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DOCTORADO EN BIOMEDICINA Y FARMACIA

FACULTAD DE FARMACIA

UNIVERSIDAD DE VALENCIA

TESIS DOCTORAL

Evaluación de la liberación de antibióticos incorporados en cementos utilizados en artroplastia

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Valencia, 2017

Departamento de Farmacia y Tecnología Farmacéutica y Parasitología

Programa de Doctorado en Biomedicina y Farmacia

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Certifican que:

El trabajo de Tesis Doctoral realizado por Javier Martínez Moreno y que lleva por título "Evaluación de la liberación de antibióticos incorporados en cementos utilizados en artroplastia", ha sido realizado bajo la dirección compartida de las mismas y reúne todos los requisitos necesarios para su presentación, juicio y calificación.

Lo que suscriben, en Valencia, a 4 de septiembre de 2017.

Matilde Merino Sanjuán Virginia Merino Sanjuán Amparo Nácher Alonso

A mis padres,

a Sara

Recuerdo los comienzos en el departamento cuando en quinto de carrera pedí la beca de colaboración en Tecnología Farmacéutica, aquel día en el que Pablo, Alex y yo fuimos y empezamos a ver qué era eso de la investigación. Recuerdo esos meses como una época dulce en la que cada día nos llevábamos algo nuevo. Y así es cómo empezó todo.

De estos años me llevo sobre todo cantidad de conocimiento y buenos amigos. En primer lugar quisiera agradecer a mis directoras de Tesis, Matilde, Virginia y Amparo por todo lo que he aprendido de vosotras, cuando creía que no había escapatoria, nos sentábamos y todo se solucionaba, habéis dado luz al proyecto. Sin vosotras esto no hubiera sido posible, gracias de nuevo.

También quiero agradecer a los compañeros con los que he pasado horas y horas en el laboratorio. Para empezar, dar las gracias a Ana por enseñarme cuando empecé, y es que aún me acuerdo los primeros días con el HPLC que sin la ayuda de Ana hubiera sido más difícil. Agradecer también a Isa por esas tardes de compañía en la que la parte experimental se hacía más amena. Gracias a Pablo, porque tu alegría en el laboratorio hacía de este un sitio mejor. Por último, gracias Alex, lo conozco desde el primer día de la carrera y siempre me ha ayudado, gracias amigo.

Tampoco puedo olvidarme de Carmina y José, los cuales siempre han estado en disposición de ayudarme en lo que era posible, siempre han estado ahí con una sonrisa.

Durante esta época, todos mis compañeros y amigos de la Farmacia del Hospital Dr. Peset han sido importantes para la consecución de esta tesis. Me llevo unos grandes amigos. Quiero agradecer a todo el personal del Servicio de Farmacia que ha hecho que me sienta muy cómodo durante mi estancia. Me llevo un gran recuerdo. Gracias a mis R mayores, Carlos, Rubén, Pau, Marina, Bea y Sandra y a mis R pequeños, Javi, Clara, Carlos, Rebeca, Ángel y Azahar porque estos años hayan sido muy productivos tanto personal como profesionalmente. Cómo no, quiero también agradecer a mi CoR, Alfons, porque me llevo un gran amigo. También agradecer a todos los farmacéuticos del Peset, gracias Marta, Ana Cris, Pilares, Ana, Juan Pablo, José Manuel, José Luis y Begoña. Finalmente estaré eternamente agradecida a mi Jefa Mónica, por enseñarme a ser un buen profesional. Gracias a todos.

Por último, quisiera agradecer a mis padres todo esto; si ahora mismo estoy escribiendo todo esto es gracias a ellos y estaré eternamente agradecidos por todo lo que me han ofrecido en la vida, os quiero. También a mis hermanos, Octa y Jorge, porque sé que cuando tengo un problema siempre están ahí para ayudarme, gracias Hermanos. Y Sara, empecé este camino a

tu lado y ahora cierro etapa a tu lado, y esto es porque me ayudas a ser cada vez mejor y espero que nada cambie. Te quiero Sara.

“Yo creo bastante en la suerte.

Y he constatado que, cuanto más duro trabajo, más suerte tengo.”

