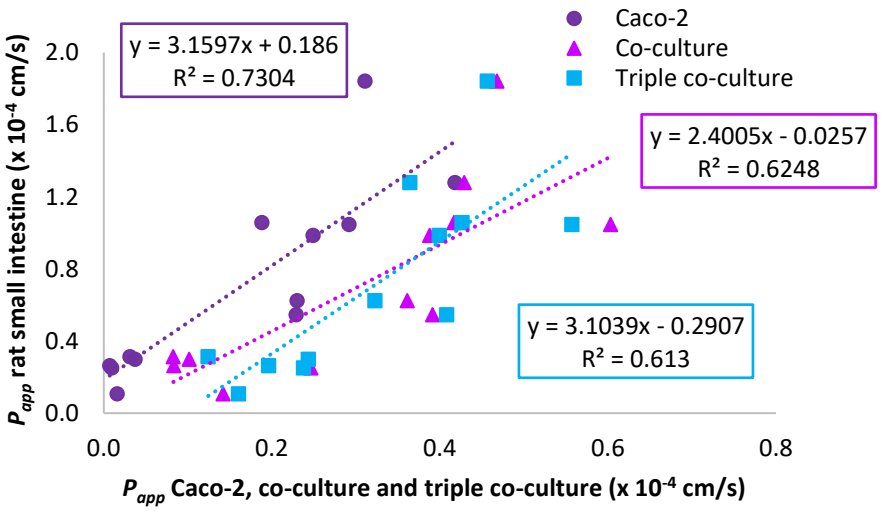

Figure 4.2.2. Correlations between the Papp of the three *in vitro* models and the Papp from rat small intestine obtained with an *in situ* technique.

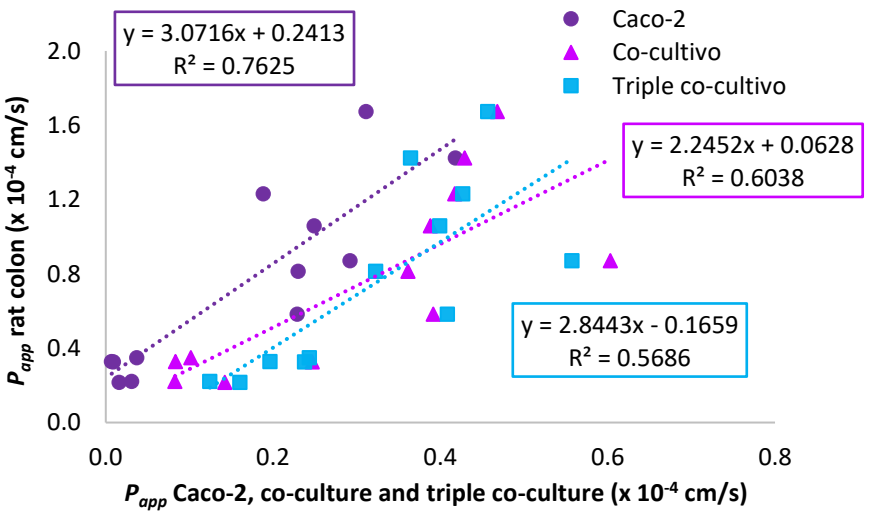

Figure 4.2.3. Correlations between the Papp of the three *in vitro* models and the Papp from rat colon obtained with an *in situ* technique.

Table 4.2.2 shows some parameters used to evaluate the validity of the correlations stablished. R^2 indicated the goodness of fit of the lineal regression, and residual sum of squares (RSS) and the significance of F ($p < 0.05$) were used to measure the statistical soundness. According to these tests, all the correlations were acceptable. However, the correlation between *in vitro* models (Figure 4.2.1) was better than those stablished between an *in vitro* and an *in situ* model (Figure 4.2.2 and Figure 4.2.3).

Table 4.2.2. Goodness of fit and statistical validity of all the correlations contained in this paper. R^2: coefficient of determination, RSS: residual sum of squares, and significance of F ($p < 0.05$).

CORRELATION	R^2	RSS	Significance F
Co-culture vs. Triple co-culture	0.913	1.68×10^{-10}	1.27×10^{-6}
Co-culture vs. small intestine	0.625	1.14×10^{-8}	2.21×10^{-3}
Triple co-culture vs. Small intestine	0.613	1.18×10^{-8}	2.60×10^{-3}
Caco-2 vs. Small intestine	0.730	8.22×10^{-9}	3.98×10^{-4}
Co-culture vs. Colon	0.604	1.09×10^{-8}	2.94×10^{-3}
Triple co-culture vs. Colon	0.569	1.19×10^{-8}	4.61×10^{-3}
Caco-2 vs. Colon	0.762	6.55×10^{-9}	2.08×10^{-4}

Figure 4.2.4 illustrates a comparison between the permeability values obtained with Caco-2 monoculture, Caco-2/HT29-MTX co-culture and Caco-2/HT29-MTX/Raji B triple co-culture. Significant statistical differences between co-culture and triple co-culture only were obtained for cimetidine and valsartan. However, between the monoculture Caco-2 and the other two *in vitro* models studied there were significant statistical differences for all the assayed compounds, except for carbamazepine.

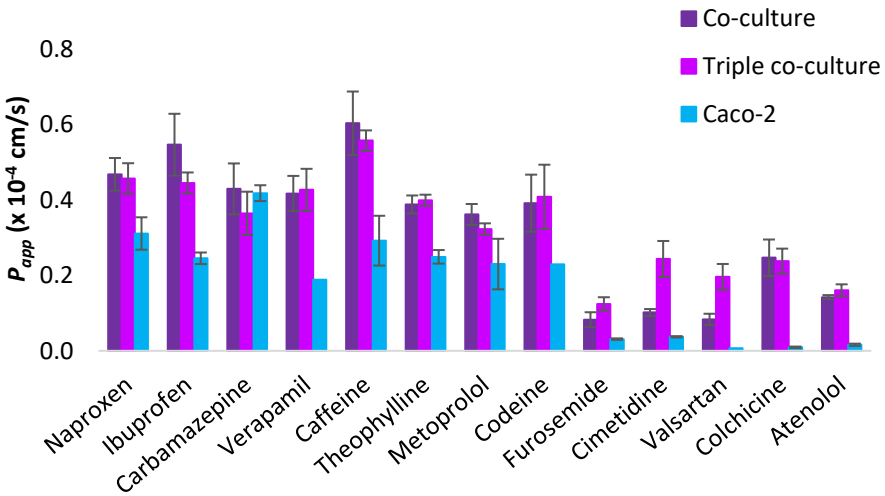

Figure 4.2.4. Permeability values of the 12 drugs assayed in three *in vitro* models: Caco-2 monoculture, Caco-2/HT29-MTX co-culture and Caco-2/HT29-MTX/Raji B triple co-culture.

Table 4.2.3 summarizes the oral fraction absorbed *in vivo,* collected from literature human data, and the permeability class (high or low) of the selected drugs according to the BCS classification [32-36]. Moreover, the classification, according to the permeability values obtained in this work, is shown to study if it corresponds with the BCS classification. The permeability values were classified considering the metoprolol as high permeability drug model, so its Papp data was used as cut-off point [35]. Figure 4.2.5 shows the correlation between the Papp values of *in vitro* models and the oral fraction absorbed.

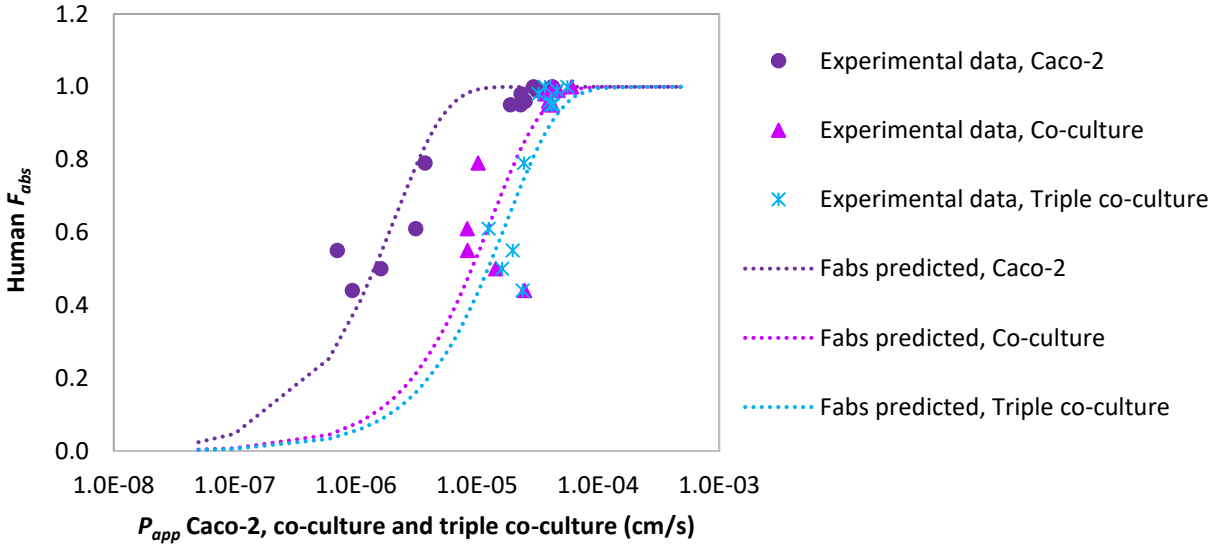

Figure 4.2.5. Correlation between the permeability values obtained with *in vitro* methods and oral fraction absorbed of the different drugs.

Table 4.2.3. Human fraction absorbed *in vivo* data, BCS permeability class data and permeability class according to co-culture and triple co-culture data obtained in this research.

Drug	Fraction absorbed (%) *in vivo*	Permeability		
		BCS	Co-culture	Triple co-culture
Caffeine	100	H	H	H
Carbamazepine	100	H	H	H
Naproxen	99	H	H	H
Metoprolol	98	H	H	H
Theophylline	96	H	H	H
Codeine	95	H	H	H
Verapamil	95	H	H	H
Cimetidine	79	L	L	L
Furosemide	61	L	L	L
Valsartan	55	L	L	L
Atenolol	50	L	L	L
Colchicine	44	L	L	L

Note: H: high permeability; L: low permeability. (Classification taking metoprolol as high permeability model).

DISCUSSION

The main objective of this research was to analyse the role of Caco-2/HT29-MTX co-culture and Caco-2/HT29-MTX/Raji B triple co-culture as predictive tools for the permeability of different drugs and also, to classify these compounds according to the BCS. To achieve this, several correlations were stablished and studied in detail.

As shown in Figure 4.2.1, the permeability values of both methods assayed correlated quite good (R^2>0.90). Moreover, there were no significant statistical differences between Papp data for each drug except for cimetidine and valsartan (see Table 4.2.1 and Figure 4.2.4). Both compounds were from low permeability class and showed more permeability in the triple co-culture

than in the co-culture. As the main difference between these two models is the performance of the paracellular route, rendering them more leaky in comparison with the standard Caco-2 model is expected that the differences of results were mainly observed in the low permeability compounds.

The 12 drugs assayed showed higher permeability values in co-culture and triple co-culture than in Caco-2 monocultures. A possible explanation could be that the tight junctions are wider in these two models therefore, the contribution to the permeation mechanism of the paracellular route is increased [19]. Moreover, this is in accordance with the TEER values obtained in this research.

In addition, correlations with Papp values from co-culture and triple co-culture and rat small intestine and rat colon were stablished (Figure 4.2.2 and Figure 4.2.3). Table 4.2.2 shows that all the correlations obtained were acceptable but, both *in vitro* models assayed in this study correlated slightly better with small intestine than with colon because, R^2 was closer to 1 for small intestine correlations than for colon correlations. This can be explained considering that the objective of the co-culture and triple co-culture development was to achieve a monolayer more similar to small intestine epithelium than to colon epithelium [19, 27]. Although, despite being more precise to predict small intestine permeability, the correlation obtained with colon data demonstrated that co-culture and triple co-culture can also be used to predict colonic permeability.

Furthermore, Figure 4.2.5 shows the correlations between the permeability values of the *in vitro* models and the oral fraction absorbed in humans. The trend of the correlations obtained with co-culture and triple co-culture was quite similar to the trend of Caco-2 correlations, which indicated that the two *in vitro* models assayed are as useful as Caco-2 to predict the human fraction absorbed. The lines represent the fitted values to the model in equation 2. As it can be observed the co-culture and triple co-culture lines shifted to the right in comparison with Caco-2, indicating co-culture and

triple co-culture models produced higher permeability values. The S factor values were 32.2, 3.8 and 2.8 from Caco-2, co-culture and triple co-culture respectively. That indicated that in the co-culture and triple co-culture the increment of surface over the transwell area is lower than in the Caco-2 model. Considering this increment of surface is due mainly to the presence of microvilli, it is clear than in the co-cultures there is a percentage of cells without this feature, thus this parameter reflects the different cell composition.

A common characteristic can be deduced from Figure 4.2.2, Figure 4.2.3, Figure 4.2.4 and Figure 4.2.5 that is drug permeability obtained with co-culture and triple co-culture is higher than that obtained with Caco-2 monocultures. This fact, as explained before, was due to the wider tight junctions because of the presence of HT29-MTX and Raji B cells. The general tendency was an increase of about two-fold in the co-culture and triple co-culture permeability versus the Caco-2 permeability, except for atenolol, colchicine and valsartan. The increment in permeability of these three drugs is more noticeable due to the contribution of the paracellular route that has more impact on low permeability compound [37].

The triple co-culture is the most physiologic one because of the presence of mucus-secreting cells and M cells. However, the results obtained here do not show an improvement of triple co-culture versus co-culture. The two techniques demonstrated to be good models to predict *in situ* permeability and oral fraction absorbed (Figure 4.2.5). The triple co-culture model is described as an efficient tool to study the intestinal transport of micro and nanoparticles [27, 38]. But, according to our results, both co-culture and triple co-culture can be used to predict permeability values and oral fraction absorbed in humans when the drug is administered as a solution.

Besides, as Table 4.2.3 shows, both models are valid to classify compounds in low or high permeability according to the BCS criteria because,

the classification obtained for co-culture and triple co-culture data is the same that the stablished classification by the BCS.

CONCLUSION

The co-culture and the triple co-culture have proved to be good and reliable *in vitro* methods to study the permeability of drugs, to predict the oral fraction absorbed and also, to classify the compounds according to the BCS criteria. However, to study the passive diffusion, co-culture and triple co-culture do not suppose any advantage over monocultures Caco-2. Therefore, for the screening stages of a drug development, the Caco-2 monocultures are more advisable than co-culture and triple co-culture, because Caco-2 monolayers are a simpler and less laborious model. However, to study thoroughly the transport mechanism of a specific drug, to use a more complex model than Caco-2 monocultures is more suitable because, co-culture and triple co-culture are more physiological models, so the results obtained with them will be closer to those obtained in the human intestine.

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4.3. Artículo científico 5

Closed-loop Doluisio (colon, small intestine) and single-pass intestinal perfusion in rat–biophysical model and predictions based on Caco-2 and PAMPA

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ABSTRACT

The effective rat intestinal permeability (P_{eff}) was dissected using a biophysical model based on parameterized paracellular, aqueous boundary layer, transcellular permeabilities, and the villus-fold surface area expansion factor (k_{VF}), with the parameters determined by regression analysis. Four types of rat *in situ* intestinal perfusion data were considered: published and freshly-measured (a) single-pass intestinal perfusion (SPIP) in the jejunum (n=40), (b) SPIP in the colon (n=15), (c) closed-loop (Doluisio type) in the small intestine (n=78), and (d) closed-loop in the colon (n=74). To determine the upper and lower boundaries of the dynamic range window (DRW), rat transcellular permeability, scaled by k_{VF}, was approximated to that of experimental Caco-2 in the pH 6.5-7.5 interval. The biophysical model predicted rat intestinal permeability data within the experimental uncertainty. This investigation revealed several surprising predictions: (i) many molecules permeate predominantly (but not exclusively) by the paracellular route, (ii) the aqueous boundary layer thickness in the small intestinal perfusion experiments is comparable to that found in unstirred *in vitro* monolayer assays and quite a bit smaller in the colon, (iii) the mucosal surface area in anaesthetized rats in the colon is 0.96-1.4 times the smooth cylinder calculated value, and in the small intestine it is 3.1-3.6 times the smooth cylinder value, and (iv) the relative "leakiness" of the intestine appeared to be greater in rat than human, with the colon surprisingly showing more leakiness than the small intestine, as indicated by the results of the Doluisio method. Given the best-fit biophysical model parameters, *in vitro* Caco-2 and PAMPA permeability values were used to predict *in situ* P_{eff} values in the three types of rat data studied.

Keywords: rat colon and small intestinal closed-loop (Doluisio) permeability; single-pass intestinal perfusion (SPIP); paracellular leakage; aqueous boundary layer resistance; dynamic range window (DRW); Caco-2; PAMPA-DS.

Abbreviations

ABL: aqueous boundary layer – adjacent to the surface of a cell monolayer or luminal side of intestine

Caco-2: human cancer of the colon epithelial cell line

D_{aq}: aqueous diffusivity ($cm \cdot s^{-1}$)

DRW: dynamic range window: observable permeability, with P_{ABL} as top limit and P_{para} as bottom limit

E($\Delta\varphi$): function due to electrical potential drop across the cell junction (dimensionless)

$f_{(0)}$, $f_{(+)}$, $f_{(-)}$: concentration fraction in uncharged, positively-, and negatively-charged forms, respectively

F(r_{HYD}/R): Renkin molecular sieving function, dimensionless fraction in the range of 0 to 1

h_{ABL}: ABL thickness (µm)

k_{VF}: villus-fold surface area expansion factor (dimensionless)

MDCK: Madin-Darby Canine Kidney epithelial cell line

PAMPA-DS: parallel artificial membrane permeability assay, Double-Sink™ intestinal model (Pion Inc.)

P_{ABL}: *in vitro* ABL permeability ($cm \cdot s^{-1}$)

P_{app}: *in vitro* apparent permeability of an epithelial cell monolayer (Caco-2) or PAMPA-DS ($cm \cdot s^{-1}$)

P_C: *in vitro* transcellular or trans-artificial membrane permeability ($cm \cdot s^{-1}$) –

either Caco-2, or PAMPA-DS

$P_C^{in\ situ}$: *in situ* rat transcellular membrane permeability ($cm \cdot s^{-1}$) (i.e., P_{eff}, corrected for k_{VF} surface expansion factor, ABL and paracellular effects)

P_{eff}: *in situ* effective luminal intestinal permeability ($cm \cdot s^{-1}$), from rat single-pass intestinal perfusion (SPIP) or closed-loop Doluisio (CLD) static perfusion technique

P_0, $P_0^{in\ situ}$: intrinsic permeability of uncharged permeant, *in vitro* and *in situ*, respectively ($cm \cdot s^{-1}$).

P_{para}: *in vitro* paracellular permeability ($cm \cdot s^{-1}$)

r_{HYD}: hydrodynamic molecular radius (Å)

R: cell junction pore radius (Å)

ε/δ: porosity of paracellular junction pores divided by the rate-limiting paracellular pathlength (size-restricted, cation-selective)

$(\varepsilon/\delta)_2$: secondary porosity-pathlength ratio (charge/size nonspecific "free diffusion" term)

$\Delta\varphi$: electrical potential drop (mV) across the electric field created by negatively-charged residues lining the junctional pores

INTRODUCTION

Intestinal perfusion studies in rats, among all non-human FDA approved methods, have proven to be a reliable and cost effective option for permeability characterization and BCS classification. A high correlation between human and rat small intestine permeability (r^2 = 0.8-0.95) was reported for drug intestinal permeability with both carrier-mediated absorption and passive diffusion mechanisms [1-4]. Correlation with human

data seems to be good even though the type of transporters and their expression levels may vary between species [5-7]. Rat perfusion studies, on the other hand, add the advantage of being useful to assess segmental-dependent permeability throughout the entire small intestine, a factor that to date cannot be measured in humans [3, 8-13].

Two standard rat perfusion techniques, the single-pass intestinal perfusion (SPIP) model and the Doluisio rat perfusion method have been recently compared [14], demonstrating a similar performance in spite of their substantial differences. The SPIP model focuses on a 10-cm intestinal segment (typically the jejunum) [15, 16], whereas the Doluisio method typically measures the absorption throughout the entire small intestine [17, 18]. With appropriate adjustments, segmental-dependent permeability can be measured by both techniques [3, 11, 13, 19-22]. Moreover, while in the SPIP model each drug molecule gets only one passage through the investigated intestinal segment, in the Doluisio method the tested drug solution remains within the intestinal lumen throughout the entire experiment. Those differences in the experimental set up could eventually produce different estimations, particularly for the low permeability drugs.

Several physiological factors influence the effective permeability (P_{eff}) of the intestinal wall, including (a) passive lipoidal transcellular resistance (indicated by P_C), (b) carrier-mediated (CM) influx/efflux (related to specificity, capacity, and density of carriers), (c) water fluxes (driven by osmotic gradients), (d) luminal pH microclimates, (e) resistance of the aqueous boundary layer (indicated by P_{ABL}), (f) leakage through the tight cellular junctions (indicated by P_{para}), (g) total tight junctional area and tortuosity of channels, as reflected by the ratio of porosity to pathlength (ε/δ), (h) total lipoidal surface area, as related by the villus-fold surface area expansion factor (k_{VF}, defined as 1 for *in vitro* methods, but can be as high as 30 in the human jejunum), and (i) regional differences in capillary blood flow [1, 14, 23-25].

It is a common practice to use *in vitro* models (e.g., excised intestinal sections; cell lines like Caco-2/MDCK; parallel artificial membrane permeability assay, PAMPA) to predict the *in vivo* permeation rates. However, methods of measuring the apparent permeability, P_{app}, are not well harmonized between different laboratories. The differing contributions from the same underlying factors mentioned above (plus *in vitro* filter porosity) make direct comparisons of P_{app} to P_{eff} values very unlikely to produce satisfactory *in vitro-in vivo* corrections (IVIVC). A recent study correlating several *in vitro* models to rodent *in situ* permeability indicated r^2 = 0.04 to 0.33 by direct P_{app} to P_{eff} comparisons. After reducing the apparent and effective values to the "intrinsic" scale (log P_0), it was found that r^2 increased to 0.58 across all models in log $P_0^{in\ vitro}$- log $P_0^{in\ vivo}$ correlations [26]. Better correlation would have been achieved at the low end of the permeability scale, were it not for the excessive leakiness of some of the *in vitro* cell models in the above comparisons.

The measured cell permeability, P_{app} or P_{eff}, is bounded by two aqueous diffusion limits: at the low end by cell junction leakage, indicated by the paracellular permeability (P_{para}), and at the high end by resistance of the aqueous boundary layer (ABL), indicated by the permeability, P_{ABL}. These two limits define the dynamic range window (DRW), as shown in Figure 4.3.1 for a model ionizable basic drug. Measured permeability values are confined within this window. Extracting cell membrane permeability (P_C) from the apparent/effective values for a system with very narrow DRW (e.g., with leaky junctions and poorly-stirred solution) is quite challenging, at times not possible.

The ABL, also known as the unstirred water layer (UWL), is a region of convectively-unstirred solution adjacent to the cell surface [27-30]. The ABL is a rate-limiting step for permeation of lipophilic compounds, resulting in reduction of the measured permeability [31-35], leading to reduced dynamic range and lower resolution in rank-ordering compound permeation. Less

often recognized, the ABL can also bias the calculated Michaelis-Menten transport kinetic parameters, K_M and J_{max} [36, 37]. Also, it can affect analysis of specific carrier-mediated (CM) transport and its dependence on inhibitors [38]. If the ABL effect is neglected or ignored, the measured *in vitro* permeability will not accurately reflect the true permeability *in vivo*. Early investigations of the ABL effect on *in vitro* cell permeability [28, 31, 39, 40] prompted awareness of the problem, but methods of accounting for the effect of the ABL are seldom applied in routine studies in current practice.

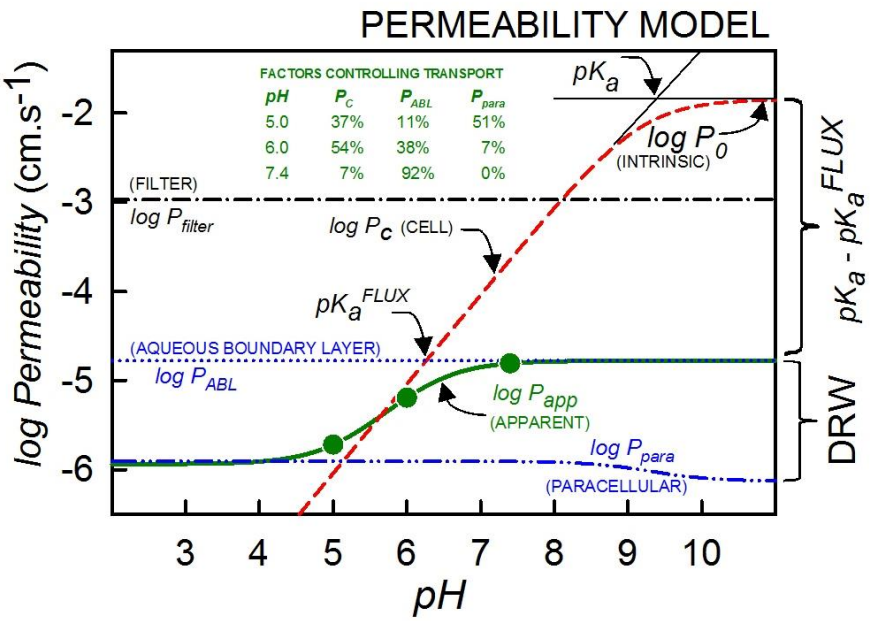

Figure 4.3.1. pK_a^{FLUX} method to derive intrinsic transcellular permeability, P_0, illustrated with an ionizable base (pK_a 9.2). The sigmoidal solid curve represents the apparent permeability, log P_{app}. The inflection point in the curve shows the apparent pK_a or pK_a^{FLUX}. The hyperbolic dashed curve represents the transendothelial (cellular) permeability of the compound, log P_C (cell), with the curve maximum indicating intrinsic transcellular permeability, log P_0. The horizontal dotted line shows the aqueous boundary layer permeability of the compound, log P_{ABL}. The horizontal dashed-double-dotted line represents the paracellular permeability, log P_{para}. The gap between the aqueous boundary layer and the paracellular permeability boundaries, DRW (dynamic range window), is a zone where the apparent permeability contains an appreciable transendothelial (cellular) contribution. The dashed-dotted line shows permeability through filter insert, log P_{filter}.

The tight junctions between epithelial cells are an important protective barrier in the intestine that regulates the diffusion of small polar/charged solutes through size-restricted and cation-selective water-filled "paracellular" channels, while blocking potentially toxic large molecules. Adson *et al.* in 1994 [40] quantitatively characterized the leakiness and size exclusion properties of such junctions in Caco-2 monolayers in terms of three parameters: (i) ε/δ (porosity-pathlength ratio; a capacity factor), (ii) R (pore radius), and (iii) $\Delta\varphi$ (electrical potential drop in the channels). The porosity, ε, is defined as the fractional area of exposed aqueous channels at the cell surface. Estimates for ε range from 0.001 to 0.1% [1, 2, 41]. The tortuous pathlength of the paracellular junctions is denoted by δ. Values of the ε/δ capacity factor in the *in vitro* cell models range from 0.1 (tight) to 70 (very leaky) cm^{-1}, with most values < 1.5 cm^{-1} [42]. The human jejunum $\varepsilon/\delta \approx 0.53$ cm^{-1}; a "secondary" capacity factor, $(\varepsilon/\delta)_2 \approx 0.027$ cm^{-1}, describing the "large hydrophilic molecule" leakiness in the human jejunum, has also been characterized [25]. Estimates of R range from 4 to 13 Å in fully-intact Caco-2 monolayers [42]. The value for the human jejunum has been estimated as 11 ± 2 Å [25]. The average paracellular voltage drop in Caco-2 models was estimated to be $\Delta\varphi$ = -43 ± 20 mV, drawing on data from several laboratories [42]. A -31 ± 16 mV value was predicted for the human jejunum [25]. Appendix A lists the various equations that frame the ABL and paracellular parameters that define the boundaries of the DRW.

To improve IVIVC analysis, Avdeef and Tam (2010) [25] used the above ABL and paracellular parameters to construct a biophysical model which was applied to the analysis of the human jejunal permeability data taken from several publications (especially from Lennernäs). The resultant model considered the combined effects of the (i) absorption-accessible surface area, (ii) dual-pore variant of the Adson paracellular model, and (iii) ABL- and paracellular-corrected Caco-2/MDCK values equated to *in vivo* transcellular permeability (P_C), as a means of determining *in vivo* ABL and paracellular

characteristics. The investigation revealed several surprises: the unstirred water layer thickness in the human *in situ* perfusion experiments ("double-balloon" type) is much greater than had been commonly thought, the absorption-accessible (effective) mucosal surface area in awake human subjects is apparently not dependent on lipophilicity of the drug, in contrast to anaesthetized-rodent studies [43], and the relative "leakiness" of the human jejunum is not so different from that observed in a number of Caco-2 studies (e.g., from Garberg *et al*., 2005 [44]).

In the current investigation, we extended the biophysical model to rat colon and small intestine data for 92 molecules (Table 4.3.1), measured by two different approaches. The SPIP method carried out in the jejunum and colon, and the closed-loop Doluisio (CLD) method [17, 18] carried out in the small intestine and colon, were compared to those estimated for the human jejunum. Two prediction models were explored, using Caco-2 and PAMPA measured P_{app} values as the descriptors.

MATERIALS AND METHODS

Materials

The new measurement compounds in rat (acetaminophen, digoxin, famotidine, fexofenadine, hydrocortisone, ibuprofen, lucifer yellow, metformin, oxprenolol, phenol red, pravastatin, propranolol and rosuvastatin) and the newly-measured molecules in PAMPA (3-phenylpropionic acid, 4-isopropylaniline, 4-phenylbutiric acid, 5-phenylvaleric acid, codeine, mepivacaine, pravastatin, rosuvastatin and valsartan) were purchased from Sigma-Aldrich.

Table 4.3.1. Physical properties of the 92 compounds studied [a].

Compound	MW (g/mol)	$pK_{a1}^{37°C}$	$pK_{a2}^{37°C}$	Type [b]	log P_0 PAMPA [c]	log P_0 Caco-2 [d]	r_{HYD} (Å) [e]	D_{aq} (10^{-6} $cm^2.s^{-1}$) [f]
1,1-Diphenylmethylamine	183.25	7.71		B	-1.70	-2.60	3.63	9.35
1,2-Diphenylethylamine	197.28	9.17		B	-0.89	-2.25	3.72	9.04
1-Naphthylamine	143.18	3.69		B	-3.31	-3.70	3.35	10.46
2,2-Diphenylethylamine	197.28	7.71		B	-1.18	-2.39	3.72	9.04
2,4-Dimethylaniline	121.18	4.67		B	-3.68	-3.91	3.18	11.28
2-Methylaniline	107.15	3.99		B	-4.04	-3.99	3.07	11.93
2-Naphthylamine	143.18	3.79		B	-3.31	-3.70	3.35	10.46
2-Phenylethylamine	121.18	9.48		B	-2.09	-2.44	3.18	11.28
3,3-Diphenylpropylamine	211.30	8.88		B	-0.46	-2.09	3.81	8.77
3-Phenylpropionic Acid	150.17	4.64		A	-3.45	-1.80	3.40	10.23
3-Phenylpropylamine	135.21	9.66		B	-1.59	-2.24	3.29	10.73
4-Aminobiphenyl	169.22	4.19		B	-2.94	-3.58	3.53	9.70
4-Butylaniline	149.23	4.68		B	-2.78	-3.65	3.39	10.26
4-Cyclohexylaniline	175.27	4.68		B	-2.44	-3.54	3.57	9.54
4-Ethylaniline	121.18	4.67		B	-3.62	-3.87	3.18	11.28
4-Isopropylaniline	135.21	4.68		B	-2.94	-3.84	3.29	10.73
4-Methylaniline	107.15	4.67		B	-4.04	-3.99	3.07	11.93
4-Phenylbutylamine	149.23	10.24		B	-1.23	-2.10	3.39	10.26
4-Phenylbutyric Acid	164.20	4.87		A	-2.94	-1.73	3.50	9.83
4-Propylaniline	135.21	4.68		B	-3.20	-3.76	3.29	10.73

Table 4.3.1. Physical properties of the 92 compounds studied [a] (continued).

Compound	MW (g/mol)	$pK_{a1}^{37°C}$	$pK_{a2}^{37°C}$	Type [b]	log P_0 PAMPA [c]	log P_0 Caco-2 [d]	r_{HYD} (Å) [e]	D_{aq} (10^{-6} $cm^2.s^{-1}$) [f]
4-t-Butylaniline	149.23	4.68		B	2.99	-3.75	3.39	10.26
5-Phenylvaleric Acid	178.23	4.59		A	-2.36	-0.75	3.59	9.47
6-Phenylhexanoic Acid	192.25	4.88		A	-2.73	-0.74	3.69	9.15
7-Phenylheptanoic Acid	206.28	4.88		A	-2.30	-0.40	3.78	8.86
8-Phenyloctanoic Acid	220.31	4.89		A	-1.88	-0.07	3.87	8.60
Acetaminophen	151.16	9.36		A	-5.81	-4.34	3.40	10.20
Aminopyrine	231.29	4.82		B	-4.10	-4.60	3.93	8.42
Amiodarone	645.31	9.37		B	1.12	-2.60	5.89	5.29
Amoxicillin	365.40	2.64	7.00	AB	-6.80	-5.70	4.67	6.84
Aniline	93.13	4.41		B	-3.71	-4.06	2.96	12.71
Antipyrine	188.23	1.33		B	-5.69	-4.05	3.66	9.24
Atenolol	266.34	9.19		B	-5.06	-4.34	4.14	7.89
Baclofen	213.66	3.72	9.49	AB	-6.34	-5.29	3.82	8.72
Barbital	184.19		7.70	A	-5.20	-4.69	3.63	9.33
Benzylamine	107.15	9.00		B	-2.68	-2.68	3.07	11.93
Bupivacaine	288.43	7.69		B	-2.07	-2.40	4.27	7.61
Caffeine	194.20	--		N	-5.55	-4.14	3.70	9.11
Carbamazepine	236.27	--		N	-3.73	-3.69	3.96	8.33
Cefadroxil	363.39	2.63	7.11	AB	-7.57	-6.07	4.66	6.86

Table 4.3.1. Physical properties of the 92 compounds studied [a] (continued).

Compound	MW (g/mol)	$pK_{a1}^{37°C}$	$pK_{a2}^{37°C}$	Type [b]	log P_0 PAMPA [c]	log P_0 Caco-2 [d]	r_{HYD} (Å) [e]	D_{aq} (10^{-6} $cm^2.s^{-1}$) [f]
Chloramphenicol	323.13	--		N	-5.58	-4.47	4.46	7.23
Cimetidine	252.30	6.78		B	-6.20	-6.06	4.06	8.09
Codeine	299.36	7.99		B	-3.64	-3.51 [g]	4.33	7.49
Colchicine	399.44	--		N	-5.40	-6.03 [g]	4.84	6.57
Desmethyletidocaine	262.39	7.93		B	-0.47	-2.11	4.12	7.95
Dexamethasone	392.46	--		N	-4.05	-4.65	4.81	6.62
Dibutylamine	129.24	10.23		B	-0.79	-1.63	3.24	10.95
Diethylamine	73.14	10.54		B	-2.40	-2.25	2.80	14.18
Digoxin	780.94	--		N	-5.78	-5.43	6.38	4.85
Dihexylamine	185.35	10.24		B	0.91	-0.97	3.64	9.30
Dimethylamine	45.08	10.38		B	-3.28	-2.60	2.59	17.65
Dipentylamine	157.29	10.24		B	0.06	-1.30	3.45	10.02
Dipropylamine	101.19	10.22		B	-1.63	-1.96	3.03	12.24
Etidocaine	276.42	7.93		B	-0.05	-1.95	4.20	7.76
Famotidine	337.44	6.55	10.67	BA	-7.75	-6.41	4.53	7.09
Fexofenadine	501.66	4.35	7.85	AB	-5.17	-6.46	5.31	5.93
Furosemide	330.74	3.53	9.90	AA	-4.03	-3.50	4.50	7.16
Glipizide	445.54	5.24		A	-3.77	-2.47	5.06	6.25
Haloperidol	375.86	8.29		B	0.05	-2.76	4.73	6.75

Table 4.3.1. Physical properties of the 92 compounds studied [a] (continued).

Compound	MW (g/mol)	$pK_{a1}^{37°C}$	$pK_{a2}^{37°C}$	Type [b]	log P_0 PAMPA [c]	log P_0 Caco-2 [d]	r_{HYD} (Å) [e]	D_{aq} (10^{-6} $cm^2.s^{-1}$) [f]
Hydrochlorothiazide	297.74	8.54	9.80	AA	-8.30	-6.32	4.32	7.51
Hydrocortisone	362.46	--		N	-4.32	-4.63	4.66	6.87
Ibuprofen	206.28	4.25		A	-2.11	-0.53	3.78	8.86
Labetolol	328.41	7.25	9.03	AB	-4.94	-4.27	4.48	7.18
Lidocaine	234.34	7.69		B	-1.42	-2.69	3.95	8.37
Lucifer Yellow	437.32	-2.6	-1.9	AA	-12.00	-9.25	5.02	6.31
Mepivacaine	246.35	7.56		B	-3.24	-2.54	4.02	8.18
Metformin	129.16	2.77	11.93	BB	-7.28	-3.51	3.24	10.96
Methyl Lidocaine	284.83	--		N	-4.17	-5.37	4.25	7.66
Metoprolol	267.40	9.19		B	-1.17	-1.85	4.15	7.88
Minoxidil	209.25	4.42		B	-4.73	-5.68	3.80	8.81
Naproxen	230.26	4.14		A	-2.30	-0.95	3.93	8.43
Nifedipine	346.33	2.08		B	-3.35	-4.10	4.58	7.01
N-Methylethylamine	59.11	10.21		B	-2.89	-2.45	2.69	15.61
N-Propylbutylamine	115.22	10.23		B	-1.21	-1.80	3.14	11.54
Octacaine	234.34	7.93		B	-1.97	-2.72	3.95	8.37
Oxprenolol	265.35	9.20		B	-0.60	-2.52	4.14	7.91
Phenol Red	352.36	0.96	7.84	AA	-3.84	-2.83	4.61	6.95
Pravastatin	424.53	4.41		A	-5.07	-3.82	4.96	6.39

Table 4.3.1. Physical properties of the 92 compounds studied [a] (continued).

Compound	MW (g/mol)	$pK_{a1}^{37°C}$	$pK_{a2}^{37°C}$	Type [b]	log P_0 PAMPA [c]	log P_0 Caco-2 [d]	r_{HYD} (Å) [e]	D_{aq} (10^{-6} $cm^2.s^{-1}$) [f]
Prochlorperazine	373.94	3.63	7.69	BB	0.84	-2.22	4.72	6.77
Progesterone	314.46	--		N	-2.55	-3.49	4.41	7.32
Propranolol	259.30	9.16		B	0.43	-1.54	4.10	7.99
Pseudoephedrine	165.23	9.39		B	-2.77	-2.92	3.50	9.80
Ranitidine	314.40	2.05	8.07	BB	-5.14	-5.27	4.41	7.32
Rodocaine	320.86	8.62		B	-0.91	-2.57	4.44	7.26
Rosuvastatin	481.54	4.63		A	-3.53	-5.27	5.22	6.04
Salicylic Acid	138.12	2.82		A	-2.64	-0.43	3.31	10.63
Sotalol	272.36	8.11	9.50	AB	-4.02	-4.60	4.18	7.81
Sulfaethidole	284.36	2.09	5.62	BA	-4.92	-3.96	4.24	7.66
Sulfasalazine	398.39	2.75	7.86	AA	-4.41	-2.66	4.84	6.58
Terbutaline	225.30	8.46	9.71	BA	-7.25	-5.23	3.90	8.52
Theophylline	180.20	8.34		A	-5.99	-4.17	3.61	9.42
Valsartan	435.52	3.46	4.54	AA	-1.92	-1.91	5.01	6.32
Verapamil	454.60	8.68		B	0.26	-2.18	5.10	6.20

Note: [a] Underlined pKa, log P_0 values were calculated using pCEL-X (in-ADME Research), where experimental values were not found. [b] B = base, A = acid, AB/BA = ampholyte, N = nonionizable. [c] Intrinsic PAMPA permeability, Double-Sink™ intestinal model (Pion Inc.). Values taken from Avdeef tabulation [45]. See text for newly measured values. [d] Intrinsic Caco-2 permeability. Values taken from Avdeef (2012) tabulation, except as noted below. [e] Calculated hydrodynamic radius of molecule, using equation (6). [f] Calculated aqueous diffusivity, using equation (2). [g] Obtained from Lozoya-Agullo et al. [46]

pK_a sources

The *Wiki-pK_a* database (www.in-adme.com/tools.html) was the source of the pK_a data used in the study. Since most of the entries are at 25 °C, conversions to 37 °C were done where necessary, using the approach described by Sun and Avdeef [47]. For 29 of the molecules, the *p*CEL-X program (*in-ADME* Research) was used to predict the pK_a at 37 °C, as indicated by underlined entries in Table 4.3.1.

PAMPA-DS measurement

Double-Sink™ PAMPA method (Pion Inc), a highly-standardized variant of that introduced by Kansy *et al.* [48], was used to measure permeability at 23 ± 2 °C of several of the studied compounds [49]. The 96-well donor-receiver pairs, separated by a filter-impregnated lecithin alkane solution, were joined into a "sandwich". The donor compartments were preloaded with 96 magnetic stirrers (Pion, PN 110243). The donor wells contained 50 to 1520 µM sample buffer solutions, along with about 0.5% v/v DMSO. The donor solutions in the Prisma HT universal buffer (Pion Inc. P/N 110151) were adjusted with NaOH to pH from 2.5 to 10, while the receiver solutions had the same pH 7.4 (Acceptor Sink Buffer, Pion Inc. P/N 110139). Optimized pH conditions were selected in the donor chambers for each compound to ensure that the pH values would be above and below the pK_a^{FLUX} value (Figure 4.3.1) of the compounds. The pH variation was necessary in order to correct the effective permeability values for ionization and aqueous boundary layer (ABL) effects [45, 49]. The receiver solutions contained a surfactant mixture ("lipophilic sink") to mitigate quick equilibration of weak bases under lower-to-higher pH gradient conditions. Vigorous stirring was employed in the assay when necessary using Gut-Box™ device (Pion), with stirring speed set to produce an ABL thickness of about 40 µm. The PAMPA sandwich was allowed to incubate for either 30 min with highly permeable molecules or for 16 hours with low permeable compounds (e.g., metformin, pravastatin, rosuvastatin, cefadroxil, baclofen). After incubation, the sandwich was then

separated, and both the donor and receiver wells were assayed for the amount of material present, by comparison with the UV spectrum (230 to 500 nm) obtained from reference standard solutions (i.e., donor solutions at t=0). Mass balance was used to determine the amount of material remaining in the membrane filter (%R) and attached to the plastic walls of the microtitre plate. The effective permeability was calculated as described previously [45, 49]

PAMPA-DS values not measured here nor found in the published literature were predicted by the *p*CEL-X program (indicated by underlined entries in Table 4.3.1).

Caco-2 data sources

For the 92 molecules considered here, measured Caco-2 permeability values for 32 compounds were obtained from published sources (tabulation in Avdeef, 2012 [45]; Lozoya-Agullo *et al.* [14]). Table 4.3.1 lists the Caco-2 log P_0 (cf., equation (3)) used in the study. Sixty of the "missing" values were predicted by the *p*CEL-X program (indicated by underlined entries in the table), factoring in passive permeability contributions. Most of the missing values were predominantly of simple amine and aniline derivatives studied here.

Rat intestinal permeability legacy data sources

Most of the rat P_{eff} data for the test compounds (Table 4.3.2 lists 74 colon closed-loop, 78 small-intestine closed-loop, 15 colon single-pass, and 40 jejunum single-pass P_{eff} values) have been published [14, 17, 21, 46, 50-59]. Those which have not been previously published are described below.

Table 4.3.2. Rat intestinal perfusion effective permeability data.

Compound	Colon (Closed-Loop Doluisio)				Small Intestine (Closed-Loop Doluisio)			
	pH	P_{eff} (10^{-6} cm.s^{-1})	SD	ref	pH	P_{eff} (10^{-6} cm.s^{-1})	SD	ref
1,1-Diphenylmethylamine	7.5	98	16	[51]	6.2	63	7	[51]
1,2-Diphenylethylamine	7.5	106	7	[51]	6.2	73	13	[51]
1-Naphthylamine	7.5	168	8	[50]	6.2	204	10	[50]
2,2-Diphenylethylamine	7.5	97	6	[51]	6.2	69	4	[51]
2,4-Dimethylaniline	7.5	154	12	[50]	6.2	156	4	[50]
2-Methylaniline	7.5	149	17	[50]	6.2	148	8	[50]
2-Naphthylamine	7.5	169	17	[50]	6.2	207	7	[50]
2-Phenylethylamine	7.5	71	8	[51]	6.2	42	6	[51]
3,3-Diphenylpropylamine	7.5	110	16	[51]	6.2	80	10	[51]
3-Phenylpropionic Acid	7.5	80	6	[58]	6.2	184	7	[56]
3-Phenylpropylamine	7.5	81	4	[51]	6.2	38	4	[51]
4-Aminobiphenyl	7.5	174	15	[50]	6.2	192	6	[50]
4-Butylaniline	7.5	179	14	[50]	6.2	163	7	[54]
4-Cyclohexylaniline	7.5	182	12	[50]	6.2	152	9	[50]
4-Ethylaniline	7.5	165	18	[50]	6.2	183	8	[54]
4-Isopropylaniline	7.5	169	9	[50]	6.2	160	13	[50]
4-Methylaniline	7.5	147	17	[50]	6.2	159	9	[54]
4-Phenylbutylamine	7.5	94	10	[51]	6.2	59	10	[51]
4-Phenylbutyric Acid	7.5	100	6	[58]	6.2	180	10	[56]

Table 4.3.2. Rat intestinal perfusion effective permeability data (continued).

Compound	Colon (Closed-Loop Doluisio)				Small Intestine (Closed-Loop Doluisio)			
	pH	P_{eff} (10^{-6} cm.s^{-1})	SD	ref	pH	P_{eff} (10^{-6} cm.s^{-1})	SD	ref
4-Propylaniline	7.5	171	12	[50]	6.2	196	5	[50]
4-t-Butylaniline	7.5	173	14	[50]	6.2	169	13	[50]
5-Phenylvaleric Acid	7.5	127	9	[58]	6.2	191	10	[56]
6-Phenylhexanoic Acid	7.5	142	6	[58]	6.2	191	5	[56]
7-Phenylheptanoic Acid	7.5	162	13	[58]	6.2	194	8	[56]
8-Phenyloctanoic Acid	7.5	170	7	[58]	6.2	196	9	[56]
Acetaminophen	7.4	91	11	a	7.0	82	12	[14]
Aminopyrine					6.2	33	--	[17]
Amoxicillin	7.0	30	5	[46]	7.0	17	4	[46]
Aniline	7.5	108	7	[50]	6.2	140	12	[50]
Antipyrine	7.0	85	9	[46]	7.0	101	11	[46]
Atenolol	7.0	21	4	[46]	7.0	11	2	[14]
Baclofen	7.5	4.3	2.7	[21]				
Barbital					6.0	55	--	[17]
Benzylamine	7.5	45	6	[51]	6.2	43	8	[51]
Bupivacaine	7.5	72	7	[52]	6.2	49	5	[53]
Caffeine	7.0	87	20	[46]	7.0	105	19	[46]
Carbamazepine	7.0	142	23	[46]	7.0	128	12	[46]
Cefadroxil	7.5	4.9	4.3	[55]	6.2	65	--	[59]

Table 4.3.2. Rat intestinal perfusion effective permeability data (continued).

Compound	Colon (Closed-Loop Doluisio)				Small Intestine (Closed-Loop Doluisio)			
	pH	P_{eff} (10^{-6} cm.s^{-1})	SD	ref	pH	P_{eff} (10^{-6} cm.s^{-1})	SD	ref
Chloramphenicol					7.0	98	3	[14]
Cimetidine	7.0	35	8	[46]	7.0	14	3	[14]
Cimetidine					7.0	30	6	[46]
Codeine	7.0	58	9	[46]	7.0	55	2	[46]
Colchicine	7.0	32	9	[46]	7.0	28	7	[14]
Colchicine					7.0	25	4	[46]
Desmethyletidocaine	7.5	79	46	[52]	6.2	49	5	[53]
Dibutylamine	7.5	47	5	[57]	6.2	30	5	[57]
Diethylamine	7.5	13	5	[57]	6.2	15	3	[57]
Digoxin	7.4	49	18	a	7.0	26	10	[14]
Dihexylamine	7.5	102	11	[57]	6.2	78	1	[57]
Dimethylamine	7.5	6.6	1.6	[57]	6.2	31	3	[57]
Dipentylamine	7.5	78	11	[57]	6.2	53	1	[57]
Dipropylamine	7.5	27	3	[57]	6.2	21	2	[57]
Etidocaine	7.5	82	9	[52]	6.2	50	6	[53]
Famotidine	7.4	51	7	a				
Fexofenadine	7.4	44	3	a	7.0	1.2	0.3	a
Furosemide	7.0	22	7	[46]	7.0	31	6	[46]
Haloperidol					6.0	42	--	[17]

Table 4.3.2. Rat intestinal perfusion effective permeability data (continued).