Thomas Jefferson

INDICE

ABREVIATURAS

AIC	Criterio de información de Akaike
C1	Compartimento referente al cemento cargado con antibiótico
C2	Compartimento referente al espacio articular
Cl	Aclaramiento local de antibiótico
CMI	Concentración mínima inhibitoria
CO_2	Dióxido de carbono
HPLC	Cromatografía líquida de alta resolución
K_d	Constante de velocidad de distribución desde el espacio articular al compartimento central
K_r	Constante de velocidad de retorno del antibiótico desde el compartimento central al espacio articular
K_{el}	Constante de eliminación del antibiótico
MRSA	Staphylococcus aureus resistentes a meticilina
MTT	Bromuro de [3-(4,5-dimethylthiazol – 2 -yl) - 2,5 - diphenyltetrazol
PBS	Suero bovino fetal
PMMA	Polimetilmetacrilato
Q_0	Velocidad de liberación del antibiótico
RGD	Arginina-glicina-ácido aspártico
THPC	Cloruro de tetrakis (hidroximetil) fosfonio
UFC	Unidades formadoras de colonias

PRÓLOGO

La presente tesis doctoral se estructura atendiendo a los requisitos establecidos por la Escuela de Doctorado de la Universitat de València para presentar la Tesis Doctoral por compendio de artículos. Consta de un resumen, 5 capítulos y un anexo. El resumen está escrito en español y en él se realiza una breve justificación del proyecto desarrollado, se resaltan los resultados obtenidos y las conclusiones del trabajo realizado. Los cinco capítulos siguientes corresponden al trabajo desarrollado en la Tesis Doctoral estructurado en artículos publicados en diferentes revistas científicas indexadas en el Journal Citation Reports (JCR). Así, el capítulo 1, corresponde a una revisión exhaustiva sobre el estado del arte acerca de la inclusión de antibióticos en cementos óseos centrando el trabajo, fundamentalmente en la eficacia para la prevención de infección tras una intervención quirúrgica de artroplastia y en la revisión de la cinética de liberación de diferentes antibióticos a partir de su inclusión en cementos óseos de diferente marca comercial. En el capítulo 2 se realiza el estudio de la liberación de ciprofloxacino a partir de tres cementos óseos comerciales y se analiza la influencia de la técnica de mezclado del antibiótico con el cemento óseo y de la especie química (base o sal) del ciprofloxacino en la cinética de liberación del antibiótico. Además, se evalúa la influencia de la inclusión de un segundo antibiótico (vancomicina) en la mezcla (antibiótico/cemento) sobre la elución de la quinolona. En el capítulo 3 se aborda la evaluación, mediante un ejercicio de simulación farmacocinética, de la bioactividad de las mezclas de ciprofloxacino y vancomicina incluidos en un cemento óseo. El capítulo 4 detalla el estudio de la cinética de liberación de los antibióticos ceftazidima y fluconazol a partir de las mezclas de antibiótico y cemento, así como los estudios de bioactividad realizados utilizando dos métodos microbiológicos (recuento de las unidades formadoras de colonias -UFC- y evaluación de halo de inhibición de crecimiento microbiano). En el capítulo 5 se describe el diseño de una nueva forma farmacéutica de administración de antibióticos en cirugía ortopédica, se evalúa la liberación del fármaco a partir de la misma y se analiza la biocompatibilidad de la formulación utilizando el método basado en la medida de la viabilidad celular utilizando la línea celular NIH3T3. Por último, en el anexo se incluye una copia completa de los artículos publicados o admitidos para su publicación hasta la fecha.

RESUMEN

Introducción

La artroplastia de cadera/rodilla consiste en la cirugía ortopédica que reemplaza de forma total o parcial la articulación por un implante artificial, llamado prótesis, en aquellos casos en los que el daño de la articulación es irreversible. En esta cirugía una de las complicaciones más grave se asocia al desarrollo de alguna infección, que, aunque presenta una baja prevalencia, entre el 0,5% y el 3%, en algunos casos puede ser de gravedad elevada y conduce al fracaso de la intervención, llegando incluso a desencadenar la muerte del paciente [1]. Para prevenir las complicaciones asociadas al desarrollo de infecciones, se ha propuesto, desde hace algún tiempo, la inclusión de antibióticos en el cemento óseo destinado a la fijación mecánica de las prótesis, ya que los sistemas de liberación local de antibiótico facilitan el aprovechamiento del fármaco, a la vez que reducen la prevalencia y gravedad de las reacciones adversas asociadas a estos fármacos cuando se administran por vía sistémica [2].

La combinación de antibióticos con los cementos poliacrílicos (polimetilmetacrilato -PMMA-) fue descrita por primera vez por Buchholz y Engelbrecht [3]. A partir de este estudio, los trabajos de investigación publicados en este contexto son numerosos y variados y aportan resultados contradictorios en cuanto a su capacidad de prevenir infecciones, debido a la incertidumbre sobre el posible desarrollo de resistencias a los antibióticos tras una exposición prolongada a bajas dosis de antibiótico, la eficacia y el coste de este sistema de administración. A pesar de ello, la evidencia clínica indica que el uso de cementos óseos cargados con antibióticos reduce significativamente el riesgo de infección profunda [4]; por ello, en la práctica clínica habitual se utilizan, aunque la cinética y el mecanismo de liberación de la mayoría de los antibióticos interpuestos en la matriz acrílica siguen siendo aspectos desconocidos. Las variables que influyen en el proceso de liberación del antibiótico desde el cemento que lo contiene son múltiples, entre ellas destacan la cantidad y el tipo de antibiótico incorporado al cemento [5, 6]. En este sentido, resaltar que la velocidad de liberación del antibiótico desde el cemento que lo contiene (cantidad de antibiótico liberada por unidad de tiempo) es mayor cuando se incorpora en forma líquida. Sin embargo, en la práctica clínica la utilización de formas líquidas está limitada debido a su influencia negativa sobre las propiedades mecánicas de los cementos. Por el contrario, los fármacos en estado sólido tienen un efecto insignificante sobre la estabilidad mecánica de cemento óseo, siempre y cuando la proporción antibiótico/cemento se mantenga por debajo del 10%. Otro factor importante a tener en cuenta es el tipo y porosidad del cemento óseo utilizado y la forma de preparación de la mezcla [7-10], ya que la porosidad del polímero facilita el acceso de los fluidos de disolución a la matriz del polímero y, en consecuencia, la liberación de los antibióticos a partir del cemento. Por otra parte, la porosidad está relacionada,

en gran medida, con el mayor o menor volumen de aire atrapado en el interior de la mezcla durante la manipulación, mezclado y amasado de la muestra. De ahí que las cantidades de antibiótico liberadas desde el cemento pueden diferir según se empleen preparados comerciales de cemento óseo impregnado de antibiótico premezclados o, por el contrario, se utilicen las mezclas preparadas de forma manual o mecánica en el momento previo a la intervención quirúrgica.

Se han comercializado cementos poliacrílicos de uso en artroplastias cargados con antibióticos aminoglucósidos, en particular gentamicina y tobramicina, y con antibióticos glucopéptidos [11], que han demostrado su utilidad clínica en términos de eficacia y seguridad del tratamiento. Sin embargo, en el momento actual ha incrementado el número de cepas multirresistentes, con capacidad de adherirse sobre el cemento, colonizándolo tras largos periodos de implantación y sobre las que los preparados comerciales disponibles no presentan cobertura antibiótica [11, 12]. Los microorganismos que causan infecciones con más frecuencia son del género *Staphylococcus,* en concreto *Staphylococcus aureus resistentes a meticilina* (MRSA) [13] y diferentes bacilos aerobios gram negativos [14]. De hecho, en el momento actual el incremento de resistencias de *Staphylococcus aureus* a los aminoglucósidos condiciona la eficacia terapéutica de este grupo de antibióticos. Se trata de una situación que genera preocupación, ya que el 30% de las infecciones de origen quirúrgico están causadas por cepas de *Staphylococcus aureus resistente a meticilina*, lo que determina las estrategias que deben utilizarse para el tratamiento y la prevención de las infecciones en las prótesis articulares [15]. Por otra parte, aunque las infecciones por hongos son raras, cuando se producen desencadenan complicaciones devastadoras de la artroplastia articular. Alrededor del 80% de las infecciones fúngicas de prótesis óseas están producidas por diversas especies del género *Candida* [16] y hasta el momento la eficacia de las mezclas de cementos óseos PMMA y fármacos antifúngicos ha sido poco estudiada [17].