Compound	Colon (Closed-Loop Doluisio)				Small Intestine (Closed-Loop Doluisio)			
	pH	P_{eff} (10^{-6} cm.s^{-1})	SD	ref	pH	P_{eff} (10^{-6} cm.s^{-1})	SD	ref
Hydrocortisone	7.4	69	8	a				
Ibuprofen	7.0	249	23	a	7.0	150	13	[14]
Labetolol					7.0	34	6	[14]
Lidocaine	7.5	65	5	[52]	6.2	39	5	[53]
Lucifer Yellow	7.0	7.2	2.7	a	7.0	9.3	2.5	a
Mepivacaine	7.5	50	4	[52]	6.2	38	4	[53]
Metformin	7.4	45	10	a				
Methyl Lidocaine	7.5	67	6	[52]	6.2	39	1	[53]
Metoprolol	7.0	81	19	[46]	7.0	66	6	[14]
Metoprolol					7.0	62	27	[46]
Naproxen	7.0	167	12	[46]	7.0	184	12	[46]
N-Methylethylamine	7.5	10	3	[57]	6.2	26	1	[57]
N-Propylbutylamine	7.5	36	4	[57]	6.2	24	3	[57]
Octacaine	7.5	42	5	[52]	6.2	31	4	[53]
Oxprenolol	7.4	111	9	a				
Phenol Red	7.0	6.6	2.4	a	7.0	10	1	a
Pravastatin	7.4	55	10	a				
Prochlorperazine					6.0	45	--	[17]
Propranolol	7.0	162	29	a	7.0	86	6	a

Table 4.3.2. Rat intestinal perfusion effective permeability data (continued).

Compound	Colon (Closed-Loop Doluisio)				Small Intestine (Closed-Loop Doluisio)			
	pH	P_{eff} (10^{-6} cm.s^{-1})	SD	ref	pH	P_{eff} (10^{-6} cm.s^{-1})	SD	ref
Ranitidine	7.0	30	7	[46]	7.0	14	3	[46]
Rodocaine	7.5	71	7	[52]	6.2	47	4	[53]
Rosuvastatin	7.4	58	9	a				
Salicylic Acid					6.0	126	--	[17]
Sulfaethidole					6.0	33	--	[17]
Terbutaline	7.0	36	7	[46]	7.0	16	3	[46]
Theophylline	7.0	106	8	[46]	7.0	99	7	[46]
Valsartan	7.0	33	6	[46]	7.0	26	2	[46]
Verapamil	7.0	123	31	[46]	7.0	106	10	[46]
Compound	**Colon (Single-Pass)**				**Jejunum (Single-Pass)**			
	pH	**P_{eff} (10^{-6} cm.s^{-1})**	**SD**	**ref**	**pH**	**P_{eff} (10^{-6} cm.s^{-1})**	**SD**	**ref**
Acetaminophen	6.5	141	31	a	6.5	56	17	[14]
Amiodarone					6.5	2000	20	[60]
Antipyrine	6.5	124	23	a	6.5	120	22	[14]
Antipyrine					6.5	148	30	a
Atenolol	6.5	2.0	0.3	a	6.5	2.8	0.4	[14]
Caffeine	6.5	115	25	a	6.5	110	24	[14]
Caffeine					6.5	163	24	a
Carbamazepine	6.5	160	10	a	6.5	160	10	[14]

Table 4.3.2. Rat intestinal perfusion effective permeability data (continued).

Compound	Colon (Single-Pass)				Jejunum (Single-Pass)			
	pH	P_{eff} (10^{-6} cm.s^{-1})	SD	ref	pH	P_{eff} (10^{-6} cm.s^{-1})	SD	ref
Carbamazepine					6.5	254	41	[61]
Chloramphenicol					6.5	110	10	[14]
Cimetidine	6.5	7.2	0.7	a	6.5	7.2	0.5	[14]
Cimetidine					6.5	13	1	[11]
Codeine	6.5	36	7	a	6.5	35	7	[14]
Codeine					6.5	49	5	[62]
Colchicine	6.5	5.0	0.4	a	6.5	5.0	0.4	[14]
Colchicine					6.5	12	39	[63]
Dexamethasone					6.5	40	2	[64]
Digoxin	6.5	78	8	a	6.5	30	4	[14]
Digoxin					6.5	34	9	a
Famotidine					6.5	3.7	0.9	[3]
Furosemide	6.5	9.0	0.5	a	6.5	9.0	0.5	[14]
Furosemide					6.5	16	1	a
Glipizide					6.5	75	6	[65]
Hydrochlorothiazide					6.5	22	13	a
Hydrocortisone	6.5	117	19	a	6.5	54	7	a
Ibuprofen	6.5	135	22	a	6.5	220	34	[14]
Ibuprofen					6.5	228	34	a

Table 4.3.2. Rat intestinal perfusion effective permeability data (continued).

Compound	Colon (Single-Pass)				Jejunum (Single-Pass)			
	pH	P_{eff} (10^{-6} cm.s^{-1})	SD	ref	pH	P_{eff} (10^{-6} cm.s^{-1})	SD	ref
Labetolol					6.5	27	2	[14]
Metformin(+)	6.5	40	12	a	6.5	16	5	a
Metoprolol	6.5	60	9	a	6.5	36	5	[14]
					6.5	43	4	a
Minoxidil					6.5	23	7	[66]
Nifedipine					6.5	700	100	[67]
Pravastatin	6.5	57	1	a	6.5	2.3	0.2	a
Progesterone					6.5	824	250	[68]
Propanolol					6.5	49	7	[69]
Pseudoephedrine					6.5	19	8	[13]
Sotalol					6.5	3.9	0.4	[12]
Sulfasalazine					6.5	7.5	2.5	[70]
Valsartan					6.5	6.0	0.7	[14]

Note: a, this work.

New rat intestinal perfusion measurement

1. Closed-loop

Male Wistar rats were used in accordance with 2010/63/EU directive of 22 September 2010 regarding the protection of animals used for scientific experimentation. The Ethics Committee for Animal Experimentation of the University of Valencia approved the experimental protocols (Spain, code A1330354541263).

The rat permeability values of the test compounds not previously published were determined by *in situ* closed-loop perfusion method based on Doluisio's technique as described in previous work [46]. Briefly, an isolated compartment was created in the intestinal segment of interest: the whole small intestine or the colon. The drug solution (10 mL for small intestine and 5 mL for colon) was introduced into the isolated segment and the samples were collected every 5 minutes up to a period of 30 minutes.

2. Single-pass

The single-pass intestinal perfusion studies were performed using protocols approved by the Ben-Gurion University of the Negev Animal Use and Care Committee (Protocol IL-60-11-2010). Male Wistar rats (250–300 g, Harlan, Israel) were housed and handled according to the Ben-Gurion University of the Negev Unit for Laboratory Animal Medicine Guidelines.

The rat permeability values of the test compounds not published before were determined by single-pass as describe in previous work [11]. Briefly, 10-cm from jejunum or the colon segment were cannulated on two ends. The drug solution was perfused through the intestinal or colonic segment at a flow rate of 0.2 $mL \cdot min^{-1}$ for 1 h without sampling, to ensure steady state conditions, followed by additional 1 h of perfusion with samples taken every 10 min.

The "intrinsic" permeability, P_0, for in vitro-in situ correlation

When rigorously comparing physicochemical properties of ionizable compounds with data compiled from different laboratories using different protocol modifications, it is a useful practice to transform the measured properties to the standard "intrinsic" state in which the molecule is uncharged. Many useful physical property descriptors (Abraham descriptors, hydrogen-bonding potentials, etc.) are only valid in reference to such a standard state. One could have defined a different standard state, e.g., pH 7.4. However, fundamental properties of molecules would be difficult to compare if the molecules had substantially different pK_a values and variable pH were used. In the "intrinsic" standard state used here, the pH effect is factored out, along with other effects, such as those associated with transport by aqueous diffusion through the paracellular channels and the aqueous boundary layer. Thus, "intrinsic" permeability refers to the passive lipoidal or carrier-mediated permeability (or to a mixture of the two) of the test compound in its uncharged form. The mathematical treatment of such "normalization" is described in detail in Appendix A. A critical step in our study was to convert the measured apparent and effective permeability, P_{app} (Caco-2 and PAMPA) and P_{eff} (SPIP and CLD small intestine and colon) to the intrinsic scale, P_0. The hydrodynamic environments of the two permeability assays (*in vitro* cell monolayer and *in situ* intestinal perfusion) are very different, necessitating the transformation to achieve improved IVIVC.

RESULTS

PAMPA-DS measurements and sources

Figure 4.3.2 shows the permeability-pH profiles of nine newly-measured molecules. Table 4.3.1 contains the list of the 92 PAMPA-DS log P_0 used here. It contains 47 predicted values (underlined entries), using the *p*CEL-X program. The remaining 45 experimental values were sourced from the compilation in Avdeef, 2012 [45], or were those reported in Figure 4.3.2.

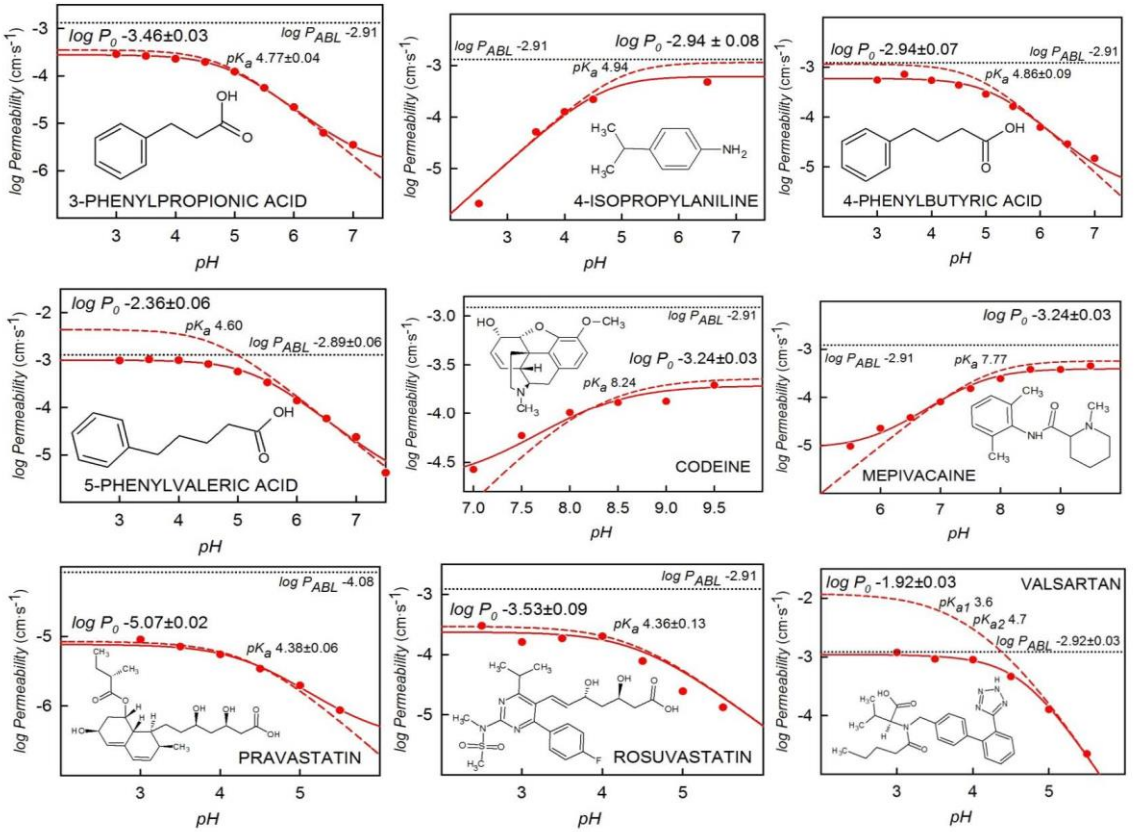

Figure 4.3.2. The PAMPA-DS permeability-pH profiles of nine newly-measured molecules.

Rat Intestinal perfusion data, P_{eff}

Table 4.3.2 lists the four types of rat *in situ* perfusion P_{eff} data used in the study, with colon and small-intestine measurements done by the CLD method, and with jejunum and colon data done by the SPIP method. Much of the data had been already published but some measurements are original to our study, as indicated in Table 4.3.2.

Dynamic Range Window (DRW) paracellular and aqueous boundary layer (ABL) analysis

It was decided to use only the *experimental* Caco-2 data as independent variables to evaluate the boundaries of the DRW, as described in Appendix A. Early exploration using both measured and predicted Caco-2 data did not appear to work well for the colon P_{eff} data, due to its high paracellular permeability. The mathematical procedure here is the same as that used to evaluate the human jejunal "double-balloon" P_{eff} data [25].

In the biophysical model (Appendix A), each P_{eff} is assumed to be a composite of three contributions: ABL, paracellular, and transcellular. The first two (protocol-sensitive) contributions are the same for all compounds for a given segment of the intestine, but the last contribution depends on the specific membrane permeability of each compound. Since P_{eff} is measured only at one pH, it is not possible to use the pK_a^{FLUX} method [33] to solve for the rat transcellular P_C, given three unknowns and one known. The computational strategy used here organizes the problem into two stages, with the obtained results detailed below.

1. First-stage analysis of system-wide DRW parameters

In the first stage, just three system-wide parameters are determined: P_{ABL}, P_{para}, and k_{VF} (the surface expansion factor). All of the P_{eff} data for a particular segment are used as dependent variables. At this stage, P_C is

approximated by the independent variable, i.e., Caco-2 permeability, for each molecule, adjusted from the intrinsic value to that appropriate at the *in situ* pH, using the pK_a of the molecule. For those molecules where the experimental Caco-2 values were not available, a zero weight was assigned during the regression analysis.

If the rat P_C estimate based on Caco-2 is well below the DRW lower limit (cf., Figure 4.3.1), then the molecule serves as a "paracellular marker". In the case of the colon CLD analysis, examples of such markers were amoxicillin, atenolol, cefadroxil, cimetidine, colchicine, furosemide, ranitidine, and terbutaline. In case of the small-intestine CLD analysis, examples of paracellular markers were amoxicillin, atenolol, cimetidine, fexofenadine, ranitidine, and terbutaline. In the case of the jejunum SPIP data, atenolol, cimetidine, colchicine, famotidine, and sotalol fell into that marker group. Paracellular markers for colon SPIP included atenolol, cimetidine, and colchicine.

On the other hand, if the rat P_C estimate based on Caco-2 is well above the DRW upper limit (cf., Figure 4.3.1), then the molecule serves as a "ABL marker". In the case of colon CLD, none of the molecules was an exclusive ABL marker, although several molecules were close to the limit and contributed to its determination. In case of the small-intestine CLD analysis, naproxen was the ABL marker. For colon SPIP data, carbamazepine was chosen as an ABL marker. It had the highest P_{eff} in the set. For jejunum SPIP data, viable ABL markers were carbamazepine and ibuprofen.

As the above indicates, most of the molecules with measured Caco-2 permeability are not predominant DRW limit markers. Even though most molecules are inside the DRW, they still may contribute to the determination of the system-wide P_{ABL}, P_{para}, and k_{VF} parameters, to varying extents [25]. It is generally not possible to determine the P_C values of molecules which serve exclusively as markers for the DRW boundary (i.e., which lie outside of the DRW window).

Table 4.3.3. Results of paracelluar analysis and comparisons to Ussing, Caco-2, rodent BBB, and human jejunum.

P_{para} (10^{-6} cm.s^{-1})	Rat Colon (Closed-Loop)	Rat Small Intestine (Closed-Loop)	Rat Colon (Single-Pass)	Rat Jejunum (Single-Pass)	Rat Colon (Single-Pass)	Rat Jejunum (Single-Pass)
urea	110	16	12	6	1.2 (19[b])	2.7 (54[b])
mannitol	67	10	7	3	0.7 (2[b])	1.7 (5[b])
sucrose	50	7	6	3	0.6	1.2
raffinose	42	6	5	2	0.5	1.0
urea/raffinose	3	3	3	3	2	3
***R* (Å)**						
(ε/δ), cm^{-1}						
$(\varepsilon/\delta)_2$, cm^{-1}	7.1 ± 2.5	1.05 ± 0.56	0.79 ± 0.44	0.37 ± 0.14	0.08 ± 0.12	0.18 ± 0.19
h_{ABL}, µm	179 ± 152	1624 ± 954	121[g]	1307 ± 673	560 ± 1151	2581 ± 2402
k_{VF}	1.0 ± 0.3	3.1 ± 1.1	1.4 ± 0.5	2.9 ± 0.9	2.1 ± 1.9 (1.9 [c])	5.9 ± 3.9 (7.8 [d])
GOF	1.8	4.5	7.9	4.6	6.8	3.7
n	23[e]	27[f]	15	42	7	7
Ref.	a	a	a	a	[71][b]	[71][b]

Table 4.3.3. Results of paracelluar analysis and comparisons to Ussing, Caco-2, rodent BBB, and human jejunum (continued).

P_{para}(10^{-6} cm.s^{-1})	Rat Colon (Ussing)	Rat Jejunum (Ussing)	Caco-2	Human Jejunum (Double-Balloon)	Rodent Blood-Brain Barrier
urea	1.8	13	5.1	3.3	2.8
mannitol	1.1	8	1.8	1.3	0.4
sucrose	0.8	6	0.7	0.6	0.07
raffinose	0.7	5	0.4	0.4	0.05
urea/raffinose	3	3	15	9	56
***R* (Å)**			11.1	11.2	4.8
(ε/δ), cm^{-1}			1.11	0.53	6.7
$(\varepsilon/\delta)_2$, cm^{-1}	0.12 ± 0.05	0.81 ± 0.03		0.027	0.009
h_{ABL}, µm	599 ± 109	2457 ± 229	200	4675	230 [h]
k_{VF}	1.0	1.0	1.0	33.5	1.0
GOF	2.2	1.6	3.0	1.3	2.2
n	11	11	11	99	23
Ref.	[24]	[24]	[42, 44]	[25]	[45]

Note: a, this work. [b] Five drugs were characterized by the SPIP method. Two markers, urea and mannitol, were done by the closed-loop method. [c] Obtained from Collet et al. [72]. [d] Obtained from Mayhew [73]. [e] For the 74 colon P_{eff} values, only 23 measured Caco-2 were available. [f] For the 78 small intestine Peff values, only 27 measured Caco-2 were available. [g] Carbamazepine was used as the sole ABL marker. [h] Flow-limit can be equated to log PABL = -3.40, which corresponds to hABL = 230 µm.

Table 4.3.3 summarizes the DRW results and compares them to several other *in vitro* and *in situ* permeability models. It was not possible to apply the full paracellular model indicate by equation (4) in the Appendix. So, the simpler equation (8) was used.

The colon data indicated surprisingly high level of leakiness, indicated by $(\varepsilon/\delta)_2$ = 7.1 ± 2.5 cm^{-1}. Also, the aqueous boundary layer thickness, h_{ABL} = 179 ± 152 µm, was surprisingly low. There was no indication of appreciable expansion of the colon surface area, above the "smooth tube" value, given that the refined expansion factors, k_{VF} = 1.0 ± 0.3 and 1.4 ± 0.5 for colon CLD and SPIP, respectively.

The rat small-intestine CLD data indicated $(\varepsilon/\delta)_2$ = 1.05 ± 0.56 cm^{-1} and h_{ABL} = 1624 ± 954 µm. These values are in appreciable agreement with those determined from the rat jejunum excised-segment Ussing measurement [24], as indicated in Table 4.3.3 (0.81 ± 0.03 cm^{-1} and 2457 ± 229 µm, resp.).

The rat jejunum SPIP data indicated tighter junctions than that of the rat CLD data, with $(\varepsilon/\delta)_2$ = 0.37 ± 0.14 cm^{-1}. This is comparable to the corresponding rat colon Ussing data (Table 4.3.3: 0.12 ± 0.14 cm^{-1}), indicating relatively tight junctions.

In order to compare the results of the DRW analysis to those reported for human jejunum and rodent blood-brain barrier (BBB), the paracellular parameters were used to calculate the expected P_{para} values of four standard hydrophilic marker molecules of variable size: urea, mannitol, sucrose, and raffinose. The extensive human jejunum and rodent BBB data allowed for application of the full paracellular equation (4). As such, it was possible to estimate the size selectivity ratio, P_{para}(urea)/P_{para}(raffinose). In the case of BBB, the ratio is 56 and in the human jejunum, it is 9. Our study of rat intestine showed a smaller size discrimination ratio of 2-3 (Table 4.3.3), suggesting that "free diffusion" was more evident in the rat data. The P_{para}(urea) value was predicted to be 110 x 10^{-6} cm·s^{-1} for the rat colon CLD

data, compared to 2.8 x 10^{-6} cm·s^{-1} expected in the BBB. The largest molecule, raffinose, is still predicted to be highly permeable in the rat colon CLD set, 42 x 10^{-6} cm·s^{-1}. By comparison, the extremely tight junctions of the rodent BBB would suggest a value of 0.05 x 10^{-6} cm·s^{-1}.

2. Second-stage analysis to estimate the rat intrinsic permeability, P_0

Given the P_{ABL}, P_{para}, and k_{VF} system-wide parameters determined in the first stage above, the individual P_{eff} data could be solved for the P_0 values, since then there would be one known and one unknown, and equation (1) could be applied, along with equation (3), assuming P_i is nil. Only the molecules that are within the DRW can be so treated, as noted above.

2.1. Colon closed-loop Doluisio

Figure 4.3.3 shows the results of the above intrinsic analysis and compares the colon CLD log P_0 to those of Caco-2 in frame (a) and to those of PAMPA-DS in frame (b). In Figure 4.3.3a, the solid circles correspond to the measured Caco-2 values used in the first-stage analysis above. The unfilled circles are based on the *p*CEL-X predicted (passive) Caco-2 log P_0 values. The linear regression between the Caco-2 and colon data indicates r^2 = 0.61 (using all 51 P_{eff} values within the DRW). A poorer fit is evident between the colon and PAMPA data, as indicated in Figure 4.3.3b. Much of the uncertainty arises out of the narrow DRW of the rat colon CLD data.

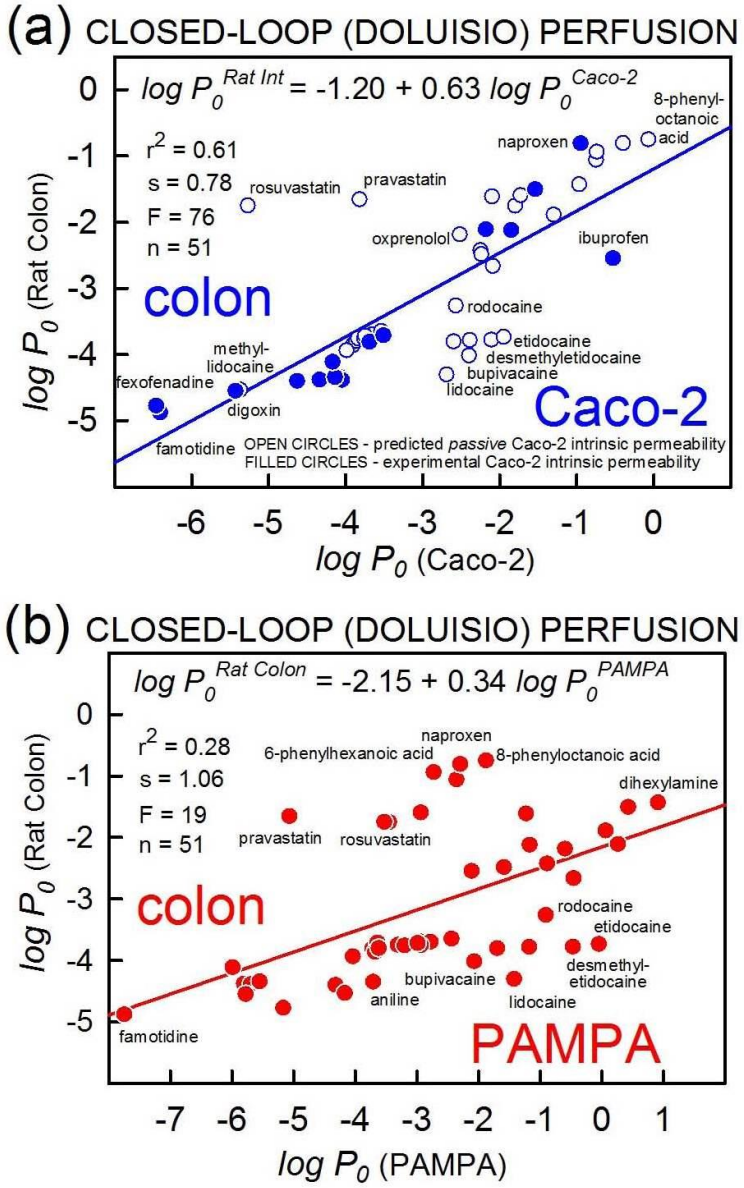

Figure 4.3.3. Rat colon closed-loop Doluisio (CLD) method correlations. The comparison of (a) Caco-2 and (b) PAMPA-DS intrinsic permeability values to those determined from the rat colon by the CLD method. The solid circles correspond to the measured Caco-2 values used in the first-stage analysis (see text). The unfilled circles are based on the *p*CEL-X predicted passive Caco-2 log P_0 values.

2.2. Small-intestine closed-loop Doluisio

Figure 4.3.4 shows the results of the intrinsic analysis and compares the small-intestine CLD log P_0 to those of Caco-2 in frame (a) and to those of PAMPA-DS in frame (b). In Figure 4.3.4a, the solid and unfilled circles have the same meaning as in Figure 4.3.3a. The linear regression between the Caco-2 and small-intestine data indicates $r^2 = 0.78$ (using all 55 P_{eff} values within the DRW). A poorer fit is evident between the small intestine and PAMPA data, as indicated in Figure 4.3.4b, but it is improved over that of the colon data. When the DRW is narrow, the calculated P_C (equation (1)) of molecules close to either limit cannot be accurately determined due to the influence of errors in the measurements of P_{eff}, and the uncertainties in the predicted P_{ABL}, and P_{para}. When the P_{eff} of a molecule is outside the DRW (i.e., of a marker molecule), P_C cannot be calculated at all. This is why it is so difficult to measure the lipoidal permeability of progesterone in Calco-2 assays, using just about any ABL marker molecule. Also, for example, the use of the diazepam cerebrovascular flow marker in the *in situ* brain perfusion method becomes problematic in cases of highly-permeable molecules, as discussed by Avdeef and Sun [74]. Likewise, it is very difficult or nearly impossible to measure the lipoidal transcellular permeability of very hydrophilic molecules, such as mannitol or raffinose, since these molecules preferentially permeate by the aqueous paracellular route.

2.3. Colon single-pass intestinal perfusion

Figure 4.3.5 shows the results of the intrinsic analysis and compares the colon SPIP log P_0 to those of Caco-2 in frame (a) and to those of PAMPA-DS in frame (b). The fit is very good for both the Caco-2 correlation (Figure 4.3.5a: $r^2 = 0.90$) and the PAMPA-DS correlation (Figure 4.3.5b: $r^2 = 0.70$).

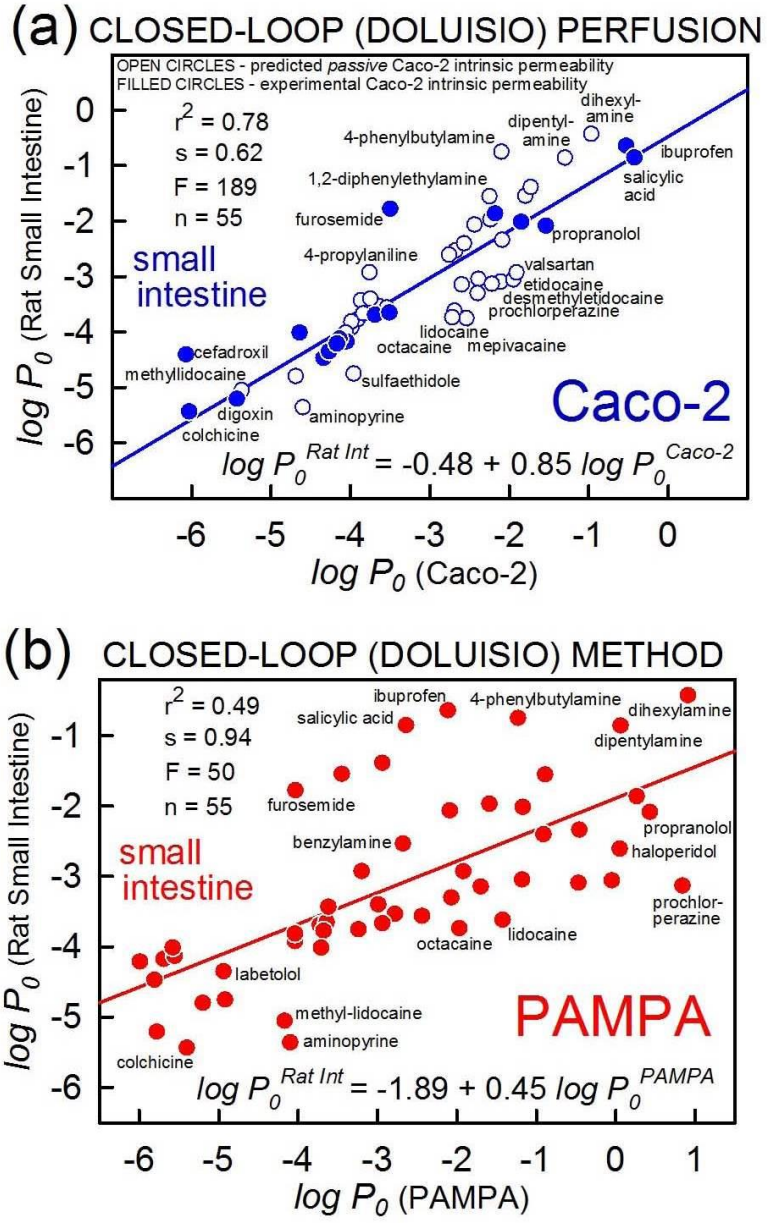

Figure 4.3.4. Rat small intestine closed-loop Doluisio (CLD) method correlations. The comparison of (a) Caco-2 and (b) PAMPA-DS intrinsic permeability values to those determined from the rat small intestine by the CLD method. The symbols have the same meaning as in Figure 4.3.3.

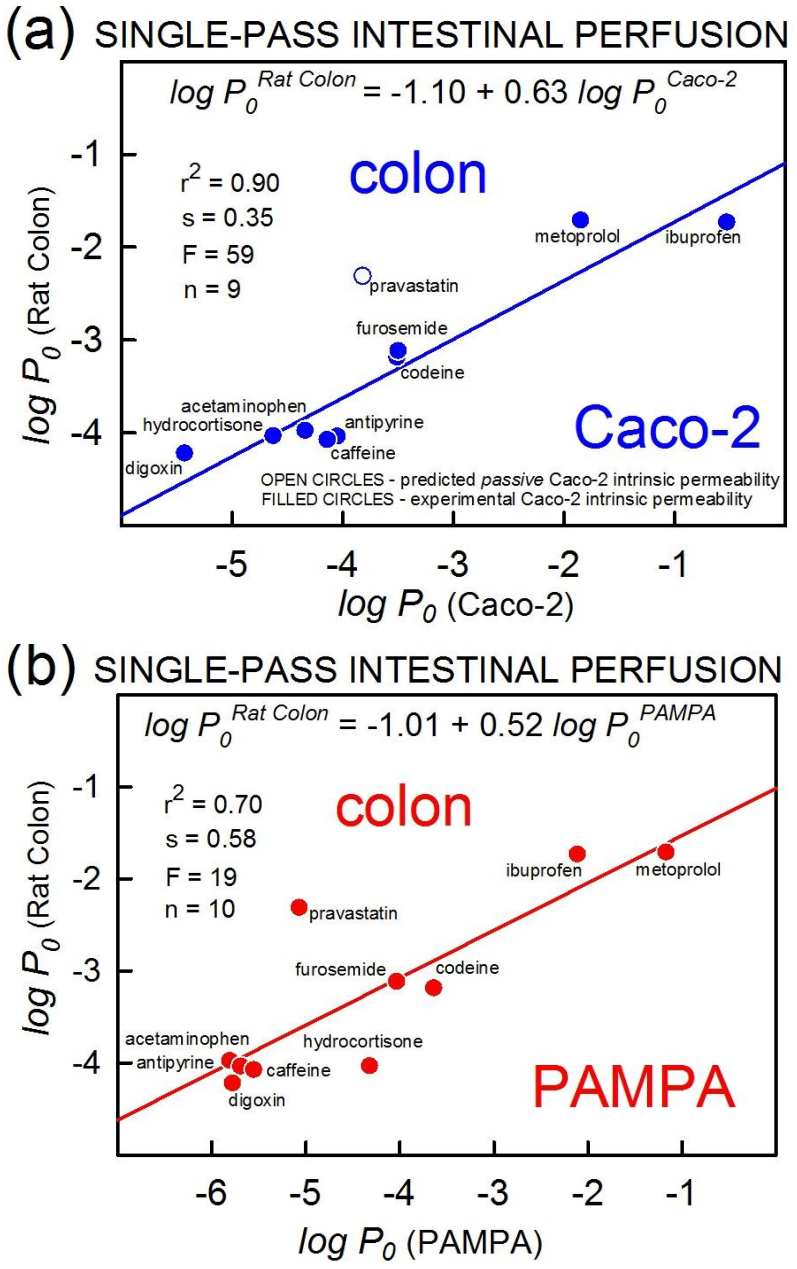

Figure 4.3.5. Rat colon single-pass intestinal perfusion (SPIP) method correlations. The comparison of (a) Caco-2 and (b) PAMPA-DS intrinsic permeability values to those determined from the rat colon by the SPIP method. The symbols have the same meaning as in Figure 4.3.3.

2.4. Jejunum single-pass intestinal perfusion

Figure 4.3.6 shows the results of the intrinsic analysis and compares the jejunum SPIP log P_0 to those of Caco-2 in frame (a) and to those of PAMPA-DS in frame (b). Just as in the case of colon SPIP, the jejunum fit is very good for both the Caco-2 correlation (Figure 4.3.6a: $r^2 = 0.92$) and the PAMPA-DS correlation (Figure 4.3.6b: $r^2 = 0.70$), for 22 molecules.

Comparisons between the rat colon and small intestine

It was of interest to compare the inter-segment permeability, i.e., that of the colon with that of the small intestine, to see if the closed-loop and the SPIP methods indicate comparable trends. Figure 4.3.7a focuses on the closed-loop method, while Figure 4.3.7b depicts that of the SPIP method. The regression equations between log P_0 in both methods have a slope of 0.74 and comparable negative intercepts. This indicates that lower-permeable molecules (e.g., digoxin, methyl lidocaine, theophylline, caffeine, and antipyrine) have higher permeability in the colon than in the small intestine. On the other hand, the higher-permeable molecules (e.g., propranolol, metoprolol, and ibuprofen) have lower permeability in the colon than in the small intestine. This may be consistent with the "villus-tip preferential absorption" jejunum model described by Oliver *et al*. [43]. A similar trend is shown by the Ussing data (Table 4.3.3), which indicates a slope of 0.87 (data not shown), where phenytoin is 1.7 times more permeable in the colon than the jejunum and where salicylic acid is 1.4 times more permeable in the jejunum than the colon.

The above comparisons suggest that there is a consistent difference between the permeability properties of the colon and the small intestine, which is indicated by each of the two assay methods without significant bias.

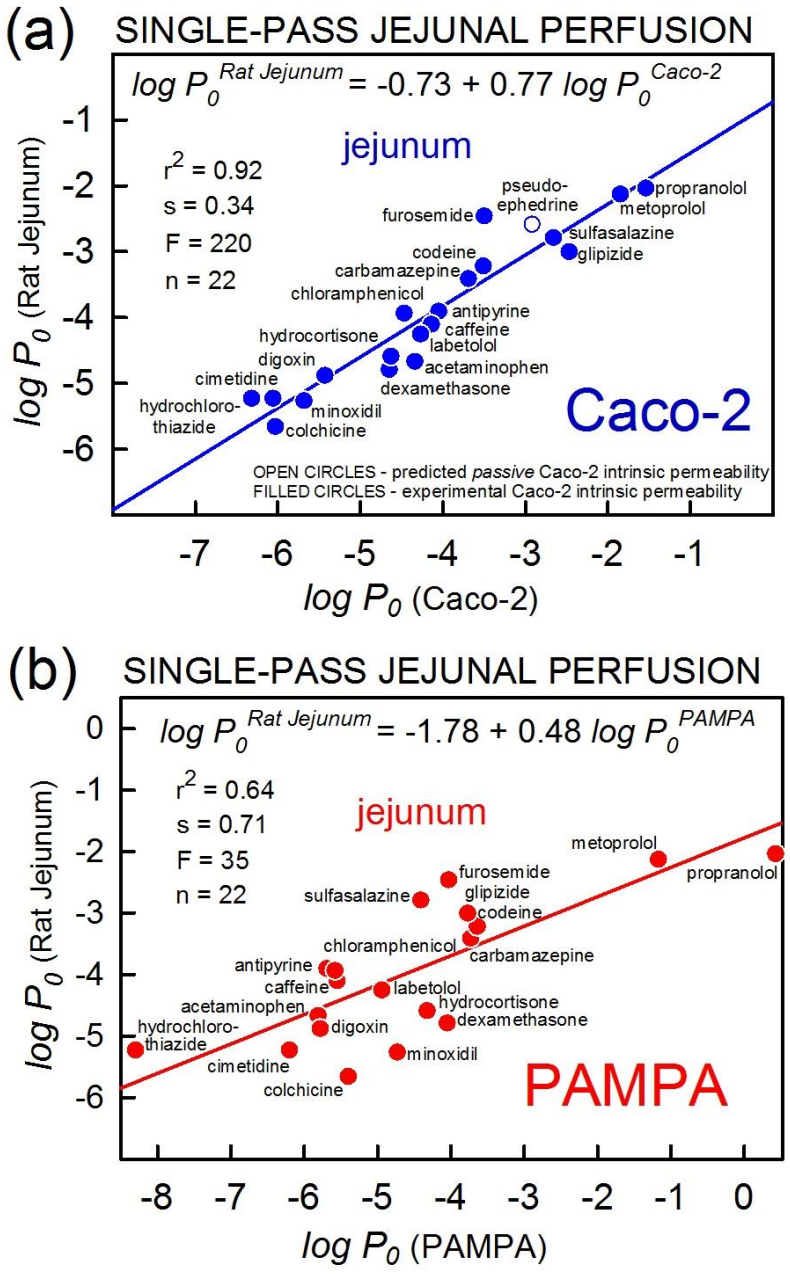

Figure 3.4.6. Rat jejunum single-pass intestinal perfusion (SPIP) method correlations. The comparison of (a) Caco-2 and (b) PAMPA-DS intrinsic permeability values to those determined from the rat jejunum by the SPIP method. The symbols have the same meaning as in Figure 4.3.3.

Figures 4.3.7c and 4.3.7d focus on intra-segment comparisons of the two methods. In both the small intestine (Figure 4.3.7c) and the colon (Figure 4.3.7d), the slope is closer to unit value and the intercept is closer to zero, in comparison to the inter-segment trends. This suggests that there is little method bias.

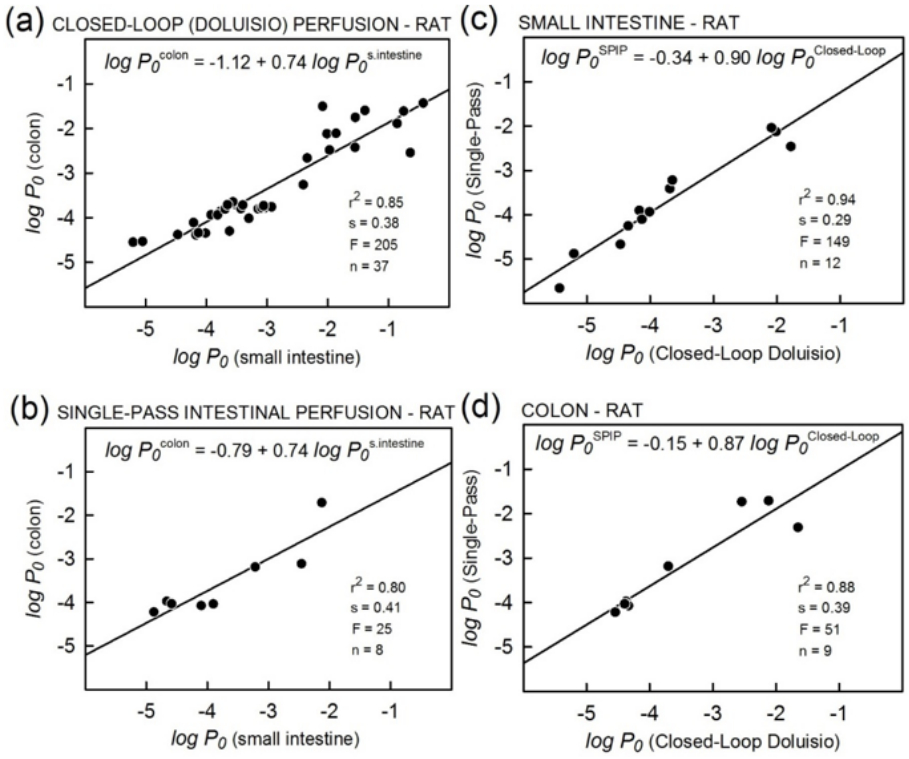

Figure 4.3.7. Inter-segment (a, b) and intra-segment (c, d) comparisons of the SPIP and CLD methods. (a) Small intestine vs. colon by CLD. (b) Small intestine vs. colon by SPIP. (c) CLD vs. SPIP in the small intestine. (d) CLD vs. SPIP in the colon. The regression equations in (a) and (b) have a slope of 0.74 and comparable negative intercepts. This indicates that lower-permeable molecules have higher permeability in the colon than in the small intestine. On the other hand, the higher-permeable molecules have lower permeability in the colon than in the small intestine. The relationships in (c) and (d) have slopes nearer unity and intercepts nearer zero, which indicates that there is nearly no method bias.

DISCUSSION

Principal findings in the study

The analysis of the DRW parameters showed a higher number of molecules that use predominantly paracellular route in colon than in small intestine (with CLD method) and also compared with small intestine in SPIP technique. This appears to indicate that in rat the relative "leakiness" of the intestine appeared to be greater in colon than in small intestine.

Previous data from human and rat small intestine and colon show inconsistent results regarding the segmental differences in intestine versus colon for different compounds. For example, Hebden *et al.* [75] performed a study in humans with an oral timed-release delivery vehicle, which allowed pulsed release within a particular site of the gut. They observed a lower transcellular permeability in colon versus intestine (quinine as a probe) and no differences in with the paracellular marker. Other authors comparing human intestinal and colon tissues in Ussing chambers observed no differences in paracellular route (using lucifer yellow as marker) as well as no marked differences in transcellular transported compounds [76]. Nevertheless, the generally accepted hypothesis is that colon presents a relatively lower paracellular permeability than small intestine. This idea was supported with rat data by Ungell *et al.* [24]. They used rat tissue from jejunum, ileum and colon in Ussing chambers and they observed a significant decrease in permeability to hydrophilic drugs delivered aborally, while hydrophobic drugs presented higher permeability in small intestine versus colon. These results were later confirmed in human intestinal tissue by Sjöberg *et al.* [77]. Regional permeability data showed a tendency for highly permeable compounds to have higher or similar P_{app} in colon as in the small intestinal segments, while the colonic regions showed a lower P_{app} for more polar compounds as well as for D-glucose and L-leucine.

The results from this study indicate that the relative "leakiness" of the intestine appeared to be greater in rat than human, with the colon surprisingly showing more leakiness than the small intestine, as indicated by the results of the Doluisio method. On the other hand, previous comparison of rat and human colon (rat perfusion studies with CLD) and human tissue in Ussing chambers already showed higher permeability in rat colon versus human colon tissue [46].

Aqueous boundary layer thickness, h_{ABL}

In many publications, the *in vivo* unstirred water layer is calculated on the basis of a "smooth-tube" surface area of the intestinal segment, not incorporating the surface area expansion factor, k_{VF}, into the resultant h_{ABL}. For example, Fagerholm and Lennernäs [78] estimated human jejunal h_{ABL} thicknesses as 83 and 188 µm for D-glucose and antipyrine, respectively, suggesting a well-mixed intestinal segment. Apparently, the underlying human P_{eff} values for the two compounds were calculated on the basis of smooth-tube surface area, as is commonly done. A re-analysis of the data, taking into account the surface-area expansion factor, k_{VF}, suggested a considerably thicker unstirred water layer [25]. In an example of rat closed-loop Doluisio small intestine permeability study [79], h_{ABL} was estimated to be 103 µm. As is customery, the smooth-tube surface area defined P_{eff} values that suggested the thin boundary layer. A re-analysis of the 2004 study, using the methods described here, indicated a thicker h_{ABL} = 2506 µm, based on the estimated k_{VF} = 4.6 (Avdeef, unpublished). Such an ABL thickness is not uncommon in unstirred Caco-2 measurements.

Table 4.3.3 indicates different leakage metrics $(\varepsilon/\delta)_2$ when comparing rat colon data from different techniques, with the lower value of 0.12 with Ussing chambers, versus 0.79 with SPIP and 7.1 with CLD. The reason for the difference obviously lies on the experimental technique but is not easy to explain. The distension of the tissue under the perfusion methods could be a factor, but in both perfusion methods the pressure of the liquid on the

intestinal wall appeared to be very similar. On the other hand, the values for in small intestine from Ussing chambers and closed-loop do not differ that much (0.81, and 1.05 respectively), while the value for SPIP in the small intestine is estimated as 0.37.

The mucosal surface area in anaesthetized rats in the colon is 1.0-1.4 times the smooth cylinder calculated value, and in the small intestine it is 2.9-3.1 times the smooth cylinder value. This values could reflect, on one hand, the absence of villi in the colon, what produces less surface available on this segment. On the other hand, the absence of folds (plicae) in rat small intestine versus the human one could be the reason for the reduction in the k_{VF} value from 33 in human to 2.9-3.1 in rats [80].

The SPIP data in Figures 4.3.5 and 4.3.6 have better correlations than those of CLD in Figures 4.3.3 and 4.3.4, because the DRW is wider in the case of SPIP than in the case of CLD. With a narrow window, the P_C values are not always well determined, being more often close to either the ABL ceiling or the paracellular floor. Leakiness in the CLD raises the level of the floor, and brings it closer to the ceiling.

Previous comparison between CLD and SPIP method [14] showed a good correlation of permeability intestinal values (raw data-uncorrected) obtained with both techniques with some differences. For example, in SPIP high permeability compounds presented higher values with SPIP compared with CLD while low permeability drugs show lower values with SPIP than with CLD. In Figure 4.3.7c the comparison of the corrected values indicates a slope closer to 1 so a good comparability of both methods in small intestine and also good in colon (Figure 4.3.7c).

CONFLICTS OF INTEREST

The authors confirm there are no conflicts of interest.

ACKNOWLEDGEMENTS

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APPENDIX A. COMPUTATIONAL METHOD – PERMEABILITY MODEL

Computational method

The model used here begins with the deconvolution of rat intestinal P_{eff}, measured at a particular physiological pH (near the microclimate value), into its three effective components: P_{eff}^{ABL}, P_{eff}^{trans}, and P_{eff}^{para} (ABL, transcellular, and paracellular permeability, respectively). It is assumed that P_{eff}^{trans} in the rat intestine can be estimated using the P_{app} values taken from Caco-2 studies, provided that the Caco-2 values are shed of any respective *in vitro* contributions from the ABL and paracellular permeability (yielding Caco-2 "cell" permeability, P_C, at the *in situ* pH), and furthermore multiplied by the villus-and-fold surface expansion factor: $P_{eff}^{trans} = k_{VF} \cdot P_C$. For molecules dominantly permeating by passive lipoidal diffusion, this may be a reasonable assumption. However, there would be increased uncertainty for drugs transported by a carrier-mediated (CM) processes, if the relative levels of expression of the transporters in the *in vitro* system and the *in situ* intestinal environment were not well matched. It is assumed that $P_{eff}^{ABL} = k_{VF} \cdot D_{aq} / h_{ABL}$, where D_{aq} is the aqueous diffusivity of the molecule and h_{ABL} is the absolute aqueous boundary layer thickness in the *in situ* perfusion experiment.

A weighted nonlinear regression method was used to determine the k_{VF}, h_{ABL}, as well as the paracellular parameters, using the rat intestinal P_{eff} as dependent and the Caco-2 P_{app} (specifically, P_C at the pH of the *in situ* assay) as independent variables [25].

$$\frac{1}{P_{eff}} = \left(\frac{1}{P_{eff}^{ABL}} + \frac{1}{P_{eff}^{trans} + P_{eff}^{para}} \right) = \frac{1}{k_{VF}} \cdot \left(\frac{h_{ABL}}{D_{aq}} + \frac{1}{P_C + P_{para}} \right) \qquad (1)$$

Values of D_{aq} ($cm^2 \cdot s^{-1}$) at 37 °C were empirically estimated from the molecular weight, MW, as

$$D_{aq} = 0.991x10^{-4} MW^{-0.453} \qquad (2)$$

which was derived from the analysis of mostly drug-like molecules [42]. The thickness of the physical boundary layer, h_{ABL}, depends on the effective agitation level in the *in situ* perfusion experiment, which is expected to depend on flow rate used and animal handling. For *in vitro* (microtitre plate) methods, typical values are about 1500-2500 µm in unstirred solutions, and about 150 µm in solutions stirred at 450 RPM ($rev \cdot min^{-1}$).