La bibliografía disponible hasta el momento únicamente muestra dos estudios de metaanálisis que evalúan la eficacia del cemento cargado con antibióticos en la artroplastia de revisión primaria. Uno de ellos es el realizado por Parvizi *et al.* [18] y otro por Wang *et al.* [4]. Los primeros autores evaluaron la eficacia del cemento cargado con gentamicina en la artroplastia de revisión primaria y concluyeron que el cemento con carga antibiótica es capaz de reducir la tasa de infección profunda aproximadamente un 50% (de un 2,3% frente a un 1,3% cuando se utilizó cemento cargado con antibiótico). Además, esta reducción alcanzó significación estadística a favor del cemento óseo cargado con antibiótico. Wang *et al.* [4], también evaluaron la tasa de infección profunda y superficial cuando el antibiótico se incorporó

en los cementos óseos en la artroplastia de revisión primaria y obtuvieron diferencias en las tasas de infección, siendo estadísticamente significativa la diferencia en la tasa de infección profunda, pero no la diferencia en la tasa de infección superficial.

En este contexto, en el que en determinadas situaciones clínicas el riesgo de infección tras una intervención de artroplastia es elevado y la disponibilidad de cementos comerciales que incorporan los antibióticos óptimos reducida, se ha considerado oportuno estudiar la cinética de liberación de algunos antibióticos incorporados a distintos cementos óseos comerciales. De esta forma se pretende obtener información relevante orientada a facilitar la selección del fármaco más adecuado, en términos de eficacia y seguridad en cada situación, ampliando así la disponibilidad de tratamientos utilizados hasta el momento en cirugía ortopédica. Además, teniendo en cuenta que los estudios realizados hasta el momento con estos sistemas apuntan a que no reducen la tasa de infección superficial, en esta Tesis Doctoral se han realizado estudios de preformulación de una forma farmacéutica encaminada a mejorar el uso de antibióticos con fines profilácticos en las intervenciones quirúrgicas de artroplastia.

Los fármacos estudiados en esta Memoria han sido el ciprofloxacino, la vancomicina, la ceftazidima y el fluconazol.

El ciprofloxacino es una fluoroquinolona efectiva frente a microorganismos Gram-positivos y Gram-negativos. La vancomicina es un glicopéptido sumamente efectivo frente a bacterias Gram-positivas. Ambos antibióticos se presentan en estado sólido, son estables a la temperatura de fraguado de los cementos y no alteran las características mecánicas de estos, por lo que reúnen características adecuadas para ser incorporados en cementos poliacrílicos utilizados en cirugía ortopédica [19].

Las cefalosporinas son antibióticos de amplio espectro de acción que se usan de forma habitual para tratar infecciones osteoarticulares. La ceftazidima es una cefalosporina de tercera generación utilizada para el tratamiento de infecciones producidas por bacterias Gram-positivas y Gram-negativas, con actividad demostrada frente diferentes especies del género *Pseudomona* [20].

El fluconazol es un fármaco antifúngico del grupo triazol, que se utiliza para tratar las infecciones producidas por *C. albicans*. Fluconazol inhibe el citocromo P450 fúngico de la enzima 14α-demetilasa, lo que evita la formación de ergosterol, aumentando así la permeabilidad de la membrana y la destrucción de células [21].

El diseño y el estudio de la nueva forma de administración de antibióticos en cirugía ortopédica que se plantea en este proyecto se fundamenta en la formación de una matriz polimérica biodegradable, en forma de lámina o de hidrogel, en la que se incluye un antibiótico. Esta formulación estaría destinada tanto para la profilaxis como el tratamiento de infecciones osteoarticulares superficiales.

Los polímeros seleccionados para la elaboración de la forma farmacéutica han sido el quitosano y la gelatina. El quitosano presenta numerosas e interesantes propiedades biológicas. Este polisacárido es un biopolímero, ampliamente utilizado en la formulación de medicamentos, bio-reabsorbible y bioactivo [22]. El quitosano pertenece al grupo de polímeros catiónicos, caracterizado por su hidrofilicidad, biocompatibilidad, buena resistencia mecánica y posibilidad de reticularse con polianiones naturales, como la gelatina [23], o sintéticos, como el cloruro de tetrakis (hidroximetil) fosfonio (THPC) [24].