P_C represents the transcellular permeability contribution, associated with cell membranes (apical, basolateral and cytosol organelle) and any contributions from CM processes. For a molecule with a single ionizable group (ionization constant, pK_a), P_C is defined by a sigmoidal function, as shown in Figure 4.3.1 by the solid curve:

$$P_C = \frac{P_0}{10^{\pm(pH - pK_a)} + 1} + \frac{P_i}{10^{\pm(pK_a - pH)} + 1} + \cdots \qquad (3)$$

where the '±' is '+' for acids and '-' is for bases. The maximum possible value of P_C is the permeability of the neutral species, P_0; the minimum possible P_C value is the permeability of the ionized species, P_i (most likely effected by CM transport). Figure 4.3.1 summarizes the relationship between these components of permeability.

P_{para} can estimated from the differential flux equation describing size- and charge-restricted diffusion through a cylindrical channel containing charged groups, under sink boundary condition [40, 81]. Avdeef and Tam [25]

extended the model to include two populations of junctional pores: (i) high-capacity ε/δ, size-restricted and cation-selective pathways, and (ii) secondary $(\varepsilon/\delta)_2$ low-capacity, "free diffusion" pathways independent on size and charge. The dual-pore population paracellular equation use in this study is:

$$P_{para} = \frac{\varepsilon}{\delta} \cdot D_{aq} \cdot F\left(\frac{r_{HYD}}{R}\right) \cdot E(\Delta\varphi) + \left(\frac{\varepsilon}{\delta}\right)_2 \cdot D_{aq} \quad (4)$$

where most of the terms have been defined already (cf., Abbreviations). $F(r_{HYD}/R)$ is the Renkin hydrodynamic sieving function for cylindrical water channels, with values ranging from 0 to 1, defined as a function of molecular hydrodynamic radii (r_{HYD}) and pore radii (R), both usually expressed in Å units:

$$F\left(\frac{r_{HYD}}{R}\right) = \left[1 - \left(\frac{r_{HYD}}{R}\right)\right]^2 \cdot \left[1 - 2.104\left(\frac{r_{HYD}}{R}\right) + 2.09\left(\frac{r_{HYD}}{R}\right)^3 - 0.95\left(\frac{R_{HYD}}{R}\right)^5\right] \quad (5)$$

Values of r_{HYD} (Å) were estimated from the Sutherland-Stokes-Einstein spherical-particle equation [42]:

$$r_{HYD} = \left(0.92 + \frac{21.8}{MW}\right) \cdot \frac{10^8 K_B T}{6\pi\eta D_{aq}} \quad (6)$$

where K_B = Boltzmann constant, T = absolute temperature, and η = solvent kinematic viscosity (0.00696 $cm^2 \cdot s^{-1}$, 37 °C).

The $E(\Delta\varphi)$ term in equation (4) is a function of the potential drop, $\Delta\varphi$, across the electric field created by negatively-charged residues lining the junctional pores [40, 81]:

$$E(\Delta\varphi) = f_{(0)} + f_{(+)} \cdot \frac{\kappa \cdot |\Delta\varphi|}{1 - e^{-\kappa \cdot |\Delta\varphi|}} + f_{(-)} \cdot \frac{\kappa \cdot |\Delta\varphi|}{e^{+\kappa \cdot |\Delta\varphi|} - 1} \quad (7)$$

where $f_{(0)}$, $f_{(+)}$, and $f_{(-)}$ are the concentration fractions of the molecule in the uncharged, cationic, and anionic forms, respectively. The constant, $\kappa = (F/N_A k_B T) = 0.037414$ mV^{-1} at 37 °C, where F is the Faraday constant, and other symbols have their usual meaning. The average $\Delta\varphi$ of -43 mV, from many Caco-2 studies. For very large channels (r << R) or for channels not lined with a high charge density, $E(\Delta\varphi) \sim 1$.

In the case of very leaky cell junctions (R > 20 Å), it may not be possible to determine all of the parameters in equation (4). A simplified "free diffusion" form may be used:

$$P_{para} \approx \left(\frac{\varepsilon}{\delta}\right)_2 \cdot D_{aq} \tag{8}$$

Refinement of the permeability parameters which define the dynamic range window

The *p*CEL-X v4.03 program (*in-ADME* Research) can be used to determine the k_{VF}, h_{ABL}, ε/δ, $(\varepsilon/\delta)_2$, R, and $\Delta\varphi$ parameters by a weighted nonlinear regression analysis based on the logarithmic form of equation (1), expanded with equations (2) - (7):

$$G\left(k_{VF}, h_{ABL}, \frac{\varepsilon}{\delta}, \frac{\varepsilon}{\delta_2}, R, \Delta\varphi\right) = log k_{VF} - \log\left[\frac{h_{ABL}}{D_{aq}} + \frac{1}{P_C^{6.5} + \frac{\varepsilon}{\delta}\cdot D_{aq}\cdot F\left(\frac{r_{HYD}}{R}\right)\cdot E(\Delta\varphi) + \frac{\varepsilon}{\delta_2}\cdot D_{aq}}\right] \tag{9}$$

The partial derivatives of G with respect to ε/δ, $(\varepsilon/\delta)_2$, R, $\Delta\varphi$, h_{ABL}, and k_{VF} are calculated explicitly in the *p*CEL X program, based on standard mathematical techniques. The weighted residuals function minimized was:

$$R_w = \sum_i^n \left(\frac{logP_{eff,i}^{obs} - G_i^{calc}}{\sigma_i(logP_{eff})} \right)^2 \quad (10)$$

where *n* is the number of P_{eff} values used in the model refinement, and σ_i(log P_{eff}) is the reported standard deviation of the logarithm of the i^{th} measured rat intestinal permeability. The effectiveness of the refinement is characterized by the "goodness-of-fit", $GOF = [R_w/(n-n_V)]^{1/2}$, where n_V refers to the number of varied parameters. The expected value of *GOF* is 1 if the model is suif for the data and the measured standard deviations accurately reflect the precision of the data.

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4.4. Artículo científico 6

Comparison between *in situ* perfusion models (SPIP vs Doluisio) in rat colon to predict permeability of drugs in human colon

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ABSTRACT

The colon is the main organ involved in the absorption of an oral controlled release (CR) product. However, there is a lack of validated methods to study the colonic absorption; but, recently, the Doluisio *in situ* perfusion method was validated to predict the absorption in colon. Nevertheless, the single-pass intestinal perfusion (SPIP) technique, widely used to predict the small intestine absorption, has not been validated to predict colonic absorption. The aim of this work was to demonstrate that SPIP is a useful method to study the permeability in rat colon. Moreover, the usefulness of both *in situ* perfusion models to predict colonic human absorption was checked. The results showed that the colon permeability values obtained with SPIP correlated well with Doluisio colon permeability and with Caco-2 permeability. Therefore, the SPIP technique is as reliable as Doluisio's method to study the absorption of drugs in rat colon. Furthermore, the rat colon data obtained with perfusion methods demonstrated to be useful to predict human colon absorption.

Keywords: colon permeability, colon permeability prediction, *in situ* perfusion methods, human colon absorption.

INTRODUCTION

The colon is considered a secondary organ in the absorption process [1]. However, in the case of drugs formulated as oral controlled release (CR) products, the importance of the absorption in colon increases [2]. A CR formulation releases the drug during 12-24 h, so the longer colonic transit time is needed to ensure the complete absorption of a CR product [3, 4].

To develop a CR formulation, it is mandatory to know the permeability of the drug in colon, nevertheless there is a lack of validated models to predict the permeability and absorption of drugs in colon [2, 5]. Recently, our group validated the closed loop perfusion technique, based on Doluisio's

method, in rat colon as a model to predict colonic absorption [6]. The Doluisio's method is an *in situ* perfusion technique that was developed to study the permeability in small intestine [7, 8]. On the other hand, there is another important *in situ* perfusion technique employed to analyse the permeability in small intestine: the single-pass intestinal perfusion (SPIP) method [9-12]. Doluisio's method and SPIP have demonstrated in recent publications to be equally reliable to determine the permeability of drugs from the different BCS classes, in rat small intestine segments (duodenum, jejunum, ileum) [13, 14]. Nevertheless, an exhaustive study of the applicability of the SPIP to analyse the permeability in rat colon has not been carried out.

The rat is the most widely used animal in intestinal absorption studies [5, 15-17]. To check the validity of a perfusion animal model to predict human absorption it is necessary to have permeability human values and/or human fraction absorbed in the studied region. Both rat perfusion methods mentioned above, Doluisio's method and SPIP, have demonstrated their predictability for human absorption in small intestine [18-21]. However, the limited availability of human colon data complicates the verification of the rat models in colon. Direct measurements of *in vivo* colonic permeability in humans is difficult to achieve [5]; but, recently, human permeability data obtained from human colonic tissue mounted in the Ussing chamber have been published by Sjoberg et al. and Rozehnal et al. [22, 23]. The Ussing chamber model is an *in vitro* method developed in 1951 by Ussing et al. [24], has been used with animal intestinal tissues and provides good prediction of human fraction absorbed [17, 25-27]. The existence of human permeability colon data and colonic human fraction absorbed from Tannergren et al. [2] allows to check the predictability of SPIP and Doluisio's method as *in situ* perfusion models in rat colon.

Thus, the first objective of this work is to demonstrate that SPIP is a useful technique to study the permeability in rat colon of compounds with

different physicochemical properties, which belong to different classes of the BCS in comparison with Doluisio's method. Secondly, the *in situ* data from rat colon will be correlated with colonic human data to check the predictability of the *in situ* perfusion models in rat colon.

MATERIALS AND METHODS

Acetaminophen, antipyrine, atenolol, caffeine, carbamazepine, cimetidine, codeine, colchicine, digoxin, famotidine, fexofenadine, furosemide, hydrocortisone, metformin, metoprolol, oxprenolol, pravastatin, propranolol, ranitidine, rosuvastatin and theophylline were purchased from Sigma-Aldrich. Methanol, acetonitrile and water were HPLC grade. All other chemicals were of analytical reagent grade.

Doluisio's (Closed loop) rat colon perfusion

Male Wistar rats were used in accordance with 2010/63/EU directive of 22 September 2010 regarding the protection of animals used for scientific experimentation. The Ethics Committee for Animal Experimentation of the University of Valencia approved the experimental protocols (Spain, code A1330354541263).

The absorption rate coefficients and the permeability values of the 14 drugs studied were determined in colon (n= 6-7) using *in situ* "closed loop" perfusion method based on Doluisio's Technique [7] modified to adapt it to the colon as described in previous work [6, 28]. Briefly, the rats were anesthetized using a mixture of pentobarbital (40 mg/kg) and butorphanol (0.5 mg/kg). An isolated compartment in the large intestine was created from the beginning of the colon, just after the cecum sac, to the end of the colon, with the aid of two syringes and two three-way stopcock valves. Before doing the second incision, the large intestine was copiously flushed with isotonic saline (pH 7.5) with 1% Sörensen phosphate buffer (v/v), 37 °C. When the surgical procedure was finished, the abdomen was covered with a cotton

wool pad avoiding peritoneal liquid evaporation and heat losses. The drug solution was introduced inside the compartment and the samples were collected every 5 min up to a period of 30 min. Perfused drug solutions were prepared in isotonic saline buffered with Sörensen phosphate buffer (66.6 mM).

At the end of the experiments the animals were euthanized. In order to separate solid components from the samples, they were centrifuged 5 minutes at 5000 r.p.m. All samples were analyzed by High Performance Liquid Chromatography (HPLC) as described in Table 4.4.1. Analytical methods were validated for each compound in terms of specificity, selectivity, linearity, precision and accuracy. All the compounds were analyzed at room temperature, with a flow rate of 1 mL/min and a 150 mm x 4.6 mm C-18 column (5 µm particle size).

There is a reduction in the volume of the perfused solutions at the end of the experiments; therefore, a correction became necessary in order to calculate the absorption rate constants accurately. Water reabsorption was characterized as an apparent zero order process. A method based on direct measurement of the remaining volume of the test solution was employed to calculate the water reabsorption zero order constant (*ko*). The volume at the beginning of the experiment (V_0) was determined on groups of three animals, while the volume at the end (V_t) was measured on every animal used. The concentration in the samples was corrected as:

$$C_t = C_e(V_t/V_0) \quad (1)$$

where C_t represents the concentration in the gut that would exist in the absence of the water reabsorption process at time t and C_e the experimental value. The C_t values (corrected concentrations) were used to calculate the actual absorption rate coefficient [29].

The absorption rate coefficients (*ka*) of compounds were determined by nonlinear regression analysis of the remaining concentrations in lumen C_t versus time.

$$C_t = C_0 e^{-k_a t} \quad (2)$$

The absorption rate coefficients (*ka*) were transformed into permeability (P_{app}) values with the following relationship:

$$P_{app} = k_a R/2 \quad (3)$$

where *R* is the effective radius of the intestinal segment. *R* value was calculated considering the colon as a cylinder with the relationship:

$$Volume = \pi R^2 L \quad (4)$$

Estimation was done using a 5 mL perfusion volume and an intestinal length (L) of 10 cm.

Single-pass rat colon prefusion

The single-pass intestinal perfusion studies were performed using protocols approved by the Ben-Gurion University of the Negev Animal Use and Care Committee (Protocol IL-60-11-2010). Male Wistar rats (250–300 g, Harlan, Israel) were housed and handled according to the Ben-Gurion University of the Negev Unit for Laboratory Animal Medicine Guidelines.

The technique used for the *in situ* single-pass perfusion experiments was published before [30]. Rats were anesthetized with an intra-muscular injection of 1 mL/kg of ketamine–xylazine solution (9%:1%, respectively), placed on a heated surface maintained at 37 °C (Harvard Apparatus Inc., Holliston, MA), and a 3-4 cm midline abdominal incision was made. Colon segment was cannulated on two ends, and were rinsed witch blank perfusion buffer. All solutions were incubated in a 37 °C water bath.

At the starting point of each experiment, perfusion solution containing the investigated drug, 10 mM MES buffer, 135 mM NaCl, 5mM KCl, and 0.01 mg/mL phenol red, with an osmolarity of 290 mOsm/L, and pH 6.5 was perfused through the intestinal segment (Watson Marlow 205S, Watson-Marlow Bredel Inc., Wilmington, MA), at a flow rate of 0.2 mL/min. The perfusion buffer was perfused for 1 h without sampling, to ensure steady state conditions, followed by additional 1 h of perfusion with samples taken every 10 min. The pH of the collected samples was measured at the outlet, to verify that there was no pH change throughout the perfusion. The samples were immediately assayed by UPLC.

The effective permeability (P_{eff}; cm/sec) through the rat gut wall was determined according to the following equation:

$$P_{eff} = \frac{-Qln(C'_{out}/C'_{in})}{2\pi RL} \quad (5)$$

where Q is the perfusion buffer flow rate (0.2 mL/min), C'_{out}/C'_{in} is the ratio of the outlet and the inlet concentration of drug that has been adjusted for water transport via the non-absorbable marker phenol red [11, 31-33], R is the radius of the intestinal segment and L is the length of the perfused intestinal segment. The average length of the segment was 10 cm.

Human colon and *in vitro* permeability data

Human colon *Papp* and *in vitro Papp*, collected from literature, have been used to stablish correlations with the *in situ Papp* obtained with Doluisio and SPIP techniques. The human colon data are from the studies developed by Sjöberg et al. and Rozehnal et al. with the Ussing chamber [22, 23]. And the *in vitro* data are from Lozoya-Agullo et al. where the *in vitro Papp* was studied with Caco-2 monolayers after 21 days of culture and after 4 days of culture [6].

Table 4.4.1. HPLC conditions for the compounds tested with Doluisio's method. MeCN: acetonitrile, MeOH: methanol; [a] UV: ultraviolet detector, F: fluorescence detector. [b] Water (pH 3) was 0.01% trifluoroacetic acid in water. [c] λ for excitation/λ for emission.

Drug	Detection[a]	Mobile phase (aqueous:organic)	λ (nm)	Retention time (min)
Acetaminophen	UV	Water pH 3: MeCN (70:30)[b]	254	4.0
Antipyrine	UV	Water (pH 3): MeCN (65:35)[b]	235	2.3
Atenolol	F	Water (pH 3): MeOH: MeCN (90:5:5)[b]	231/307[c]	7.0
Caffeine	UV	Water(pH 3): MeOH (65:35)[b]	273	3.6
Carbamazepine	UV	50 mM KH_2PO_4 (pH 7): MeCN (50:50)	280	2.9
Cimetidine	UV	Water pH 3: MeCN (87:13)[b]	220	4.9
Codeine	UV	0.05 mM acid phosphoric (pH 3): MeCN (75:25)	212	6.5
Colchicine	UV	10 mM NaH_2PO_4 (pH 3): MeCN (65:35)	245	4.8
Digoxin	UV	Water pH3:MeCN (60:40)[b]	225	4.5
Famotidine	UV	Water pH 3: MeCN (80:20)[b]	300	4.4
Fexofenadine	UV	Water pH3:MeCN (60:40)[b]	210	5.0
Furosemide	UV	25 mM Na_2HPO_4 (pH 3): MeCN (45:55)	254	2.5
Hydrocortisone	UV	Water pH 3: MeCN (60:40)[b]	245	3.0
Metformin	UV	10 mM KH_2PO_4 pH 3.5 : MeCN (90:10)	234	3.0
Metoprolol	F	Water (pH 3): MeOH: MeCN (60:20:20)[b]	231/307[c]	4.3
Oxprenolol	UV	Water pH 3.4:MeOH:MeCN (39:44:17)	226	5.5
Pravastatin	UV	Water pH 3: MeCN (50:50)[b]	280	3.7
Propranolol	F	0.1% triethylamine:MeOH:MeCN (50:25:25)	251/350	5.5
Ranitidine	UV	10 mM Na_2HPO_4 pH 7: MeOH (30:70)	320	2.0
Rosuvastatin	UV	Water pH 3: MeCN (50:50)[b]	240	6.5
Theophylline	UV	Water pH 3: MeCN (85:15)[b]	280	6.0

RESULTS

Table 4.4.2 summarizes the apparent permeability values (P_{app}, cm/s) in colon of the 14 drugs assayed with two *in situ* perfusion methods: Doluisio's method and SPIP. As was done with whole small intestine and with different segments throughout the small intestine [13, 14], the correlation between Doluisio's method and SPIP has been stablished for colon in this paper. A good linear correlation is obtained with a $R^2 > 0.80$ (Figure 4.4.1).

Table 4.4.2. Apparent permeability values (*Papp*, cm/s) in colon with the two *in situ* perfusion methods assayed. S.D.: Standard deviation.

Drug	SPIP		Doluisio's method	
	P_{app}	S.D.	P_{app}	S.D.
Acetominophen	1.41E-04	3.05E-05	9.13E-05	1.15E-05
Antipyrine	1.24E-04	2.28E-05	8.48E-05	8.60E-06
Atenolol	1.96E-06	3.35E-07	2.11E-05	4.30E-06
Caffeine	1.15E-04	2.46E-05	8.70E-05	1.96E-05
Carbamazepine	1.60E-04	9.70E-06	1.42E-04	2.30E-05
Cimetidine	7.20E-06	7.40E-07	3.46E-05	7.80E-06
Codeine	3.55E-05	7.14E-06	5.83E-05	9.10E-06
Colchicine	5.00E-06	4.00E-07	3.20E-05	8.50E-06
Digoxin	7.75E-05	7.69E-06	4.89E-05	2.14E-05
Furosemide	8.97E-06	5.06E-07	2.22E-05	6.90E-06
Hydrocortisone	1.17E-04	1.89E-05	6.91E-05	7.99E-06
Metformin	3.96E-05	1.16E-05	4.51E-05	1.00E-05
Metoprolol	6.03E-05	9.46E-06	8.14E-05	1.87E-05
Pravastatin	5.72E-05	7.23E-07	5.49E-05	1.01E-05

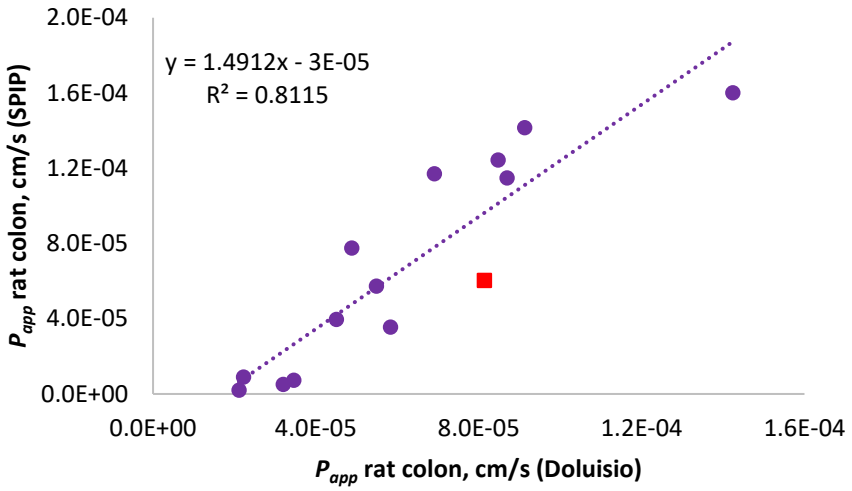


Figure 4.4.1. Correlation between *Papp* in colon obtained with Doluisio's method and *Papp* in colon obtained with SPIP. Red symbol corresponds to metoprolol.

Several correlations have been explored to study the validity of the Single Pass Intestinal Perfusion method (SPIP) in rat colon. Permeability values in colon obtained *in situ* and permeability values *in vitro* with Caco-2 cells kept in culture for 21 days (Figure 4.4.2) and 4 days (Figure 4.4.3) have been correlated.

A correlation with *in situ* rat colon data with both perfusion techniques and permeability values obtained in human colonic tissue with Ussing chamber have also been stablished. In Figure 4.4.4 some *in situ* perfusion data from rat colon obtained with Doluisio's method and SPIP have been correlated with human colon permeability values from literature, these human data have been obtained with the Ussing chamber technique and human colonic tissue [22, 23].

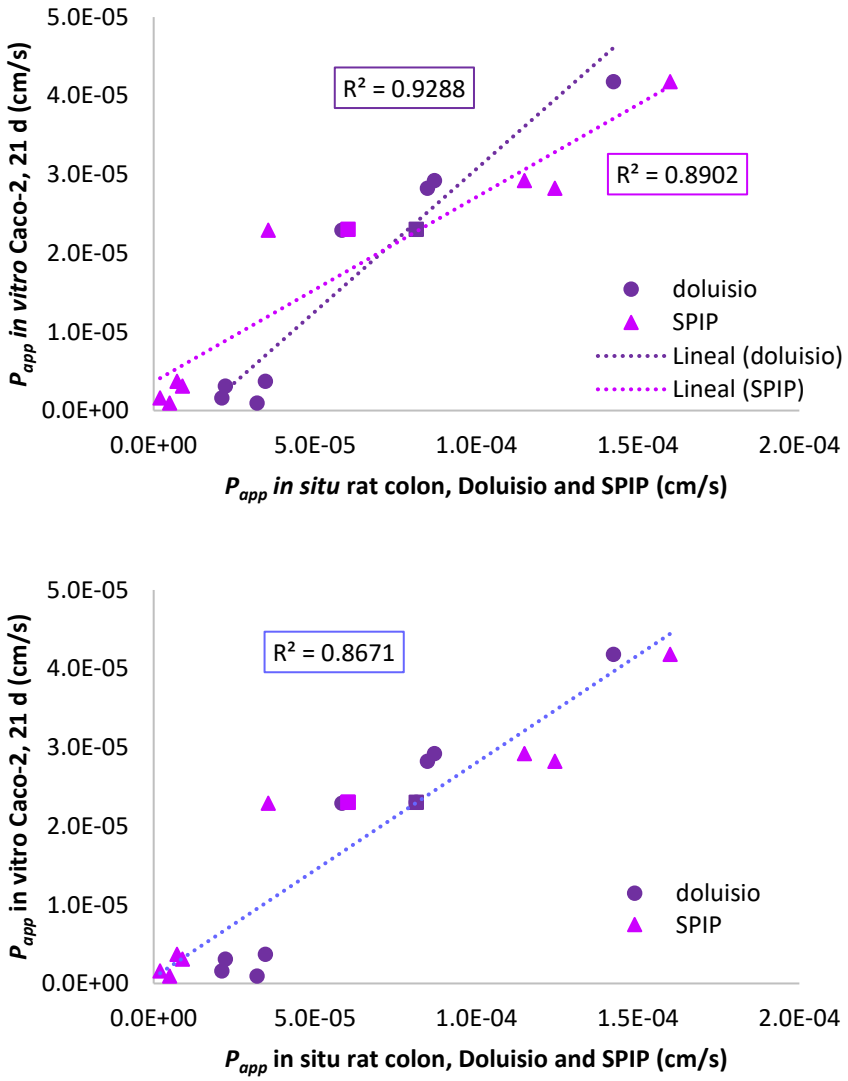
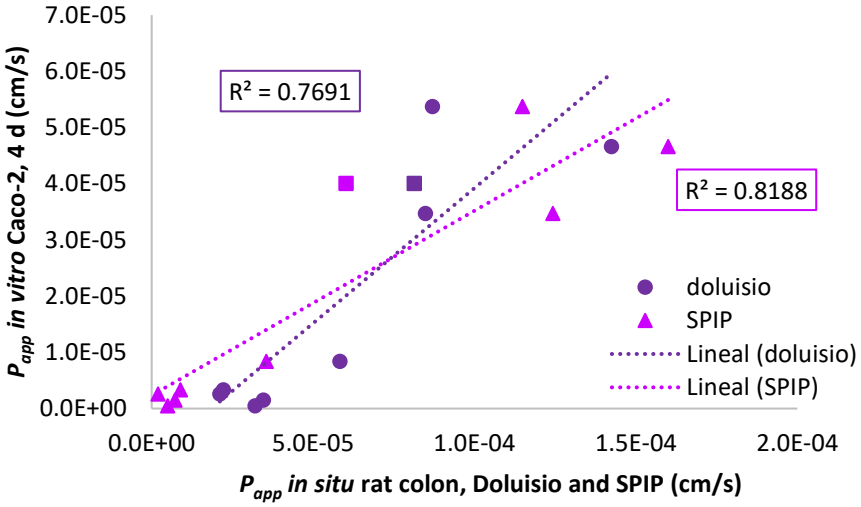


Figure 4.4.2. Correlation between colon *in situ* data and Caco-2 *in vitro* data, 21 days model. The correlation between permeability colon data with Doluisio's method and *in vitro* data has been obtained from Lozoya-Agullo et al. [6]. Squares correspond to metoprolol.



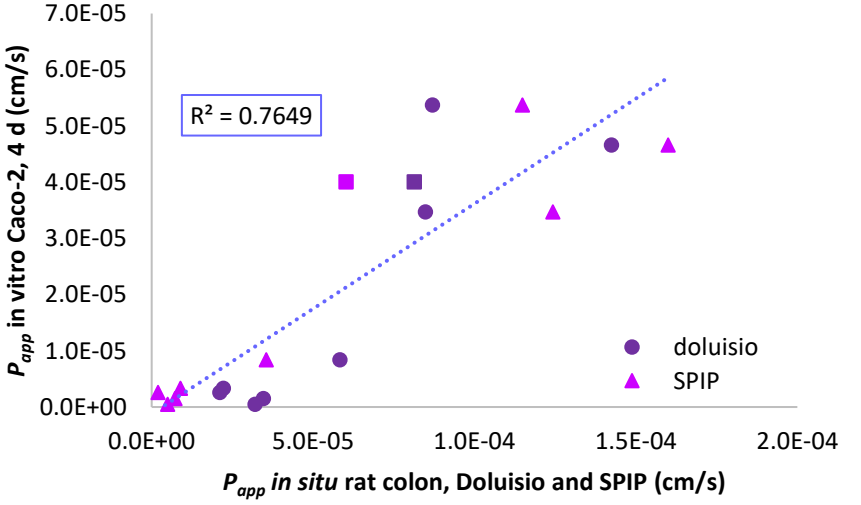
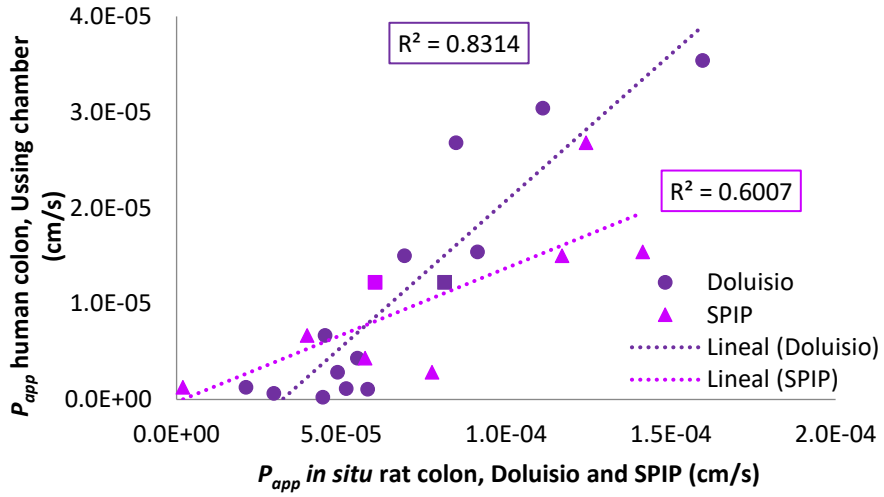


Figure 4.4.3. Correlation between colon *in situ* data and Caco-2 *in vitro* data, 4 days model. The correlation between permeability colon data with Doluisio's method and *in vitro* data have been obtained from Lozoya-Agullo et al. [6]. Squares correspond to metoprolol.



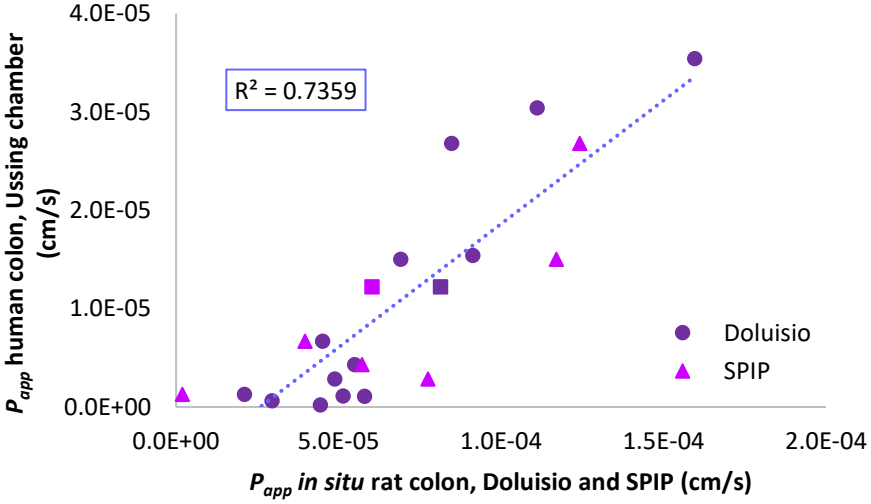


Figure 4.4.4. Correlation between *in situ* rat colon permeability values obtained with perfusion methods and human colon permeability values obtained with Ussing chamber. Squares correspond to metoprolol.

In order to study the ability to predict the oral fraction absorbed in colon in humans of the *in situ* methods tested in colon. Figure 4.4.5 shows a correlation between *in situ* colon permeability values and Fabs in colon obtained from Tannergren et al. [2]. Moreover, Table 4.4.3 presents the oral human fraction absorbed in small intestine and oral fraction absorbed in colon and the permeability class (high or low) according to the expected BCS classification in colon [5, 22, 23, 34-37]. SPIP and Doluisio colon permeability values have been classified into high and low permeability, according to metoprolol permeability, to compare with BCS classification.

Table 4.4.3. Human fraction absorbed in small intestine and colon, expected BCS permeability classification in colon and permeability class according to the results of the *in situ* perfusion methods assayed in this research. H: high permeability; L: low permeability; N/A: not available data. * classification expected to colon.

Drug	F_{abs} (%) *in vivo* small intestine	F_{abs} (%) *in vivo* colon	Permeability		
			BCS*	Doluisio	SPIP
Caffeine	100	N/A	N/A	H	H
Carbamazepine	100	N/A	N/A	H	H
Metoprolol	98	100	H	H	H
Antipyrine	97	N/A	N/A	H	H
Codeine	95	N/A	N/A	H	L
Hydrocortisone	89	N/A	N/A	H	H
Digoxin	81	N/A	N/A	H	H
Acetominophen	80	N/A	N/A	H	H
Cimetidine	79	19	L	L	L
Furosemide	61	22	L	L	L
Metformin	53	N/A	N/A	L	L
Atenolol	50	28	L	L	L
Colchicine	44	N/A	N/A	L	L
Pravastatin	34	N/A	N/A	L	L
Ranitidine	50	9	L	L	N/A
Theophylline	96	85	H	H	N/A
Oxprenolol	90	74	H	H	N/A
Fexofenadine	13	13	L	L	N/A

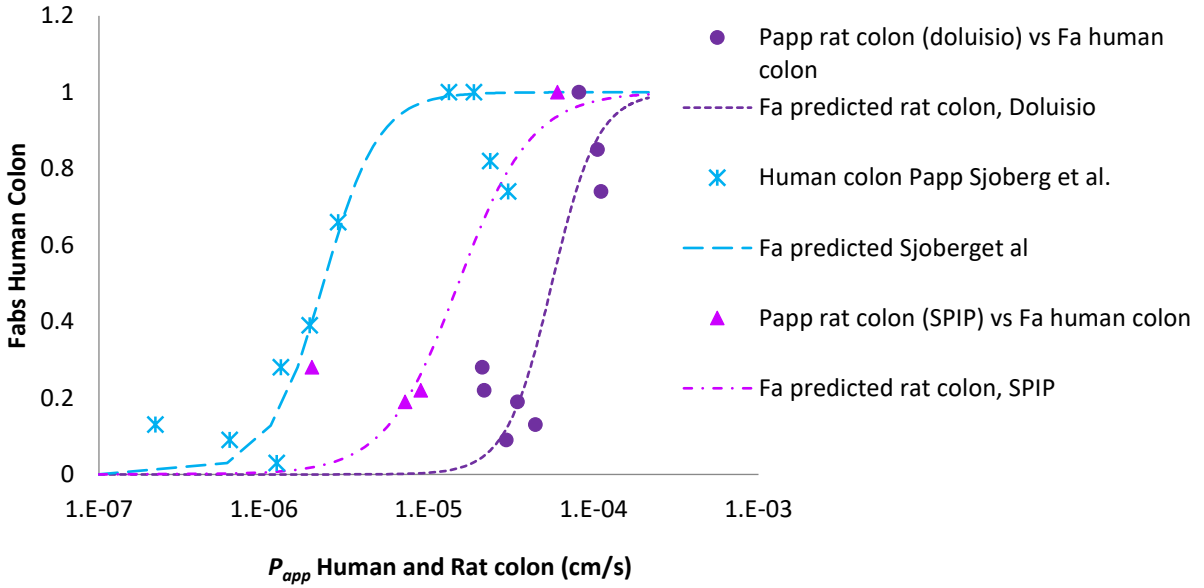


Figure 4.4.5. Correlation between permeability values obtained in rat colon with *in situ* perfusion methods and oral fraction absorbed in colon taken from Tannergren et al. and Sjoberg et al. [2, 22]. Fitted lines were obtained with a four parameter logistic model y=B/(1+(x/C)^D) where B is 1, C is *Papp* at Fa=0.5 ($5.59 \cdot 10^{-5}$cm/s); D is the slope factor= -3.13 (R^2=0.89, p<0.05) for Doluisio's method and B is 1, C is *Papp* at Fa=0.5 ($1.52 \cdot 10^{-5}$ cm/s); D is the slope factor= -1.96 (R^2=0.89, p<0.05) for SPIP. The sigmoidal correlation between human colonic *Papp* and human Fa in colon obtained by Sjoberg et al. [22] is overlapped for comparison.

DISCUSSION

The single-pass intestinal perfusion (SPIP) model and the Doluisio rat perfusion method are two *in situ* techniques employed in intestinal absorption studies. Despite the differences in their execution, the final objective is the same: to measure the intestinal absorption of a drug [7, 9]. Recently, the relationship between both have been analysed showing good correlations between them. Those studies have been developed with whole small intestine and different segments from small intestine, and they concluded that the two *in situ* methods are equally reliable for absorption studies in small intestine [13, 14]. However, the relationship between both techniques in colon have not been studied; therefore, one of the objectives of this work was to check if the good relationship between SPIP and Doluisio's method was also maintained in colon. Figure 4.4.1 shows the correlation between colon permeability values obtained with SPIP and colon permeability values obtained with Doluisio's method. The correlation was good ($R^2 > 0.80$), so the trend observed in small intestine remained in colon, which means that both techniques also correlates well in this distal part of the gastrointestinal tract.

The Doluisio rat perfusion method employed to determine the permeability of drugs in colon has been validated in a previous work [6]. It also showed good correlations between *in vitro* permeability data obtained with Caco-2 cells monolayers cultured during 4 days and during 21 days [6]. Now, SPIP data have been correlated with *in vitro* Caco-2 permeability values as well. The correlations obtained in this research with SPIP and Caco-2 21 days model (Figure 4.4.2), and SPIP and Caco-2 4 days model (Figure 4.4.3) were good. Due to the relationship shown in Figure 4.4.1, the good correlations between SPIP and Caco-2 permeability values (Figure 4.4.2 and Figure 4.4.3) was the expected result because, in our previous work [6], the Doluisio data in colon correlated well with the *in vitro* data. Correlation between Doluisio colon permeability and Caco-2 monolayers permeability

from Lozoya-Agullo et al. [6] were included in these plots for an easier comparison.

In the literature, the closed loop method, as Doluisio's method, is recommended to analyse the absorption in colon with an *in situ* technique. The reason given is that the colonic absorption can be too slow to achieve a significant absorptive extraction after a single passage [38, 39]. But, the results presented in this paper indicated that the SPIP is as reliable as Doluisio's method to study the absorption of drugs in rat colon.

However, to validate a perfusion method in rat colon it is necessary to have human colon data. Sjoberg et al. and Rozehnal et al. published data from human colon obtained with Ussing chamber technique [22, 23]. These data were used in our previous work to stablished a correlation between rat colon permeability obtained with Doluisio's method and human colon permeability obtained with Ussing chamber [6]. The correlation was good, but only 4 drugs were employed, so a reliable conclusion could not be achieved. Therefore, a similar correlation has been stablished in this research, but with more drugs and with data obtained from rat colon using both *in situ* perfusion methods (Figure 4.4.4). In this case, the correlation was even better ($R^2 > 0.8$), which corroborated the validity of rat colon data to predict human colon absorption. Besides, Figure 4.4.5 shows the relationship between rat colon perfusion data obtained in this work and human fraction absorbed in colon obtained from Tannergren et al. [2]. In addition, in the same plot (Figure 4.4.5), the human permeability of colon correlated with the human fraction absorbed in colon [22] was superimposed to compare human data with rat data. The permeability values from rat colon were higher than those obtained from human colon, this difference was likely due to wider tight junctions in rat colon membrane than in human colon membrane.

Spite of the higher permeability values in rat colon than in human colon, the three correlations showed a similar sigmoidal relationship (Figure 4.4.5). Eventually, that means that rat colon permeability values can be used

for classification purposes in high-low colon permeability compounds. In that case the boundary value for classification has to be selected by considering the objective of the classification. i.e. if the objective is to explore CR developability a permeability value ensuring Fabs> 50% could be reasonable, (in our rat model that corresponds to a permeability value of $5.59 \cdot 10^{-5}$ cm/s for Doluisio and $1.52 \cdot 10^{-5}$ cm/s for SPIP). According to this fact, oxprenolol should be considered high permeability in colon due to its Fabs in colon was higher than 50% (74%). Table 4.4.3 shows the permeability class assigned to the different drugs assayed in this work based on the different experimental models and considering metoprolol as high permeability model [36], and the classification for CR developability based on our rat colon permeability values and the correlation with human colon Fabs values (from Figure 4.4.5).

CONCLUSION

SPIP is an *in situ* perfusion method as reliable as Doluisio's method to determine the permeability of different drugs in colon. Moreover, both methods have good correlations with human colonic data and could be used for BCS classification. Therefore, permeability data obtained with the two rat perfusion methods studied in this research are useful to select compounds for CR development.

ACKOWLEDGMENTS

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5. ESTRATEGIAS PARA MODIFICAR LA ABSORCIÓN EN COLON

La validación del modelo de perfusión *in situ* en colon de rata expuesta en el Capítulo 4 es útil para realizar una clasificación y cribado de moléculas candidatas a desarrollarse en formas de liberación controlada. Las formas de liberación controlada son formas farmacéuticas con las que el principio activo se absorberá principalmente en colon [1]. Este tipo de formulaciones presentan una serie de ventajas descritas en el apartado de generalidades del Capítulo 1 de la presente Tesis Doctoral.

En las formas farmacéuticas convencionales de liberación inmediata es conveniente, pero no imprescindible, que el principio activo posea una permeabilidad elevada. Por el contrario, un compuesto que se desea formular en forma de liberación controlada necesita poseer una permeabilidad intermedia o alta para ser eficaz, ya que la liberación actúa como factor limitativo de la absorción del fármaco.

Debido al motivo mencionado en el párrafo anterior, para los compuestos que presentan baja permeabilidad en colon, sería recomendable valorar diferentes estrategias y/o modificaciones para aumentar su permeabilidad antes de ser formulados en forma de liberación controlada. Existen diferentes opciones para solucionar el problema de la baja permeabilidad de un principio activo. Por ejemplo, incluir en la formulación determinados excipientes, desarrollar un profármaco, formular un par iónico o modificar la forma farmacéutica en la que se administra el compuesto [2-6].

Por otro lado, los compuestos que muestran una permeabilidad alta o moderada en colon se absorberán sin problemas al ser liberados por la forma farmacéutica. En estos casos no es necesario aumentar la permeabilidad del principio activo, el objetivo principal es prolongar la liberación a lo largo del tiempo para conseguir que el fármaco alcance el colon.

Para el desarrollo de este capítulo se ha seleccionado un compuesto modelo de baja permeabilidad y otro de alta, para modificar su absorción en

colon. Como compuesto de baja permeabilidad se ha elegido el atenolol y se ha estudiado en profundidad el desarrollo de un par iónico. Como compuesto representante de alta permeabilidad se ha escogido el ibuprofeno y se ha analizado el desarrollo de nanopartículas poliméricas para prolongar su liberación.

En ambos casos una forma farmacéutica de liberación controlada podría aportar ventajas para el paciente ya que, además de reducir el número de dosis diarias disminuye la probabilidad de que se manifiesten efectos adversos. Además, este tipo de formulaciones son útiles para fármacos usados en el tratamiento de enfermedades que exhiben síntomas acordes con un ritmo circadiano, como la hipertensión y arritmias cardiacas, para los que se puede prescribir atenolol entre otros; o la artritis reumatoide u osteoartritis, para la que se suele prescribir ibuprofeno a altas dosis [7].

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5.1. Artículo científico 7

Development of an ion-pair to improve the colon permeability of a low permeability drug: atenolol

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ABSTRACT

To ensure the optimal performance of oral controlled release formulations, drug colon permeability is one of the critical parameters. Consequently, developing this kind of formulations for low permeability molecules requires strategies to increase their ability to cross the colonic membrane. The objective of this work is to show if an ion-pair formation can improve the colon permeability of atenolol as a low permeability drug model. Two counter ions have been tested: brilliant blue and bromophenol blue. The Distribution coefficients at pH 7.00 (D_{pH7}) of atenolol, atenolol + brilliant blue and atenolol + bromophenol blue were experimentally determined in n-octanol. Moreover, the colonic permeability was determined in rat colon using *in situ* closed loop perfusion method based in Doluisio's Technique. To check the potential effects of the counter ions on the membrane integrity, a histological assessment of colonic tissue was done. The results of the partitioning studies were inconclusive about ion-pair formation; nevertheless, colon permeability was significantly increased by both counter ions (from 0.232 ± 0.021 cm/s to 0.508 ± 0.038 cm/s in presence of brilliant blue and to 0.405 ± 0.044 cm/s in presence of bromophenol blue). Nor damage on the membrane was observed on the histological studies, neither any change on paracellular permeability suggesting that the permeability enhancement could be attributed to the ion-pair formation.

Keywords: atenolol, colonic permeability, counter ion, ion-pair, low permeability drugs.

INTRODUCTION

The oral route is the preferred choice for drug administration because it is the most physiological and convenient for the patient. Passive diffusion is one of the main mechanism for oral absorption, thus there are many drugs with low permeability, due to the selectivity of biological membranes: narrow range of molecular weight, lipophilicity and charge state [1, 2].

Hydrophilic and ionic molecules have difficulties to permeate the intestinal membrane because of its lipid nature [3].

Small intestine is considered to be the main site for absorption after oral administration due to its anatomical and, physiological features. Nevertheless, almost the whole gastrointestinal tract can absorb nutrients and drugs. In fact, large intestine and, in particular, colon can contribute significantly to the absorption of many compounds [4-7]. The larger residence time in colon compared with small intestine helps to the complete absorption of some drugs. Moreover, absorption in colon has great relevance for oral controlled release formulations. Controlled release products have many advantages compared to oral immediate release products: reduced number of daily doses, reduced plasma fluctuations which is reflected in less side effects and, therefore better patient compliance [5, 8]. Low permeability drugs in controlled release formulations could not be completely absorbed, so it is necessary to study strategies to increase their permeability through the colonic membrane.

There are several strategies to overcome low membrane permeability; for example, the use of excipients or surfactants as permeability enhancers [9-11]; Prodrug strategy has been also successfully used nevertheless a prodrug is a new molecular entity with different characteristics which may affect the drug function [12-16]. Another strategy is ion-pair formation; an ion-pair is a pair of oppositely charged ions held together by Coulomb attraction without formation of a covalent bond. They behave like a single unit and partition into the membrane as a more lipophilic unit. Hydrophobic ion pairing technique has been used to increase the hydrophobicity of molecules containing ionisable groups. The pair dissociates when diluted or displaced after absorption in blood. An ion-pairing approach has several advantages: it can improve stability and permeability across biological membranes, become a valuable tool for enhancing solubility and stability in an organic solvent, the formation of ion-pairs normally does not

entail an alteration in the structure and function of drug and eliminates the need for prodrug uptake by transports and activation by specific enzymes [17, 18].

In the literature many successful studies about the application of ion-pair approaches through several administration routes can be found. An ion-pair can improve drug permeation by transdermal, ocular, parenteral or inhalation route [19-23]. Of course, it can also improve oral absorption of drugs with poor intestinal permeability [17, 24, 25] and to assist in designing better dosage forms for alternative routes of administration [18]. Highly polar drugs, ionized in the physiological pH range and poorly permeable are ideal candidates for an ion-pair development. Atenolol accomplishes these characteristics, shows low permeability in small intestine and colon [26], moreover atenolol is considered as a low permeability marker [5, 27].

The aim of this paper was to study the colonic absorption of the basic drug atenolol in presence of two acidic counter ions: brilliant blue and bromophenol blue, using an *in situ* rat perfusion method in order to explore the feasibility of the ion-pair strategy for the colon membrane. Previously, the octanol-buffer partitioning studies were carried out for atenolol, atenolol-brilliant blue and atenolol-bromophenol blue. In order to support the hypothesis of ion-pair formation and to exclude membrane damage due to the counter ions, colon histological studies by microscopy were performed. The results obtained will be very useful for the development of controlled release formulation of low permeability drugs as atenolol.

MATERIALS AND METHODS

Atenolol, brilliant blue, bromophenol blue, 1-octanol and trifluoroacetic acid were purchased from Sigma-Aldrich. Methanol, acetonitrile and water were HPLC grade. All other chemicals were of analytical reagent grade.

Figure 5.1.1 shows the major micro species at pH 7.4 for atenolol (pKa: 9.60); bromophenol blue (pKa: 4.0) and brilliant blue (pKa: 5.63, 6.58) [28-32].

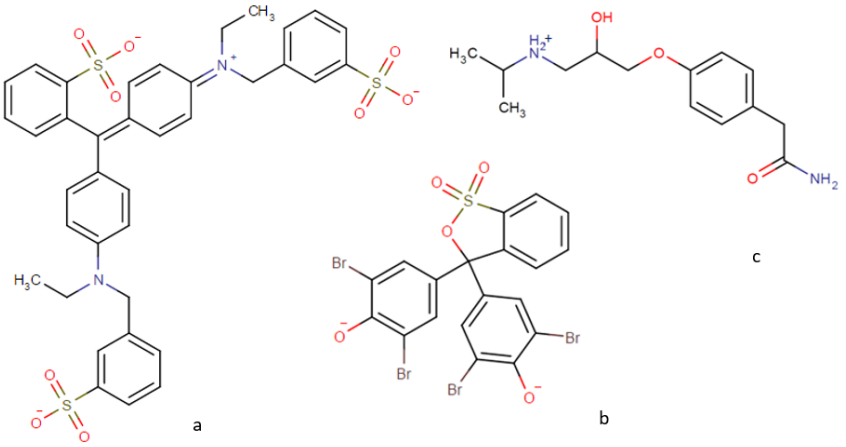

Figure 5.1.1. Major micro species at pH 7.4. a: brilliant blue, b: bromophenol blue, c: atenolol.

HPLC analysis

Atenolol samples in absence and in presence of counter ions were analyzed using a Perkin-Elmer HPLC with fluorescence detection at 231 nm for excitation λ and 307 nm for emission λ. Mobile phase consisted of a mixture of 75:12.5:12.5 (v/v/v) water with 0.01% (v/v) of trifluoroacetic acid (pH 3): methanol: acetonitrile. Stationary phase was a µBondapak 250 mm x 4.6 mm C-18 column (5 µm particle size) at room temperature. Flow rate was 1 mL/min and the injection volume was 10 µL. Retention time of atenolol was 4.5 min. The standard calibration curves were prepared by dilution from the drug solution assayed. Sample concentrations were within the linear range of quantitation for all the assays. Analytical method was validated in terms of specificity, selectivity, linearity, precision and accuracy.