La gelatina es un biopolímero prometedor para la preparación de soportes celulares debido a que es biocompatible y biodegradable además de que presenta una elevada similitud con la matriz extracelular de los tejidos de la piel, los cartílagos y los huesos [25]. Además, presenta baja inmunogenicidad y podría facilitar el aumento de la adhesión, proliferación y diferenciación celular debido a que en su composición contiene la secuencia de aminoácidos arginina-glicina-ácido aspártico (RGD). Esta secuencia de aminoácidos se encuentra en las proteínas de adhesión de la matriz extracelular, principalmente en la fibronectina, que, al interactuar con las integrinas de la membrana celular, se ha demostrado que facilita la adhesión de las células y desencadena diferentes respuestas biológicas [26].

El THPC es un compuesto organofosforado soluble en agua, relativamente económico, compuesto por cuatro grupos hidroximetilo unidos a un átomo de fósforo electronegativo. Chung *et al.* [24] han propuesto este compuesto como un agente reticulante para los materiales proteicos, ya que permite el enlace covalente entre el agente reticulante y grupos amina. Además, estos mismos autores estudiaron el uso de THPC en el desarrollo de hidrogeles y demostraron su citocompatibilidad.

Objetivos

1. Evaluar los factores que condicionan la velocidad y la magnitud de liberación de fármacos, seleccionando ciprofloxacino como antibiótico modelo, incorporados a cementos óseos.

2. Caracterizar la cinética de liberación de ciprofloxacino, ceftazidima y fluconazol incorporados al cemento comercial Palacos® en las proporciones utilizadas en cirugía ortopédica para profilaxis y para tratamiento antibiótico.
3. Evaluar la bioactividad de las mezclas del cemento Palacos® y los antibióticos ciprofloxacino, ceftazidima y fluconazol en las proporciones utilizadas en cirugía ortopédica para profilaxis y para tratamiento antibiótico.
4. Diseñar y evaluar una nueva forma farmacéutica para la administración de antibióticos en la cirugía de artroplastia.

Material y métodos

Muestras ensayadas

Se ha evaluado la cinética de liberación de los fármacos ensayados a partir de mezclas fármaco-cemento óseo comercial e incorporado en sistemas poliméricos.

A continuación, se describen con detalle las muestras ensayadas en cada caso.

1. Preparación de las mezclas de antibiótico y cemento óseo

La preparación de las mezclas de antibiótico y cemento óseo (tabla 1) se realizó bajo campana de extracción de gases con el objeto de evitar la inhalación de los vapores del componente líquido de los cementos.

Tabla 1.- Composición, tipo de mezclado y condiciones del ensayo de liberación de fármaco de las muestras seleccionados.

Condición del ensayo de liberación	Mezclado	Antibiótico	Cemento óseo	Proporción		Identificación de muestras
				1/40	4/40	
Estática	Manual	Ciprofloxacino clorhidrato	Simplex®	✓		A
			Lima®	✓		B
			Palacos®	✓		C
		Ciprofloxacino base	Lima®	✓		D
		Ciprofloxacino clorhidrato y vancomicina	Simplex®	✓		E
			Lima®	✓		F
			Palacos®	✓		G
	Mecánico	Ciprofloxacino clorhidrato	Simplex®	✓		H
			Lima®	✓		I
			Palacos®	✓		J
Dinámica	Manual	Ciprofloxacino clorhidrato	Palacos®	✓		K
		Ciprofloxacino clorhidrato			✓	L
		Ceftazidima		✓		M
		Ceftazidima			✓	N
		Fluconazol		✓		O
		Fluconazol			✓	P

Para la obtención de estas muestras se siguió el siguiente procedimiento:

- En un vaso de precipitados de vidrio (componente inerte para los cementos acrílicos), se depositaron las cantidades necesarias del componente sólido del cemento comercial (Palacos®, Simplex® o Lima CMT 1®) y del antibiótico seleccionado, mezclando los polvos con ayuda de una varilla de vidrio hasta obtener una mezcla homogénea de los sólidos. Las proporciones (antibiótico/cemento) estudiadas han sido 1/40 y 4/40. La primera es la proporción utilizada en cirugía ortopédica con fines profilácticos y la segunda es la proporción seleccionada cuando se requiere tratamiento antibiótico en el caso de que se haya diagnosticado la infección.