Partitioning studies

Solutions of atenolol in presence and absence of counter ions were evaluated by partitioning experiments for 4 h between an organic phase:1-octanol and an aqueous phase. The assays were performed with two different aqueous phases: pure water and sodium phosphate buffer (50 mM), the pH of aqueous phase was adjusted to 7 [17]. 1-octanol was saturated with the aqueous phase and the aqueous phase was saturated with 1-octanol. Atenolol with and without counter ions was dissolved in the saturated aqueous phase and an equivalent volume of saturated 1-octanol was added. Assays were performed in three conditions: atenolol, atenolol+ brilliant blue and atenolol+ bromophenol blue. The solutions were prepared at 0.5 mM concentration for both atenolol and for the counter ions. i.e. the molar relationship drug:counter ion was 1:1. The experiments were carried out at 37 °C under constant stirring at 100 r.p.m. for 4 h. Three samples were taken of each solution to assess reproducibility, at 0.5 h, 1 h, 2 h and 4 h. The octanol and aqueous phases were then separated by centrifugation and the atenolol concentrations in the aqueous phase were determined by HPLC. The amount disappearing from the aqueous phase was considered as partitioned into the organic phase. The distribution coefficient (D_{pH7}) was calculated as the atenolol concentration ratio between the organic phase and the aqueous phase according to the following equation:

$$D = \frac{(Qaq_i - Qaq_f)/Vorg}{Caq_f} \quad (1)$$

where Qaq_i is the atenolol initial amount in the aqueous phase, Qaq_f is the atenolol amount in the aqueous phase at the end of the experiment, Ca_{qf} is the final drug concentration in the aqueous phase and *Vorg* is the volume of the organic phase.

Absorption studies

Male Wistar rats were used in accordance with 2010/63/EU directive of 22 September 2010 regarding the protection of animals used for scientific experimentation. The Ethics Committee for Animal Experimentation of the University of Valencia approved the experimental protocols (Spain, code A1330354541263).

The absorption rate coefficients and the permeability values of atenolol were determined in colon (n= 6-7) in absence and presence of counter ions by using *in situ* "closed loop" perfusion method based in Doluisio's Technique [33] modified to adapt it to the colon [26, 34]. Male Wistar rats (body weight, 250-300 g) fasted 4 h and with free access to water were used for these studies. Rats were anesthetized using a mixture of pentobarbital (40 mg/kg) and butorphanol (0.5 mg/kg). A midline abdominal incision was made. The intestinal segment was manipulated with care in order to minimize any intestinal blood supply disturbances. The perfusion technique consists of creating an isolated compartment in the intestinal segment of interest, with the aid of two syringes and two three-way stopcock valves. An incision was performed at the beginning of the colon just after the cecum sac. Surgical ligature to a catheter was placed at the incision. In order to remove all intestinal contents, the large intestine was copiously flushed with a physiologic solution: isotonic saline (pH 7.5) with 1% Sörensen phosphate buffer (v/v), 37 °C. After that, a second incision was performed at the end of the colon and was tied up and connected to a second catheter. Both catheters were connected to glass syringes using stopcock three-way valves. The colon was carefully placed back into the peritoneal cavity and the abdomen was covered with a cotton wool pad avoiding peritoneal liquid evaporation and heat losses. Once this system is set up, the colon is an isolated compartment where the drug solution (5 mL) can be introduced and sampled with the aid of the syringes and stopcock valves. The samples were collected every 5 min up to a period of 30 min.

Perfused drug solutions were prepared in isotonic saline buffered with Sörensen phosphate buffer (66.6 mM), pH was adjusted to 7 and the concentration was 500 µM for atenolol and counter ions.

At the end of the experiments the animals were euthanized. In order to separate solid components (mucus, intestinal contents) from the samples, they were centrifuged 5 min at 5000 r.p.m. All samples were analyzed by HPLC with the method described above.

There is a reduction in the volume of the perfused solutions at the end of the experiments; therefore, a correction became necessary in order to calculate the absorption rate constants accurately. Water reabsorption was characterized as an apparent zero order process. A method based on direct measurement of the remaining volume of the test solution was employed to calculate the water reabsorption zero order constant (*ko*). The volume at the beginning of the experiment (V_0) was determined on groups of three animals, while the volume at the end (V_t) was measured on every animal used. The concentration in the samples was corrected as:

$$C_t = C_e(V_t/V_0) \tag{2}$$

where C_t represents the concentration in the gut that would exist in the absence of the water reabsorption process at time t and C_e the experimental value. The C_t values (corrected concentrations) were used to calculate the actual absorption rate coefficient [35].

The absorption rate coefficients (*ka*) of compounds were determined by nonlinear regression analysis of the remaining concentrations in lumen C_t versus time.

$$C_t = C_0 e^{-k_a t} \tag{3}$$

The absorption rate coefficients (*ka*) were transformed into permeability values with the following relationship:

The absorption rate coefficients (k_a) were transformed in to permeability values with the following relationship:

$$P_{app} = k_a R/2 \quad (4)$$

where *R* is the effective radius of the intestinal segment. *R* value was calculated considering the intestinal segment as a cylinder with the relationship:

$$Volume = \pi R^2 L \quad (5)$$

Estimation was done using a 5 mL perfusion volume and an intestinal length of 10 cm.

Histological assessment of colonic tissue

Colonic tissue was examined under the microscope in order to evaluate the possible physiological changes due to the dyes used as counter ions. The tissues were examined and compared after the perfusion with the three work solutions: atenolol 0.5 mM solution, atenolol 0.5 mM with brilliant blue 0.5 mM solution and atenolol 0.5 mM with bromophenol blue 0.5 mM solution. Colonic tissue without any drug solution perfused was taken as reference of healthy tissue (control).

After the *in situ* experiments the animals were sacrificed and the colon was extracted. 1 cm from the proximal colon, 1 cm from the medial colon and 1 cm from the distal colon were caught and placed into a microscope slide cassette. These cassettes were put in a formaldehyde solution (4.5 % (v/v) dissolved in PBS) during 24 h. Then the colon segments were processed (dehydrated, cleared and infiltrated with paraffin wax), embedded and cut into 5 µm sections using a microtome. The paraffin was then removed using xylene, and the samples were hydrated, stained with haematoxylin and eosin, dehydrated and examined under the microscope.

Data analysis

Permeabilities determined *in situ* for the three different assayed solutions (atenolol, atenolol with brilliant blue and atenolol with bromophenol blue) were compared using ANOVA and Scheffé Post Hoc test to detect the existence of significant differences at the 0.05 probability level. The statistical comparison was made using the statistical package SPSS, V.20.

RESULTS

Table 5.1.1 summarizes the results of the partitioning studies of atenolol alone, atenolol with brilliant blue and atenolol with bromophenol blue, for the two aqueous phases assayed.

Table 5.1.1. Partitioning studies of the three tested solutions between 1-octanol and two different aqueous phases (sodium phosphate buffer and pure water). The results correspond to the data obtained after 4 h partitioning between the two phases (average ± standard deviation; n=3).

	Partitioning studies 1-octanol/phosphate buffer		**Partitioning studies 1-octanol/water**	
	D	% C_0 atenolol in aqueous phase ± SD	D	% C_0 atenolol in aqueous phase ± SD
Atenolol	0.001	99.99±0.95	0.016	96.28±0.26
Atenolol + brilliant blue	0.055	94.55±0.80	0.056	93.77±1.98
Atenolol + bromophenol blue	0.001	99.99±0.24	0.128	87.57±3.04

Table 5.1.2 shows the absorption rat coefficients, *Ka*, calculated with equation 3, and the apparent permeability values, calculated with equation 4, of the 3 assayed solutions determined in rat colon with the Doluisio's

method. Table 5.1.2 shows as well as the statistical analysis to compare the absorption of atenolol alone and atenolol absorption in the presence of the counter ions.

Figure 5.1.2 presents a comparative graphic of P_{app} values in colon and Figure 5.1.3 shows the colon lumen concentrations of atenolol vs time in the three assayed conditions. Both figures show a higher absorption of atenolol when it is perfused with counter ions.

Table 5.1.2. Absorption rate coefficients and apparent permeability values obtained by in situ perfusion method in rat colon (average ± standard deviation; n=6-7).

	Ka ± SD (h^{-1})	**P_{app} ± SD ($x10^{-4}$ cm/s)**	**Ratio[a]**
Atenolol	0.429 ± 0.038	0.232 ± 0.021	-
Atenolol + brilliant blue	0.937 ± 0.070	0.508 ± 0.038*	2.189
Atenolol + bromophenol blue	0.747 ± 0.081	0.405 ± 0.044*	1.746

[a] ratio between *Papp* obtained with ion-pair and *Papp* with atenolol. * Denotes significant differences versus permeability of atenolol alone (p<0.05).

Table 5.1.3 shows the phenol red permeability data perfused with increasing concentrations of brilliant blue to check if the paracellular route is altered. Phenol red is a non-absorbed marker with a negligible absorption when the intestinal membrane is not altered [36].

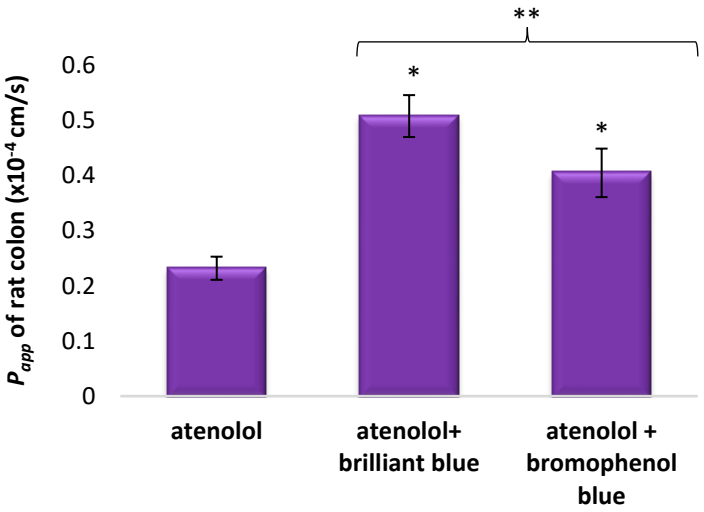

Figure 5.1.2. Permeability values of atenolol solutions and atenolol with counter ions in rat colon with standard deviation. * Denotes significant differences versus atenolol (p<0.05). ** Denote significant differences between atenolol + brilliant blue and atenolol + bromophenol blue.

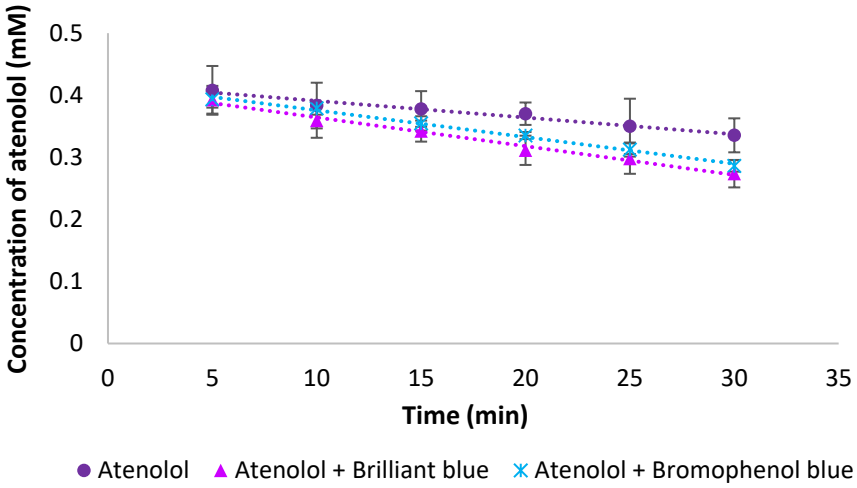

Figure 5.1.3. Representative plots of lumen atenolol concentrations vs time in all conditions.

Table 5.1.3. Phenol red permeability values (*Papp*) in rat colon with increasing concentrations of brilliant blue (average ± standard deviation). S.D.: Standard deviation.

	P_{app} **phenol red ± S.D. (x 10^{-4} cm/s)**
Phenol red (0.5 mM) + brilliant blue (0.5 mM)	0.069 ± 0.024
Phenol red (0.5 mM) + brilliant blue (2.5 mM)	0.088 ± 0.024
Phenol red (0.5 mM) + brilliant blue (5 mM)	0.040 ± 0.022

Table 5.1.4 summarizes the final water volumes measured at the end of the perfusion experiments in absence and presence of counter ions. There are not significant differences within the three groups assayed.

Table 5.1.4. Final water volumes (Vf) measured at the end of the perfusion experiments and absorption rate coefficient (Ka) for every animal used, in absence and presence of counter ions. S.D.: Standard deviation. CV%: Coefficient of variation. NS: not statistically significant differences versus atenolol alone. S: statistically significant differences versus atenolol alone (p<0.05).

Animal	**Atenolol**		**Atenolol + brilliant blue**		**Atenolol + bromophenol blue**	
	Vf (mL)	Ka (h^{-1})	Vf (mL)	Ka (h^{-1})	Vf (mL)	Ka (h^{-1})
1	3.7	0.43	3.5	0.96	4.1	0.81
2	4.7	0.39	4.1	0.99	4.4	0.63
3	4.6	0.46	4.4	0.88	4.3	0.66
4	4.2	0.45	3.5	1.06	4.2	0.80
5	4.5	0.38	4.1	0.93	4.0	0.82
6	4.6	0.47	4.1	0.86	3.9	0.80
7			3.6	0.89		
mean	4.39	0.43	3.95	0.94	4.14	0.75
S.D.	0.37	0.04	0.33	0.07	0.18	0.08
CV%	8.58	8.97	8.45	7.48	4.50	10.84
Significance	-	-	NS	S	NS	S

Figure 5.1.4 shows the microscopy images of the histological assessment of colonic tissue for four different conditions: a blank without drug solution, atenolol solution, atenolol-brilliant blue solution and atenolol-bromophenol blue solution. Different sections of colon were examined: proximal, medial and distal colon. This assay allows to compare the structure of the colonic membrane in standard conditions and the potential changes in presence of any xenobiotic.

DISCUSSION

Atenolol is a β-blocker widely used for the treatment of hypertension, angina pectoris, cardiac arrhythmia and heart attack. It is commercially available as conventional tablet and is usually administered once daily. But, exceptionally, it can be administered two or three times a day [37-39]. Taking many doses would lead to large fluctuation in drug plasma concentration and side effects on patients. Therefore, is desirable to use controlled released systems which solve these problems [37, 38]. Moreover, the controlled release approach is advantageous for the treatment of diseases that have peak symptoms in the early morning and exhibit circadian rhythms, such as cardiac arrhythmias or hypertension [40, 41].

Atenolol shows a human oral bioavailability of 50% and a human fraction absorbed of 57% and is classified as a low permeability drug in small intestine and colon [5, 26] . The problem of applying controlled released formulations is that low permeability drugs, as atenolol, are not completely absorbed. In order to ensure their complete absorption, it is necessary to study strategies to increase their permeability through the colonic membrane where the residence time is enough to allow a complete absorption in favorable conditions. On the other hand, atenolol has a secondary amino group, it is a basic drug (pKa=9.6) ionized with positive charge in the physiological pH range. It is a hydrophilic drug and its high polarity is the main reason of its low permeability. Due to these features,

atenolol is a perfect candidate to enhance its permeability through an ion-pair development.

Literature described the formation ion-pairs of drugs with a basic functional group using anionic dyes [42-44]. Therefore, in our research, two acidic dyes, negatively charged at colonic pH, were selected as counter-ions: brilliant blue and bromophenol blue. Bromophenol blue was chosen because Al-Ghannam et al. describe the successful ion-pair formation with this dye and atenolol [42]. The use of bromophenol blue in humans is limited to the field of ophthalmology, particularly in ocular surgery to improve the visualization of preretinal tissues and membranes during vitrectomy, thus, reduces surgical risks; but high concentrations (≥ 1%) may have significant toxic effects [45-47].

Brilliant blue was chosen as a counter ion because it is an acidic dye, like the bromophenol blue, but, in addition, it is approved as a food additive. Previous works have concluded that a suitable counter ion for atenolol was bromophenol blue [42], but this acidic counter ions is toxic. For this reason, a less toxic counter ion was selected (brilliant blue). Brilliant blue (E133) it is a colorant for foods and other substances so should be consider as GRAS (generally regarded as safe). Brilliant blue is a colorant food additive widely used all over the world, it is employed in many foods such as canned peas, dairy products, drinks, packet soups, sweets, icings and ice cream. The EFSA (European Food Safety Authority) accept a daily intake for brilliant blue of 6.0 mg/kg bodyweight per day. Moreover, data available on the absorption, distribution, metabolism and excretion of this dye, show that it is poorly absorbed and mainly excreted unchanged in faeces [48].

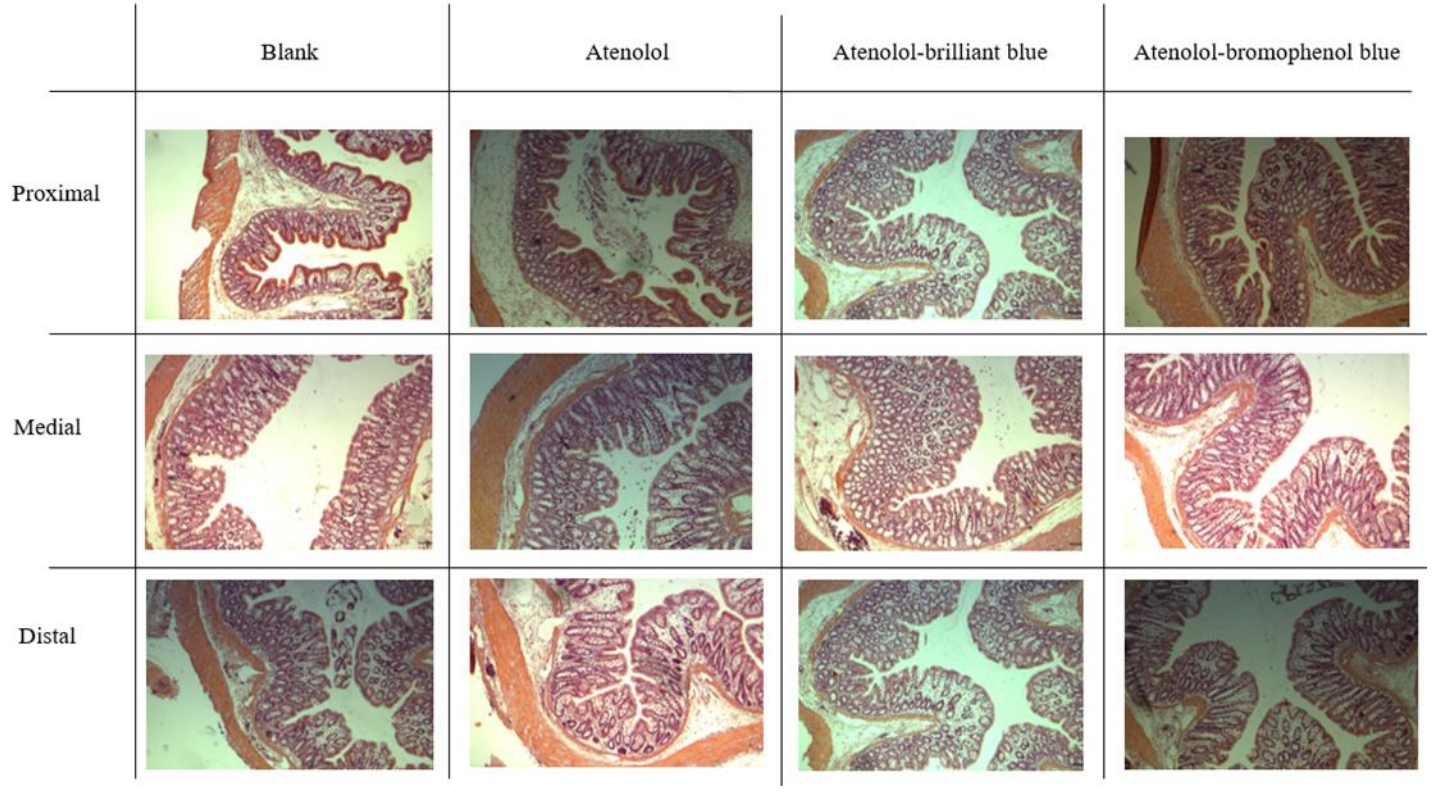

Figure 5.1.4. Proximal, medial and distal segments of colon for four different conditions: a blank without drug solution, atenolol solution, atenolol-brilliant blue solution and atenolol-bromophenol blue solution. All the images are 4x.

The *in situ* rat perfusion technique can be used to predict human oral fraction absorbed according to the following equation [49-52]:

$$F_a = 1 - e^{\left(-P_{app}\frac{2}{R}T\right)} \quad (6)$$

where *Fa* is the fraction absorbed, *Papp* is the permeability obtained in a particular intestinal segment (either whole small intestine or rat colon), *R* represents the effective radius of the intestinal segment, (where the term 2/*R* derives from the ratio between the area of transport and the volume at the absorption site) and *T* is the effective absorption time, i.e. the transit time in the intestinal segment. Therefore, with higher values of *Papp,* higher *Fa* will be expected.

In the case of human absorption from colon, we have recently demonstrated a good correlation from rat permeability values in colon and human oral fraction absorbed from colon [26].

The permeability of atenolol in presence of bromophenol blue and brilliant blue is increased due to the ion-pair formation, consequently the fraction absorbed achieved with ion-pair would be higher than the fraction absorbed without ion-pair.

Ion-pair is a “neutral entity” formed when ions with opposite charge (cation and anion) interact and remains together in solution thanks to electrostatic attraction but without formation of a covalent bond. As it is shown in Figure 5.1.1 at the pH used for the perfusion studies, Atenolol is positively charged while both counter ions are in anionic form, consequently the conditions for the formation of ion-pairs are optimal.

In this study, the ion-pair formation was evaluated as a strategy to enhance the colonic permeability of atenolol. The results of preliminary partitioning experiments performed with two different aqueous phases are shown in Table 5.1.1. The ion-pair formation should increase the atenolol

affinity for the lipophilic solvents, as 1-octanol. However, our results indicate that the increase of the distribution coefficient (D_{pH7}) is negligible for atenolol-bromophenol blue when the aqueous phase is phosphate buffer; but there is a notable increase of D when the aqueous phase is water instead of phosphate buffer. The increment of D for atenolol-brilliant blue is similar with both aqueous phases. A similar effect was observed by Van Gelder et al. who described an increased partition in the octanol phase and a significant decrease of drug remaining in the aqueous phase with water but not with isotonic buffer. This fact indicates that ion pair formation diminish or even disappear when other ions are present, due to competition between counter-ion and other ions present in the medium [53].

There are many studies with important increases of *in vitro* permeabilities using ion pairs but only a few of them are able to demonstrate in vivo effectivity. The reason of these differences remain unclear [25]. For these reasons, the next step was to evaluate the *in situ* behavior. In our assays, nevertheless, the results of *in situ* experiments (Table 5.1.2) are pleasantly positive. The colon permeability of atenolol improves considerably when it is perfused with brilliant blue or bromophenol blue. Table 5.1.2 and Figure 5.1.2 show that the differences obtained between the solutions tested are statistically significant. Moreover, Figure 5.1.3 presents the decrease of the atenolol concentration in the colonic lumen vs time due to the absorption process for the three assayed conditions, this decrease is bigger with the ion-pairs.

The highest increase of permeability values has been obtained with brilliant blue, probably, because at pH 7 (the pH of the perfused solution) 99.97% has negative charge [54] but the bromophenol blue presents less ionized percentage (88.57%) at same pH [55]. Steric reasons could also favor a stronger interaction with brilliant blue than with bromophenol blue. According to the remaining amounts of atenolol in the aqueous phases after partitioning experiments, a small increase of permeability with *in situ* assays

should be expected. However, the increase in absorption coefficients and apparent permeability achieved in rat colon are significant. The differences observed between the results of *in vitro* and *in situ* methods could be explained with the different features of 1-octanol and the phospholipid bilayer. The propensity for ion-pair formation and the general effect of ionic strength on membrane-buffer distribution and 1-octanol-buffer distribution are different [18, 55].

In order to check that the increase of atenolol permeability was due to ion-pair formation and not for membrane disturbances, a histological assessment of colonic tissue was made (Figure 5.1.4). No important damages in the colonic membrane for the different solutions were observed. The most relevant difference was observed in the goblet cells. These cells are present in the colonic mucosa and they secrete mucus. The mucus layer protects the epithelial mucosal surface with an essential barrier function, defending the body from pathogens and irritants [56, 57]. When comparing the two ion-pair solutions with the blank and atenolol solutions, morphological and size differences in goblet cells are observed; these changes are indicative of an inflammatory response. This tendency is larger with the bromophenol blue in the distal colon, because the distal part is the most sensitive to variations [58]. In spite of the differences in goblet cells, the alterations are not important. Some authors have indicated that with the changes induced by an inflammation, the colonic function in terms of fluid absorption, secretion and epithelial barrier function, are largely preserved [59]. Therefore, it is unlikely that the increased permeability is caused by a membrane disruption. In order to check that paracellular permeability was not increased, permeability of phenol red was measured in presence of increasing concentrations of brilliant blue (from 1:1 to 1:10). Phenol red permeability remained unchanged and almost negligible at all brilliant blue concentrations (Table 5.1.3), suggesting the paracellular route was not affected. The water reabsorption process observed in all the experimental conditions characterized by the final remaining water volume (4.39 mL for atenolol, 3.94

mL for atenolol + brilliant blue and 4.14 mL for atenolol + bromophenol blue) confirms that the increased permeability is not due to intestinal membrane alterations as there were not statistical differences among groups of the final water volumes. In Table 5.1.4 the average water reabsorption final volumes were summarized as well as their statistical comparison. Differences obtained between the water absorption final volumes are not statistically significant between results obtained with atenolol and both ion-pair. In addition, the water absorption process showed a similar variability in all animal groups.

CONCLUSION

In summary, it has been possible to overcome the colonic permeability limitations of atenolol using an ion pair strategy. The *in situ* perfusion assays of the ion-pairs have shown a permeability increase of atenolol in rat colon. Moreover, the histological assessment from colon tissue suggests that this increase is due to the ion-pair formation and not to membrane damage. Between the two counter ions tested, the brilliant blue seems more effective because the increase in permeability is higher. In addition, this compound is approved as a food additive, which means that it is considered safe. Available data of brilliant blue absorption show that it is a poorly absorbed compound, probably because it is charged at physiological pH. Nevertheless, it would be advisable to study brilliant blue toxicity when it is administered as an ion-pair because, the dye permeability could also be increased achieving higher plasma concentration that may cause adverse effects.

This study confirms the possibility to increase colonic permeability of atenolol wit ion-pair formation. The data obtained can be useful to improve the absorption of low permeability class drugs, with similar features to atenolol, and to further design and develop more efficient and intelligent controlled release formulations.

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5.2. Artículo científico 8

PLGA nanoparticles of ibuprofen for oral controlled release approach

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Draft version

ABSTRACT

The oral controlled release (CR) formulations have become more important in recent years. Among them, the polymeric nanoparticles are outlined, consequently they are used and studied for several applications and drugs. The objective of this research was to develop polymeric nanoparticles (NPs) of ibuprofen with poly(lactic-co-glycolic) acid (PLGA) as polymer, and to study their application for oral CR formulations development. Different proportion of drug/polymer was employed to develop the ibuprofen NPs and their release profiles were analysed. Moreover, the *in vitro* permeability of the encapsulated ibuprofen was tested in different cell cultures and the permeability values obtained were compared with *in vitro* and *in situ* permeability of ibuprofen in free solution. The results showed that the release of the ibuprofen from the NPs was pH-dependent because the release is bigger at colonic pH. Moreover, the proportion of drug/polymer also affected the drug release. 7.5% ibuprofen/92.5% PLGA was the best proportion to obtain the higher release. Furthermore, the *in situ* data showed high permeability of ibuprofen in colon. Therefore, the ibuprofen PLGA-NPs will be a good CR formulation to achieve a controlled release focused to the colon, where the release rate of the drug from the NPs will be the limiting factor for the absorption process.

Keywords: colon permeability, controlled release, ibuprofen, oral absorption, PLGA nanoparticles.

INTRODUCTION

In last decades, has been an increasing development of oral controlled release (CR) products. For these formulations the colon is the main site of absorption, because a CR pharmaceutical form is designed to release the active ingredient during 12-24 h, so the small intestine transit time (2-5 h) is not time enough to ensure a complete absorption of the drug [1-5]. The CR products have several benefits over the immediate release (IR) products, for

instance, a CR drug focused to the colon can be used to avoid gastric irritation, to avoid the degradation in stomach or small intestine or to reduce the intestinal first pass metabolism or of the active pharmaceutical ingredient. Moreover, the prolonged release allows once daily dose of drugs with short elimination half-life; as a result, the patient will experience a reduction of side effects and an extend of the effect duration [1, 6-8]. There are a lot of approaches and different strategies to develop CR formulations: coated tablets, drugs embedded in matrices, the employ of a carrier, microparticles, nanoparticles…[9]. Indeed, the strategy of the CR formulations development has been applied to several active pharmaceutical ingredients. For example, the Osmet™ device is available commercially for anti-inflammatory and anti-hypertensive drugs; Entocort® EC can be found for treatments with budesonine and Chronset™, for acetaminophen administration [10].

Nanoparticles have become in an important question in the field of drug delivery because they can deliver a wide range of drugs to different target organs and with different objectives for a long period. Some of the target organs for the drug delivery using nanoparticles are: brain, arterial, pulmonary, vaginal or oral delivery; moreover, can be used for tumour therapy, topical applications, lymphatic systems, and vaccines [11-19]. The oral delivery is the most physiological and preferred route for drugs administration. Polymer nanoparticles are particles with a diameter lower than 1 μm and they can be prepared from natural or synthetic polymers. These nanoparticles have shown good results for protein delivery by the oral route [20, 21]. Peptides and protein drugs, such as insulin, are degraded in stomach and small intestine, but colon provides a more suitable environment. Therefore, a CR formulation could become a solution for IR drugs that cannot be administered by oral route. However, the nanoparticles development can be applied to different drugs, not only proteins and peptides [22, 23]. They can be used to avoid the release of an active pharmaceutical ingredient in the upper part of the gastrointestinal tract,

independently if its absorption in small intestine is good or not. Thus, the nanoparticles will reach the colon without releasing the drug and an extended release will be achieved.

So, this approach has been developed with a high permeability model drug ibuprofen. Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID) widely used, it has a rapid and complete oral absorption of IR formulations, the maximum plasma concentration is reached at 1.4-2.9 h; so, it acts quickly as an anti-inflammatory analgesic [24]. Its mechanism of action consists in the nonselective inhibition of the cyclo-oxigenase (COX) activity. There are two different COX enzimes: COX-1 is responsible of some physiological functions regulation, such as blood flow, vascular functions, platelet aggregation, acid-pepsin mucus and other gastrointestinal functions; on the other hand, COX-2 is responsible of the inflammatory reactions [25]. The biggest impediment to a frequent use of ibuprofen is their potential adverse effects, mainly the gastrointestinal negative effects: abdominal pain, petechial, erosions and peptic ulcers which can cause gastrointestinal bleeding and perforation. The NSAIDs, included ibuprofen, can damage the gastroduodenal mucosa by two different mechanisms, by local effect and systemic effect. The local effect is due to the alteration of the gastric mucosa, that is pH dependent; and the systemic effect is due to the COX-1 inhibition, because COX-1 protects the gastric mucosa under normal conditions [26-30]. At doses ≤ 1200 mg ibuprofen per day for acute treatments, the risk of adverse effects is low. Nevertheless, this risk increases in chronic treatments with high doses (≥ 2400 mg) of ibuprofen per day, such as the treatments of osteoarthritis and arthritis rheumatoid. Moreover, the incidence of side effects is bigger with age, history of gastrointestinal problems, concurrent glucocorticoids and Helicobacter pylori infection [31-33]. Therefore, to develop an ibuprofen CR formulation can be useful for risk patients who take ibuprofen for chronic treatments. Thereby, the gastrointestinal local effects will be avoided, the dosage will be more comfortable because it could be reduced to once daily dosing. In addition, with a prolonged release, there will

be an increase of effect duration; so, the mg of ibuprofen per day could be reduced, which would mean a decrease of systemic side effects.

The objective of this work is to develop polymeric nanoparticles of ibuprofen with PLGA (poly(lactic-co-glycolic) acid) as polymer in order to limit ibuprofen delivery, then, to and evaluate their application as controlled release formulations.

MATERIALS AND METHODS

Materials

Poly(vinyl alcohol) (PVA) and ibuprofen were purchased from Sigma-Aldrich. PLGA 50:50 (poly(lactic-co-glycolic) acid) was kindly provided by Corbion (Gorinchem, the Netherlands). Methanol, acetonitrile and water were HPLC grade. All other chemicals were of analytical reagent grade**.**

Preparation of nanoparticles

The ibuprofen nanoparticles (NPs) were prepared with the nanoprecipitation method based on the method described by Bonelli et al. [34]. In brief, 5 mg of ibuprofen and 95 mg of the polymer, PLGA 50:50 (poly(lactic-co-glycolic) acid), were dissolved in 5 mL of organic solvent, acetone. The acetone solution was added dropwise to a 50 mL of 1% (w/v) PVA (polyvinyl alcohol) solution under magnetic stirring. The magnetic stirring was maintained during 3-4 h at 300 r.p.m. to ensure the acetone evaporation. The resulting suspension was subjected to high-speed centrifugation (40,000 g, 20 min) to recover the nanoparticles.

Three different sets of NPs were prepared. Set A: 5 mg ibuprofen and 95 mg PLGA; set B: 7.5 mg ibuprofen and 92.5 mg PLGA; set C: 10 mg ibuprofen and 90 mg PLGA. Unloaded nanoparticles, without drug, were also performed.

Nanoparticle characterization

The ibuprofen NPs were characterized with a zetasizer nano ZS regarding size, polydispersity index and zeta potential. The association efficiency (AE), amount of ibuprofen encapsulated, was calculated by difference between the total amount used to prepare the NPs and the remaining drug in the supernatant after centrifugation. Ibuprofen was measured by High Performance Liquid Chromatography (HPLC) as described in "HPLC analysis" section.

Moreover, the morphology and the surface of the NPs were observed using a scanning electron microscope (SEM).

Ibuprofen *in vitro* release studies from nanoparticles

To estimate ibuprofen release from NP an excess of NPs was added in a standard buffer solution, under magnetic stirring at 37 °C (pH 1.2, 4.5, 6.8 and 7.4) and samples were taken during 48 h. But, physiologically the pH value changes throughout the gastrointestinal tract, from acid pH in the proximal segments to basic pH in the distal segments [35]. Therefore, this assay was also performed simulating the physiological changes of pH along the gastrointestinal tract (Table 5.2.1), this simulation was done changing the pH in the same vessel. Sample concentration was determined using HPLC.

***In vitro* permeability studies**

Ibuprofen solution and ibuprofen NPs were tested in Caco-2 monocultures, in Caco-2/HT29-MTX co-cultures and Caco-2/HT29-MTX/Raji B triple co-culture model. The techniques employed to obtain the models used for the experiments are described in previous works [36], ["Artículo científico 4"]. The transepithelial electrical resistance (TEER) was measured every time the medium was replaced and before and after the transport

experiments to check the confluence evolution and to ensure the monolayer integrity.

To prepare the experimental solutions a high concentration of ibuprofen (0.242 mM) was used to ensure saturating conditions in order to get the diffusional component of the transport and a negligible contribution of the transporters. Before starting permeability assays, the culture medium was removed and the cell monolayers were washed. Hank's balanced salt solution (HBSS) was used to do the washes, to prepare the ibuprofen solutions and to resuspend the nanoparticles pellet obtained after the centrifugation. Basolateral chamber was filled with 2.5 mL of HBSS and apical chamber was filled with 1.5 mL of ibuprofen solution or nanoparticles suspension. The permeability studies were carried out from apical-to-basolateral (A-to-B) direction in an orbital environmental shaker at 37 °C and 100 r.p.m. At different times, basolateral samples of 200 µL were collected and analysed the drug concentration by HPLC.

The apparent permeability coefficient (P_{app}, cm/s) was calculated for both, solution and NPs, according to the following equation:

$$C_{receiver,t} = \frac{Q_{total}}{V_{receiver}+V_{donor}} + \left(\left(C_{receiver,t-1} \cdot f \right) - \frac{Q_{total}}{V_{receiver}+V_{donor}} \right) \cdot e^{-P_{app\ 0,1} \cdot S \cdot \left(\frac{1}{V_{receiver}} + \frac{1}{V_{donor}} \right) \cdot \Delta t} \quad (1)$$

where $C_{receiver,t}$ is the drug concentration in the receiver chamber at time t, Q_{total} is the total amount of drug in both chambers, $V_{receiver}$ and V_{donor} are the volumes of each chamber, $C_{receiver,t-1}$ is the drug concentration in the receiver chamber at previous time, f is the sample replacement dilution factor, S is the surface area of the monolayer, Δt is the time interval and P_{app} is the permeability coefficient which might be $P_{app\ 0}$ or $P_{app\ 1}$. This equation considers a continuous change of the donor and receiver concentrations, and it is valid in either sink or non-sink conditions. Its main feature is the option to estimate two permeability coefficients ($P_{app\ 0}$ and $P_{app\ 1}$) to account for

atypical profiles in which the initial rate is different [37], but ibuprofen doesn't show an atypical profile.

Table 5.2.1. Scheme of release assay according to gastrointestinal tract conditions.

Time (h)	pH	composition	Simulated segment	Sample time (h)
1.5	1.2	A) 78 mL HCl 0.1 N + NaOH c.s.p. pH 1.2	Stomach	0.083, 0.25, 0.5, 1, 1.5
0.25	4.5	B) A + 112 mL phosphate buffer pH 7.5 (50 mM) + 2.6 mL NaOH 1M	Duodenum	1.58, 1.75
2.75	6.8	C) B + 2.6 mL NaOH 1 M	Jejunum and ileum	2, 3, 4, 4.5
Until 48	7.4	D) C + 1.7 mL NaOH 1 M	Colon	4.58, 6, 8, 10, 24, 48

***In situ* permeability studies**

Male Wistar rats were used in accordance with 2010/63/EU directive of 22 September 2010 regarding the protection of animals used for scientific experimentation. The Ethics Committee for Animal Experimentation of the University of Valencia approved the experimental protocols (Spain, code A1330354541263).

The permeability (*Papp*, cm/s) values of ibuprofen in solution (0.242 mM) were determined in different 10-cm segments of small intestine (jejunum, medium and ileum) and colon (n= 6-7) using *in situ* "closed loop" perfusion method based on Doluisio's Technique [38] modified as described in Lozoya-Agullo et al. [39, 40]. Briefly, the perfusion technique consists of creating an isolated compartment in the intestinal segment of interest with the aid of two syringes and two three-way stopcock valves. To remove all intestinal contents, each segment was flushed with a physiologic solution: isotonic saline with 1% Sörensen phosphate buffer (v/v) at 37 °C. The physiologic solution pH was adjusted depending on the segment perfused:

6.5 for the jejunal segment, 7.0 for the middle segment, and pH 7.5 for the ileal segment and colon. The drug solution was introduced in the correct segment and the samples were collected every 5 min up to a period of 30 min. All the animal samples were analysed by HPLC.

Water flux during the experiment was significant (up to 20%), so a correction was necessary to calculate the absorption rate constants accurately. A method based on direct measurement of the remaining solution volume was employed to calculate the water reabsorption zero order constant (k_0). For each segment, the initial volume (V_0) was determined, and the endpoint volume (V_t) was measured. The drug concentration in the samples was corrected as:

$$C_t = C_e(V_t/V_0) \quad (2)$$

where C_t represents the concentration in the absence of water reabsorption at time t, and C_e the experimental value. The corrected concentration, C_t, was then used as the actual absorption rate coefficient calculations [41].

The absorption rate coefficients (k_a) of ibuprofen was determined by nonlinear analysis of remaining concentrations in lumen C_t versus time:

$$C_t = C_0 e^{-k_a t} \quad (3)$$

These absorption rate coefficients (k_a) were then transformed in to permeability values (P_{app}) using the relationship:

$$P_{app} = k_a R/2 \quad (4)$$

where R is the effective radius of the intestinal segment calculated considering the intestinal segment as a cylinder with:

$$Volume = \pi R^2 L \quad (5)$$

Considering a 2-mL volume for each intestinal segment and 5 mL for colon, the length was approximately 10 cm, but it was exactly measured at the end of the experiments in each segment and each animal.

HPLC analysis

The experimental samples were analysed using a Perkin-Elmer HPLC according to the published method [42]. Briefly, a fluorescence detector at 225 nm for excitation λ and 535 nm for emission λ was used. The mobile phase consisted of a mixture of 60:40 (v/v) 30 mM Na_2HPO_4 pH 7.0: acetonitrile. The stationary phase was a µBondapak 150 mm x 4.6 mm C-18 column (5 µm particle size) at room temperature. Flow rate was 1 mL/min and the injection volume was 10 µL. Retention time of ibuprofen was 3.5 min. The standard calibration curves were prepared by dilution from the drug solution assayed. Sample concentrations were within the linear range of quantitation for all the assays. Analytical method was validated in terms of specificity, selectivity, linearity, precision and accuracy.

Data analysis

To compare the ibuprofen permeability values, in solution and encapsulated, with the different techniques contrasted (Caco-2/HT29-MTX co-culture, Caco-2/HT29-MTX/Raji B triple co-culture, rat small intestine, rat colon and monoculture Caco-2), the permeability values were compared using ANOVA to detect the existence of significant differences at the 0.05 probability level. The statistic Levene was calculated to test the homogeneity of variances and, depending on the result of the statistic Levene, Scheffé or Dunnett's T3 *Post Hoc* test were applied to determine statistical significative difference between groups. The statistical comparison was made using the statistical package SPSS, V.22.

RESULTS

Below are show the results obtained in the tests described in the materials and methods section.

Table 5.2.2 shows the characterization PLGA nanoparticles regarding size (diameter), zeta potential, polydispersity index and association efficiency. Set A is 5 mg ibuprofen and 95 mg PLGA, set B is 7.5 mg ibuprofen and 92.5 mg PLGA and set C is 10 mg ibuprofen and 90 mg PLGA. The data suggest that the size increase when the amount of drug increases. In addition, Figure 5.2.1 shows the morphology of the nanoparticles.

Table 5.2.2. Characterization of PLGA nanoparticles in terms of size, zeta potential (ZP), polydispersity index (PDI) and ibuprofen association efficiency (AE%) (n=3, mean ± standard deviation).

	Size (nm)	ZP (mV)	PDI	AE%
Unloaded NPs	211.9 ± 1.67	-15.6 ± 1.22	0.05 ± 0.01	-
Ibuprofen NPs-Set A	227.8 ± 5.81	-18.8 ± 0.92	0.08 ± 0.05	64.29 ± 0.97
Ibuprofen NPs-Set B	235.1 ± 3.04	-18.0 ± 0.26	0.08 ± 0.04	58.01 ± 0.47
Ibuprofen NPs-Set C	339.4 ± 7.80	-17.0 ± 0.20	0.06 ± 0.04	68.07 ± 0.78

Figure 5.2.2, Figure 5.2.3 and Figure 5.2.4 show the time-dependent release rate of ibuprofen from PLGA-NPs of the three sets of NPs assayed under the conditions explained in material and methods. The plots indicate that the proportion drug/polymer and the pH of the solution employed for the delivery study affect the release of ibuprofen.

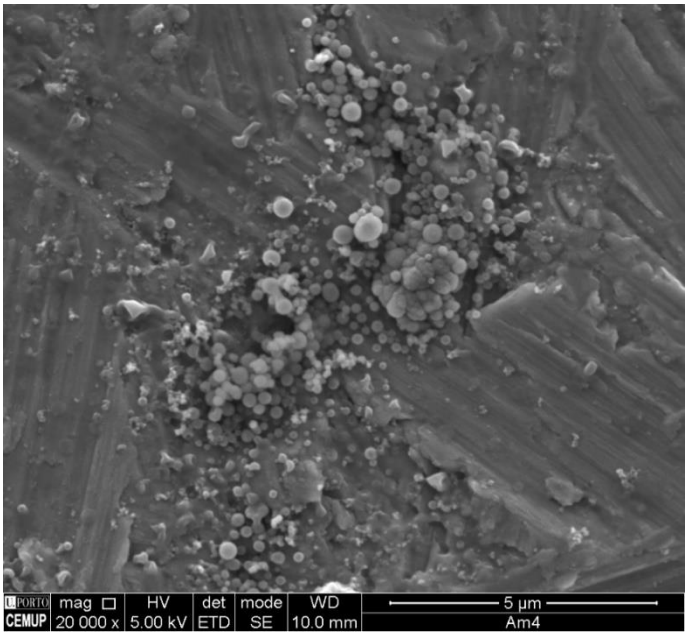

Figure 5.2.1. Scanning electron microscope image of ibuprofen nanoparticles.

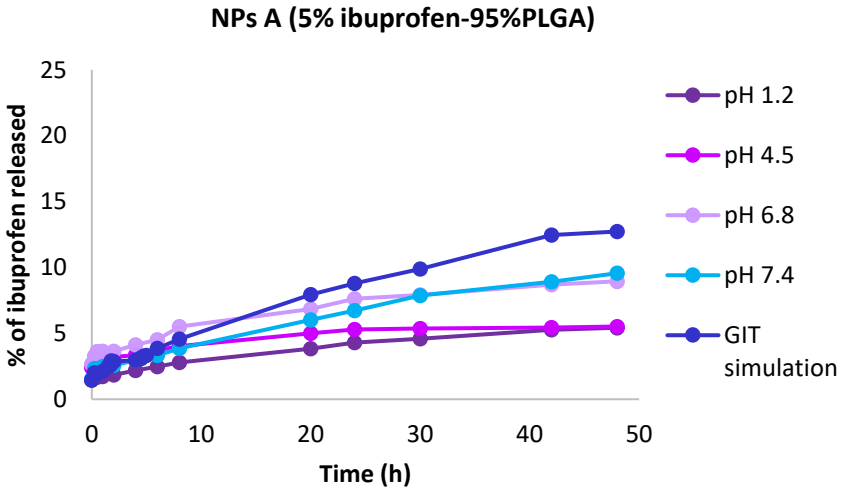

Figure 5.2.2. Percentage of drug released vs time of the NPs A at the different conditions assayed. GIT: Gastrointestinal tract.

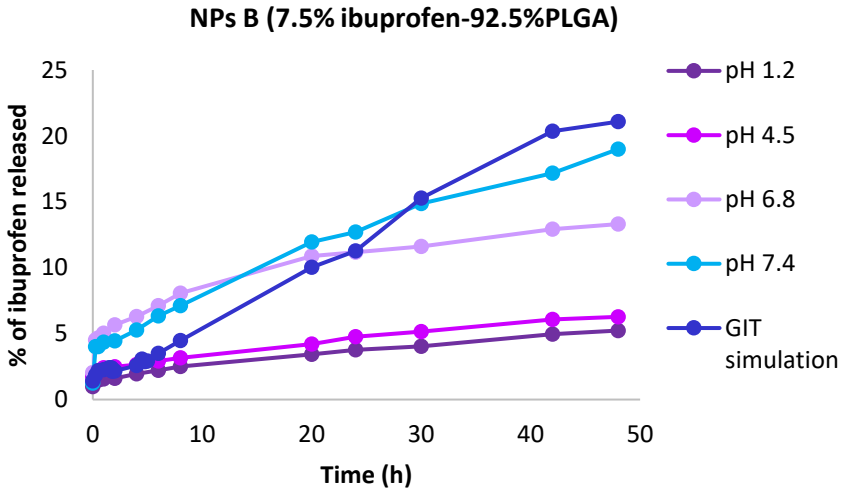

Figure 5.2.3. Percentage of drug released vs time of the NPs B at the different conditions assayed. GIT: Gastrointestinal tract.

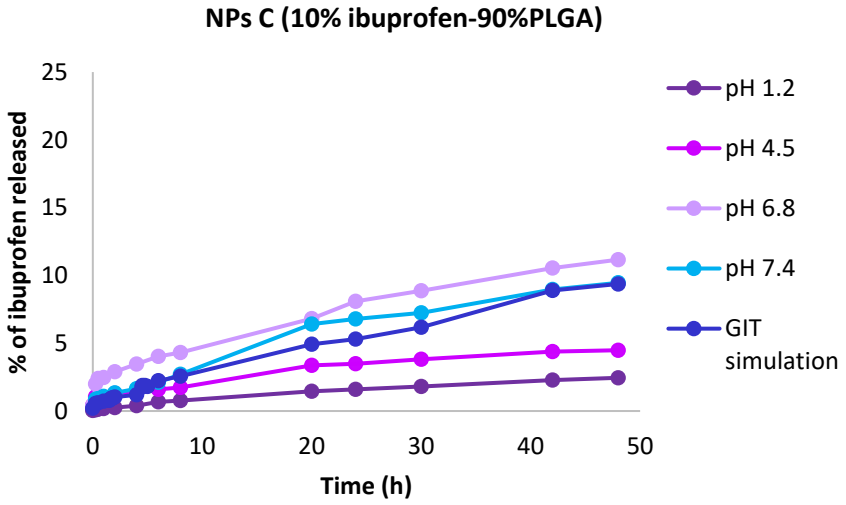

Figure 5.2.4. Percentage of drug released vs time of the NPs C at the different conditions assayed. GIT: Gastrointestinal tract.

Table 5.2.3 and Figure 5.2.5 show the permeability values obtained with *in vitro* studies, *Papp* (x10^{-4} cm/s) of ibuprofen in solution and NPs (Set A: 5 mg ibuprofen-95 mg PLGA) can be observed for the three *in vitro* models assayed. Figure 5.2.5 presents a comparative graphic of the exposed permeability in Table 5.2.3, a marked difference can be seen between solution and NPs permeability. For ibuprofen in solution, *Papp* of Caco-2 monoculture has statistically significant differences compared with co-culture and triple co-culture. However, for ibuprofen in NPs the *Papp* values are similar for the three *in vitro* models.

Table 5.2.3. *In vitro* apparent permeability coefficient (*Papp*, x10^{-4} cm/s) of ibuprofen. (n=3, mean ± standard deviation).

	Caco-2	**Co-culture**	**Triple co-culture**
	Papp (x10^{-4} cm/s)	*Papp* (x10^{-4} cm/s)	*Papp* (x10^{-4} cm/s)
Solution	0.245 ± 0.015	0.546 ± 0.082	0.445 ±0.028
NPs	0.065 ± 0.004	0.064 ± 0.010	0.065 ± 0.006

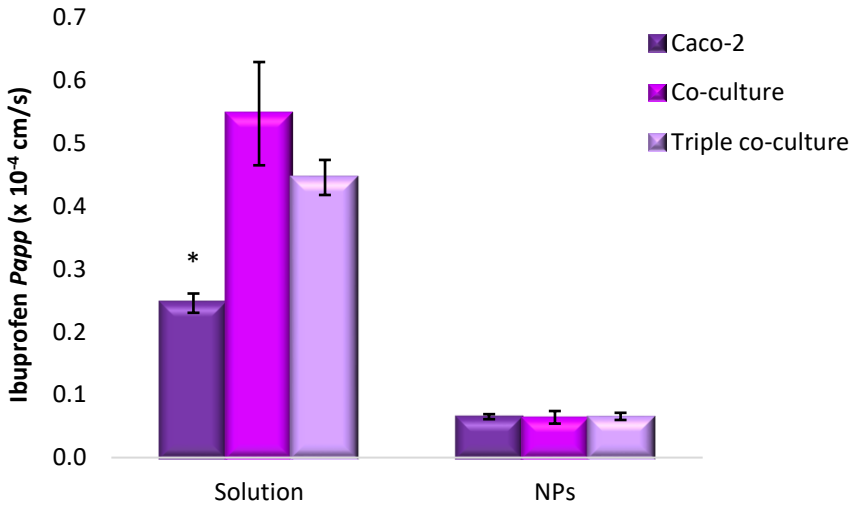

Figure 5.2.5. Permeability values of ibuprofen in solution and NPs assayed in three *in vitro* models: Caco-2 monoculture, Caco-2/HT29-MTX co-culture and Caco-2/HT29-MTX/Raji B triple co-culture. * Statistically significant differences between the Caco-2 *Papp* and *Papp* of the other two models ($p < 0.05$).

Table 5.2.4 shows the *in situ* permeability values of ibuprofen in solution obtained in rat segments of small intestine (jejunum, medium, ileum) and rat colon. A comparative graphic between *in situ* permeability data in different intestinal segments can be observed in Figure 5.2.6. The statistical comparison reveals that there are statistically significant differences between ileum and colon.

Table 5.2.4. *In situ* apparent permeability coefficient (*Papp*, x10^{-4} cm/s) of ibuprofen in solution. (n=6, mean ± standard deviation).

	***Papp* ibuprofen (x10^{-4} cm/s)**
Jejunum	2.310 ± 0.348
Middle	2.020 ± 0.193
Ileum	1.750 ± 0.299
Colon	2.491 ± 0.232

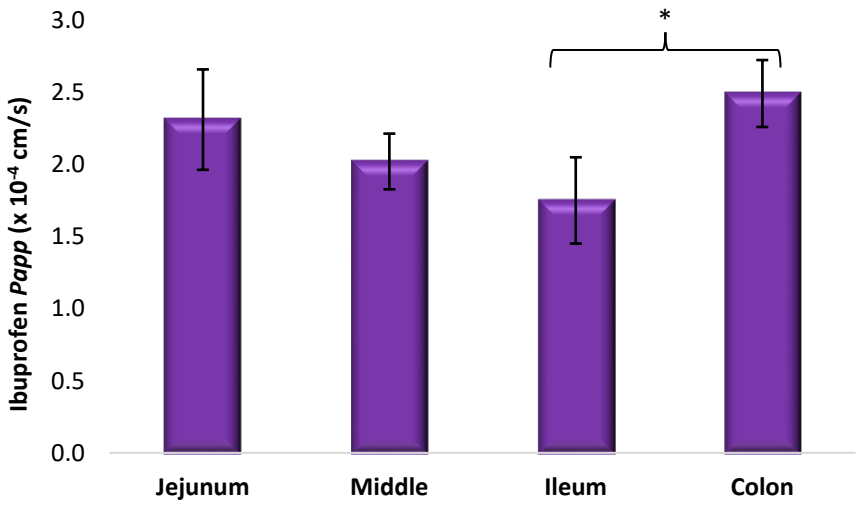

Figure 5.2.6. Permeability values of ibuprofen solution in the different intestinal segments assayed. Middle: the medium part of the small intestine. * Statistically significant differences between ileum and colon ($p < 0.05$).

DISCUSSION

The main objective of this research was to develop PLGA nanoparticles loaded with ibuprofen to study their applicability to controlled release formulation development. Even though ibuprofen as controlled release formulation already exists, this work shows a proof of concept of new approach to achieve a CR formulation focused on the colon [43, 44].

However, an advantage of NPs is that water-insoluble drugs, as ibuprofen, can be transported more efficiently in the aqueous physiological environment when formed into stable NPs [34]; NPs also allow a controlled drug delivery [45]. Moreover, PLGA is one of the most successfully polymer employed for NPs development because it is biodegradable and biocompatible, a minimal systemic toxicity is associated with the use of PLGA and it is approved by the FDA (Food and Drug Administration) and EMA (European Medicine Agency) [46].

Table 5.2.2 shows the characterization of PLGA-NPs. The size observed was less than 1 μm diameter, so they were inside the range of nanoparticles. Moreover, the NPs obtained had a uniform surface without irregularities (Figure 5.2.1).

The release profiles of the three NPs sets developed can be seen in Figure 5.2.2, Figure 5.2.3 and Figure 5.2.4. The three sets show a pH-dependent release because the release in acidic medium (pH 1.2 and pH 4.5) not overcame the 5%, however with higher pH (6.8 and 7.4) the release was bigger. Therefore, most of the release happened under colonic conditions. This result is positive because to achieve the maximum release at the end of the gastrointestinal tract is the objective of a CR formulation with a release focused to ileum and colon. Moreover, the result of the gastrointestinal simulation assay also supported the suitability of the NPs developed because they showed that the amount of released drug was maximum after 48 h (pH 7.4), when the pH was changed according to the gastrointestinal variations.

In addition, the release at pH 1.2 is minimum, so the gastric and duodenal side effects related with ibuprofen action could be avoid.

The different ratio drug/PLGA also affected the release of ibuprofen. It seems that the optimal proportion was 7.5 mg ibuprofen/92.5 mg PLGA (Figure 5.2.3). Employing more amount of drug to perform the NPs no implied a higher release (Figure 5.2.4). Considering the drug solubility, all the release assays were done under sink conditions.

Nevertheless, the highest percentage of drug release was 21% with the NPs B (Figure 5.2.3) and lower with NPs A (Figure 5.2.2) and NPs C (Figure 5.2.4), which is not a great rate. So, these results suggest that after 48 h not all the amount of ibuprofen has been released and it needs more time to achieve a complete release. But, this is a problem because the total gastrointestinal transit time in human is around 20-30 h [2]. Thus, after 48 h the dosage form will have been eliminated from the organism, and the patient will present underdosing. In order to avoid the underdosing, the necessary amount of NPs to achieve the required plasma concentration of drug would have to be calculated taking into account the percentage of released ibuprofen. In addition, this problem of the formulation could be easily overcome by combining it with an adhesive strategy in order to increase to residence time.

Table 5.2.3 and Figure 5.2.5 show the ibuprofen *Papp* values obtained *in vitro*. When the drug was tested in solution the *Papp* in Caco-2 monocultures was less than co-culture and triple co-culture, but between these two models there were not differences. Therefore, the *Papp* values obtained with co-culture and triple co-culture were closer to those obtained with *in situ* method, this fact can be explain because co-culture and triple co-culture are more physiological models than Caco-2 [36]. Nevertheless, when the ibuprofen was administered as PLGA-NPs, there were not differences in the *Papp* values of the three *in vitro* models and their permeability was markedly lower than that obtained with the drug in free solution. The low *in*

vitro permeability obtained with encapsulated ibuprofen agrees with the results of the *in vitro* release studies. This fact may be indicative that the absorption limiting factor is the release rate of the ibuprofen by the nanoparticles and this is the main condition to obtain a CR formulation.

The permeability obtained with *in situ* techniques is higher than that obtained *in vitro*, because although co-culture and triple co-culture are more physiological models, they still have a lower available surface of absorption [47]. Table 5.2.4 and Figure 5.2.6 show the permeability values obtained with *in situ* method for each segment assayed. Ibuprofen has a pKa=4.91, so the different pH of the studied segments could modify the ionized fraction of the drug and hence affect the permeability. Moreover, these results (Table 5.2.4 and Figure 5.2.6) agree with the literature data, because ibuprofen is an OATP-B substrate. OATP-B is an uptake transporter highly expressed in jejunum and colon [48, 49]. On the other hand, the high *Papp* value of free ibuprofen obtained in colon (2.491 $x10^{-4}$ cm/s) indicates that there will be not problems in the ibuprofen absorption when a CR formulation release the loaded drug in this distal segment of the gastrointestinal tract.

CONCLUSION

The developed PLGA-NPs show a pH-dependent release with a higher release at typical colonic pH. Therefore, these nanoparticles are optimal for CR formulation and, moreover, the low release at pH 1.2 and 4.5 minimize the gastric and duodenal side effects of ibuprofen. In addition, the optimal proportion of drug/polymer to obtain a better release rate is 7.5% ibuprofen/92.5% PLGA. Furthermore, the permeability of free ibuprofen is high in colon, so the drug will have no problems in the absorption process when it will be release from a CR formulation.

FUTURE WORK

The permeability of ibuprofen NPs will be determined *in vivo* using Wistar rats. To carry out the *in vivo* assays, twenty-four hours prior to dose administration, a silicone catheter will be implanted in the jugular vein of all the animals, following a previously validated technique [50]. Parameters as C_{max}, T_{max} and AUC will be compared to determine if the ibuprofen NPs decrease the intestinal side effects and the daily dosing.

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6. RESULTADOS Y DISCUSIÓN GENERAL

La presente Tesis Doctoral tiene como objetivo general validar el uso en colon de rata del modelo de absorción desarrollado por Doluisio y colaboradores [1] para cuantificar la absorción *in situ* de compuestos en intestino delgado, ya que disponer de una técnica experimental capaz de predecir la absorción de compuestos en este tramo distal del tracto gastrointestinal contribuiría en el diseño de formulaciones de liberación modificada para fármacos que pueden absorberse en colon.

A lo largo de los años el colon se ha considerado un lugar inespecífico para la absorción sistémica de fármacos, ya que se ha atribuido al intestino delgado la prioridad en la absorción de xenobióticos administrados por vía oral [2]. Este es el motivo por el que la mayoría de estudios científicos, que abarcan desde la determinación de la permeabilidad intestinal de moléculas con actividad terapéutica hasta el desarrollo y la validación de métodos para realizar estudios de absorción, tanto en modelos desarrollados en humanos como en los desarrollados en animales (*in vivo* e *in situ*) y en tejidos aislados o en cultivos celulares (*in vitro)*, se hayan centrado principalmente en evaluar la capacidad de absorción del intestino delgado. Por el contrario, la cantidad de datos generados utilizando modelos de absorción en colon es considerablemente menor y además no se ha abordado de modo sistemático [3-6].

Sin embargo, el desarrollo de las formas farmacéuticas de liberación controlada (retardada o prolongada) para la administración de fármacos por vía oral ha propiciado que en la actualidad se profundice en el estudio del colon como órgano de absorción. Este tipo de formulaciones están diseñadas para liberar el principio activo que contienen durante un periodo de tiempo que puede prolongarse hasta las 24 horas [5], por lo que la absorción de las moléculas administradas utilizando estos sistemas de formulación se produce preferentemente en el colon, que es donde los productos ingeridos permanecen durante un periodo de tiempo mayor [7]. Las formas farmacéuticas de liberación controlada presentan varias ventajas, como la

posibilidad de incrementar el intervalo de dosificación; además, se evitan las fluctuaciones en la concentración plasmática del fármaco, por lo que se consigue una respuesta farmacológica más uniforme y una reducción de los efectos adversos. Cabe resaltar el aumento de la adherencia terapéutica al tratamiento por parte del paciente.

Dado que el colon es el lugar de absorción principal cuando el fármaco se administra utilizando una forma farmacéutica de liberación modificada, conocer la permeabilidad del mismo en este tramo del tracto gastrointestinal se convierte en la fase del diseño de la forma de dosificación en una necesidad. Sin embargo, hasta el momento no se ha caracterizado de forma sistemática la capacidad de los fármacos de atravesar la membrana intestinal colónica, ya que en múltiples ocasiones, se ha aceptado que los compuestos que presentan una buena absorción en intestino delgado, presentan, a su vez, una absorción suficiente en intestino grueso lo que, en principio, garantizaría una absorción oral completa cuando el compuesto se administre en una forma de dosificación oral de liberación controlada [8].

El hecho de aceptar una buena absorción en intestino delgado como garantía de una absorción idónea en colon, explica, al menos en parte, la inexistencia de un método validado para determinar la permeabilidad en este tramo del tracto gastrointestinal. La validación de un método es el proceso por el cual se confirma que el procedimiento utilizado para una prueba determinada es adecuado para el uso previsto. La importancia de trabajar con un método validado es innegable; además es un requisito esencial cuando se desean obtener resultados válidos, precisos, confiables y que puedan ser comparados con datos procedentes de otros laboratorios.

Esta situación justifica el objetivo principal de esta Tesis Doctoral que es validar la técnica de perfusión *in situ* en colon de rata para determinar la permeabilidad de compuestos y confirmar que a partir de los valores de permeabilidad intestinal proporcionados con esta técnica experimental se puede predecir, con exactitud y precisión, la fracción de dosis administrada

por vía oral que será absorbida cuando el fármaco se administre en humanos. Para tal fin se ha utilizado una amplia batería de moléculas activas seleccionadas de acuerdo a distintos criterios. El principal de ellos fue disponer de fármacos de distintas propiedades fisicoquímicas y en consecuencia de distinta permeabilidad intestinal (baja, intermedia y alta). Además, se tuvo en cuenta si para los compuestos que cumplían estas condiciones existían datos publicados de permeabilidad intestinal en colon y/o de la fracción absorbida en humanos. Por último, se tuvo en cuenta la recomendación de la FDA en la que hace hincapié en que el número mínimo de fármacos modelo evaluados debe ser de 20 en aquellas situaciones en las que el objetivo del estudio es demostrar la aptitud de un método destinado a medir la permeabilidad intestinal mediante estudios de perfusión en animales [9].

6.1. Validación de la técnica de perfusión *in situ*

La técnica experimental utilizada para llevar a cabo los ensayos fue un modelo de perfusión *in situ* en rata sin recirculación basado en el método descrito por Doluisio y colaboradores en 1969 [1]. Los métodos de perfusión *in situ* no presentan la complejidad propia de los métodos *in vivo,* pero mantienen intacto el aporte sanguíneo, endocrino y linfático de la zona de interés, por tanto, son modelos sensibles a influencias fisiológicas y farmacológicas, lo que permite observar mayor variabilidad en los resultados obtenidos en comparación con los sistemas *in vitro.*

Los métodos de perfusión *in situ* que se utilizan con mayor frecuencia para caracterizar la permeabilidad intestinal de moléculas son el método de paso simple (SPIP) [10] y el método de Doluisio. Ambos modelos presentan diferencias en su procedimiento, que se encuentran descritas en el Capítulo 3, pero su objetivo final es el mismo: determinar la permeabilidad del compuesto ensayado midiendo la desaparición del mismo en el lumen intestinal.

No obstante, aunque presenten un objetivo común y ambos permitan determinar la permeabilidad intestinal, en la literatura científica disponible se encuentra un número de publicaciones más elevado en las que se usa el método de paso simple en rata, tanto si los parámetros de permeabilidad se utilizan para predecir la absorción intestinal en humanos como si estos parámetros tienen la finalidad de clasificar los compuestos evaluados de acuerdo con los criterios que se establecen en el Sistema de Clasificación Biofarmacéutico (BCS) [4, 11-16]. Por este motivo, el primer paso realizado en esta Tesis Doctoral, encaminado a conseguir el objetivo general del trabajo, estuvo dirigido a discernir si ambos métodos son idóneos para determinar la permeabilidad intestinal de los compuestos. Para ello, se compararon los coeficientes de permeabilidad obtenidos en distintas secciones del tracto gastrointestinal utilizando ambos métodos y se obtuvieron las correlaciones entre los valores de permeabilidad que proporcionan ambas técnicas experimentales para cada tramo intestinal ensayado (yeyuno, tramo intermedio, íleon y colon). La selección de los segmentos en intestino delgado se hizo en base a los tramos seleccionados en los ensayos realizados con el método de referencia (SPIP) [17], que corresponden a la parte proximal del yeyuno, la parte distal del íleon y el tramo intermedio abarca la parte comprendida entre el final del yeyuno y el inicio del íleon. Asimismo, se obtuvo la correlación entre los valores de permeabilidad de distintos fármacos en intestino delgado completo de rata, obtenidos utilizando la metodología sin recirculación propuesta por Doluisio y colaboradores [1], y los obtenidos en yeyuno mediante el método de SPIP. En esta última correlación no se utiliza la permeabilidad determinada en intestino delgado completo utilizando la técnica de SPIP porque esta técnica emplea el valor de permeabilidad determinado en yeyuno como valor representativo de la absorción en intestino delgado completo. En todas las correlaciones realizadas que tuvieron como finalidad clasificar los compuestos en alta o baja permeabilidad se utilizó el metoprolol como fármaco de referencia [18].

A continuación, se muestran las representaciones gráficas en las que se resumen los resultados obtenidos. La Figura 6.1 representa la correlación entre la permeabilidad determinada en intestino delgado completo de rata determinada con el método de Doluisio y la permeabilidad en yeyuno de rata, tomada como referencia de la permeabilidad en intestino delgado completo, determinada mediante la técnica de SPIP. La Figura 6.2 muestra la correlación entre las permeabilidades en yeyuno de rata determinadas mediante ambas técnicas de perfusión. En la Figura 6.3 se observa la correlación entre las permeabilidades en el tramo medio del intestino delgado determinadas con ambos métodos. La Figura 6.4 representa la correlación entre las permeabilidades obtenidas en íleon con los dos métodos ensayados. Por último, la Figura 6.5 muestra la correlación existente entre los valores de permeabilidad en colon determinados con los dos métodos indicados.

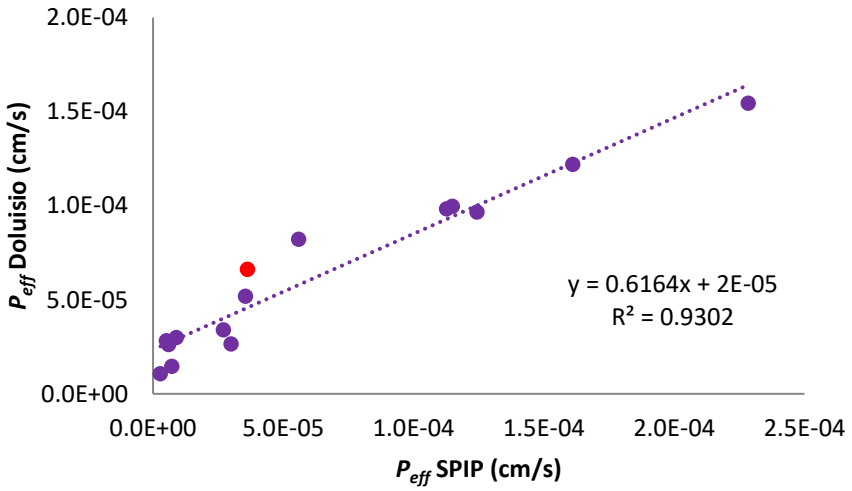

Figura 6.1. Correlación entre las dos series de datos, permeabilidad obtenida utilizando SPIP en yeyuno (tomada en representación de la permeabilidad en intestino delgado completo) vs. permeabilidad obtenida con el método de Doluisio en intestino delgado completo de rata. El símbolo rojo corresponde al metoprolol.

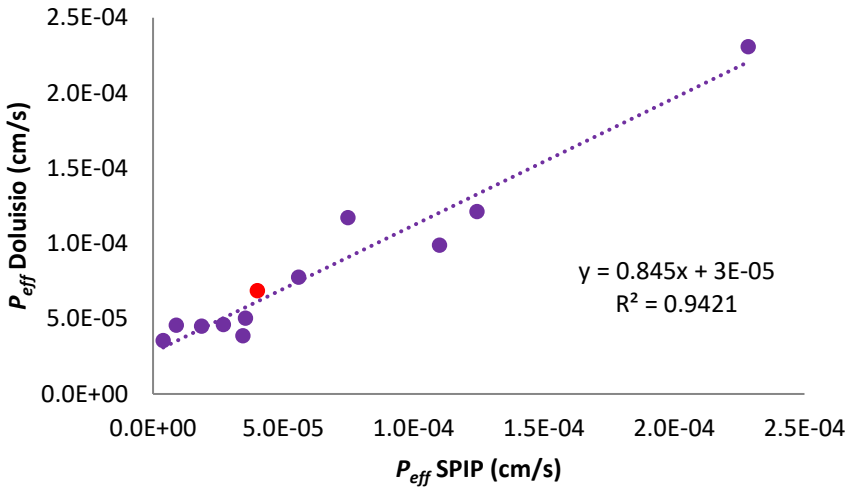

Figura 6.2. Correlación entre las dos series de datos, permeabilidad obtenida mediante el método SPIP vs. permeabilidad obtenida con el método de Doluisio en yeyuno de rata. El símbolo rojo corresponde al metoprolol.

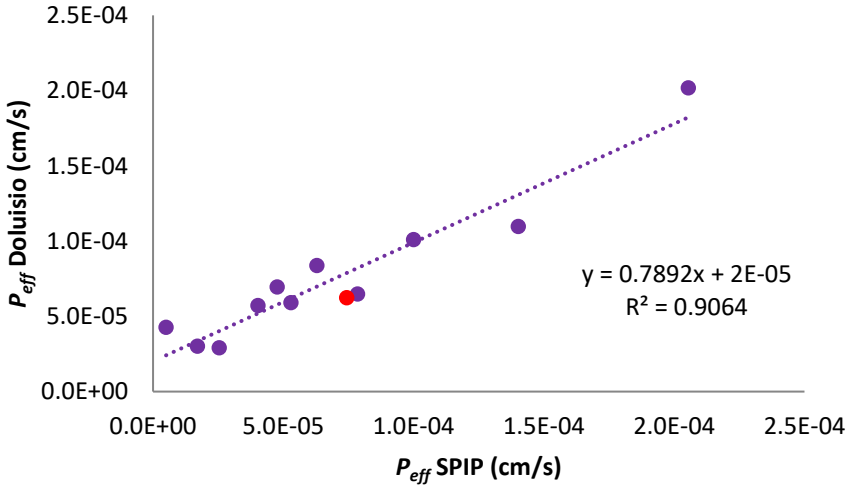

Figura 6.3. Correlación entre las dos series de datos, permeabilidad obtenida utilizando el método SPIP vs. permeabilidad obtenida mediante el método de Doluisio en el tramo medio del intestino delgado de rata. El símbolo rojo corresponde al metoprolol.

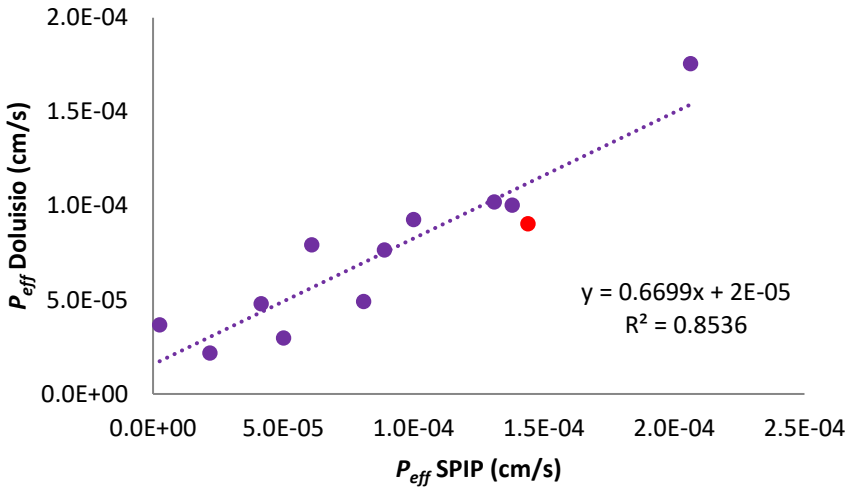

Figura 6.4. Correlación entre las dos series de datos, permeabilidad obtenida utilizando el método SPIP vs. permeabilidad obtenida con el método de Doluisio en íleon de rata. El símbolo rojo corresponde al metoprolol.

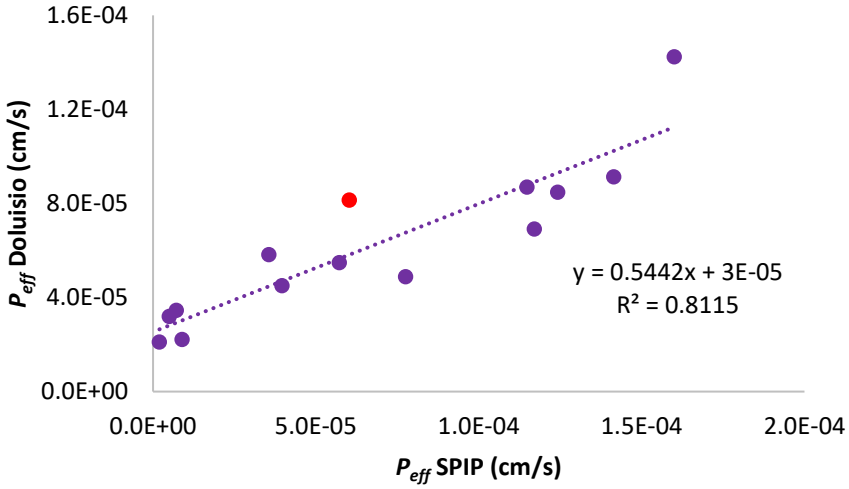

Figura 6.5. Correlación entre las dos series de datos, permeabilidad obtenida con el método SPIP vs. permeabilidad obtenida con el método de Doluisio en colon de rata. El símbolo rojo corresponde al metoprolol.

Como se aprecia en las gráficas anteriores todas las correlaciones establecidas son buenas, ya que las cinco presentan un coeficiente de determinación (R^2) mayor a 0,8, siendo las mejores aquéllas obtenidas entre las permeabilidades determinadas utilizando el método de Doluisio y la técnica de SPIP en intestino delgado completo y en yeyuno (Figuras 6.1 y 6.2). Cabe recordar que la técnica de SPIP nunca utiliza el intestino completo, sino que en el ensayo estándar la perfusión tiene lugar en 10 cm del yeyuno [19]; sin embargo, en el método de Doluisio se utiliza el intestino delgado completo [1]. La similitud existente entre las dos primeras correlaciones (Figura 6.1 y Figura 6.2) podría significar que el yeyuno presenta una contribución dominante en la permeabilidad global obtenida tras usar el intestino completo en el método de Doluisio.

Además, se aprecia una disminución del coeficiente de determinación, R^2, en las correlaciones obtenidas para los valores de permeabilidad determinados en las secciones del tracto intestinal más distales, aunque en todos los casos los valores obtenidos se encuentran entre 0,81 y 0,94 lo que indica que la correlación entre ambas técnicas es excelente.

Otro hecho que merece destacarse es la diferencia existente entre los valores de permeabilidad obtenidos con ambos métodos para los compuestos poco permeables. En la Figura 6.6 se observa claramente cómo los valores obtenidos para los compuestos de baja permeabilidad son mayores cuando se determinan con la técnica de Doluisio. No obstante, para aquéllos compuestos de permeabilidad media y alta el coeficiente de permeabilidad que proporcionan ambas técnicas es similar. Esta característica podría deberse a las diferencias inherentes al procedimiento experimental de los dos métodos estudiados. Así, en el método de Doluisio la solución de fármaco permanece en el intestino delgado (compartimento estanco) durante el transcurso del experimento (30 minutos), pero con el método de SPIP la solución atraviesa el segmento intestinal a un flujo constante y no permanece retenida en el tramo estudiado. Este aspecto

diferencial condiciona el tiempo de contacto del fármaco con la membrana absorbente, que para los compuestos que presentan dificultades para atravesar la membrana intestinal absorbente podría ser el factor determinante. Otro aspecto que podría justificar la diferencia de valores de permeabilidad entre ambos métodos podría estar relacionado con el protocolo de toma de muestras experimentales utilizado, ya que la toma de muestras en el método de SPIP comienza una vez se ha alcanzado el estado de equilibrio, mientras que en el método de Doluisio se toma la primera muestra transcurridos cinco minutos desde que se introduce la solución a ensayar en el segmento intestinal de interés. Este aspecto implica que los fármacos de baja permeabilidad podrían presentar con el método de Doluisio una mayor permeabilidad que con la técnica de SPIP.

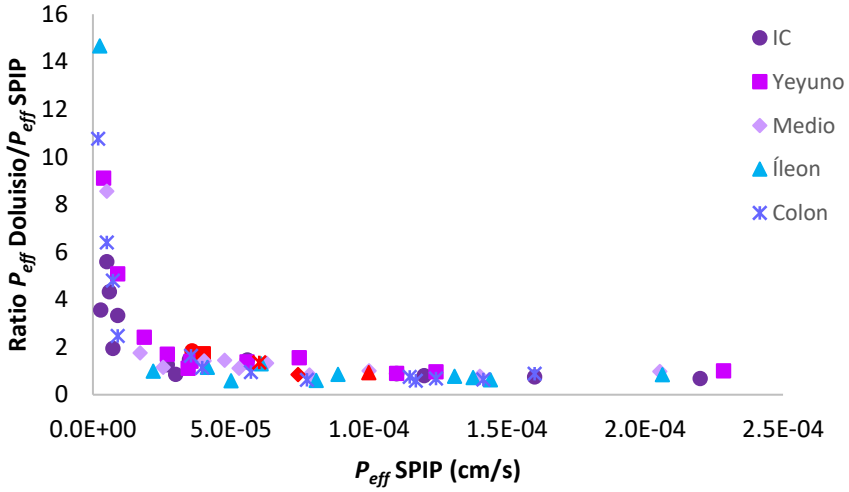

Figura 6.6. Relación entre los valores de permeabilidad obtenidos para los compuestos ensayados con ambos métodos en los distintos tramos intestinales frente al coeficiente de permeabilidad obtenido mediante el método SPIP. IC: intestino completo; Medio: tramo medio del intestino delgado. Los símbolos rojos corresponden al metoprolol.

A pesar de las diferencias de los resultados obtenidos con ambos métodos, las correlaciones establecidas indican que los resultados que proporciona el método de Doluisio son equiparables a los obtenidos con el

método SPIP y, por tanto, ambos pueden considerarse igual de útiles y válidos para determinar, utilizando como modelo animal la rata, la permeabilidad intestinal de los compuestos de interés. Además, los valores de permeabilidad intestinal determinados mediante cualquiera de las dos técnicas podrían utilizarse para realizar la clasificación de los compuestos de acuerdo con los criterios de clasificación establecidos en el Sistema de Clasificación Biofarmacéutica (BCS) [20, 21].

6.2. Predicción de la absorción en colon

Una vez demostrado que el método de perfusión *in situ* propuesto por Doluisio y col. proporciona parámetros de absorción en intestino delgado similares a los que proporciona en este mismo lugar del tracto gastrointestinal el método SPIP, se procedió a validar el método de perfusión de Doluisio para su uso en colon de rata y a estudiar su utilidad para predecir la absorción en colon de humanos. Para ello, se analizaron las correlaciones entre los parámetros de absorción obtenidos en intestino delgado y en colon de rata utilizando el método de perfusión de Doluisio, entre los últimos (parámetros de absorción en colon de rata) y los datos de absorción en colon obtenidos en humanos de acuerdo con la bibliografía publicada y, entre los parámetros obtenidos *in vitro* utilizando diferentes líneas celulares (Caco-2, co-cultivo de Caco-2/HT29-MTX y el triple co-cultivo de Caco-2/HT29-MTX/Raji B).

6.2.1. Correlaciones entre parámetros de absorción

Parámetros de absorción obtenidos *in situ* en intestino delgado y colon de rata

La Figura 6.7 muestra la correlación obtenida entre la permeabilidad determinada para los 16 compuestos estudiados mediante el método de perfusión *in situ* en intestino delgado y en colon de rata. La excelente bondad de ajuste de esta correlación (R^2 = 0,94) indica que los valores de permeabilidad obtenidos en intestino delgado de rata pueden ser útiles para predecir la permeabilidad en colon. Además, pone de manifiesto que la clasificación de compuestos de acuerdo con los criterios del BCS realizada a partir de los valores de permeabilidad obtenidos en intestino delgado, también podría establecerse a partir de los valores determinados en colon.

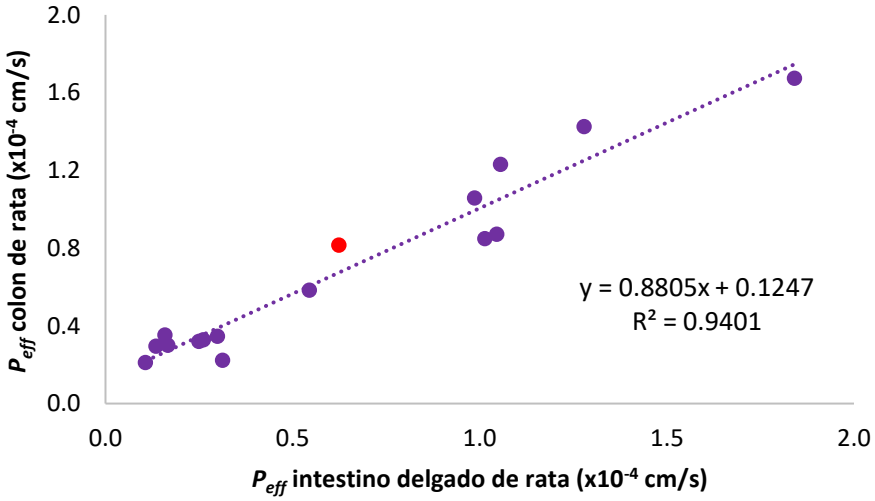

Figura 6.7. Correlación entre los valores de permeabilidad obtenidos en intestino delgado y en colon de rata mediante el método de Doluisio para los 16 compuestos ensayados. El símbolo rojo corresponde al metoprolol.

Es conveniente resaltar que existen numerosos trabajos publicados en los que la técnica de perfusión propuesta por Doluisio y colaboradores ha sido utilizada para medir la permeabilidad en colon. No obstante, los estudios realizados, un gran número de ellos liderados por Plá-Delfina [22-30], estaban encaminados a esclarecer los modelos biofísicos de absorción usando series homólogas de compuestos. Sin embargo, en estos trabajos no se usaron fármacos con distintas propiedades fisicoquímicas, ni se realizaron los estudios oportunos para validar la técnica de perfusión *in situ* utilizada en colon. Por ello, en esta Tesis Doctoral se han recopilado los resultados publicados por Plá-Delfina y colaboradores [22-30] y se han correlacionado de forma global con los obtenidos en esta Memoria. La correlación global obtenida (Figura 6.8) refuerza la validez de la técnica de perfusión basada en el método de Doluisio para determinar la permeabilidad en colon de rata, así como para clasificar a los compuestos a partir de los valores de permeabilidad determinados en colon de rata de acuerdo con los criterios establecidos por el BCS.

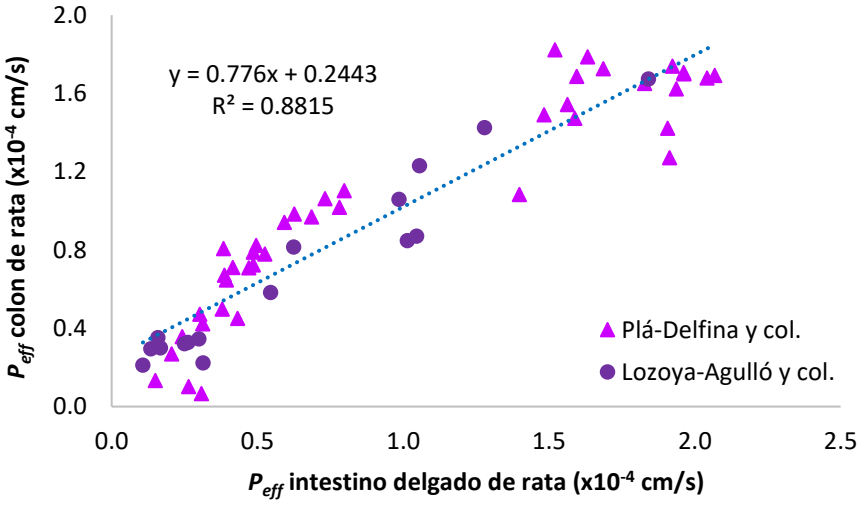

Figura 6.8. Correlación entre los valores de permeabilidad en intestino delgado y en colon de rata obtenidos mediante el método de Doluisio para 57 compuestos [22-30].

Parámetros de absorción obtenidos *in situ* en colon de rata y parámetros de absorción obtenidos *in vitro*

Tras demostrar una correcta correlación entre la permeabilidad en colon y en intestino delgado de rata para una gran cantidad de compuestos, los valores de permeabilidad obtenidos en colon mediante la técnica de perfusión *in situ* propuesta por Doluisio y col. se correlacionaron con los parámetros de permeabilidad determinados usando membranas artificiales y cultivos celulares. Este trabajo se realizó con el fin de establecer si las técnicas *in vitro*, más sencillas, menos costosas en términos económicos y exentas de problemas éticos, son útiles para predecir la absorción de compuestos en colon de rata.

La Figura 6.9 muestra la correlación entre la permeabilidad de 51 compuestos obtenida *in situ* mediante el método de Doluisio e *in vitro* usando membranas artificiales (PAMPA). En este caso, para establecer la correlación se ha utilizado la permeabilidad intrínseca (P_0). Este parámetro facilita la comparación de datos de permeabilidad obtenidos en diferentes laboratorios y/o utilizando distintos métodos experimentales para su estimación, ya que para su cálculo utiliza factores de ponderación que permiten corregir la variabilidad entre métodos y entre laboratorios [31]. En estas condiciones es de esperar que las correlaciones presenten una bondad de ajuste mayor en relación con la que se obtiene al correlacionar los valores de permeabilidad obtenidos por medida directa [32]. Sin embargo, en este trabajo la correlación obtenida utilizando la permeabilidad intrínseca se desvía de las previsiones iniciales (Figura 6.9, $R^2 = 0,28$), lo que parece indicar que las membranas artificiales no constituyen, en principio, un método de elección para predecir la permeabilidad de compuestos en colon de rata.

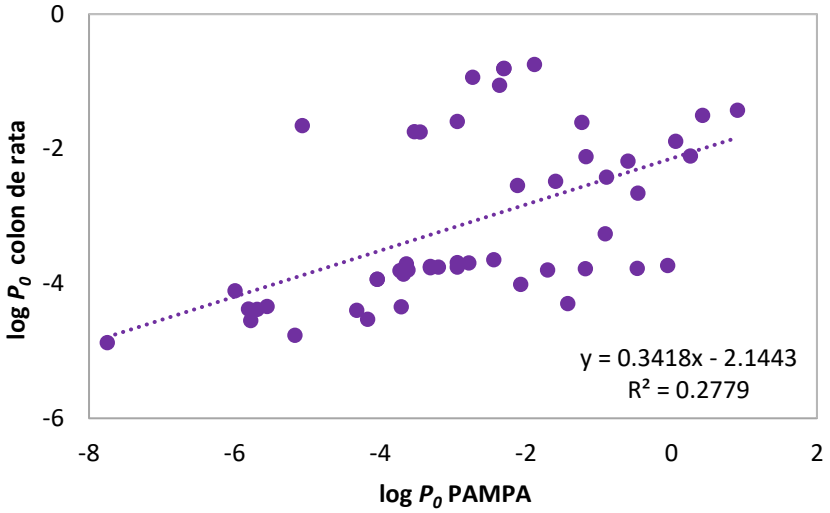

Figura 6.9. Correlación entre los valores de permeabilidad obtenidos en colon de rata mediante la técnica de perfusión *in situ* y los valores de permeabilidad determinados *in vitro* utilizando membranas artificiales.

En la Figura 6.10 se muestra la correlación entre los valores de permeabilidad obtenidos en colon mediante la técnica de perfusión *in situ* y los obtenidos *in vitro* utilizando monocapas celulares Caco-2. Este último, sistema ampliamente utilizado para abordar estudios de transporte transepitelial. Asimismo, la permeabilidad *in vitro* se ha determinado en dos condiciones diferentes: tras mantener las células en cultivo durante 21 o 4 días. Esta última condición fue descrita por Lentz y colaboradores [33] como un método más rápido y menos costoso económicamente que el tradicional cultivo de 21 días. Además, Lentz y colaboradores [33], demostraron que es un método válido para clasificar los compuestos según su permeabilidad por difusión pasiva, ya que los sistemas de 4 días de cultivo presentan una monocapa perfectamente formada en la que no se han expresado todos los transportadores responsables de los mecanismos involucrados en los procesos de transporte activo que acontecen en el intestino delgado. El hecho de presentar una menor expresión de transportadores intestinales convierte al modelo propuesto por Lentz y colaboradores en un buen sistema predictor de la permeabilidad en colon, ya que en este segmento del tracto

gastrointestinal la expresión de transportadores es menor que en intestino delgado.

A la vista de los resultados que se muestran en la Figura 6.10 queda patente que las correlaciones son similares. Por tanto, ambos sistemas *in vitro* basados en monocapas celulares Caco-2 se podrían utilizar para predecir la permeabilidad de compuestos en colon de rata. La mayor permeabilidad que se aprecia cuando se utiliza la línea celular de 4 días de cultivo puede atribuirse a que el espacio existente entre las células que forman la monocapa es mayor en este caso que trascurridos 21 días de cultivo, contribuyendo a obtener una mayor permeabilidad en las moléculas susceptibles de atravesar las membranas por transporte paracelular.

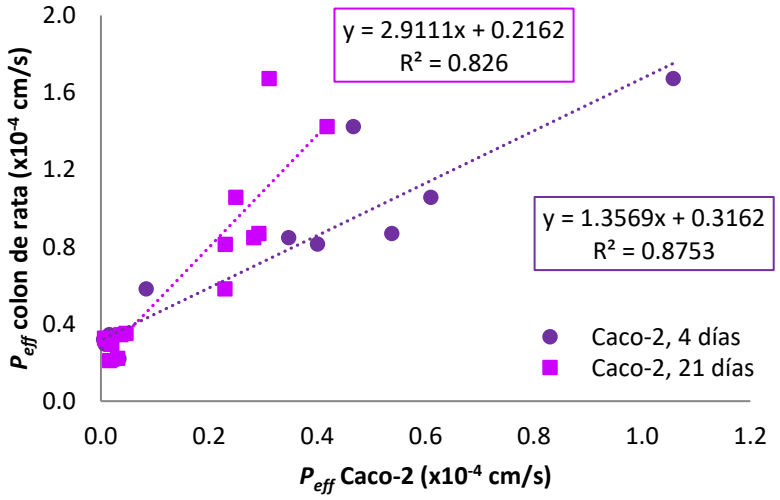

Figura 6.10. Correlación entre los valores de permeabilidad obtenidos *in situ* en colon de rata y los valores de permeabilidad determinados *in vitro* utilizando cultivos celulares Caco-2.

Asimismo, para finalizar el estudio sobre la idoneidad de los métodos *in vitro* para la predicción de la absorción en colon de rata, se ha analizado el alcance que puede tener en dicha predicción el empleo de sistemas *in vitro* basados en monocapas celulares de distinta complejidad. Para ello, se han

realizado ensayos de permeabilidad usando dos modelos que combinan varias líneas celulares: el co-cultivo de Caco-2/HT29-MTX y el triple co-cultivo de Caco-2/HT29-MTX/Raji B. En la Figura 6.11 se muestran las correlaciones que se han establecido a partir de los valores de permeabilidad de 12 compuestos determinados usando tres modelos *in vitro* (monocultivo de Caco-2, co-cultivo de Caco-2/HT29-MTX y el triple co-cultivo de Caco-2/HT29-MTX/Raji B) y la permeabilidad de estos mismos compuestos en colon de rata determinada con el método de perfusión *in situ* de Doluisio y colaboradores.

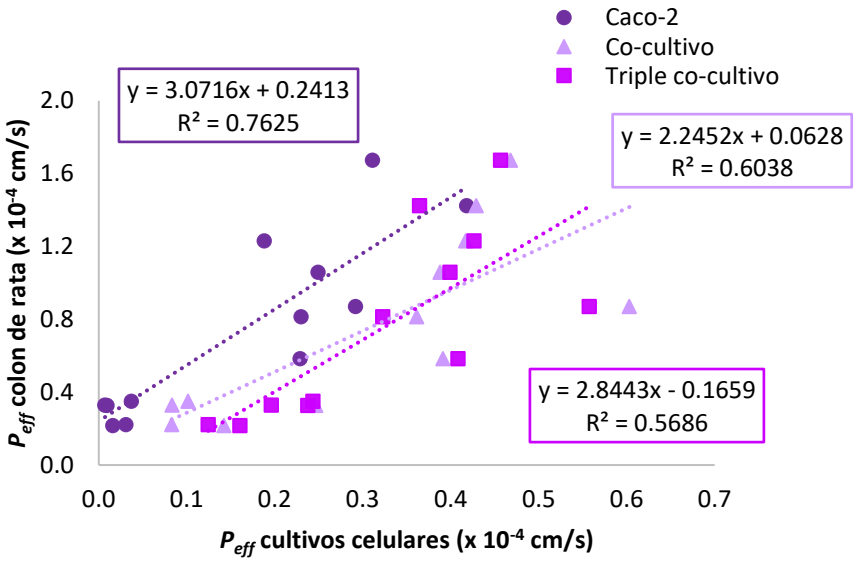

Figura 6.11. Correlaciones entre los valores de permeabilidad obtenidos en colon de rata mediante el método *in situ* de Doluisio y los valores de permeabilidad obtenidos *in vitro* con los tres modelos celulares indicados.

Los resultados que se observan en la Figura 6.11 indican que la bondad de ajuste de las correlaciones con los parámetros de absorción determinados con los modelos celulares más complejos es inferior a la que se obtiene con los parámetros obtenidos en monocultivos de Caco-2. Hay que tener en cuenta que tanto el co-cultivo de Caco-2/HT29-MTX como el triple co-cultivo

de Caco-2/HT29-MTX/Raji B han sido desarrollados para mimetizar las condiciones fisiológicas del intestino delgado de una forma más aproximada que los monocultivos de Caco-2 [34] por lo que es de esperar que las correlaciones obtenidas entre los datos de permeabilidad en estos modelos celulares y la permeabilidad en colon no sean tan prometedoras como las que se obtendrían al correlacionar los datos de permeabilidad de estos modelos más complejos con la permeabilidad determinada en intestino delgado. Por tanto, se puede concluir que, aunque los modelos *in vitro* con mayor complejidad son útiles para determinar los aspectos mecanísticos del proceso de absorción de moléculas con actividad terapéutica, no serían los más apropiados para predecir la permeabilidad en colon en rata o para realizar la clasificación de compuestos de acuerdo con los criterios BCS.

6.2.2. Predicción de absorción en humanos

Para validar la técnica de perfusión *in situ* propuesta por Doluisio y desarrollada en colon de rata de forma completa es necesario comprobar su utilidad para predecir la absorción en colon de humanos. Para conseguir este objetivo se necesitan datos de permeabilidad en colon y fracción absorbida, ambos obtenidos en humanos. Sin embargo, debido a la dificultad que conllevan los estudios de absorción en humanos en este tramo del tracto gastrointestinal, los resultados proporcionados por la bibliografía consultada son escasos [4]. No obstante, en los últimos años se han publicado datos de permeabilidad en colon de humanos obtenidos mediante la técnica de la cámara Ussing [35, 36]. Esta técnica originalmente estuvo desarrollada para determinar la permeabilidad *in vitro* utilizando diferentes tejidos de procedencia animal [37]. Sin embargo, recientemente, Rozehnal y colaboradores y Sjoberg y colaboradores [35, 36] han usado este método con tejido colónico humano, lo que ha permitido disponer de valores de permeabilidad en el tramo distal del tracto gastrointestinal de humanos de fármacos modelo.

Los datos publicados por Rozehnal y colaboradores y Sjoberg y colaboradores han sido utilizados en esta Tesis Doctoral para obtener la correlación entre dichos valores y los obtenidos en colon de rata con el método *in situ* de Doluisio, tal y como se muestra en la Figura 6.12. La bondad de ajuste obtenida indica que ambos datos de permeabilidad se correlacionan bien.

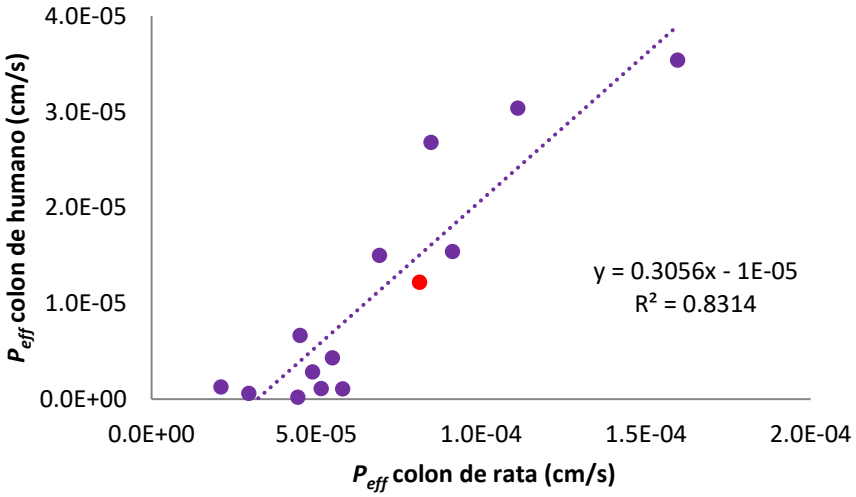

Figura 6.12. Correlación entre los valores de permeabilidad en colon de rata obtenidos con la técnica de perfusión *in situ* propuesta por Doluisio y los valores obtenidos en colon de humano usando la técnica *in vitro* de la cámara Ussing.

La Figura 6.13 muestra la correlación entre la permeabilidad obtenida *in vitro* en monocapas celulares (Caco-2), la obtenida en colon de rata y la obtenida en colon humano y la fracción de dosis absorbida en colon de humanos, estos últimos datos han sido extraídos del estudio de Tannergren y colaboradores [5].

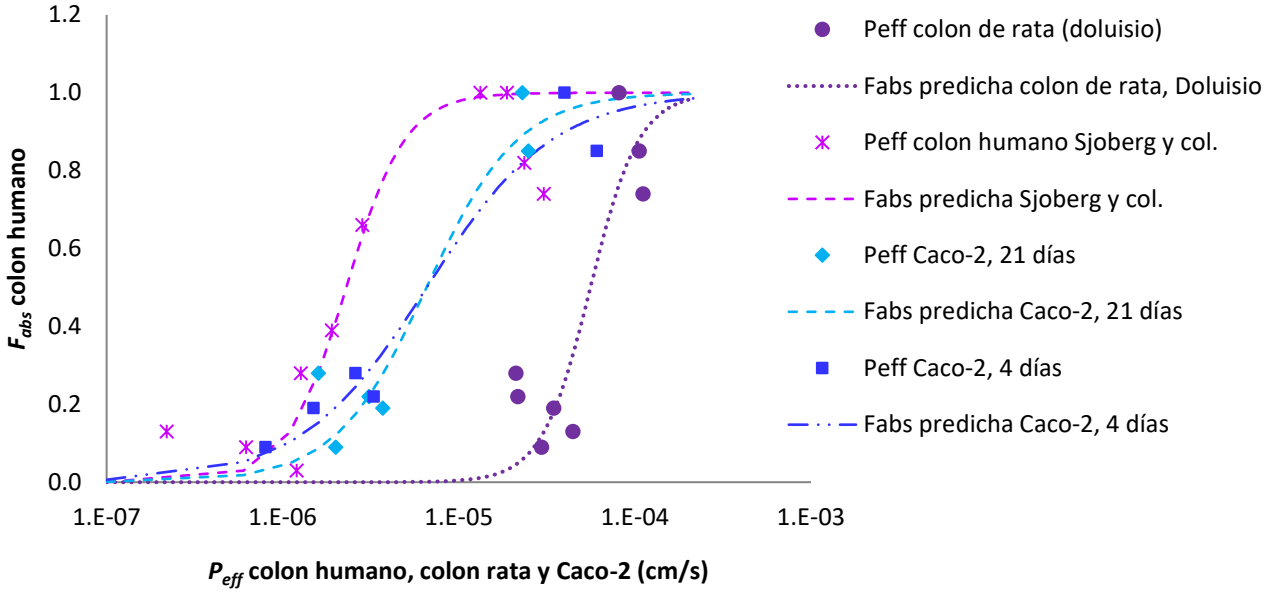

Figura 6.13. Correlaciones entre los valores de permeabilidad obtenidos con las técnicas indicadas (*in situ* en colon de rata, *in vitro* en cultivos celulares y en colon de humanos) y los valores de fracción absorbida en colon de humanos.

Llama la atención que la permeabilidad en colon humano sea la más baja, esto podría ser indicativo de que las uniones intercelulares en humanos en este tramo del tracto gastrointestinal son más estrechas que las uniones intercelulares existentes en el resto de modelos ensayados. Se puede apreciar que las curvas de la Figura 6.13 se podrían superponer si se corrige por el factor de superficie de absorción disponible en cada sistema. No obstante, la cantidad de datos en humanos sigue siendo escasa; por ejemplo, se necesitan valores de un mayor número de compuestos que se caractericen por presentar una fracción absorbida en colon en el ámbito intermedio; a pesar de ello, todos los modelos comparados (Figura 6.13) presentan una relación bastante similar, lo que indica que todos serían útiles para predecir la fracción absorbida en colon en humanos.

6.3. Estrategias para modificar la absorción en colon

El modelo de perfusión *in situ* propuesto por Doluisio utilizado en colon de rata ha demostrado ser útil para clasificar los compuestos de acuerdo con su permeabilidad en colon, lo que puede ser de gran utilidad en etapas tempranas del desarrollo de formas farmacéuticas de liberación controlada. Como se ha comentado anteriormente, el principio activo que se administre en una formulación de liberación controlada se absorberá principalmente en colon. Por tanto, una molécula de permeabilidad moderada o alta no tendrá problemas para atravesar la membrana permeable del colon por lo que si se administra en una forma farmacéutica de liberación controlada parece que su absorción completa está garantizada. Por ello, el objetivo en los compuestos de permeabilidad alta o moderada sería prolongar su liberación un tiempo suficientemente elevado para garantizar que el fármaco alcanza el colon y, de esta forma, conseguir un efecto terapéutico sostenido. Sin embargo, para aquellos compuestos que presenten baja permeabilidad en este tramo del tracto gastrointestinal, la absorción podría no ser completa. Por este motivo, sería interesante valorar

estrategias que permitieran aumentar su permeabilidad a través de las membranas absorbentes. Por todo esto, en esta Memoria se han seleccionado dos fármacos modelo, uno de baja permeabilidad y otro de alta permeabilidad, y se ha estudiado su permeabilidad tras la estrategia de modificación realizada.

Baja permeabilidad

El atenolol es un fármaco de baja permeabilidad, tanto en intestino delgado como en colon, modelo. La estrategia adoptada para aumentar su permeabilidad colónica fue la formación de un par iónico. El desarrollo de un par iónico consiste, a grandes rasgos, en la unión de un compuesto ionizado con una molécula de carga opuesta, conocida como contra ion, formando un complejo de carga neta neutra. Este complejo sin carga será más lipófilo y, debido a que la lipofilia es una de las características más importantes en el transporte de xenobióticos a través de membranas biológicas, facilitará el paso del principio activo a través de la membrana absorbente.

El atenolol es un fármaco hidrófilo de carácter básico que se encuentra ionizado en todo el rango de pH fisiológico del tracto gastrointestinal, propiedad que parece ser la responsable de su baja permeabilidad. Tras una conveniente búsqueda bibliográfica se seleccionaron dos colorantes ácidos (azul brillante y azul de bromofenol) como posibles candidatos a contra ion y se realizaron los ensayos pertinentes, descritos en el artículo científico 7 [38], para determinar el efecto de la formación del par iónico sobre los parámetros de permeabilidad del atenolol.

El incremento de permeabilidad del atenolol en colon cuando se administró en forma de par iónico fue notable, siendo mayor cuando el par iónico se formó con el azul brillante, como puede observarse en la Figura 6.14. Además, en la Tabla 6.1 se indican los valores exactos de permeabilidad obtenida en colon y el ratio calculado dividiendo la permeabilidad del atenolol determinada a partir de los pares iónicos y la determinada en

solución libre. Al observar los ratios puede apreciarse que la permeabilidad del principio activo estudiado aumenta más del doble cuando se administra formando un par iónico con el azul brillante.

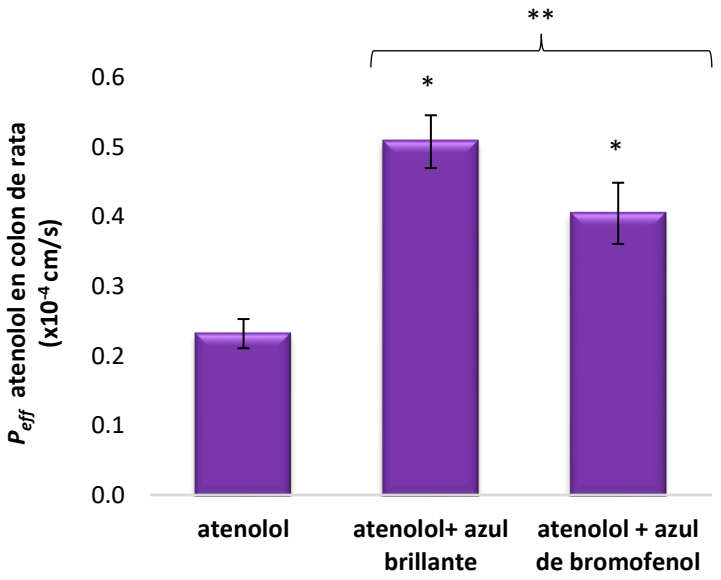

Figura 6.14. Permeabilidad del atenolol en colon de rata en ausencia y en presencia de los dos contra iones seleccionados. * diferencias estadísticamente significativas frente al atenolol sin contra ion (p<0.05). ** diferencias estadísticamente significativas entre atenolol + azul brillante y atenolol + azul de bromofenol (p<0.05).

La predicción de la permeabilidad del atenolol en intestino delgado tras la perfusión del fármaco con ambos contra iones se ha realizado usando la correlación que se muestra en la Figura 6.7, los resultados indican que si se formara un par iónico el aumento de permeabilidad del atenolol en intestino delgado sería similar al que se obtiene en colon. Estos resultados indican que el desarrollo de un par iónico también podría aplicarse a formulaciones de liberación inmediata, ya que tras la administración de estas formas de dosificación el fármaco se absorbe preferentemente en el intestino delgado. No obstante, esta afirmación merece una confirmación experimental, ya que los cambios de pH existentes a lo largo del intestino

delgado podrían afectar a la ionización de las moléculas administradas y con ello modificar la capacidad de formación del par-iónico.

Tabla 6.1. Permeabilidad en colon de rata del atenolol en ausencia y en presencia de los contra iones (media ± desviación estándar, n = 6-7). Permeabilidad del atenolol a partir de la perfusión del fármacos formando los pares iónicos estimada en intestino delgado usando la correlación obtenida en Lozoya-Agulló y colaboradores [39]. * Existencia de diferencias estadísticamente significativas frente al atenolol sin contra ion (p<0.05).

	P_{eff} colon ± SD (x10^{-4} cm/s)	**Ratio[a]**	**P_{eff} intestino delgado (x10^{-4} cm/s)**
Atenolol	0.232 ± 0.021	-	0.107 [b]
Atenolol + Azul brillante	0.508 ± 0.038*	2.189	0.435 [c]
Atenolol + Azul de bromofenol	0.405 ± 0.044*	1.746	0.318 [c]

$P_{eff:}$ Permeabilidad

[a] Ratio calculado entre permeabilidad del atenolol perfundido en par-iónico y en solución libre.

[b] Dato obtenido de Lozoya-Agulló y colaboradores [39].

[c] Valores estimados usando: P_{eff} colon = 0.8805· P_{eff} intestino delgado + 0.1247 [39].

El proceso llevado a cabo en este estudio en principio podría ser aplicable a cualquier molécula de baja permeabilidad que presente características similares al atenolol. Pero no hay que olvidar que son múltiples los factores que deben tenerse en cuenta para formar un par iónico, entre ellos el pk_a de la molécula, el pH del medio, los sustituyentes que posee el compuesto de interés y su estructura tridimensional. Además, es importante prestar atención a las características de la molécula elegida para la complejación, entre ellas su pk_a, la capacidad para reaccionar con el compuesto objetivo y la lipofilia que pueden aportar sus sustituyentes al complejo formado [40].

Alta permeabilidad

En este caso fue el ibuprofeno el fármaco seleccionado como representante de alta permeabilidad en colon. Como se ha comentado anteriormente, el objetivo para este tipo de compuestos es conseguir una liberación controlada y de este modo tener la capacidad de modular la rápida absorción que presentan estas moléculas. Por ello, se desarrollaron nanopartículas de ibuprofeno usando PLGA (ácido poli(láctico co-glicólico)) por ser un polímero biodegradable y biocompatible [41]. De esta manera se puede conseguir una liberación lenta y prolongada del principio activo a lo largo del tiempo, de tal forma que la mayor parte del fármaco alcance el colon.

Como puede observarse en la Figura 6.15, en la que se muestra la permeabilidad del ibuprofeno en distintos tramos intestinales, este fármaco es un compuesto de alta permeabilidad a lo largo del tracto intestinal y, por tanto, para asegurar una buena absorción cuando se administre en forma de liberación controlada no requiere ninguna modificación química, ya que se absorberá de manera rápida conforme se vaya liberando de la forma farmacéutica que lo contiene.

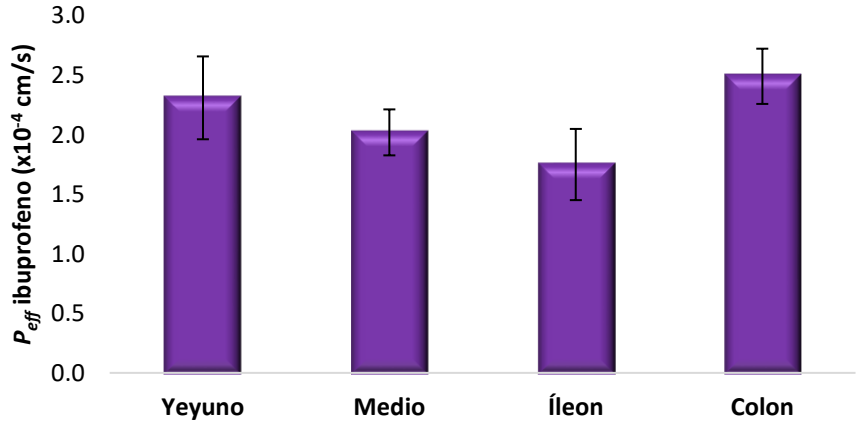

Figura 6.15. Permeabilidad del ibuprofeno en los distintos tramos intestinales ensayados: yeyuno, tramo medio del intestino delgado, íleon y colon.

La Figura 6.16 muestra los perfiles de liberación del ibuprofeno durante 48 horas a partir de las nanopartículas desarrolladas. El ensayo de liberación se llevó a cabo a diferentes pHs para simular los distintos tramos del tracto gastrointestinal. Además, este experimento también se realizó modificando el pH a lo largo de las 48 horas para simular el paso de las nanopartículas a través del tracto gastrointestinal humano, de forma que en el tiempo preciso se ajustó al pH adecuado con el fin de representar las condiciones de cada segmento gastrointestinal. Los resultados del ensayo de liberación (Figura 6.16) muestran una liberación del principio activo mínima a los pHs más ácidos (pH 1.2 y 4.5). Esto implica que a partir de las nanopartículas el ibuprofeno apenas se liberará en los tramos superiores del tracto gastrointestinal, lo cual permitirá que el fármaco alcance el colon consiguiendo así mantener su absorción durante el tiempo de permanencia del fármaco en todo el tracto gastrointestinal.

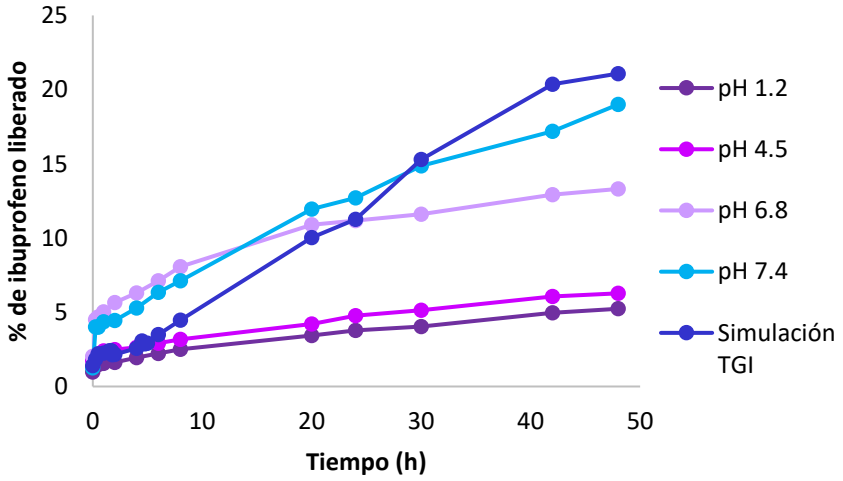

Figura 6.16. Perfiles de liberación del ibuprofeno a partir de las nanopartículas elaboradas a los distintos pHs ensayados. TGI: tracto gastrointestinal.

Las nanopartículas de PLGA desarrolladas han permitido disminuir significativamente la permeabilidad del ibuprofeno en los tres modelos *in vitro,* basados en cultivos celulares, ensayados: monocultivo de Caco-2, co-cultivo de Caco-2/HT29-MTX y triple co-cultivo de Caco-2/HT29-MTX/Raji B. En la Figura 6.17 se muestran los valores de permeabilidad para el ibuprofeno en solución y en nanopartículas determinados en los tres modelos *in vitro*. Las diferencias que se aprecian entre la permeabilidad del ibuprofeno encapsulado y del ibuprofeno en solución, junto con los resultados del ensayo de liberación (Figura 6.16), indican que las nanopartículas liberan el fármaco lentamente dando lugar a una liberación sostenida en el tiempo. Por tanto, la velocidad de liberación del fármaco a partir de las nanopartículas será el factor limitante del proceso de absorción, provocando una absorción prolongada y localizada principalmente en el colon.

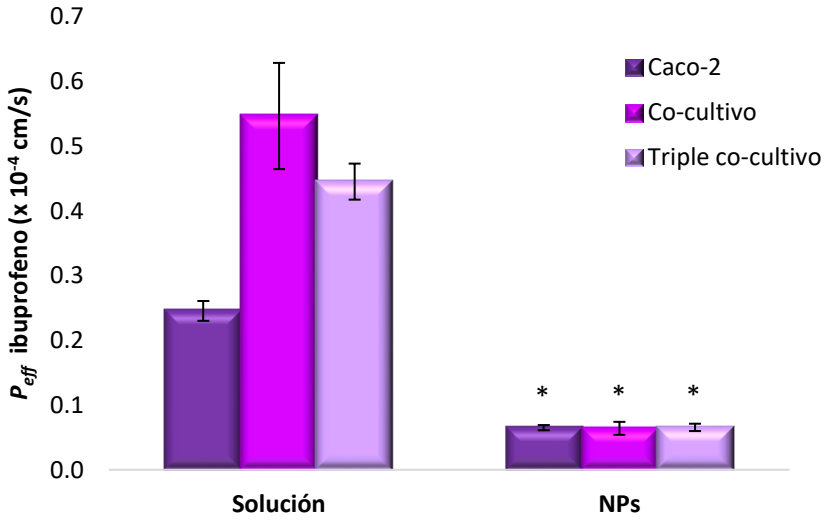

Figura 6.17. Valores de permeabilidad del ibuprofeno obtenidos en los tres modelos *in vitro* tras su administración en solución libre y en nanopartículas. * diferencias estadísticamente significativas frente al ibuprofeno administrado en solución en la misma línea celular (p<0.05).

Este planteamiento podría aplicarse a otras moléculas de similares características de permeabilidad que el ibuprofeno. Ya sea usando nanopartículas o cualquier otra forma farmacéutica de liberación controlada, acorde a las características propias de cada fármaco, lo importante es conseguir una liberación prolongada del principio activo en el tiempo para mantener concentraciones plasmáticas estables durante un largo periodo de tiempo y, de este modo, poder reducir el número de dosis diarias así como los efectos adversos que pueda sufrir el paciente derivados de la fluctuación elevada de las concentraciones plasmáticas en estado estacionario que se obtienen tras la administración de los fármacos utilizando formas farmacéuticas convencionales de liberación rápida del principio activo.

Los resultados alcanzados en la presente Tesis Doctoral han supuesto un avance de gran relevancia científica. Con el trabajo realizado se ha validado la técnica de perfusión *in situ* en colon de rata; además, se ha demostrado que los datos de permeabilidad obtenidos en colon de rata son útiles para predecir la absorción colónica en humanos. También, se ha probado que existe una buena correlación entre la permeabilidad en colon y la permeabilidad en monocapas celulares Caco-2, por lo que este modelo *in vitro*, más sencillo, también puede utilizarse para estimar la fracción oral absorbida en colon humano. La importancia de disponer de un modelo validado es indiscutible, ya que a partir de ahora se podrá predecir con seguridad la permeabilidad de un compuesto en cualquier tramo intestinal partiendo de un modelo *in vitro* y, por tanto, minimizando el uso de animales.

Asimismo, se ha profundizado en las estrategias de modificación del paso de sustancias a través de membranas biológicas. Los resultados obtenidos pueden servir de pautas generales en las etapas tempranas de la formulación de formas farmacéuticas de liberación controlada.

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7. CONCLUSIONES

1. La técnica de perfusión *in situ* sin recirculación en rata Wistar se ha validado en todos los tramos intestinales (intestino delgado completo, yeyuno, tramo medio e íleon, y colon). La validación se ha basado en la correlación entre los valores de permeabilidad de más de 10 fármacos modelo obtenidos con el método objeto de estudio y el método de paso simple. El coeficiente de determinación, utilizado como parámetro de bondad de ajuste, de todas las correlaciones realizadas ha sido muy satisfactorio, superior a 0,80.

2. A juzgar por los parámetros de bondad de ajuste de las correlaciones establecidas en este trabajo se puede afirmar que la permeabilidad de fármacos en colon de rata puede predecirse de forma satisfactoria a partir de la determinación de los valores de permeabilidad en intestino delgado de la misma especie animal o en monocultivos celulares Caco-2.

3. Se han obtenido excelentes correlaciones entre las permeabilidades *in situ* y las permeabilidades *in vitro* de monocultivos de células Caco-2 y modelos celulares de mayor complejidad (co-cultivo Caco-2/HT29-MTX y triple co-cultivo Caco-2/HT29-MTX/Raji B). En consecuencia, el modelo Caco-2 resulta de elección para cribado o clasificación BCS mientras que los co-cultivos serían de elección para estudios mecanísticos por sus características más próximas a la realidad fisiológica humana.

4. Las membranas artificiales (PAMPA) han demostrado ser útiles para predecir la permeabilidad de compuestos en intestino delgado. Las correlaciones entre permeabilidad en PAMPA y colon de rata son de menor utilidad debido a la mayor porosidad del modelo en colon. El modelo biofísico establecido ha permitido comparar permeabilidades de diferentes laboratorios, técnicas y segmentos en una escala común. Estas comparaciones permiten identificar las características de cada modelo como las resistencias difusionales limitantes o la porosidad del sistema.

5. El método de perfusión *in situ* sin recirculación desarrollado en colon de rata es útil para predecir la absorción colónica en humanos, ya que la permeabilidad obtenida en colon de rata con el uso de esta técnica correlaciona bien con los datos de absorción obtenidos en colon de humanos (permeabilidad y fracción oral absorbida en colon).

6. La formación de un par iónico con el atenolol produce un incremento de la permeabilidad en colon de este fármaco de un 74% al usar azul de bromofenol como contra ion y de un 119% cuando el contra ion usado es azul brillante.

7. Las nanopartículas poliméricas sintetizadas con ibuprofeno han permitido obtener un sistema de liberación controlada que podría ser útil para facilitar la absorción de este fármaco en colon, ya que la liberación del principio activo ocurre principalmente cuando el pH del medio es superior a 6,8.

7. CONCLUSIONS

1. The *in situ* closed-loop perfusion technique in Wistar rat has been validated in different intestinal sections (whole small intestine, jejunum, middle small intestine, ileum, and colon). Validation was based in the correlation of closed-loop permeability values of a set of model drugs with single-pass ones. All the correlations showed coefficients of determination higher than 0.80.

2. Drug permeability values in rat colon can be predicted satisfactorily from the rat small intestine permeability values or Caco-2 monocultures permeability data based on the correlation models established in this work and their excellent goodness of fit indexes.

3. Excellent correlations have been found between *in situ* permeability values and *in vitro* permeability values from Caco-2 monocultures and more complex co-cultures (co-culture Caco-2/HT29-MTX and triple co-culture Caco-2/HT29-MTX/Raji B). Consequently Caco-2 model would be the selected model for drug screening and BCS classification while the co-cultures could be used for mechanistic studies thanks to their closer characteristics to human intestinal physiology.

4. The parallel artificial membrane assay (PAMPA) has shown to be a useful method to predict the drug permeability in small intestine. Correlation between PAMPA and colon permeabilities is less useful due to the higher leakiness of the colon model. The biophysical model applied has allowed the comparison of experimental permeability values from different methods/laboratories and intestinal segments. These comparisons help to identify the different characteristics of each experimental systems as the limiting diffusional resistances or the system leakiness.

5. The good correlation between the rat colon permeability values and human colon absorption data (permeability and oral fraction absorbed in colon) demonstrates that the *in situ* closed-loop perfusion technique in rat colon is useful to predict the human colonic absorption.

6. The ion-pair development with atenolol produces a 74% increase of the atenolol permeability in colon with bromophenol blue as counter ion, and a 119% increase of the atenolol permeability in colon with brilliant blue as counter ion.

7. The ibuprofen polymeric nanoparticles synthesized have allowed to obtain a controlled release system which may be useful to provide a sustained release of ibuprofen in colon, because the drug release happens mainly at pH higher than 6.8.

8. ANEXOS

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In-situ intestinal rat perfusions for human F_{abs} prediction and BCS permeability class determination: Investigation of the single-pass vs. the Doluisio experimental approaches

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ABSTRACT

Intestinal drug permeability has been recognized as a critical determinant of the fraction dose absorbed, with direct influence on bioavailability, bioequivalence and biowaiver. The purpose of this research was to compare intestinal permeability values obtained by two different intestinal rat perfusion methods: the single-pass intestinal perfusion (SPIP) model and the Doluisio (closed-loop) rat perfusion method. A list of 15 model drugs with different permeability characteristics (low, moderate, and high, as well as passively and actively absorbed) was constructed. We assessed the rat intestinal permeability of these 15 model drugs in both SPIP and the Doluisio methods, and evaluated the correlation between them. We then evaluated the ability of each of these methods to predict the fraction dose absorbed (F_{abs}) in humans, and to assign the correct BCS permeability class membership. Excellent correlation was obtained between the two experimental methods ($r^2 = 0.93$). An excellent correlation was also shown between literature F_{abs} values and the predictions made by both rat perfusion techniques. Similar BCS permeability class membership was designated by literature data and by both SPIP and Doluisio methods for all compounds. In conclusion, the SPIP model and the Doluisio (closed-loop) rat perfusion method are both equally useful for obtaining intestinal permeability values that can be used for F_{abs} prediction and BCS classification.

1. Introduction

Amidon et al. (1995) revealed that the two fundamental parameters controlling the rate and extent of drug absorption following oral administration are the permeability of the drug through the gastrointestinal (GI) membrane and the solubility/dissolution of the drug dose in the GI milieu, and developed the Biopharmaceutics Classification System (BCS). Based on this work, the FDA initiated the replacement of *in-vivo* bioequivalence (BE) studies with *in-vitro* dissolution tests for immediate-release (IR) solid oral dosage forms when formulated as rapidly dissolved drug product (CDER/FDA, 2000). To date, only BCS class I, high-solubility high-permeability drugs, may be eligible as candidates for this biowaiver according to the American FDA. Under the EMA jurisdiction, class III drugs may also be eligible for biowaivers under certain conditions (Morais and Lobato, 2010).

While the solubility parameter is fairly straightforward when assigning a BCS classification, intestinal permeability is not routinely measured, and difficulties to prove high-permeability classification may limit the broad regulatory application of the BCS-based biowaiver concept (Amidon et al., 2011; Dahan et al., 2010, 2012; Fairstein et al., 2013; Zur et al., 2014b). The permeability class of a drug can be determined in human subjects using mass balance, systemic bioavailability, or intestinal perfusion approaches. Recommended methods not involving human

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subjects include *in-vivo* or *in-situ* intestinal perfusion in a suitable animal model, and/or *in-vitro* permeability methods using excised intestinal tissues, or monolayers of suitable epithelial cells (CDER/FDA, 2000).

While human data is more convincing than pre-clinical or *in-vitro* experiments, the difficulty in obtaining human results is tremendous and cannot be ignored (Amidon et al., 2011; Knutson et al., 2009; Lennernäs et al., 1992). In fact, in many cases it is probably less difficult to run the traditional human bioequivalence study than to obtain human intestinal permeability data, so the whole purpose of the biowaiver concept is missed. Among all non-human FDA approved methods for BCS permeability classification, intestinal perfusion studies in rats emerge as the most reliable and cost effective option. A high correlation between human and rat small intestine permeability (r^2 = 0.8–0.95) was reported for drug intestinal permeability with both carrier-mediated absorption and passive diffusion mechanisms (Dahan and Amidon, 2009b; Dahan et al., 2009a; Lennernäs, 2007a,b). Perfusion studies in the rat intestine appear to correlate best with human data, even though the type of transporters and their expression levels may vary between species (Cao et al., 2006; Fagerholm et al., 1996; Kim et al., 2006). Rat perfusion studies even allow to assess segmental-dependent permeability throughout the entire small intestine, a factor that to date cannot be measured in humans (Dahan and Amidon, 2009b; Dahan et al., 2009d, 2010; Fairstein et al., 2013; Lennernäs, 1998, 2014a,b).

The purpose of this research was to compare two different intestinal rat perfusion methods: the single-pass intestinal perfusion (SPIP) model and the Doluisio rat perfusion method. While both of these experimental methods measure disappearance from the intestinal lumen as a measure for drug absorption, they have substantial differences between them; the SPIP model focuses on a 10 cm intestinal segment (typically the jejunum) (Amidon et al., 1988; Lennernäs et al., 1995), whereas the Doluisio method typically measures the absorption throughout the entire small intestine (Doluisio et al., 1969a,b), although with appropriate adjustments segmental-dependent permeability can be measured by both techniques (Dahan and Amidon, 2009b; Dahan et al., 2009d; Fairstein et al., 2013; Fernandez-Teruel et al., 2005; González-Alvarez et al., 2007; Merino et al., 1989; Zur et al., 2014a). Moreover, in the SPIP model each drug molecule gets only one passage through the investigated intestinal segment, while in the Doluisio method the tested drug solution remains within the intestinal lumen throughout the entire experiment. We constructed a list of 15 model drugs with different permeability characteristics: low, moderate, and high permeability, as well as passively and actively absorbed compounds. We assessed the rat intestinal permeability of these 15 model drugs in both SPIP and the Doluisio experimental methods, and evaluated the correlation between them. We then evaluated the ability of each of these methods to predict the fraction dose absorbed (F_{abs}) in humans, and to assign the correct BCS permeability class membership. Overall, this research can aid and guide the correct assignment of BCS permeability classification using FDA approved animal models.

2. Materials and methods

2.1. Materials

Antipyrine, atenolol, caffeine, carbamazepine, chloramphenicol, cimetidine, colchicine, codeine, ibuprofen, digoxin, furosemide, labetalol, metoprolol, paracetamol and valsartan were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, methanol and water (Merck KGaA, Darmstadt, Germany) were UPLC grade. All other chemicals were of analytical reagent grade.

2.2. The model drugs

A list of 15 model drugs was constructed: antipyrine, atenolol, caffeine, carbamazepine, chloramphenicol, cimetidine, colchicine, codeine, ibuprofen, digoxin, furosemide, labetalol, metoprolol, paracetamol and valsartan. These model drugs were chosen while making sure to include drugs with different permeability characteristics: low, moderate, and high permeability, as well as passively and actively absorbed compounds, as will be discussed hereinafter.

2.3. Single-pass rat intestinal perfusion

The effective permeability coefficient (P_{eff}) of the 15 model drugs using the single-pass rat intestinal perfusion model was conducted using protocols approved by the Ben-Gurion University of the Negev Animal Use and Care Committee (Protocol IL-60-11-2010). Male Wistar rats (250–300 g, Harlan, Israel) were housed and handled according to the Ben-Gurion University of the Negev Unit for Laboratory Animal Medicine Guidelines.

Rats were anesthetized with an intra-muscular injection of 1 mL/kg of ketamine–xylazine solution (9%:1%, respectively), placed on a heated surface maintained at 37 °C (Harvard Apparatus Inc., Holliston, MA), and a 3 cm midline abdominal incision was made. A proximal 10 cm jejunal segment, starting 2 cm below the ligament of Treitz, was cannulated on two ends, and was rinsed with blank perfusion buffer. All solutions were incubated in a 37 °C water bath (Beig et al., 2012; Dahan and Amidon, 2010; Miller et al., 2009).

At the starting point of each experiment, perfusion solution containing the investigated drug, 10 mM MES buffer, pH 6.5, 135 mM NaCl, 5 mM KCl, and 0.01 mg/mL phenol red, with an osmolarity of 290 mOsm/L, was perfused through the intestinal segment (Watson Marlow 205S, Watson-Marlow Bredel Inc., Wilmington, MA), at a flow rate of 0.2 mL/min. The perfusion buffer was perfused for 1 h without sampling, to ensure steady state conditions, followed by additional 1 h of perfusion with samples taken every 10 min. The pH of the collected samples was measured at the outlet, to verify that there was no pH change throughout the perfusion (pH 6.5). The samples were immediately assayed by UPLC. The length of the perfused intestinal segment was measured at the endpoint of the experiment.

The effective permeability (P_{eff}; cm/sec) through the rat gut wall was determined according to the following equation:

$$P_{eff} = \frac{-Q\ln(C'_{out}/C'_{in})}{2\pi RL}$$

where Q is the perfusion buffer flow rate (0.2 mL/min), C'_{out}/C'_{in} is the ratio of the outlet and the inlet concentration of drug that has been adjusted for water transport via the non-absorbable marker phenol red (Dahan and Amidon, 2009a; Dahan and Miller, 2012; Dahan et al., 2009c; Tuğcu-Demiröz et al., 2014), R is the radius of the intestinal segment (set to 0.2 cm), and L is the length of the perfused intestinal segment.

2.4. Doluisio's (closed-loop) rat intestinal perfusion

The studies were approved by the Scientific Committee of the Faculty of Pharmacy, Miguel Hernandez University, and followed the guidelines described in the EC Directive 86/609, the Council of the Europe Convention ETS 123 and Spanish national laws governing the use of animals in research.

Fasted male Wistar rats weighing ~250 g with free access to water were used in these studies. Rats were anesthetized using a mixture of diazepam (1.67 mg/kg, Valium, Roche), ketamine (50 mg/kg, Ketolar, Parke-Davis), and atropine (1 mg/kg, Atropina

sulfato, Braun), and placed on heated surface maintained at 37 °C. A midline abdominal incision was made, and the small intestine was exposed. The bile duct was ligated in order to avoid drug enterohepatic circulation and the presence of bile salts in lumen. The method consists of creating a small intestinal compartment with the aid of two syringes and two three-way stopcock valves. Two incisions were made in the intestine, the first at the beginning of the duodenal segment, and the second at the end of the ileum segment, just before the cecum, and the entire small intestine was cannulated through these incisions (Casabó et al., 1987; Ferrando et al., 1999). Care was taken to avoid disturbance of the intestinal blood supply. In order to remove all intestinal contents, the small intestine was thoroughly flushed with a warm physiologic solution. The catheters were then connected to a glass syringe using a stopcock three-way valve. The intestine was carefully placed back into the peritoneal cavity, and the abdomen was covered with cotton wool pads to prevent peritoneal liquid evaporation and heat loss. This set up ensures the isolation of the small intestine, and drug solution can be introduced and sampled with the aid of the syringes and stopcock valves. To take a sample, the luminal content is pushed out from one syringe to the other. This procedure is done alternatively from the proximal syringe to the distal one, assuring the mixing of the solution in the intestinal lumen. The samples were collected every 5 min up to a period of 30 min (Bermejo et al., 1999; Ruiz-Garcı'a et al., 1999).

Water flux throughout the experiment may be significant, and hence must be accounted for (Tuğcu-Demiröz et al., 2014). A method based on direct measurement of the remaining solution volume was employed to calculate the water reabsorption zero order constant (k_0). For each tested compound, the initial volume (V_0) was determined on groups of three animals, while the the endpoint volume (V_t) was measured on every animal used. The drug concentration in the samples was corrected as: $C_t = C_e(V_t/V_0)$, where C_t represents the concentration in the absence of water reabsorption at time t, and C_e the experimental value. The corrected concentration, C_t, was then used as the actual absorption rate coefficient calculations (Martín-Villodre et al., 1986).

The absorption rate coefficients (k_a) of the compounds were determined by nonlinear regression analysis of the remaining concentrations in the intestinal lumen (C_t) vs. time:

$$C_t = C_0 \times e^{-k_a t}$$

These absorption rate coefficients were then transformed into permeability values using the relationship: $P_{app} = k_a \times R/2$, where R is the effective radius of the intestinal segment, calculated from the area/volume relationship considering a 10 mL volume and an intestinal length of 100 cm.

2.5. Analytical methods

Analytical analyses of the SPIP and the Doluisio samples were performed on a Waters (Milford, MA) Acquity UPLC H-Class system, and a Waters 2695HPLC Separation Module, respectively. Both systems were equipped with photodiode array detector and Empower software. The simultaneous determination of the investigated drug and the non-absorbable marker phenol red in the SPIP samples was achieved using a Waters (Milford, MA) Acquity UPLC BEH C_{18} 1.7 μm 2.1 × 100 mm column. The determination of the investigated drug in the Doluisio samples was carried out using a Waters C_{18} 3.5 μm 4.6 × 250 mm XTerra column. A general gradient mobile phase consisted of 90:10 going to 20:80 (v/v) water:acetonitrile (both with 0.1% TFA) over 7 min was suitable for all analyses. Injection volumes ranged from 2 to 50 μL.

2.6. Statistical analysis

All animal experiments were replicated with $n = 4–6$. Values are expressed as mean ± standard deviation (SD). To determine statistically significant differences among the experimental groups, the nonparametric Kruskal–Wallis test was used for multiple comparisons, and the two-tailed nonparametric Mann–Whitney U-test for two-group comparison where appropriate. A p value of less than 0.05 was termed significant.

3. Results

The permeability values (cm/sec) obtained for the different drugs in both the SPIP and the Doluisio experimental methods are shown in Fig. 1, and summarized in Table 1. The correlation between the two sets of data, the SPIP vs. the Doluisio P_{eff} values, is shown in Fig. 2. It can be seen that excellent correlation was obtained between the two experimental methods, as evident by a coefficient of determination (r^2) of 0.93.

The correlation between the permeability values obtained in this study and literature oral fraction dose absorbed, F_{abs}, in humans, of the different drugs, was evaluated using the relationship:

$$F_{abs} = 1 - e^{-P_{eff}\frac{2}{R}T}$$

where R represents the intestinal radius and T the effective absorption time (Salphati et al., 2001; Sánchez-Castaño et al., 2000). Figs. 3–5 illustrate the correlations between F_{abs} and SPIP P_{eff}, Doluisio P_{eff}, and all P_{eff} data, respectively. It can be seen that in all cases a good fit was obtained between the predicted and the literature F_{abs} values, indicating the high capabilities of both rat perfusion models to predict human fraction dose absorbed.

Table 2 summarizes the literature human F_{abs} data, and the corresponding SPIP and Doluisio predicted F_{abs} and BCS permeability class. With the exception of one drug, digoxin, for which definite permeability classification could not be found, similar BCS permeability class membership was designated by literature data and by both SPIP and Doluisio methods. The predicted F_{abs} obtained by both SPIP and Doluisio methods were also in very good agreement to literature human F_{abs} values, and for both the SPIP and the Doluisio methods, all experimental values were within the 95% confidence band of the predicted fit.

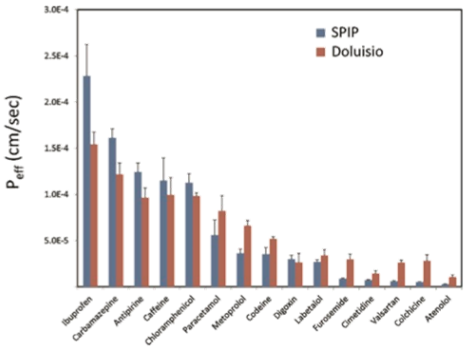

Fig. 1. The permeability values (cm/sec) obtained for the different drugs in both the SPIP and the Doluisio experimental methods.

Table 1
The concentrations (μM) and permeability values (cm/sec) of the different drugs in both experimental methods. Data presented as means (SD).

The drug	Single-pass rat jejunal perfusion		Doluisio's rat perfusion	
	Conc. (μM)	P_{eff} (cm/sec)	Conc. (μM)	P_{eff} (cm/sec)
Ibuprofen	250	2.2×10^{-4} (3.4×10^{-5})	500	1.5×10^{-4} (1.3×10^{-5})
Carbamazepine	500	1.6×10^{-4} (9.7×10^{-6})	500	1.2×10^{-4} (1.2×10^{-5})
Antipirine	200	1.2×10^{-4} (2.2×10^{-5})	500	9.7×10^{-5} (1.0×10^{-5})
Caffeine	500	1.1×10^{-4} (2.4×10^{-5})	500	9.9×10^{-5} (1.8×10^{-5})
Chloramphenicol	1000	1.1×10^{-4} (9.6×10^{-6})	500	9.8×10^{-5} (3.4×10^{-6})
Paracetamol	330	5.6×10^{-5} (1.7×10^{-5})	500	8.2×10^{-5} (1.6×10^{-5})
Metoprolol	500	3.6×10^{-5} (4.8×10^{-6})	500	6.6×10^{-5} (5.6×10^{-6})
Codeine	750	3.5×10^{-5} (7.1×10^{-6})	750	5.2×10^{-5} (2.3×10^{-6})
Digoxin	150	3.0×10^{-5} (4.2×10^{-6})	500	2.6×10^{-5} (9.6×10^{-6})
Labetalol	500	2.7×10^{-5} (2.3×10^{-6})	500	3.4×10^{-5} (6.1×10^{-6})
Furosemide	500	9.0×10^{-6} (5.0×10^{-7})	500	3.0×10^{-5} (5.7×10^{-6})
Cimetidine	500	7.2×10^{-6} (4.7×10^{-7})	500	1.4×10^{-5} (3.0×10^{-6})
Valsartan	250	6.0×10^{-6} (6.7×10^{-7})	500	2.6×10^{-5} (2.7×10^{-6})
Colchicine	15	5.0×10^{-6} (4.0×10^{-7})	500	2.8×10^{-5} (6.5×10^{-6})
Atenolol	200	2.8×10^{-6} (3.6×10^{-7})	500	1.0×10^{-5} (2.3×10^{-6})

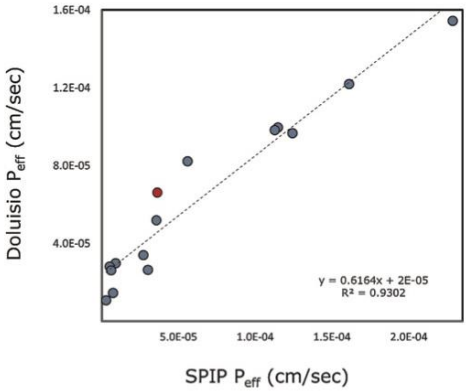

Fig. 2. The correlation between the two sets of data, the SPIP vs. the Doluisio P_{eff} values. Metoprolol, the traditional low/high permeability class boundary marker, is highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Rat intestinal perfusions represent an efficient and reliable experimental tool in human F_{abs} predictions, BCS classification, regional-dependent absorption, permeability mechanisms, and absorption related drug–drug interactions. The purpose of this study was to compare two different intestinal rat perfusion methods: the single-pass intestinal perfusion (SPIP) model and the Doluisio (closed-loop) rat perfusion method. A list of 15 model drugs was constructed to allow the comparison between the two experimental methods.

A coefficient of determination (r^2) of 0.93 was obtained when correlating the P_{eff} data from both experimental methods (Fig. 2). This excellent correlation was achieved in spite of very different experimental settings. First and foremost, with the SPIP model, only a 10-cm long jejunal segment was perfused and used to determine the drugs' permeability. On the other hand, the entire small intestine was used to generate the P_{eff} values in the Doluisio method. Since permeability is position dependent and pertains to a specific point along the intestinal membrane (Dahan and Amidon, 2009b; Dahan et al., 2012; Fairstein et al., 2013; Lennernäs, 2014a, b), this difference could potentially lead to very different results between the two models. However, the SPIP and the Doluisio methods actually agreed with each other very well, and were able

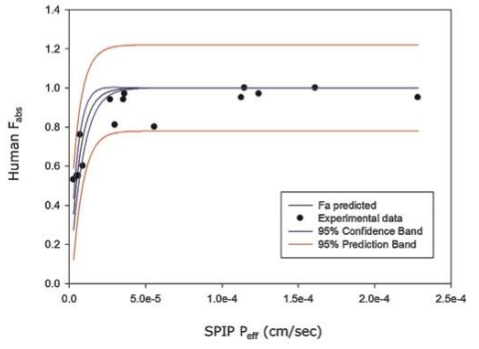

Fig. 3. The correlation between the SPIP permeability values obtained in this study and literature oral fraction dose absorbed, F_{abs}, in humans, of the different drugs.

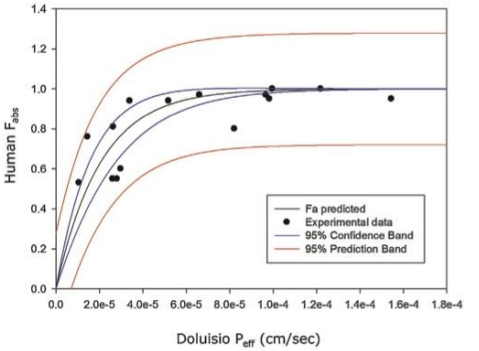

Fig. 4. The correlation between the Doluisio permeability values obtained in this study and literature oral fraction dose absorbed, F_{abs}, in humans, of the different drugs.

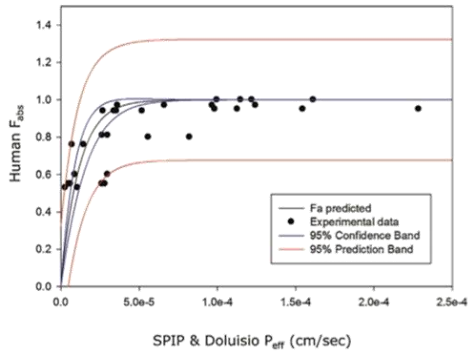

Fig. 5. The correlation between the SPIP and Doluisio permeability values obtained in this study and literature oral fraction dose absorbed, F_{abs}, in humans, of the different drugs.

to correctly classify 14 of the 15 investigated drugs. This result is in corroboration with the good correlation that was found between human jejunal P_{eff} and the overall human fraction of dose absorbed (Dahan et al., 2013; Lennernäs, 1997, 1998, 2007b), indicating that in general the jejunum can be predictive for the overall absorption. Of course, there are cases in which looking solely at the jejunum cannot predict the overall absorption, as will be discussed hereinafter.

For 14 out of the 15 investigated drugs, similar BCS permeability class membership was designated by literature data and by both SPIP and Doluisio methods (Table 2). The predicted fraction dose absorbed (F_{abs}) obtained by both SPIP and Doluisio methods (Figs. 3–5) were also in very good agreement with literature human F_{abs} values (Table 2). It should be noted that very frequently, literature human F_{abs} values do not include any indication for the margin of error, and only the average value is reported. Taking into account the expected variability in the human F_{abs} data, the good correlation between the experimental and the SPIP/Doluisio predicted F_{abs} can be even more appreciated. The only difficult case to analyze was that of digoxin; reports of its F_{abs} had large variability, no clear BCS permeability classification could be found in the literature for this drug, and different predictions were obtained by the two experimental models. While almost complete absorption was predicted by the SPIP model, F_{abs} value of only 72% was indicated by the Doluisio method. The transport of digoxin is very well known to be mediated by P-gp, and together with the drug's low dose, and intermediate physicochemical properties, inconsistent and unpredictable absorption can be expected (Cao et al., 2005; Dahan et al., 2009b; Doppenschmitt et al., 1999; Shirasaka et al., 2008). In this case, the different settings of the two experimental methods may become significant; the expression of P-gp throughout the rat small intestine was shown to follow a gradient, increasing from the proximal to the distal segments (Dahan and Amidon, 2009b; Dahan et al., 2009c; MacLean et al., 2008). Similar trend, with higher variability, was shown in humans too (Mouly and Paine, 2003; Thorn et al., 2005; Zimmermann et al., 2005). By considering only the upper jejunum, an intestinal region with lower P-gp expression, the SPIP model may give a higher P_{eff} value for digoxin than the Doluisio method that takes into account the entire small intestine, including segments with high P-gp expression. This explains the single disagreement between the two experimental methods in the case of digoxin. This case also highlights that the correlation between the two techniques could be improved with standardization of the perfused intestinal segment, the pH/composition of the perfusion solution, and the drug concentration used.

Both experimental techniques follow the disappearance of drug from the intestinal lumen as a measure for drug absorption. Nevertheless, the experimental settings lead to significantly different kinetic conditions in each method. In the SPIP model, the drug solution is perfused through the intestinal segment until steady-state is reached in the segment, i.e., the overall input rate equals to the overall output rate. In this case, the drug output from the perfused intestinal segment corresponds to the absorption process and to the drug leaving the intestine without being absorbed. In the closed-loop technique, the drug solution is kept in the segment, and the change in drug concentration with time corresponds to the rate of drug permeation. As a consequence, a different mathematical treatment is necessary in order to estimate the drug permeability. Nevertheless, an excellent correlation was obtained between the two methods.

A noticeable difference in the obtained data was related to the range of the P_{eff} values; while in the SPIP model, the permeability values ranged ~100-fold between 2.8×10^{-6} and 2.2×10^{-4} cm/sec, in the Doluisio method only ~10-fold range was obtained, from 1.0×10^{-5} and 1.5×10^{-4} cm/sec (Table 1). As a consequence, it may be easier to distinguish between drugs with close (but not similar) intestinal permeability using the SPIP model. This trend may be attributable to the use of short segment (SPIP) vs. the entire small intestine (Doluisio), as well as to the steady-state

Table 2
Literature human F_{abs} data (Pham-The et al., 2013; Skolnik et al., 2010; Takagi et al., 2006; Varma et al., 2005; Wolk et al., 2014), and the corresponding SPIP and Doluisio predicted F_{abs} and BCS permeability class. H, high; L, low; N/A, not available.

The drug	Literature human data		Single-pass rat jejunal perfusion		Doluisio's rat perfusion	
	F_{abs} (%)	Permeability class	Predicted F_{abs}	Permeability class	Predicted F_{abs}	Permeability class
Ibuprofen	95	H	100	H	99.9	H
Carbamazepine	100	H	100	H	99.7	H
Antipirine	97	H	100	H	99.1	H
Caffeine	100	H	100	H	99.2	H
Chloramphenicol	100	H	100	H	100	H
Paracetamol	80	H	99.9	H	98.2	H
Metoprolol	98	H	100	H	100	H
Codeine	94	H	99.5	H	92.0	H
Digoxin	81	N/A	99	H	72.5	L
Labetalol	94	H	98.4	H	88.0	H
Furosemide	60	L	74.8	L	76.7	L
Cimetidine	76	L	66.9	L	50.9	L
Valsartan	55	L	60.4	L	72.2	L
Colchicine	55	L	54.3	L	74.6	L
Atenolol	50	L	35.4	L	40.6	L

(SPIP) vs. non-steady-state (Doluisio) conditions. In any case, the drugs' P_{eff} rank was similar with both experimental models, and both of them were able to correctly classify all of the investigated drugs.

5. Conclusions

The single-pass intestinal perfusion (SPIP) model and the Doluisio (closed-loop) rat perfusion method are both equally useful for obtaining intestinal permeability values that can be used for F_{abs} prediction and BCS classification. A very good correlation was shown between the two experimental techniques, as well as between literature human F_{abs} values and the predictions made by the rat perfusions. Similar BCS permeability class membership was designated by literature data and by both SPIP and Doluisio methods for all compounds.

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Segmental-dependent permeability throughout the small intestine following oral drug administration: Single-pass *vs.* Doluisio approach to *in-situ* rat perfusion

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ABSTRACT

Intestinal drug permeability is position dependent and pertains to a specific point along the intestinal membrane, and the resulted segmental-dependent permeability phenomenon has been recognized as a critical factor in the overall absorption of drug following oral administration. The aim of this research was to compare segmental-dependent permeability data obtained from two different rat intestinal perfusion approaches: the single-pass intestinal perfusion (SPIP) model and the closed-loop (Doluisio) rat perfusion method. The rat intestinal permeability of 12 model drugs with different permeability characteristics (low, moderate, and high, as well as passively and actively absorbed) was assessed in three small intestinal regions: the upper jejunum, mid-small intestine, and the terminal ileum, using both the SPIP and the Doluisio experimental methods. Excellent correlation was evident between the two approaches, especially in the upper jejunum ($R^2 = 0.95$). Significant regional-dependent permeability was found in half of drugs studied, illustrating the importance and relevance of segmental-dependent intestinal permeability. Despite the differences between the two methods, highly comparable results were obtained by both methods, especially in the medium-high P_{eff} range. In conclusion, the SPIP and the Doluisio method are both equally useful in obtaining crucial segmental-dependent intestinal permeability data.

1. Introduction

Although oral drug absorption is a complex cascade of processes influenced by many physicochemical, physiological and pharmaceutical factors, the work of Amidon et al. revealed that the solubility of the drug dose in the intestinal milieu, and the drug permeability through the intestinal membrane are the two key factors governing the fraction of dose absorbed (Amidon et al., 1995; Dahan et al., 2009a; Martinez and Amidon, 2002). However, these two parameters are not constant throughout the gastrointestinal tract (GIT) (Dahan et al., 2010; Lennernas, 2014a). It is well recognized that the solubility of some drugs may differ dramatically as a function of the location throughout the GIT, for instance due to pH changes, or bile salts content. The regulatory requirement to supply solubility data in all luminal conditions before assigning a BCS (biopharmaceutics classification system) solubility classification is a reflection of this fact (Amidon et al., 2011; Polli et al., 2008; Varma et al., 2012; Yu et al., 2002). On the other hand, no parallel thinking currently exists for the BCS permeability classification, which is based merely on the jejunal permeability value. This policy may miss the complexity behind the permeability measure, considering the whole of the intestine; permeability is location dependent, and pertaining to each point throughout the GIT (Dahan et al., 2012b; Lennernas, 2014b; Sjogren et al., 2015; Zur et al., 2015, 2014a). The mechanisms behind segmental-dependent intestinal drug permeability may include ionization variations due to pH changes (Dahan et al., 2010, 2014;

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Fairstein et al., 2013; Zur et al., 2014b), different expression levels of various influx/efflux transporters (Dahan and Amidon, 2009b; Dahan et al., 2009b), local water absorption, membrane structure/composition variations, *etc.* (Dahan et al., 2012a; Gonzalez-Alvarez et al., 2007; Olivares-Morales et al., 2015; Ungell et al., 1998).

Human data on site-specific intestinal permeability would be highly appreciated and convincing. Recently, Sjögren et al. indirectly estimated such human information from clinical trials that administered drug solutions to different intestinal regions (Sjögren et al., 2014; Sjogren et al., 2015), but still, human regional-dependent intestinal permeability data is unfortunately very rare and hard to get (Lennernas, 2007, 2014a). Cell culture based experimental approaches (Caco-2, MDCK, *etc.*) are also very limited in representing the intestinal membrane permeability process in all of its complexity. Currently, the main source for segmental-dependent permeability data is animal studies, typically in rats, which allow a fairly straightforward investigation of the different GIT regions. Given the lack of direct experimental data in humans, this experimental approach represents the best we can do today, providing a good indication of the expected situation in humans (Cao et al., 2006; Chiou and Barve, 1998; Kim et al., 2006). For instance, we have shown that the permeability of the H_2 blockers and P-gp substrates cimetidine varies along the rat intestine, and is inversely correlated with the P-gp expression levels in the different segments (Dahan and Amidon, 2009b; Dahan et al., 2009c), while the same segmental-dependent permeability, at the same magnitude (approximately 2-fold), was reported for cimetidine intestinal absorption in humans (Sutcliffe et al., 1988).

The aim of this research was to compare segmental-dependent permeability data obtained from two different rat intestinal perfusion methods: the single-pass intestinal perfusion (SPIP) model and the Doluisio rat perfusion method. While both of these experimental methods measure disappearance from the intestinal lumen as an indication for drug absorption (Lennernas et al., 1995), they have substantial differences between them (Lozoya-Agullo et al., 2015). For instance, while in the SPIP model each drug molecule gets only one passage through the investigated intestinal segment, in the Doluisio method the tested drug solution remains within the intestinal lumen throughout the entire experiment. We have constructed a list of 12 model drugs with different permeability characteristics: low, moderate, and high permeability, as well as passively and actively absorbed compounds. We assessed the rat intestinal permeability of these 12 model drugs in three small intestinal regions: the upper jejunum, mid-small intestine, and the terminal ileum, using both SPIP and the Doluisio experimental methods, and evaluated the correlation between them. While we have recently presented analysis concerning the upper small intestine (Lozoya-Agullo et al., 2015), this is the first time to analyze segmental-dependent permeability using this two experimental approaches. Overall, this research provides a deeper insight into segmental-dependent permeability following oral drug administration, with emphasize on the two experimental approaches investigated.

2. Material and methods

2.1. Materials

Acetaminophen, antipyrine, caffeine, codeine, digoxin, furosemide, glipizide, ibuprofen, labetalol, metoprolol, pseudoephedrine and sotalol were purchased from Sigma-Aldrich. Methanol, acetonitrile and water were HPLC grade. All other chemicals were of analytical reagent grade.

2.2. Single-pass rat intestinal perfusion

The single-pass intestinal perfusion studies were performed using protocols approved by the Ben-Gurion University of the Negev Animal Use and Care Committee (Protocol IL-08-01-2015). Male Wistar rats (280–320 g, Harlan, Israel) were housed and handled according to the Ben-Gurion University of the Negev Unit for Laboratory Animal Medicine Guidelines.

The technique used for the *in situ* single-pass perfusion experiments followed previous reports (Dahan et al., 2009c). Rats were anesthetized with an intra-muscular injection of 1 mL/kg of ketamine–xylazine solution (9%:1%, respectively), placed on a heated surface (37 °C; Harvard Apparatus Inc., Holliston, MA), and a 3 cm midline abdominal incision was made. Three individual 10 cm segments of the small intestine were simultaneously perfused in each rat: (1) the proximal jejunum, starting 2 cm distal to the ligament of Treitz; (2) mid-small intestine, between the end of the upper and the start of the lower segments; and (3) the distal ileum, ending 2 cm proximal to the caecum. The three segments were cannulated on two ends, and were rinsed witch blank perfusion buffer. All solutions were incubated in a 37 °C water bath.

To closely mimic the physiological intestinal conditions, each segment was perfused with a phosphate buffer containing the investigated drug and the physiological pH relevant to each segment: 6.5 for the upper jejunal segment, 7.0 for the mid-small intestine, and pH 7.5 for the ileal segment. These perfusion buffers (freshly prepared 30 min prior to starting the experiments) were prepared by adding different ratios of potassium phosphate monobasic and sodium phosphate dibasic to give the pH values of 6.5, 7.0 and 7.5, while smolality (290 mOsm/L) and ionic strength (50 mM) were similar in all buffers. The perfusion buffer was perfused through the intestinal segment (Watson Marlow 205S, Watson-Marlow Bredel Inc., Wilmington, MA) at a flow rate of 0.2 mL/min, first for 1 h without sampling, to ensure steady state conditions, followed by additional 1 h of perfusion with samples (2 mL) taken every 10 min. The pH of the collected samples was measured at the outlet, to verify that the pH did not change throughout the perfusion. The samples were immediately assayed for drug content by UPLC. The exact length of each perfused intestinal segment was measured at the endpoint of the experiment.

The effective permeability (P_{eff}; cm/s) through the rat gut wall was determined according to the following equation:

$$P_{eff} = \frac{-Q\ln\left(C'_{out}/C'_{in}\right)}{2\pi RL}$$

where Q is the perfusion buffer flow rate (0.2 mL/min), C'_{out}/C'_{in} is the ratio of the outlet and the inlet concentration of drug that has been adjusted for water transport *via* the non-absorbable marker phenol red (Dahan and Amidon, 2009a; Dahan and Miller, 2012; Dahan et al., 2009b; Tugcu-Demiroz et al., 2014), R is the radius of the intestinal segment (set to 0.2 cm), and L is the length of the perfused intestinal segment.

2.3. Doluisio's (closed-loop) rat intestinal perfusion

The Doluisio studies were approved by the Scientific Committee of the Faculty of Pharmacy, Miguel Hernandez University, and followed the guidelines described in the EC Directive 86/609, the Council of the Europe Convention ETS 123 and Spanish national laws governing the use of animals in research. The experimental protocols was also approved by the Ethics Committee for Animal Experimentation of the University of Valencia (Spain, code A1330354541263).

The absorption rate coefficients and the permeability values of each model drug were evaluated by the *in-situ* close-loop perfusion method based on Doluisio's technique (Doluisio et al., 1969), in the same three 10 cm segments appointed in the single-pass intestinal perfusion method: the upper jejunum, mid-small intestine, and the distal ileum. Male Wistar rats (body weight, 260–300 g), fasted 4 h prior to the experiment with free access to water, were used in these studies. Rats were anesthetized using a mixture of diazepam (1.67 mg/kg, Valium, Roche), ketamine (50 mg/kg, Ketolar, Parke-Davis), and atropine (1 mg/kg, Atropina sulfato, Braun), and were placed on heated surface maintained at 37 °C. A midline abdominal incision was made, and the small intestine was exposed. The perfusion technique consists of creating three isolated compartments in the three intestinal segments of interest with the aid of six syringes and six three-way stopcock valves. Care was taken to avoid disturbance of the intestinal blood supply. In order to remove

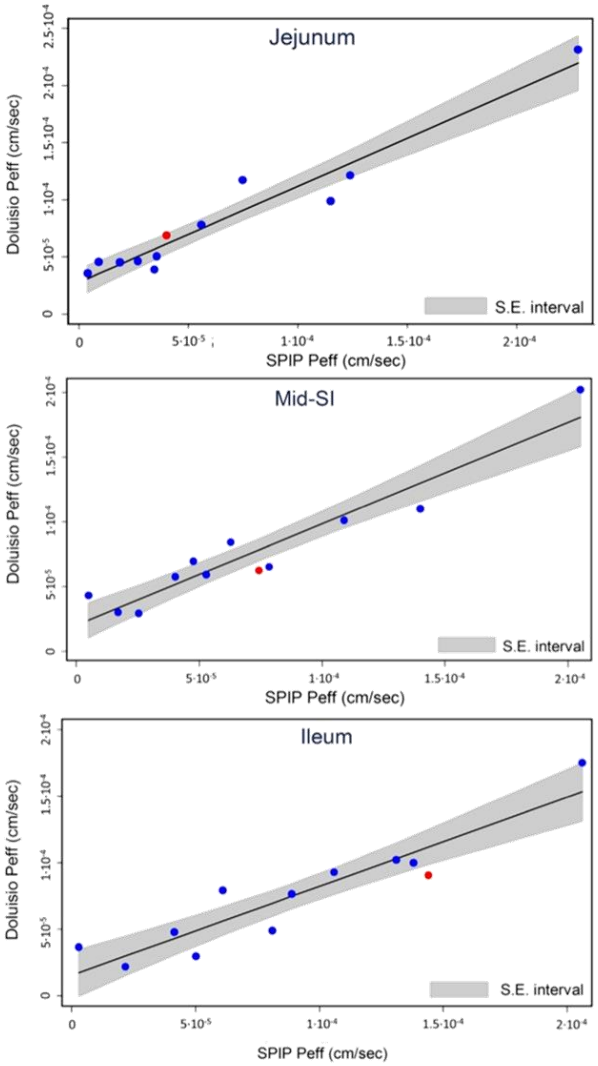

Fig. 1. The correlation between the permeability values (cm/s) obtained for the 12 model drugs using the SPIP *vs.* the Doluisio experimental methods in the jejunum (upper panel), mid-small intestine (middle panel) and in the ileum (lower panel). The red circle indicates the permeability value of metoprolol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

all intestinal contents, the three segments were thoroughly flushed with a warm physiologic solution at the physiologic pH of each segment. The catheters were then connected to a glass syringe using a stopcock three-way valve. This set up ensures the isolation of the three compartments, and drug solution (pH 6.5 for the upper segment, 7.0 for the middle segment, and pH 7.5 for the distal segment, similar to the SPIP buffers) were then introduced into each segment and sampled with the aid of the syringes and stopcock valves. The samples were collected every 5 min up to a period of 30 min (Bermejo et al., 1999; Ruiz-Garcia et al., 1999). The length of the perfused intestinal segment was accurately measured at the endpoint of the experiment, as well as the pH.

Water flux during the experiment may be significant, and hence must be accounted for (Tugcu-Demiroz et al., 2014). A method based on direct measurement of the remaining solution volume was employed to calculate the water reabsorption zero order constant (k_0). For each segment, the initial volume (V_0) was determined, and the endpoint volume (V_t) was measured. The drug concentration in the samples was corrected as: $C_t = C_e(V_t/V_0)$, where C_t represents the concentration in the absence of water reabsorption at time t, and C_e the experimental value. The corrected concentration, C_t, was then used as the actual absorption rate coefficient calculations (Martin-Villodre et al., 1986).

The absorption rate coefficients (k_a) of the compounds were determined by nonlinear regression analysis of the remaining concentrations in the intestinal lumen (C_t) *vs.* time:

$$C_t = C_0 \times e^{-k_a t}$$

These absorption rate coefficients were then transformed into permeability values using the relationship: $P_{app} = k_a \times R/2$, where R is the effective radius of the intestinal segment, calculated from the area/volume relationship considering a 2 mL volume and the measure of each segment length in each animal.

2.4. Analytical methods

Analytical analyses of the SPIP and the Doluisio samples were performed on a Waters (Milford, MA) Acquity UPLC H-Class system, and a Waters 2695 HPLC Separation Module, respectively. Both systems were equipped with photodiode array (PDA) detector and Empower software.

The simultaneous determination of the investigated drug and the non-absorbable marker phenol red in the SPIP samples was achieved using a Waters (Milford, MA) Acquity UPLC BEH C_{18} 1.7 μm 2.1 × 100 mm column. The determination of the investigated drugs in the Doluisio samples were carried out using a Waters C_{18} 3.5 μm 4.6 × 250 mm XTerra column. A general gradient mobile phase consisted of 90:10 going to 20:80 (v/v) water:acetonitrile (both with 0.1% TFA) over 7 min was suitable for all analyses. Injection volumes ranged 5–20 μL in all analyses.

2.5. Statistical analysis

All animals experiments were replicated with $n = 4–7$. Values are expressed as mean ± standard deviation (SD). Permeability values determined in each intestinal segment and perfusion method were compared using Student's *t*-test to detect the existence of significant differences at the 0.05 probability level. All statistical analyses were made using the statistical package SPSS V.20, as well as STATA software.

3. Results

The correlation between the permeability values (cm/s) obtained for the 12 model drugs using the SPIP *vs.* the Doluisio experimental methods in the jejunum (upper panel), mid small intestine (middle panel) and in the ileum (lower panel) are presented in Fig. 1. The shadowed area around the trendline represents the standard error (SE). These results illustrate that excellent correlation exists between the SPIP and the Doluisio approaches in the jejunum ($R^2 = 0.95$). A good correlation between the two experimental methods was also obtained in the mid-SI ($R^2 = 0.9$), as well as in the lower ileum ($R^2 = 0.85$), yet not as good as the upper segment.

A broader look on the correlation between the SPIP and the Doluisio approaches is illustrated in Fig. 2, where all of the 36 permeability values gathered in these experiments (12 drugs × 3 small intestinal segments) are shown altogether. A very high correlation between the two sets of data can be seen, as evident by a coefficient of determination (R^2) of 0.9. This good correlation highlights that in spite of the significant differences between the two methods, *e.g.* single-pass *vs.* closed-loop, and equilibrium *vs.* non-equilibrium, both approaches track the same fundamental process, and use disappearance of drug from the gut lumen as a measurement for absorption, which eventually governs the final result.

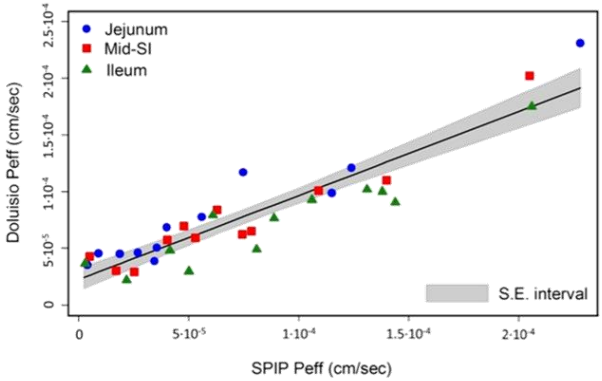

Fig. 2. The correlation between all of the 36 permeability values obtained in this study using the SPIP *vs.* the Doluisio experimental methods.

In Fig. 3 we have examined the Doluision/SPIP ratio of the 36 permeability values obtained in this work. This Figure reveals that in the medium-high P_{eff} range, the permeability values obtained by the two methods resembled a lot (as evident by Doluision/SPIP ratio close to 1). On the other hand, in the low-permeability range the Doluisio method produces higher values than the SPIP approach, as evident by Doluisio/SPIP ratio significantly higher than 1. This trend was irrespective of the small intestinal segment being examined, and was shown to be stronger as the permeability value gets lower.

The permeability values (cm/s) obtained for the 12 model drugs in the three small-intestinal segments (jejunum, upper panel; mid-SI, middle panel; ileum, lower panel) using both the SPIP approach (blue bars) and the Doluisio experimental method (red bar) are shown in Fig. 4. The 12 drugs were organized from the lowest to the highest permeability, according to the proximal jejunum (upper panel), and this order was maintained in the other panels; it can be seen that already at the mid-SI, and more profoundly at the lower ileum, this order failed to represent the lowest to the highest P_{eff} order. This illustrates the importance of segmental-dependent permeability throughout the gastrointestinal tract; it shows that low-permeability in the jejunum does not necessarily indicate that this drug will show low-permeability in other intestinal regions, as will be further discussed hereinafter.

4. Discussion

In this work, a comparison of two different rat intestinal perfusion methods, the single-pass intestinal perfusion (SPIP) approach and the Doluisio rat perfusion model, is presented. Specifically, we aimed to study the ability of these experimental approaches to investigate segmental-dependent permeability throughout the small intestine.

Traditionally, the Doluisio (closed-loop) method is performed on the entire small intestine, and we have previously evaluated permeability results of 15 model drugs obtained in this manner (Lozoya-Agullo et al., 2015). An excellent correlation ($R^2 = 0.93$) was obtained between the jejunal SPIP and the Doluisio results, in spite of the fact that the entire SI was used in the Doluisio method. In the current work, even better correlation was evident for the upper intestinal segment ($R^2 = 0.95$) between the SPIP and the Doluisio results, since this time the Doluisio method was focused solely on a 10-cm jejunal segment, similarly to the SPIP approach. Yet, the excellent correlation obtained previously reveals that when the entire SI was used in the Doluisio method, the jejunal segment was the dominant one, dictating the overall result. This may be attributable to the fact that a jejunal buffer (pH 6.5) was used in the entire SI Doluisio experiments, and hence those studies closely represented the upper SI and were highly correlated to the jejunal SPIP results. This analysis highlights that using too long intestinal segment in permeability studies may miss the complexity of the small intestine, as a complicated organ with a diverse environment throughout.

Permeability is position dependent and pertains to a specific point along the intestinal membrane (Dahan et al., 2012a; Lennernas, 2014a; Ozawa et al., 2015). A variety of mechanisms may result in the phenomenon of segmental-dependent permeability, in which a certain drug may move from low- to high-permeability, and *vice versa*, during the travel throughout the gastrointestinal tract. As demonstrated in this work, this may happen not only due to differences between the small and the large intestine, but even between the different segments of the small intestine itself. Table 1 summarizes the permeability classification of the 12 model drugs in the three small intestinal regions investigated, using the SPIP *vs.* the Doluisio method. The classification was determined compared to the jejunal P_{eff} value of metoprolol (for each experimental method); values lower than metoprolol's jejunal P_{eff} indicated low-permeability, while values similar/higher than metoprolol's jejunal P_{eff} indicated high-permeability. It can be seen that for five drugs (over 40%) a permeability classification switch was detected throughout the small intestine, highlighting the relevance and significance of accounting for regional variations throughout the GIT in drug permeability classification. It should be noted that significant different permeability values in the different small intestinal regions were very likely to occur in two situations: (1) when active transporters are known to play a significant role in the overall absorption process, *e.g.* digoxin, furosemide, and glipizide; and (2) when the pK_a and Log *P* of the drug molecule support a significant ionization state variations in the small intestinal pH range, *e.g.* labetalol, sotalol, and pseudoephedrine. Occasionally, both mechanisms may be relevant for the same drug molecule, *e.g.* glipizide

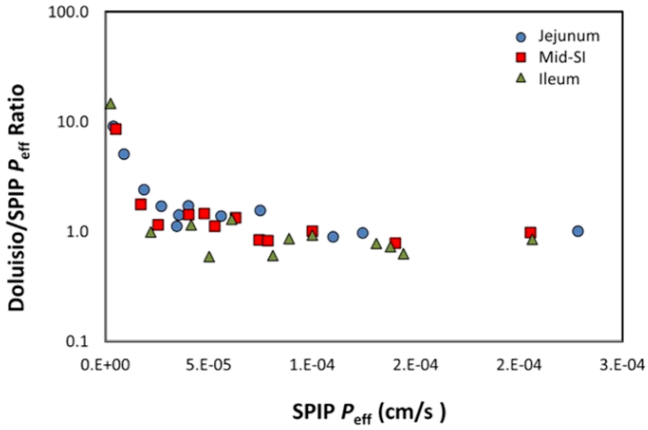

Fig. 3. Examination of the Doluisio/SPIP ratio of the 36 permeability values obtained in this work.

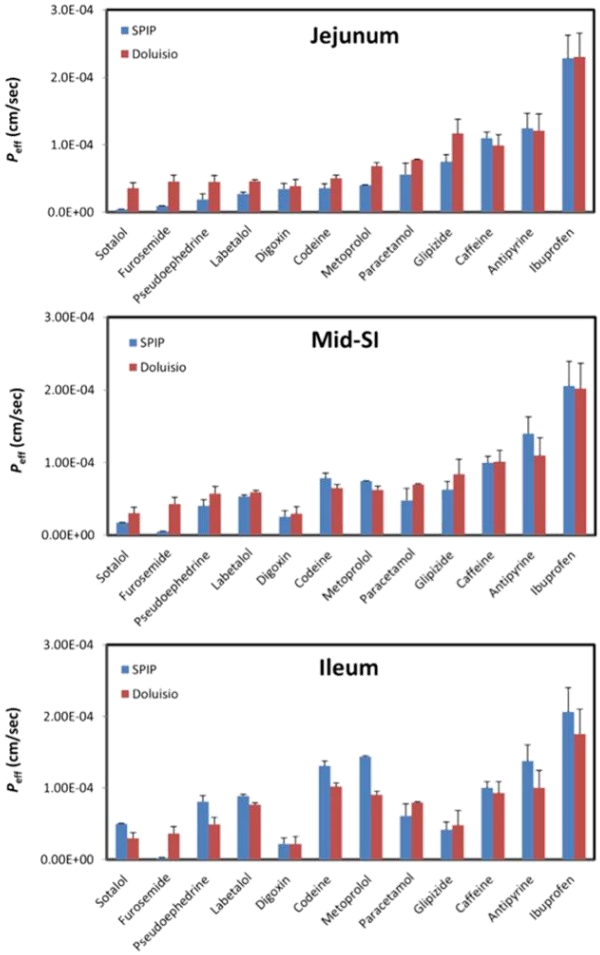

Fig. 4. The permeability values (cm/s) obtained for the 12 model drugs in the three small-intestinal segments (jejunum, upper panel; mid-SI, middle panel; ileum, lower panel) using both the SPIP approach (blue bars) and the Doluisio experimental method (red bar). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and furosemide. An interesting phenomenon was observed for the cases where segmental-dependent passive permeability was significant due to pH-dependent lipophilicity: while the segmental differences obtained with the SPIP approach were in accordance with the theoretical distribution coefficients (Log D), the Doluisio's method showed much lower differences than expected. This may be attributable to the critical difference between the two models: single-pass of the solution through the intestinal segment *vs.* closed loop. With the SPIP approach, fresh buffered solution is continuously perfused through the intestinal segment, and thus, the pH is kept at the initial value. On the other hand, with the Doluisio technique it is possible that during the experiment, since the same buffer remains constantly within the investigated intestinal segment, the relatively long contact time of the perfusion solution with the intestinal compartment, the water reabsorption and the intestinal secretions interchange, alter the initial buffer pH. This may increase and decrease the buffer pH in the proximal and distal segments, respectively, thus masking the regional-dependent permeability in these cases. For this reason the segmental P_{eff} differences in the Doluisio method were attenuated, and even when permeability trend similar to that of the SPIP approach was observed, no statistical significant difference could be detected. This analysis reveals another technical difference between the two methods: with the SPIP approach a fresh drug solution is continuously perfused through the investigated intestinal segment, and hence, a large volume of

Table 1
The permeability classification of the 12 model drugs in the 3 small intestinal segments, using the SPIP approach *vs.* the Doluisio method. The classification was determined compared to the jejunal P_{eff} value of metoprolol (for each experimental method). H, high; L, low; N/A, not available.

Drug	F_{abs}	Literature P_{eff} Classification	SPIP			Doluisio		
			Jejunum	Mid-SI	Ileum	Jejunum	Mid-SI	Ileum
Glipizide	100	H	H	H	H	H	H	L
Caffeine	100	H	H	H	H	H	H	H
Metoprolol	98	H	H	H	H	H	H	H
Antipyrine	97	H	H	H	H	H	H	H
Ibuprofen	95	H	H	H	H	H	H	H
Sotalol	95	H	L	L	H	L	L	L
Pseudoephedrine	95	H	L	H	H	L	L	L
Labetalol	94	H	L	H	H	L	L	H
Codeine	94	H	H	H	H	L	L	H
Digoxin	81	N/A	H	L	L	L	L	L
Paracetamol	80	N/A	H	H	H	H	H	H
Furosemide	60	L	L	L	L	L	L	L

Table 2
Relevant literature physicochemical properties of the 12 model drugs, the solubility classification, and the concentrations (mM) used in all of the permeability experiments. H, high; L, low; N/A, not available.

Drug	Concentration (μM)	pK_a	Log *P*	Literature Solubility Classification
Glipizide	100	5.9	1.9	L
Caffeine	500	14; 0.6	−0.07	L
Metoprolol	500	9.7	1.9	H
Antipyrine	200	2.2; 1.4	−1.1	H
Ibuprofen	250	5.2	4	L
Sotalol	500	9.8, 8.3	0.2	H
Pseudoephedrine	100	9.9	1.1	H
Labetalol	500	7.3; 8.8	1.3	H
Codeine	750	7.9	1.1	H
Digoxin	100	7.1	1.2	L
Paracetamol	330	9.9	0.2	N/A
Furosemide	500	4.7; 3.9	2	L

drug solution is required for these experiments. On the other hand, since with the Doluisio technique the same drug solution remains constantly within the investigated intestinal segment, a small volume of drug solution is required for this method. This may be critical when the investigated drug is very expensive, or during initial stages of drug development, when only small quantity of the drug is available (Table 2).

A close look at Fig. 3 reveals that in the medium-high P_{eff} range, the permeability values obtained by the 2 methods resembled a lot (as evident by Doluision/SPIP ratio close to 1), while in the low-permeability range the Doluisio method produces higher values than the SPIP approach, as evident by Doluisio/SPIP ratio significantly higher than 1. This trend was irrespective of the small intestinal segment being examined, and was shown to be stronger as the permeability value gets lower. This phenomenon may be related to the equilibrium/non-equilibrium state of the drug transport; while with the SPIP approach steady-state is assured before starting the permeability measurements, the Doluisio method measures the initial, non-equilibrium phase of the drug transport. It should be noted that in our previous SPIP-Doluisio comparison when the entire SI was used in the Doluisio method, this phenomenon was even more profound; in that work both the low-permeability and the high-permeability range were narrowed in the Doluisio technique. As a consequence, while in the SPIP model the permeability values ranged 100-fold between the lowest to the highest P_{eff}, in the Doluisio method only 10-fold range was obtained (Lozoya-Agullo et al., 2015). In this current work, where the Doluisio method was focused solely on a 10-cm intestinal segment, the high-permeability range was similar to that obtained with the SPIP method, highlighting the advantage of measuring the permeability in individual restricted intestinal regions rather than in the entire SI. In spite of all of the discussed differences between the two approaches, the correlation between both methods was found to be very good in the three small intestinal segments, with minimal variations. Moreover, the BCS permeability classification relative to metoprolol's jejunal permeability was highly comparable with both methods, supporting our previous analysis that states that if a compound has high fraction of dose absorbed, it will have high-permeability, not necessarily in the jejunum, but somewhere along the GIT.

5. Conclusions

In conclusion the SPIP and the Doluisio methods presented a very good correlation in the three studied small intestinal segments, and were demonstrated to be valuable approaches to elucidate segmental permeability differences. Both methods were able to correctly classify drugs according to the BCS. Irrespective of the experimental method, it is advisable to measure the permeability in individual restricted intestinal segments, rather than using wider regions.

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RESEARCH ARTICLE – *Pharmacokinetics, Pharmacodynamics and Drug Transport and Metabolism*

In Situ Perfusion Model in Rat Colon for Drug Absorption Studies: Comparison with Small Intestine and Caco-2 Cell Model

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ABSTRACT: Our aim is to develop and to validate the *in situ* closed loop perfusion method in rat colon and to compare with small intestine and Caco-2 cell models. Correlations with human oral fraction absorbed (Fa) and human colon fraction absorbed (Fa_colon) were developed to check the applicability of the rat colon model for controlled release (CR) drug screening. Sixteen model drugs were selected and their permeabilities assessed in rat small intestine and colon, and in Caco-2 monolayers. Correlations between colon/intestine/Caco-2 permeabilities versus human Fa and human Fa_colon have been explored to check model predictability and to apply a BCS approach in order to propose a cut off value for CR screening. Rat intestine perfusion with Doluisio's method and single-pass technique provided a similar range of permeabilities demonstrating the possibility of combining data from different laboratories. Rat colon permeability was well correlated with Caco-2 cell-4 days model reflecting a higher paracellular permeability. Rat colon permeabilities were also higher than human colon ones. In spite of the magnitude differences, a good sigmoidal relationship has been shown between rat colon permeabilities and human colon fractions absorbed, indicating that rat colon perfusion can be used for compound classification and screening of CR candidates. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:3136–3145, 2015
Keywords: colon absorption; fraction absorbed; permeability; absorption; colonic drug delivery; in vitro models; Caco-2 cells; site-specific absorption

INTRODUCTION

The small intestine is the main site of absorption, because of its anatomical, physiological, physicochemical environment, and biopharmaceutical features.[1] Traditionally, the colon has been considered less important than the small intestine for drug absorption. Anatomically, colon is shorter and wider than small intestine; colonic lumen has no extra surface area provided by villi; moreover, epithelial cell layer junctions are tighter in colon than in small intestine. So, the available surface for drug absorption in colon is smaller and morphologically less well equipped than that of the small intestine.[1–4] Differences also affect the expression of efflux and uptake transporters. Intestinal drug transporters are predominant in the jejunum and ileum[5,6] and, according to Drozdzik et al.[7] the transporter protein abundance was similar in the jejunum and ileum but markedly different in the colon. In fact the major transporter protein in the small intestine (around 50%) was the uptake carrier PEPT1, and in the colon, the major transporter was the basolateral efflux carrier ABCC3.[8] In addition, the gastrointestinal tract is populated with a large number of bacteria and most of them reside in the large intestine; the stability of a drug to the microbiota is clinically relevant; the metabolism can transform a drug in pharmacologically active, inactive, or toxic. Many drugs are substrates for colonic bacteria, which could result in degradation and influence their bioavailability.[9,10] These arguments also support that the permeability and the absorptive capacity for drugs of the colonic membrane are considered lower than the small intestine. However, this is not always so simple. Distal regions of intestine, including the colon, can significantly contribute to the overall absorption as well. Several studies have demonstrated that some drugs show fairly high permeability in colon.[11–14]

Colon has one main advantage for absorption: the transit time of drugs. The colonic transit time is longer than 24 h, whereas the small intestinal transit time is shorter about 2–5 h.[15,16] Thus, colonic absorption can be beneficial to ensure the performance of oral controlled release (CR) products. As CR formulations are designed to release the drug during 12–24 h, it is obvious that drug absorption in colon is a prerequisite for oral CR development.[3,13] Despite the fact that there are various *in vitro* and *in situ* models to predict the absorption and permeability in small intestine, there is less knowledge regarding the large intestine.[1,17] There is a need for exploratory *in vivo* studies to clarify regional drug absorption along the intestine, and especially from the colon in order to validate animal or *in vitro* biopharmaceutical colon models as these models would have a clear impact for the study of oral CR formulations.[4]

It is well known that oral drug absorption is defined mainly by two parameters: solubility and permeability. These parameters are the basis for the Biopharmaceutics Classification System (BCS) for immediate-release (IR) products developed by Amidon et al.[18] The premise of BCS is that the rate and extent of drug absorption depends on the solubility and permeability

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of the drug. The applicability of BCS for CR formulations and colonic absorption has been discussed.[19–21] Some authors have remarked that to extend the BCS model to the CR formulations with a simple measure of permeability is not adequate,[21] but on the contrary, Tannergren et al.[1] demonstrated that compounds with high permeability had good absorption in colon, whereas low permeability drugs presented unfavorable colon absorption, that is, permeability data could be used for early assessment of the colonic absorption potential during CR development.

The first purpose of this work is to develop and to validate the technique of *in situ* perfusion in rat colon based on Doluisio's method and to correlate the colon permeability values with permeability data obtained in small intestine to characterize the segmental differences in the rat model. Moreover, in order to obtain a simpler model for screening drug absorbability in colon, the second objective is to compare the animal model with an *in vitro* model based on Caco-2 monolayers. For those purposes, 16 model drugs with different permeability characteristics have been selected and the permeability has been assessed in both segments of the rat gastrointestinal tract: small intestine and colon, and in Caco-2 monolayers. Small intestine/colon and *in situ/in vitro* correlations and the comparison with literature *in vivo* data will be used to apply a BCS approach to colon absorption in order to propose a cut off value for screening during CR product development.

MATERIALS AND METHODS

Antipyrine, caffeine, carbamazepine, metoprolol, naproxen, theophylline, verapamil, amoxicillin, atenolol, cimetidine, codeine, colchicine, furosemide, ranitidine, terbutaline, and valsartan were purchased from Sigma–Aldrich. Methanol, acetonitrile, and water were HPLC grade. All other chemicals were of analytical reagent grade.

Table 1 summarizes the contribution of transporters that can affect the permeability of the test compounds and some physicochemical properties.[1,6,22–42] In this work, a high concentration of drugs (500 μM) was used to ensure saturating conditions in order to get the diffusional component of the transport and a negligible contribution of the transporters.

Absorption Studies

Male Wistar rats were used in accordance with 2010/63/EU directive of September 22, 2010 regarding the protection of animals used for scientific experimentation. The Ethics Committee for Animal Experimentation of the University of Valencia approved the experimental protocols (Spain, code A1330354541263).

The absorption rate coefficient and the permeability value in the whole small intestine of each compound were evaluated by *in situ* "close loop" perfusion method based on Doluisio's Technique.[43] Male Wistar rats (body weight, 250–300 g) fasted 4 h and with free access to water were used for these studies. Rats were anesthetized using a mixture of pentobarbital (40 mg/kg) and butorphanol (0.5 mg/kg). A midline abdominal incision was made. The intestinal segment was manipulated in order to minimize any intestinal blood supply disturbances. The bile duct was always tied up in order to avoid drug enterohepatic circulation and the presence of bile salts in lumen. The perfusion technique consists of creating an isolated compartment in the intestinal segment of interest, with the aid of two syringes and two three-way stopcock valves. Two incisions were performed in the intestine, the first one at the beginning of the duodenal portion and the second one at the end of the ileum portion just before the cecum sac. Surgical ligature to a catheter was placed at the duodenal incision. In order to remove all intestinal contents, the small intestine was copiously flushed with a physiologic solution: isotonic saline (pH 6.9) with 1% Sörensen phosphate buffer (v/v), 37°C. After that, the end of the segment was tied up and connected to a second catheter. Both catheters were connected to a glass syringe using a stopcock three-way valve. The intestinal segment was carefully placed back into the peritoneal cavity and the abdomen was covered with a cotton wool pad avoiding peritoneal liquid evaporation and heat losses. Once this system is set up, the intestinal segment is an isolated compartment where the drug solution (10 mL) can be introduced and sampled with the aid of the syringes and stopcock valves. The samples were collected every 5 min up to a period of 30 min.

The absorption rate coefficient and the permeability value in the colon were evaluated with a similar technique to that used for the whole small intestine.[3] The colon was flushed with a physiologic solution: isotonic saline (pH 7.5) with 1% Sörensen phosphate buffer (v/v), 37°C. The drug solution (5 mL) was introduced in the isolated compartment and it was sampled at fixed times.

Drug solutions for experiments in colon and intestine were prepared in isotonic saline buffered with Sörensen phosphate buffer (pH 7.0). For furosemide and valsartan, 1% DMSO (dimethyl sulfoxide) was used to achieve complete dissolution of these compounds.

At the end of the experiments, the animals were euthanized. In order to separate solid components (mucus, intestinal contents) from the samples, they were centrifuged for 5 min at 2991 g. All samples were analyzed by HPLC.

The absorption rate coefficient (K_a) and permeability values (P_{app}) for 16 drugs were determined in two segments of gastrointestinal tract: entire small intestine and colon ($n = 5$–7). The perfusion solution of each drug was prepared at 500 μM. After dissolving the drugs, the pH of the solution was readjusted to 7.0.

The reduction in the volume of the perfused solutions at the end of the experiments was significant (up to 20%), and a correction became necessary in order to calculate the absorption rate constants accurately. Water reabsorption was characterized as an apparent zero-order process. A method based on direct measurement of the remaining volume of the test solution was employed to calculate the water reabsorption zero-order constant (k_o). The volume at the beginning of the experiment (V_0) was determined on groups of three animals, whereas the volume at the end (V_t) was measured on every animal used. The concentration in the samples was corrected as

$$C_t = C_e\,(V_t/V_o) \tag{1}$$

where C_t represents the concentration in the gut that would exist in the absence of the water reabsorption process at time t, and C_e represents the experimental value. The C_t values (corrected concentrations) were used to calculate the actual absorption rate coefficient.[44]

The absorption rate coefficients (k_a) of compounds were determined by nonlinear regression analysis of the remaining

Table 1. Transporters Involved on the Intestinal Permeation of Each Drug and Physicochemical Properties

		Efflux Transporters			Uptake Transporters			Physicochemical Properties		
DRUG	*CYP 3A4*	P-gp	MRP	BCRP	OATP	PEPT1	OCT	MW	log *P*	p*Ka*
Antipyrine[22]	X							188.2	0.38	1.50[a]
Caffeine[23,24]		x						212.2	−0.07	10.4[b]
Carbamazepine[25,26]								236.3	2.45	13.9[b]
Metoprolol[27]								267.4	1.88	9.70[b]
Naproxen[28]					x			230.3	3.18	4.15[a]
Theophylline[1]								180.2	−0.02	8.81[b]
Verapamil[6,28–30]	X	x	x	x	x	x	x	491.1	3.79	8.92[b]
Amoxicillin[1,6]		x				x		365.4	0.87	2.40[a]
Atenolol[1,6,31]			x		x			266.3	0.16	9.60[b]
Cimetidine[1,32–34]		x		x			x	252.3	0.40	6.80[b]
Codeine[35,36]	X						x	299.4	1.19	8.21[b]
Colchicine[6,37]	X	x	x					399.4	1.30	1.85[a]
Furosemide[6,38,39]			x	x			x	330.7	2.03	3.80[a]
Ranitidine[1,6]		x					x	314.4	0.27	8.20[b]
Terbutaline[40]							x	274.3	0.90	8.80[b]
Valsartan[18,41,42]		x			x			435.5	3.65	4.90[a]

[a] Acid drugs.
[b] Basic drugs.
CYP 3A4, cytochrome P450; P-gp, P-glycoprotein; MRP, multidrug-resistance-associated protein; BCRP, breast cancer-resistance protein; OATP, organic anion transporting polypeptide; PEPT1, peptide transporter protein; OCT, organic cation transporter; MW, molecular weight (g/mol).

concentrations in lumen C_t versus time.

$$C_t = C_0 e^{-k_a t} \tag{2}$$

The absorption rate coefficients (k_a) were transformed in to permeability values with the following relationship:

$$P_{app} = K_a R/2 \tag{3}$$

where R is the effective radius of the intestinal segment. R value was calculated considering the intestinal segment as a cylinder with the relationship:

$$\text{Volume} = \pi R^2 L \tag{4}$$

Estimation was carried out using a 10-mL perfusion volume and an intestinal length of 100 cm for the whole small intestine, and a 5-mL perfusion volume and a length of 10 cm for the colon. The effective radius of the intestinal segment was fixed at a value of 0.1784 cm for small intestine and 0.3989 cm for colon.

The relationship between absorption rate coefficient and permeability value has been described in detail in Supporting Information.

Cell Culture and Transport Studies

Caco-2 cells were grown in Dubelcco's modified Eagle's media containing L-glutamine, fetal bovine serum, and penicillin–streptomycin. Cell monolayers were prepared by seeding 250,000 cells/cm^2 on the polycarbonate membrane whose surface of the insert placed in each well. They were maintained at 37°C temperature, 90% humidity, and 5% CO_2 for 4 days until confluence. Afterwards, the integrity of the each cell monolayer was evaluated by measuring the transepithelial electrical resistance (TEER). Hank's balanced salt solution supplemented with HEPES was used to fill the receiver chamber and to prepare the drug solution that was placed in the donor chamber.

Transport studies were conducted in an orbital environmental shaker at constant temperature (37°C) and agitation rate (50 rpm). Four samples were taken from the receiver side at prefixed sample times 15, 30, 45, and 90 min. Samples of the donor side were taken at the beginning and at the end of the experiment. The amount of compound in cell membranes and inside the cells was determined at the end of experiments in order to check the mass balance and the percentage of compound retained in the cell compartment as always less than 5%.

Drug transport studies were performed from apical-to-basal (A-to-B) direction using the same concentration that in the *in situ* assays (500 μM). The TEER values were measured before and after the transport experiments and were always between 800 and 1100 ohmios/cm^2, which ensure the integrity of the cell monolayer at assayed concentrations (500 μM).

The apparent permeability coefficient was calculated according the following equation:

$$C_{\text{receiver},t} = \frac{Q_{\text{total}}}{V_{\text{receiver}} + V_{\text{donor}}} + \left(\left(C_{\text{receiver},t-1} f \right) - \frac{Q_{\text{total}}}{V_{\text{receiver}} + V_{\text{donor}}} \right) \times e^{-P_{\text{eff}0,1} \cdot S \left(\frac{1}{V_{\text{receiver}}} + \frac{1}{V_{\text{donor}}} \right) \Delta t} \tag{5}$$

where $C_{\text{receiver},t}$ is the drug concentration in the receiver chamber at time t, Q_{total} is the total amount of drug in both chambers, V_{receiver} and V_{donor} are the volumes of each chamber, $C_{\text{receiver},t-1}$ is the drug concentration in receiver chamber at previous time, f is the sample replacement dilution factor, S is the surface area of the monolayer, Δt is the time interval, and P_{eff} is the permeability coefficient that might be $P_{\text{eff}\,0}$ or $P_{\text{eff}\,1}$. This equation considers a continuous change of the donor and receiver concentrations, and it is valid in either sink or nonsink conditions. The new feature is the option to estimate two permeability

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Table 2. Apparent First-Order Absorption Rate Coefficients (K_a, h^{-1}) and Apparent Permeability Values (P_{app}, 10^{-4} cm/s) of the 16 Drugs in Whole Small Intestine and Colon

	Small Intestine				Colon			
Drugs	K_a	SD	P_{app}	SD	K_a	SD	P_{app}	SD
Naproxen	7.434	0.492	1.841	0.116	3.016	0.227	1.673	0.123
Carbamazepine	5.164	0.510	1.279	0.120	2.575	0.425	1.424	0.230
Verapamil	4.304	0.470	1.056	0.101	2.221	0.556	1.230	0.308
Caffeine	4.221	0.790	1.046	0.186	1.565	0.363	0.870	0.196
Antipyrine	4.094	0.435	1.014	0.108	1.527	0.155	0.848	0.086
Theophylline	3.983	0.254	0.986	0.067	1.907	0.150	1.058	0.081
Metoprolol	2.518	0.239	0.624	0.273	1.505	0.344	0.814	0.187
Codeine	2.200	0.090	0.545	0.023	1.053	0.165	0.583	0.091
Furosemide	1.265	0.242	0.313	0.057	0.401	0.127	0.222	0.069
Cimetidine	1.211	0.229	0.299	0.057	0.639	0.146	0.346	0.078
Valsartan	1.050	0.087	0.262	0.022	0.606	0.115	0.328	0.062
Colchicine	1.010	0.158	0.250	0.039	0.591	0.157	0.320	0.085
Amoxicillin	0.708	0.172	0.167	0.041	0.553	0.095	0.299	0.051
Terbutaline	0.672	0.114	0.159	0.027	0.650	0.132	0.352	0.071
Ranitidine	0.573	0.136	0.135	0.032	0.546	0.135	0.295	0.073
Atenolol	0.430	0.096	0.107	0.024	0.390	0.079	0.211	0.043

Values represent mean ± SD of five to seven rats for each drug and region.

coefficients ($P_{eff\,0}$ and $P_{eff\,1}$) to account for atypical profiles in which the initial rate is different.[45]

The nonlinear regression to fit the equation to data was performed in Excel® using Solver tool for minimization of the Sum of Squared Residuals.

HPLC Analysis

All the samples were analyzed using a Perkin-Elmer HPLC with an UV detector or with a fluorescence detector depending on the characteristics of each drug. The standard calibration curves were prepared by dilution from the drug solution assayed. Sample concentrations were within the linear range of quantitation for all the assays. Analytical methods were validated for each compound in terms of specificity, selectivity, linearity, precision, and accuracy. All the compounds were analyzed with a flow rate of 1 mL/min and at room temperature. μBondapak 150 × 4.6 mm^2 C-18 column (5 μm particle size) was used for antipyrine, caffeine, carbamazepine, naproxen, verapamil, amoxicillin, furosemide, ranitidine, and terbutaline; and a μBondapak 250 × 4.6 mm^2 C-18 column (5 μm particle size) was required for metoprolol, colchicine, valsartan, atenolol, theophylline, codeine, and cimetidine. An injection volume of 10 μL was used for the compounds analyzed with fluorescence detection, and 20 μL for the drugs analyzed with UV detection. Analytical details are presented in Table S1 of Supporting Information file.[46]

Data Analysis

Permeabilities determined in each intestinal region were compared using Student's *t*-test to detect the existence of significant differences at the 0.05 probability level. The statistical comparison was made using the statistical package SPSS, V.20.

In order to characterize the relationship between permeability values (P_{app}) and oral fraction absorbed (Fa), the following equations were used for small intestine and colon (Eq. 6), and Caco-2 (Eq. (7), respectively:

$$Fa = 1 - e^{\left(-P_{app}\frac{2}{R}T\right)} \quad (6)$$

$$Fa = 1 - e^{\left(-P_{app}\frac{A}{V}T\right)} \quad (7)$$

where R represents the effective radius of the intestinal segment that was fixed at a value of 0.1784 cm for small intestine and 0.3989 cm for colon, A and V represent the surface area of the well and the volume in the donor chamber in cell culture experiments, and T is the effective absorption time.

Additionally, a simultaneous fit of both equation to data from Caco-2 cells (21 days model) and rat colon and intestine permeability was performed, keeping a shared T parameter and including a surface factor correction factor, S, in the exponent of Eq. (7), to account for the surface difference between the monolayer model and the intestine and colon (because of villi and folds).

RESULTS

Table 2 summarizes the apparent first-order absorption rate coefficients (K_a, h^{-1}) and apparent permeability values (P_{app}, 10^{-4} cm/s) of the 16 drugs in whole small intestine and colon.

Figure 1 shows a comparative graphic of P_{app} values in small intestine and colon of each assayed drug. A good linear correlation was established between rat colon and intestinal permeability with $R^2 = 0.94$ (see Fig. S1 in Supporting Information).

Figure 2 shows a comparative graphic of P_{app} values in colon and Caco-2 cells; 4 days (top panel) and 21 days (bottom panel), respectively, of each assayed drug. P_{app} Caco-2 data of theophylline, metoprolol, furosemide, and atenolol (4 days) and codeine and valsartan (21 days) were obtained from literature.[32,47] Data are summarized in Table 3.

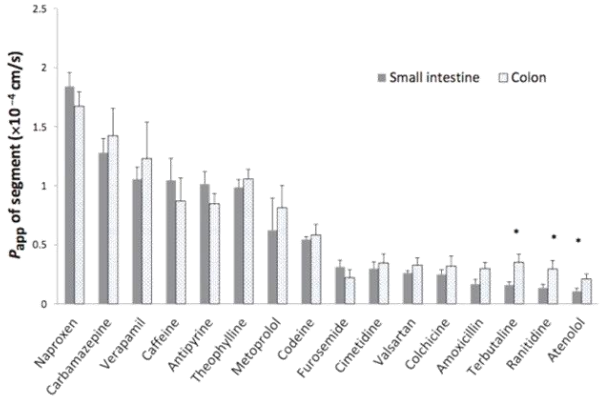

Figure 1. Permeability values of the drugs in small intestine and colon. *Significant differences between both segments ($p < 0.05$).

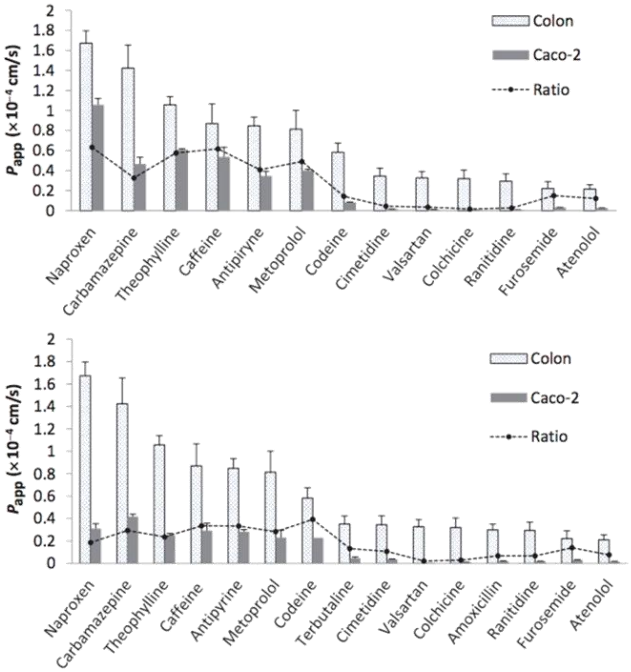

Figure 2. Top panel: permeability values of the drugs in colon and Caco-2, 4 days model. Bottom panel: permeability values of the drugs in colon and Caco-2, 21 days model.

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Table 3. Permeability Values in Colon and in Caco-2 Monolayers (4 and 21 Days)

			P_{app} Caco-2 ($\times 10^{-4}$ cm/s)					
			4 Days Model			21 Days Model		
Drug	P_{app} Colon ($\times 10^{-4}$ cm/s)	SD	P_{app}	SD	Ratio	P_{app}	SD	Ratio
Naproxen	1.673	0.123	1.058	0.063	0.632	0.311	0.043	0.186
Carbamazepine	1.424	0.230	0.466	0.067	0.328	0.418	0.021	0.294
Theophylline	1.058	0.081	0.610	0.009	0.576	0.249	0.018	0.236
Caffeine	0.870	0.196	0.537	0.096	0.618	0.292	0.066	0.336
Antipiryne	0.848	0.086	0.347	0.047	0.409	0.282	0.019	0.333
Metoprolol	0.814	0.187	0.400	0.014	0.491	0.230	0.067	0.283
Codeine	0.583	0.091	0.084	0.003	0.143	0.229		0.393
Terbutaline	0.352	0.071				0.047	0.010	0.132
Cimetidine	0.346	0.078	0.015	0.002	0.045	0.037	0.003	0.107
Valsartan	0.328	0.062	0.012	0.002	0.036	0.007		0.021
Colchicine	0.320	0.085	0.005	0.001	0.016	0.009	0.002	0.029
Amoxicillin	0.299	0.051				0.020	0.003	0.067
Ranitidine	0.295	0.073	0.008	0.001	0.027	0.020	0.001	0.067
Furosemide	0.222	0.069	0.033	0.001	0.150	0.031	0.002	0.140
Atenolol	0.216	0.043	0.026	0.002	0.121	0.016	0.003	0.076

P_{app} Caco-2 data of theophylline, metoprolol, furosemide, and atenolol (4 days) and codeine and valsartan (21 days) were obtained from literature.[32,47] Values represent mean ± SD. Ratio between P_{app} obtained in Caco-2 and P_{app} obtained in colon.

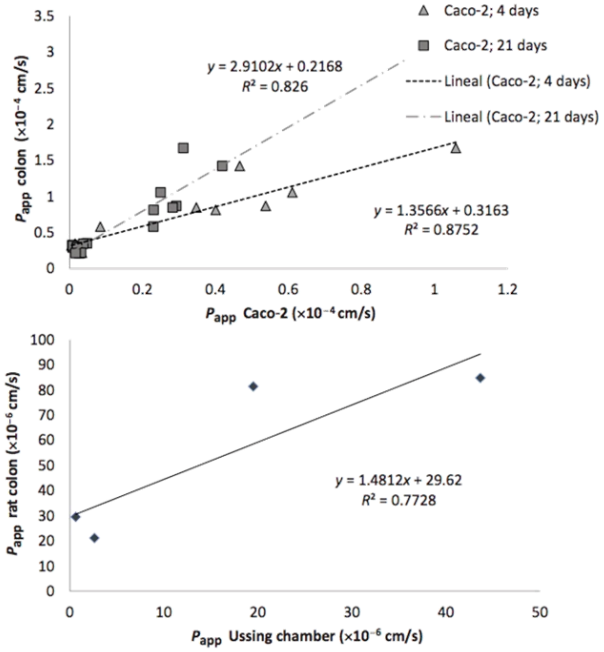

Figure 3. Top panel: correlation between apparent permeability coefficients in rat colon and Caco-2, 21 days model, and Caco-2, 4 days model. Bottom panel: correlation between rat colon permeability obtained in this study and human colon tissue in Ussing chambers (average values from references[48,49]).

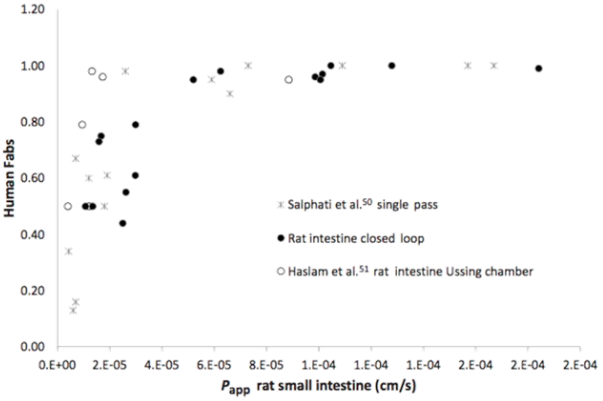

Figure 4. Correlation between permeability values obtained in rat small intestine with closed loop method and oral fraction absorbed of the different drugs. Asterisk represents permeability data obtained with single-pass method in rat small intestine by Salphati et al.[50] Hollow circles correspond to Ussing chamber data with rat intestine obtained by Haslam et al.[51]

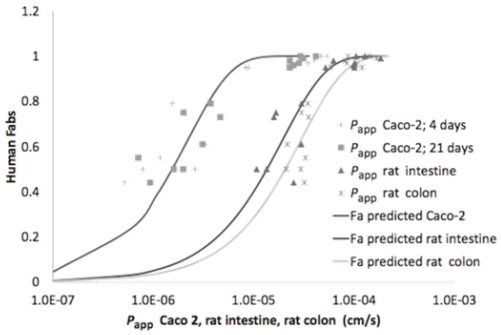

Figure 5. Simultaneous fit of permeability values obtained in different models (rat intestine, rat colon, and Caco-2; 21 and 4 days) to estimate the difference in surface area correction factor (47.0 for small intestine and 32.0 for rat colon).

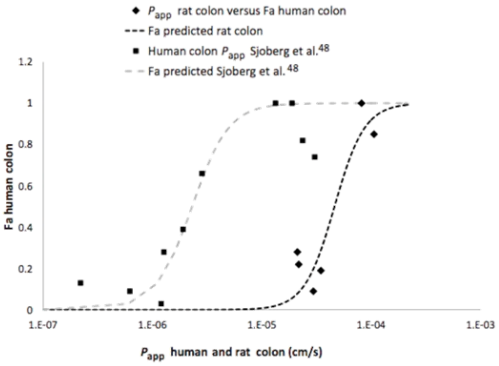

Figure 6. Correlation between permeability values obtained in rat colon and oral fraction absorbed in colon taken from Tannergren et al.[1] and Sjoberg et al.[48] Fitted lines were obtained with a four parameter logistic model $y = B/[1+(x/C)^{\wedge}D]$ where B is 1, C is P_{app} at Fa = 0.5 (4.6×10^{-5} cm/s); D is the slope factor = −3.15 ($R^2 = 0.87$, $p < 0.05$). The sigmoidal correlation between human colonic P_{app} and human Fa in colon obtained by Sjoberg et al.[48] is superimposed for comparison.

Figure 3 shows in the top panel the correlation obtained between *in situ* (colon of the rat) and *in vitro* (Caco-2) models, whereas the bottom panel displays the correlation among the permebility values of antipyrine, metoprolol, atenolol, and ranitidine obtained in rat colon and permeabiliy values obatined in human colonic tissue with Ussing chamber.[48,49]

Figure 4 displays the rat intestinal permeability obtained with our closed loop method versus human oral fraction absorbed. Data obtained in rat small intestine with single-pass perfusion method by Salphati et al.[50] and in rat intestine with Ussing chambers[51] are represented in Figure 4 to explore the similarity of data from different laboratories and experimental techniques. The human fractions absorbed were obtained from literature.[32,52–54]

In Figure 5, a simultaneous fit of fraction absorbed versus permeability in Caco-2 model (21 days and 4 days) rat small intestine and colon is represented to show the shift of the curves because of the differences in surface area for transport in cell system versus the *in situ* models.

Fraction absorbed in human colon[48] were included in Figure 6 to explore the predictability of rat colon permeability to classify compounds in high/low colon absorbability. A previous correlation of Human colon P_{app} and human colon Fa is overlapped for comparison.[47]

Table S2 of Supporting Information shows the permeability class of the different compounds assayed according to the BCS classification (literature data by comparison with

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metoprolol)[32,52,54,55] and the permeability class according to the results of this research in small intestine and colon (*in situ* method) and Caco-2 monolayers (*in vitro* method) obtained by comparison with metoprolol as high permeability model drug. A BCS-based classification derived from Figure 6 and considering a cut-off value of fraction absorbed in colon of 50% is also shown.

DISCUSSION

The main purpose of this study is to validate the technique of *in situ* perfusion in colon and intestine of Wistar rat based on Doluisio's method and to compare the permeability values in both segments with permeability values in two Caco-2 cells-based models. A second objective is to explore whether the BCS system could be applicable for colonic absorption, at least for permeability classification and screening for CRS development. To reach these objectives, several drugs with different physicochemical characteristics (acids, bases, and lipophilicity) and transport mechanism have been chosen (see Table 1). Compounds have been assayed at a high concentration (500 μM) to ensure saturating conditions in the case that the tested compound is absorbed through a specialized, carrier-mediated mechanism. In these conditions (high concentration), the contribution of the transporters in both intestinal segments (small intestine and colon) would be negligible and absorption process could be described as a passive diffusion mechanism.

Our research shows that the correlation obtained between the permeability values in small intestine and colon is excellent ($R^2 > 0.9$). There are not statically significant differences in the permeability of both segments assayed for most of the drugs but for three low permeability compounds: terbutaline, ranitidine, and atenolol. Interestingly, for these basic drugs, colonic permeability was higher in our rat model. These results are not because of differences in ionization degree in intestine versus colon as the perfusion solution were prepared and buffered at the same pH (7.00) for both segments.

Other authors have shown that polar drugs are less permeable in colon than in small intestine, whereas high permeability drugs present similar or higher permeability in colon in a rat model and in human tissue in Ussing chambers[48,56]; thus, our results present a different trend. On the contrary, Rozehnal et al.[49] did not found significant differences between human small intestine and human colon in Ussing chambers. From our results in rat colon and the comparison with the two models of Caco-2 cells monolayers, it seems that paracellular pathway in rat colon is less restrictive than in human intestine or at least there are less regional differences in the rat. Rat colon showed better correlation with Caco-2, 4 days model in which the paracellular pathway is less tight. On the contrary, the correlation between human colon permeability obtained in Ussing chambers and rat colon permeability is good (see Fig. 3), but demonstrated higher permeability values in rat colon compared with human tissue (slope close to 1.5). This linear correlation also reflects the difference in surface area for transport that is higher in human tissue (represented by the intersection with Y axis ~30). The higher permeability values in rat colon versus human tissue in spite of the higher surface area available in the human tissue could reflect higher paracellular permeability in the rat model.

As it can be seen in Figure 4, the relationship between permeability values obtained in rat small intestine with Doluisio's technique and human fraction absorbed can be combined with permeability data obtained in rat small intestine with single-pass perfusion method as well as with rat tissue in Ussing chambers.[50,51] In accordance with these results, recently a good correlation between permeability values obtained in rat small intestine using these two experimental techniques (single-pass and Doluisio's method) in different laboratories has been demonstrated.[57] This indicates the robustness of the rat model for predicting human Fa and the possibility of using this curve of combined data to predict oral fraction absorbed without the need of developing a full correlation with 20 model compounds but instead, running one or two model drugs of low, intermediate, and high permeability and checking whether the data fall in the same line. A similar approach has been proposed for the human permeability versus human Fa correlation.[48]

Figure 5 shows that a good sigmoidal relationship can be obtained with all the experimental systems used in this work between permeability values and human fractions absorbed but with a shift to higher permeability values in the *in situ* models (rat intestine or colon). This difference in the permeability ranges in cell culture versus whole tissue models reflects the difference in surface area available for transport as in the cell model only microvilli are present, whereas in the tissue or in the animal model, villi and folds contribute to a higher area availability.

Figure 5 also makes evident the higher permeability values obtained in rat colon versus small intestine in particular for low permeability drugs. Even if the differences in permeability between intestine and rat colon were statistically different only for three compounds, the fitted curve permits to point out the difference between both regions. These results could be further explored by performing experiments with markers of paracellular permeability with a wider range of molecular weights.

Finally, to validate the rat colon perfusion method to predict human colon absorption, it was necessary to have data availability from permeability values in human colon and/or human fraction absorbed in this region. Only recently, these data have been published,[48,49] making this comparison possible. In Figure 3, a good correlation between human colon tissue and rat is shown. In Figure 6, a sigmoidal correlation between rat colon permeability and human fraction absorbed in colon is shown. In the same figure, the correlation between human colon tissue and human colon Fa have been represented to show the shift to higher permeability values in rat colon, which nevertheless show a similar relationship.

Eventually, that means that rat colon permeability values can be used for classification purposes in high–low colon permeability compounds. In that case, the boundary value for classification has to be selected by considering the objective of the classification, that is, if the objective is to explore CR developability, a permeability value ensuring Fa > 50% could be reasonable (in our rat model that corresponds to a permeability value of 4.6×10^{-5} cm/s). Table S2 in Supporting Material shows the permeability class assigned to the different drugs assayed in this work based on the different experimental models and considering metoprolol as high permeability model, and the classification for CR developability based in our rat colon permeability values and the correlation with human colon Fa values (from Fig. 6).

CONCLUSION

In situ rat perfusion technique in colon and the same technique in small intestine are both equally useful to predict human oral fraction absorbed. Rat perfusion with Doluisio's method and single-pass provided a similar range of values demonstrating the robustness of the animal model and the possibility of combining data from different laboratories.

Rat colon permeability is well correlated with Caco-2 cell 4 days model reflecting higher paracellular permeability. Rat colon permeability values are also higher than human colon permeability values. In spite of the magnitude differences between rat colon and human colon, a good sigmoidal relationship has been shown between rat colon values and human fraction absorbed in colon indicating that rat colon perfusion can be used for compound classification and screening of candidates for CR development.

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Development of an ion-pair to improve the colon permeability of a low permeability drug: Atenolol

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ABSTRACT

To ensure the optimal performance of oral controlled release formulations, drug colon permeability is one of the critical parameters. Consequently developing this kind of formulations for low permeability molecules requires strategies to increase their ability to cross the colonic membrane. The objective of this work is to show if an ion-pair formation can improve the colon permeability of atenolol as a low permeability drug model. Two counter ions have been tested: brilliant blue and bromophenol blue. The Distribution coefficients at pH 7.00 (D_{pH7}) of atenolol, atenolol + brilliant blue and atenolol + bromophenol blue were experimentally determined in n-octanol. Moreover, the colonic permeability was determined in rat colon using *in situ* closed loop perfusion method based in Doluisio's Technique. To check the potential effects of the counter ions on the membrane integrity, a histological assessment of colonic tissue was done. The results of the partitioning studies were inconclusive about ion-pair formation; nevertheless colon permeability was significantly increased by both counter ions (from 0.232 ± 0.021 cm/s to 0.508 ± 0.038 cm/s in the presence of brilliant blue and to 0.405 ± 0.044 cm/s in the presence of bromophenol blue). Neither damage on the membrane was observed on the histological studies, nor any change on paracellular permeability suggesting that the permeability enhancement could be attributed to the ion-pair formation.

1. Introduction

The oral route is the preferred choice for drug administration because it is the most physiological and convenient for the patient. Passive diffusion is one of the main mechanism for oral absorption, thus there are many drugs with low permeability, due to the selectivity of biological membranes: narrow range of molecular weight, lipophilicity and charge state (Dahan et al., 2009; Martinez and Amidon, 2002). Hydrophilic and ionic molecules have difficulties to permeate the intestinal membrane because of its lipid nature (Shen, 2009).

Small intestine is considered to be the main site for absorption after oral administration due to its anatomical and, physiological features. Nevertheless, almost the whole gastrointestinal tract can absorb nutrients and drugs. In fact, the large intestine and, in particular, the colon can contribute significantly to the absorption of many compounds (Masaoka et al., 2006; Sjogren et al., 2014; Tannergren et al., 2009; Ungell et al., 1998). The larger residence time in the colon compared with the small intestine helps in the complete absorption of some drugs. Moreover, absorption in the colon has great relevance for oral controlled release formulations. Controlled release products have many advantages compared to oral immediate release products: reduced number of daily doses, reduced plasma fluctuations which is reflected in less side effects and, therefore better patient compliance (Tannergren et al., 2009; Thombre, 2005). Low permeability drugs in controlled release formulations could not be completely absorbed, so it is necessary to study strategies to increase their permeability through the colonic membrane.

There are several strategies to overcome low membrane permeability; for example, the use of excipients or surfactants as permeability enhancers (El-Maghraby and Alomrani, 2011; Shrestha et al., 2014; Takizawa et al., 2013). Prodrug strategy has been also successfully used nevertheless a prodrug is a new molecular entity with different characteristics which may affect the drug function (Cao et al., 2012; Ettmayer et al., 2004; Han and Amidon, 2000; Nofsinger et al., 2014; Zhang et al., 2013). Another strategy is ion-pair formation; an ion-pair is a pair of oppositely charged ions held together by Coulomb attraction without formation of a covalent bond. They behave like a single unit and partition into the membrane as a more lipophilic unit. Hydrophobic ion pairing technique has been used to increase the hydrophobicity of molecules containing ionizable groups. The pair dissociates when diluted or displaced after absorption in blood. An ion-pairing approach has several

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advantages: it can improve stability and permeability across biological membranes, become a valuable tool for enhancing solubility and stability in an organic solvent, the formation of ion-pairs normally does not entail an alteration in the structure and function of drug and eliminates the need for prodrug uptake by transports and activation by specific enzymes (Miller et al., 2009; Suresh, 2011).

In the literature many successful studies about the application of ion-pair approaches through several administration routes can be found. An ion-pair can improve drug permeation by transdermal, ocular, parenteral or inhalation route (Sarveiya et al., 2004; Tan et al., 2009; Trotta et al., 2003; Zara et al., 1999; Zhou et al., 2002). Of course, it can also improve oral absorption of drugs with poor intestinal permeability (Miller et al., 2009, 2010; Samiei et al., 2013) and to assist in designing better dosage forms for alternative routes of administration (Suresh, 2011). Highly polar drugs, ionized in the physiological pH range and poorly permeable are ideal candidates for an ion-pair development. Atenolol accomplishes these characteristics, shows low permeability in small intestine and colon (Lozoya-Agullo et al., 2015), moreover atenolol is considered as a low permeability marker (Lennernas, 2014; Tannergren et al., 2009).

The aim of this paper was to study the colonic absorption of the basic drug atenolol in the presence of two acidic counter ions: brilliant blue and bromophenol blue, using an *in situ* rat perfusion method in order to explore the feasibility of the ion-pair strategy for the colon membrane. Previously, the octanol-buffer partitioning studies were carried out for atenolol, atenolol-brilliant blue and atenolol-bromophenol blue. In order to support the hypothesis of ion-pair formation and to exclude membrane damage due to the counter ions, colon histological studies by microscopy were performed. The results obtained will be very useful for the development of controlled release formulation of low permeability drugs as atenolol.

2. Materials and methods

Atenolol, brilliant blue, bromophenol blue, 1-octanol and trifluoroacetic acid were purchased from Sigma-Aldrich. Methanol, acetonitrile and water were HPLC grade. All other chemicals were of analytical reagent grade.

Fig. 1 shows the major micro species at pH 7.4 for atenolol (pKa: 9.60); bromophenol blue (pKa: 4.0) and brilliant blue (pKa: 5.63, 6.58) (Flury and Flühler, 1995; Parvin et al., 2012; http://www.chemicalize.org/).

2.1. HPLC analysis

Atenolol samples in the absence and in presence of counter ions were analyzed using a Perkin-Elmer HPLC with fluorescence detection at 231 nm for excitation λ and 307 nm for emission λ. Mobile phase consisted of a mixture of 75:12.5:12.5 (v/v/v) water with 0.01% (v/v) of trifluoroacetic acid (pH 3): methanol:acetonitrile. Stationary phase was a μBondapak 250 mm × 4.6 mm C-18 column (5 μm particle size) at room temperature. Flow rate was 1 mL/min and the injection volume was 10 μL. Retention time of atenolol was 4.5 min. The standard calibration curves were prepared by dilution from the drug solution assayed. Sample concentrations were within the linear range of quantitation for all the assays. Analytical method was validated in terms of specificity, selectivity, linearity, precision and accuracy.

2.2. Partitioning studies

Solutions of atenolol in the presence and absence of counter ions were evaluated by partitioning experiments for 4 h between an organic phase:1-octanol and an aqueous phase. The assays were performed with two different aqueous phases: pure water and sodium phosphate buffer (50 mM), the pH of aqueous phase was adjusted to 7 (Miller et al., 2009). 1-Octanol was saturated with the aqueous phase and the aqueous phase was saturated with 1-octanol. Atenolol with and without counter ions was dissolved in the saturated aqueous phase and an equivalent volume of saturated 1-octanol was added. Assays were performed in three conditions: atenolol, atenolol + brilliant blue and atenolol + bromophenol blue. The solutions were prepared at 0.5 mM concentration for both atenolol and for the counter ions. *i.e.* the molar relationship drug:counter ion was 1:1. The experiments were carried out at 37 °C under constant stirring at 100 rpm for 4 h. Three samples were taken of each solution to assess reproducibility, at 0.5 h, 1 h, 2 h and 4 h. The octanol and aqueous phases were then separated by centrifugation and the atenolol concentrations in the aqueous phase were determined by HPLC. The amount disappearing from the aqueous phase was considered as partitioned into the organic phase. The distribution coefficient (D_{pH7}) was calculated as the atenolol concentration ratio between the organic phase and the aqueous phase according to the following equation:

$$D = \frac{(Qaq_i - Qaq_f)/Vorg}{Caq_f} \quad (1)$$

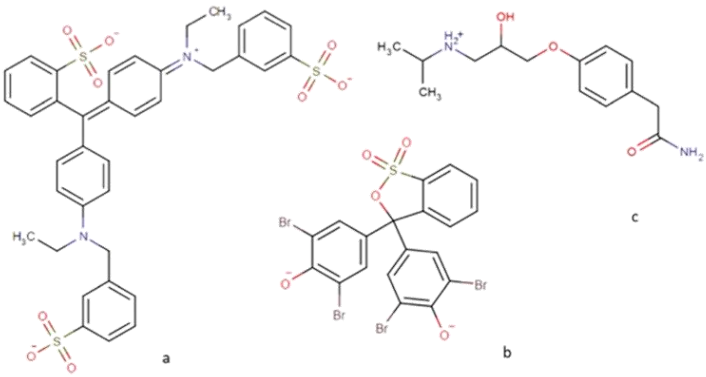

Fig. 1. Major micro species at pH 7.4. a: brilliant blue, b: bromophenol blue, c: atenolol.

Where Qaq_i is the atenolol initial amount in the aqueous phase, Qaq_f is the atenolol amount in the aqueous phase at the end of the experiment, Ca_{qf} is the final drug concentration in the aqueous phase and *Vorg* is the volume of the organic phase.

2.3. Absorption studies

Male Wistar rats were used in accordance with 2010/63/EU directive of 22 September 2010 regarding the protection of animals used for scientific experimentation. The Ethics Committee for Animal Experimentation of the University of Valencia approved the experimental protocols (Spain, code A1330354541263).

The absorption rate coefficients and the permeability values of atenolol were determined in the colon ($n = 6–7$) in the absence and presence of counter ions by using *in situ* "closed loop" perfusion method based in Doluisio's Technique (Doluisio et al., 1969) modified to adapt it to the colon (Lozoya-Agullo et al., 2015; Yuasa, 2008). Male Wistar rats (body weight, 250–300 g) fasted 4 h and with free access to water were used for these studies. Rats were anesthetized using a mixture of pentobarbital (40 mg/kg) and butorphanol (0.5 mg/kg). A midline abdominal incision was made. The intestinal segment was manipulated with care in order to minimize any intestinal blood supply disturbances. The perfusion technique consists of creating an isolated compartment in the intestinal segment of interest, with the aid of two syringes and two three-way stopcock valves. An incision was performed at the beginning of the colon just after the cecum sac. Surgical ligature to a catheter was placed at the incision. In order to remove all intestinal contents, the large intestine was copiously flushed with a physiologic solution: isotonic saline (pH 7.5) with 1% Sörensen phosphate buffer (v/v), 37 °C. After that, a second incision was performed at the end of the colon and was tied up and connected to a second catheter. Both catheters were connected to glass syringes using stopcock three-way valves. The colon was carefully placed back into the peritoneal cavity and the abdomen was covered with a cotton wool pad avoiding peritoneal liquid evaporation and heat losses. Once this system is set up, the colon is an isolated compartment where the drug solution (5 mL) can be introduced and sampled with the aid of the syringes and stopcock valves. The samples were collected every 5 min up to a period of 30 min.

Perfused drug solutions were prepared in isotonic saline buffered with Sörensen phosphate buffer (66.6 mM), pH was adjusted to 7 and the concentration was 500 μM for atenolol and counter ions.

At the end of the experiments the animals were euthanized. In order to separate solid components (mucus, intestinal contents) from the samples, they were centrifuged 5 min at 5000 rpm. All samples were analyzed by HPLC with the method described above.

There is a reduction in the volume of the perfused solutions at the end of the experiments; therefore, a correction became necessary in order to calculate the absorption rate constants accurately. Water reabsorption was characterized as an apparent zero order process. A method based on direct measurement of the remaining volume of the test solution was employed to calculate the water reabsorption zero order constant (ko). The volume at the beginning of the experiment (V_0) was determined on groups of three animals, while the volume at the end (V_t) was measured on every animal used. The concentration in the samples was corrected as

$$C_t = C_e(V_t/V_o) \quad (2)$$

where C_t represents the concentration in the gut that would exist in the absence of the water reabsorption process at time t and C_e the experimental value. The C_t values (corrected concentrations) were used to calculate the actual absorption rate coefficient (Martin-Villodre et al., 1986).

The absorption rate coefficients (ka) of compounds were determined by nonlinear regression analysis of the remaining concentrations in lumen C_t *versus* time.

$$C_t = C_0 e^{-ka \cdot t} \quad (3)$$

The absorption rate coefficients (ka) were transformed into permeability values with the following relationship:

$$\text{Papp} = \text{Ka } R/2 \quad (4)$$

where R is the effective radius of the intestinal segment. R value was calculated considering the colon as a cylinder with the relationship:

$$\text{Volume} = \pi R^2 L \quad (5)$$

Estimation was done using a 5 mL perfusion volume and an intestinal length of 10 cm.

2.4. Histological assessment of colonic tissue

Colonic tissue was examined under the microscope in order to evaluate the possible physiological changes due to the dyes used as counter ions. The tissues were examined and compared after the perfusion with the three work solutions: atenolol 0.5 mM solution, atenolol 0.5 mM with brilliant blue 0.5 mM solution and atenolol 0.5 mM with bromophenol blue 0.5 mM solution. Colonic tissue without any drug solution perfused was taken as reference of healthy tissue (control).

After the *in situ* experiments the animals were sacrificed and the colon was extracted. 1 cm from the proximal colon, 1 cm from the medial colon and 1 cm from the distal colon were caught and placed into a microscope slide cassette. These cassettes were put in a formaldehyde solution (4.5% (v/v) dissolved in PBS) for 24 h. Then the colon segments were processed (dehydrated, cleared and infiltrated with paraffin wax), embedded and cut into 5 μm sections using a microtome. The paraffin was then removed using xylene, and the samples were hydrated, stained with hematoxylin and eosin, dehydrated and examined under the microscope.

2.5. Data analysis

Permeabilities determined *in situ* for the three different assayed solutions (atenolol, atenolol with brilliant blue and atenolol with bromophenol blue) were compared using ANOVA and Scheffé *Post Hoc* test to detect the existence of significant differences at the 0.05 probability level. The statistical comparison was made using the statistical package SPSS, V.20.

3. Results

Table 1 summarizes the results of the partitioning studies of atenolol alone, atenolol with brilliant blue and atenolol with bromophenol blue, for the two aqueous phases assayed.

Table 2 shows the absorption rat coefficients, Ka, calculated with Eq. (3), and the apparent permeability values, calculated with Eq. (4), of the 3 assayed solutions determined in rat colon with the Doluisio's method. Table 2 shows as well as the statistical analysis to compare the absorption of atenolol alone and atelonol absorption in the presence of the counter ions.

Fig. 2 presents a comparative graphic of Papp values in the colon and Fig. 3 shows the colon lumen concentrations of atenolol *vs* time in the three assayed conditions. Both figures show a higher absorption of atenolol when it is perfused with counter ions.

Table 3 shows the phenol red permeability data perfused with increasing concentrations of brilliant blue to check if the paracellular route is altered. Phenol red is a non-absorbed marker with a negligible absorption when the intestinal membrane is not altered (Chowdhury and Islam, 2011).

Table 1
Partitioning studies of the three tested solutions between 1-octanol and two different aqueous phases (sodium phosphate buffer and pure water). The results correspond to the data obtained after 4 h partitioning between the two phases (average ± standard deviation; $n = 3$).

	Partitioning studies 1-octanol/phosphate buffer		Partitioning studies 1-octanol/water	
	D	% C_0 atenolol in aqueous phase ± SD	D	% C_0 atenolol in aqueous phase ± SD
Atenolol	0.001	99.99 ± 0.95	0.016	96.28 ± 0.26
Atenolol + brilliant blue	0.055	94.55 ± 0.80	0.056	93.77 ± 1.98
Atenolol + bromophenol blue	0.001	99.99 ± 0.24	0.128	87.57 ± 3.04

Table 4 summarizes the final water volumes measured at the end of the perfusion experiments in the absence and presence of counter ions. There are no significant differences within the three groups assayed.

Fig. 4 shows the microscopy images of the histological assessment of colonic tissue for four different conditions: a blank without drug solution, atenolol solution, atenolol-brilliant blue solution and atenolol-bromophenol blue solution. Different sections of colon were examined: proximal, medial and distal colon. This assay allows comparing the structure of the colonic membrane in standard conditions and the potential changes in the presence of any xenobiotic.

4. Discussion

Atenolol is a β-blocker widely used for the treatment of hypertension, angina pectoris, cardiac arrhythmia and heart attack. It is commercially available as conventional tablet and is usually administered once daily. But, exceptionally, it can be administered two or three times a day (Liu and Wang, 2008; Xue et al., 2015). Taking many doses would lead to large fluctuation in drug plasma concentration and side effects on patients. Therefore, it is desirable to use controlled released systems which solve these problems (Liu and Wang, 2008; Xue et al., 2015). Moreover, the controlled release approach is advantageous for the treatment of diseases that have peak symptoms in the early morning and exhibit circadian rhythms, such as cardiac arrhythmias or hypertension (Kumar et al., 2013; Patel, 2011).

Atenolol shows a human oral bioavailability of 50% and a human fraction absorbed of 57% and is classified as a low permeability drug in the small intestine and colon (Lozoya-Agullo et al., 2015; Tannergren et al., 2009). The problem of applying controlled released formulations is that low permeability drugs, as atenolol, are not completely absorbed. In order to ensure their complete absorption, it is necessary to study strategies to increase their permeability through the colonic membrane where the residence time is enough to allow a complete absorption in favorable conditions. On the other hand, atenolol has a secondary amino group, it is a basic drug (pKa = 9.6) ionized with positive charge in the physiological pH range. It is a hydrophilic drug and its high polarity is the main reason of its low permeability. Due to these features, atenolol is a perfect candidate to enhance its permeability through an ion-pair development.

Literature described the formation ion-pairs of drugs with a basic functional group using anionic dyes (Al-Ghannam, 2006; Cardoso et al., 2007; Onal and Caglar, 2007). Therefore, in our research, two acidic dyes, negatively charged at colonic pH, were selected as counter-ions: brilliant blue and bromophenol blue. Bromophenol blue was chosen because Al-Ghannam et al. describe the successful ion-pair formation with this dye and atenolol (Al-Ghannam, 2006). The use of bromophenol blue in humans is limited to the field of ophthalmology, particularly in ocular surgery to improve the visualization of preretinal tissues and membranes during vitrectomy, thus, reduces surgical risks; but high concentrations (≥1%) may have significant toxic effects (Balaiya et al., 2011; Dib et al., 2009; Morales et al., 2010).

Brilliant blue was chosen as a counter ion because it is an acidic dye, like the bromophenol blue, but, in addition, it is approved as a food additive. Previous works have concluded that a suitable counter ion for atenolol was bromophenol blue (Al-Ghannam, 2006), but this acidic counter ions is toxic. For this reason, a less toxic counter ion was selected (brilliant blue). Brilliant blue (E133) it is a colorant for foods and other substances so should be consider as GRAS (generally regarded as safe). Brilliant blue is a colorant food additive widely used all over the world, it is employed in many foods such as canned peas, dairy products, drinks, packet soups, sweets, icings and ice cream. The EFSA (European Food Safety Authority) accepts a daily intake for brilliant blue of 6.0 mg/kg bodyweight per day. Moreover, data available on the absorption, distribution, metabolism and excretion of this dye, show that it is poorly absorbed and mainly excreted unchanged in feces (EFSA, 2010).

The *in situ* rat perfusion technique can be used to predict human oral fraction absorbed according to the following equation (Amidon et al., 1995; Lennernas, 2007; Prieto et al., 2010; Sanchez-Castano et al., 2000):

$$Fa = 1 - e^{(-Papp\frac{2}{R}T)} \quad (6)$$

Where *Fa* is the fraction absorbed, *Papp* is the permeability obtained in a particular intestinal segment (either whole small intestine or rat colon), *R* represents the effective radius of the intestinal segment, (where the term 2/R derives from the ratio between the area of transport and the volume at the absorption site) and *T* is the effective absorption time,

Table 2
Absorption rate coefficients and apparent permeability values obtained by *in situ* perfusion method in rat colon (average ± standard deviation; $n = 6$–7).

	Ka ± SD (h^{-1})	Papp ± SD ($\times 10^{-4}$ cm/s)	Ratio[a]
Atenolol	0.429 ± 0.038	0.232 ± 0.021	–
Atenolol + brilliant blue	0.937 ± 0.070	0.508 ± 0.038*	2.189
Atenolol + bromophenol blue	0.747 ± 0.081	0.405 ± 0.044*	1.746

[a] Ratio between Papp obtained with ion-pair and Papp with atenolol.
* Denotes significant differences *versus* permeability of atenolol alone ($p < 0.05$).

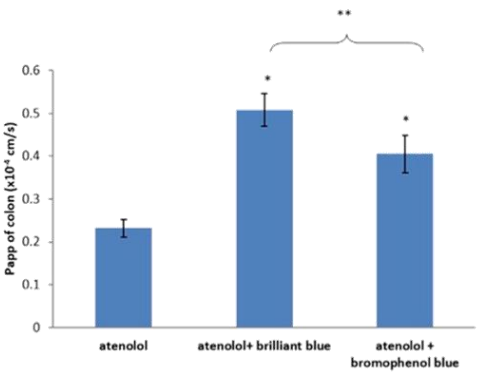

Fig. 2. Permeability values of atenolol solutions and atenolol with counter ions in rat colon with standard deviation. * Denotes significant differences *versus* atenolol ($p < 0.05$).
** Denote significant differences between atenolol + brilliant blue and atenolol + bromophenol blue.

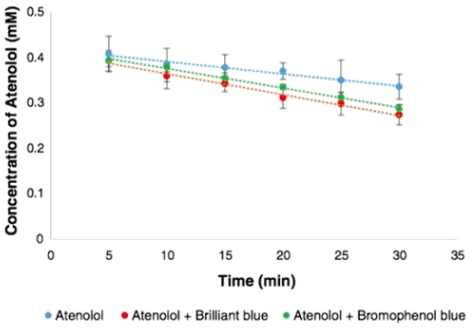

Fig. 3. Representative plots of lumen atenolol concentrations *vs* time in all conditions.

i.e. the transit time in the intestinal segment. Therefore, with higher values of *Papp*, higher *Fa* will be expected.

In the case of human absorption from the colon, we have recently demonstrated a good correlation from rat permeability values in the colon and human oral fraction absorbed from the colon (Lozoya-Agullo et al., 2015).

The permeability of atenolol in the presence of bromophenol blue and brilliant blue is increased due to the ion-pair formation, consequently the fraction absorbed achieved with ion-pair would be higher than the fraction absorbed without ion-pair.

Ion-pair is a "neutral entity" formed when ions with opposite charge (cation and anion) interact and remains together in solution thanks to electrostatic attraction but without formation of a covalent bond. As it is shown in Fig. 1 at the pH used for the perfusion studies. Atenolol is positively charged while both counter ions are in anionic form; consequently the conditions for the formation of ion-pairs are optimal.

In this study, the ion-pair formation was evaluated as a strategy to enhance the colonic permeability of atenolol. The results of preliminary partitioning experiments performed with two different aqueous phases are shown in Table 1. The ion-pair formation should increase the atenolol affinity for the lipophilic solvents, as 1-octanol. However, our results indicate that the increase of the distribution coefficient (D_{pH7}) is negligible for atenolol-bromophenol blue when the aqueous phase is phosphate buffer; but there is a notable increase of D when the aqueous phase is water instead of phosphate buffer. The increment of D for atenolol-brilliant blue is similar with both aqueous phases. A similar effect was observed by Van Gelder et al. who described an increased partition in the octanol phase and a significant decrease of drug remaining in the aqueous phase with water but not with isotonic buffer. This fact indicates that ion pair formation diminishes or even disappears when other ions are present, due to competition between counter-ion and other ions present in the medium (Van Gelder et al., 1999).

There are many studies with important increases of *in vitro* permeabilities using ion pairs but only a few of them are able to demonstrate *in vivo* effectivity. The reason of these differences remain unclear (Miller et al., 2010). For these reasons, the next step was to evaluate the *in situ* behavior. In our assays, nevertheless, the results of *in situ* experiments

Table 3
Phenol red permeability values (Papp) in rat colon with increasing concentrations of brilliant blue (average ± standard deviation). SD: Standard deviation.

	Papp phenol red ± SD ($\times 10^{-4}$ cm/s)
Phenol red (0.5 mM) + brilliant blue (0.5 mM)	0.069 ± 0.024
Phenol red (0.5 mM) + brilliant blue (2.5 mM)	0.088 ± 0.024
Phenol red (0.5 mM) + brilliant blue (5 mM)	0.060 ± 0.022

Table 4
Final water volumes (Vf) measured at the end of the perfusion experiments and absorption rate coefficient (Ka) for every animal used, in absence and presence of counter ions. S.D.: Standard deviation. CV%: Coefficient of variation. NS: not statistically significant differences *versus* atenolol alone. S: statistically significant differences *versus* atenolol alone ($p < 0.05$).

	Atenolol		Atenolol + brilliant blue		Atenolol + bromophenol blue	
Animal	Vf (mL)	Ka (h^{-1})	Vf (mL)	Ka (h^{-1})	Vf (mL)	Ka (h^{-1})
1	3.7	0.43	3.5	0.96	4.1	0.81
2	4.7	0.39	4.1	0.99	4.4	0.63
3	4.6	0.46	4.4	0.88	4.3	0.66
4	4.2	0.45	3.5	1.06	4.2	0.80
5	4.5	0.38	4.1	0.93	4	0.82
6	4.6	0.47	4.1	0.86	3.9	0.80
Mean	4.39	0.43	3.95	0.94	4.14	0.75
S.D.	0.37	0.04	0.33	0.07	0.18	0.08
CV%	8.58	8.97	8.45	7.48	4.50	10.84
Significance	–	–	NS	S	NS	S

(Table 2) are pleasantly positive. The colon permeability of atenolol improves considerably when it is perfused with brilliant blue or bromophenol blue. Table 2 and Fig. 2 show that the differences obtained between the solutions tested are statistically significant. Moreover, Fig. 3 presents the decrease of the atenolol concentration in the colonic lumen *vs* time due to the absorption process for the three assayed conditions, this decrease is bigger with the ion-pairs.

The highest increase of permeability values has been obtained with brilliant blue, probably, because at pH 7 (the pH of the perfused solution) 99.97% has negative charge, but the bromophenol blue presents less ionized percentage (88.57%) at the same pH. Steric reasons could also favor a stronger interaction with brilliant blue than with bromophenol blue. According to the remaining amounts of atenolol in the aqueous phases after partitioning experiments, a small increase of permeability with *in situ* assays should be expected. However, the increase in absorption coefficients and apparent permeability achieved in rat colon are significant. The differences observed between the results of *in vitro* and *in situ* methods could be explained with the different features of 1-octanol and the phospholipid bilayer. The propensity for ion-pair formation and the general effect of ionic strength on membrane-buffer distribution and 1-octanol-buffer distribution are different (Obata et al., 2005; Suresh, 2011).

In order to check that the increase of atenolol permeability was due to ion-pair formation and not for membrane disturbances, a histological assessment of colonic tissue was made (Fig. 4). No important damages in the colonic membrane for the different solutions were observed. The most relevant difference was observed in the goblet cells. These cells are present in the colonic mucosa and they secrete mucus. The mucus layer protects the epithelial mucosal surface with an essential barrier function, defending the body from pathogens and irritants (Gersemann et al., 2009; Kroger-Lui et al., 2015). When comparing the two ion-pair solutions with the blank and atenolol solutions, morphological and size differences in goblet cells are observed; these changes are indicative of an inflammatory response. This tendency is larger with the bromophenol blue in the distal colon, because the distal part is the most sensitive to variations (Yan et al., 2009). In spite of the differences in goblet cells, the alterations are not important. Some authors have indicated that with the changes induced by an inflammation, the colonic function in terms of fluid absorption, secretion and epithelial barrier function, is largely preserved (Thiagarajah et al., 2014). Therefore, it is unlikely that the increased permeability is caused by a membrane disruption. In order to check that paracellular permeability was not increased, permeability of phenol red was measured in the presence of increasing concentrations of brilliant blue (from 1:1 to 1:10). Phenol red permeability remained unchanged and almost negligible at all brilliant blue concentrations (Table 3), suggesting that the paracellular route was not affected. The water reabsorption process observed in all

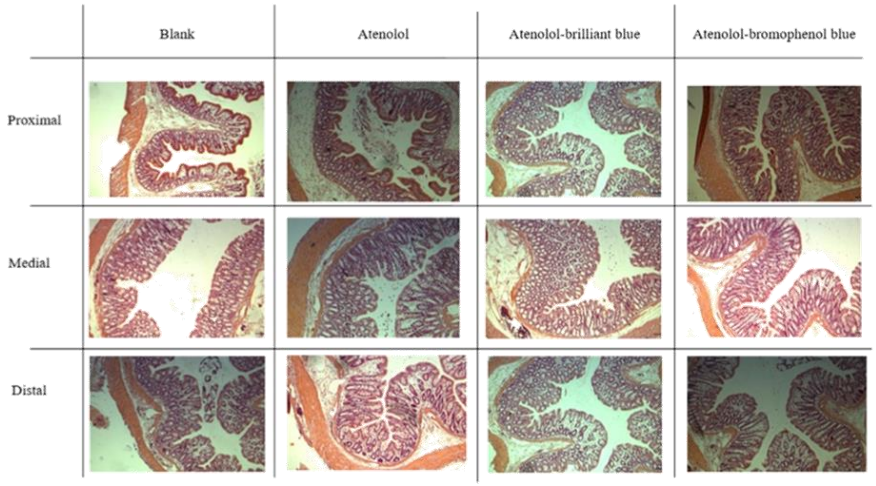

Fig. 4. Proximal, medial and distal segments of colon for four different conditions: a blank without drug solution, atenolol solution, atenolol-brilliant blue solution and atenolol-bromophenol blue solution. All the images are 4×.

the experimental conditions characterized by the final remaining water volume (4.39 mL for atenolol, 3.94 mL for atenolol + brilliant blue and 4.14 mL for atenolol + bromophenol blue) confirms that the increased permeability is not due to intestinal membrane alterations as there were no statistical differences among groups of the final water volumes. In Table 4 the average water reabsorption final volumes as well as their statistical comparison were summarized. Differences obtained between the water absorption final volumes are not statistically significant between results obtained with atenolol and both ion-pair. In addition, the water absorption process showed a similar variability in all animal groups.

5. Conclusion

In summary, it has been possible to overcome the colonic permeability limitations of atenolol using an ion pair strategy. The *in situ* perfusion assays of the ion-pairs have shown a permeability increase of atenolol in rat colon. Moreover, the histological assessment from colon tissue suggests that this increase is due to the ion-pair formation and not to membrane damage. Between the two counter ions tested, the brilliant blue seems more effective because the increase in permeability is higher. In addition, this compound is approved as a food additive, which means that it is considered safe. Available data of brilliant blue absorption show that it is a poorly absorbed compound, probably because it is charged at physiological pH. Nevertheless, it would be advisable to study brilliant blue toxicity when it is administered as an ion-pair because, the dye permeability could also be increased achieving higher plasma concentration that may cause adverse effects.

This study confirms the possibility to increase colonic permeability of atenolol with ion-pair formation. The data obtained can be useful to improve the absorption of low permeability class drugs, with similar features to atenolol, and to further design and develop more efficient and intelligent controlled release formulations.

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