- Finalizada la mezcla de los componentes sólidos, se incorporó el componente líquido del cemento y se procedió al amasado de la mezcla, bien de forma manual, con ayuda de una varilla de vidrio, o de forma mecánica, con el mezclador comercial de vacío (Palamix Uno®).

- Tras 1-2,5 minutos de mezclado, tiempo necesario para que la temperatura de la mezcla permitiera su manipulación, se extrajo la masa del vaso de precipitados, o del mezclador comercial, y se completó el amasado con ayuda de las manos. Este proceso se dió por finalizado cuando la mezcla dejó de adherirse a los guantes del operador.

- A continuación, la masa formada se depositó en moldes de teflón de dimensiones conocidas. Esta fase debe realizarse con rapidez y finalizarse antes de que se produzca el fraguado completo del cemento, ya que de lo contrario la rigidez que alcanza la masa impediría la obtención de las muestras de forma y dimensión controlada.

- Por último, las muestras se mantuvieron en el molde durante un periodo de 15 minutos, tiempo necesario para completar la fase de fraguado. A continuación, se extrajeron con sumo cuidado, se pesaron y se midieron sus dimensiones. Finalmente, se mantuvieron protegidas en un medio adecuado para evitar su contaminación hasta su posterior análisis.

2. Preparación de las láminas e hidrogeles

En la tabla 2 se detalla la composición de las muestras poliméricas elaboradas. Para la preparación se dispersó el quitosano en una solución acuosa acidificada a una temperatura de 45°C. A continuación, se disolvió en ella el antibiótico y, posteriormente, se incorporó el agente reticulante. Esta mezcla se agitó con ayuda de un *vortex* durante aproximadamente 10 segundos. Finalmente, se depositaron 27 g de algunos de los hidrogeles en una placa petri y se mantuvieron en estufa a 40°C durante 48 horas, hasta su deshidratación.

Tabla 2.- Composición de los hidrogeles y láminas poliméricas formuladas.

		Identificación muestras																								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Fármaco	Ciprofloxacino	X	X	X	X	X	X	X	X									X	X	X	X	X	X	X	X	X	X
	Gentamicina									X	X	X	X	X	X	X	X										
Concentración de fármaco	0,5 mg/cm^2	X		X		X		X		X		X		X		X		X	X			X					
	1 mg/cm^2																						X				
	2 mg/cm^2		X		X		X		X		X		X		X		X			X	X			X	X	X	X
Acidificante	Ác. Acético 0,5%	X	X			X	X			X	X			X	X							X	X	X	X	X	X
	Ác. láctico 1%			X	X			X	X			X	X			X	X	X	X	X	X						
Polímero	Quitosano 1,5%	X	X	X	X					X	X	X	X					X		X							
	Quitosano 2%					X	X	X	X					X	X	X	X		X		X	X	X	X	X	X	X
Reticulante	Gelatina 6·%																	X	X	X	X						
	THPC 12%																					X	X	X	X		
	THPC 24%																									X	
	THPC 36%																										X
Forma farmacéutica	Lámina	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X			
	Hidrogel																								X	X	X

Determinación analítica

La cuantificación del fármaco (ciprofloxacino, ceftazidima y fluconazol) en las muestras experimentales se ha realizado mediante cromatografía líquida de alta resolución (HPLC) de fase inversa con detección por ultravioleta (Perkin Elmer® Series 200 equipado con un detector Waters 484®), utilizando como fase estacionaria una columna cromatográfica Kromasil® C18, de 150 mm x 4,6 mm y las condiciones de ensayo para cada antibiótico que se indican en la tabla 3 [27-29]. La determinación de vancomicina se realizó mediante inmunoanálisis de micropartículas quimioluminiscentes utilizando Architect i1000SR (Abbot Laboratories) [30].

Tabla 3.- Condiciones cromatográficas utilizadas para cada fármaco ensayado.

Fármaco	Fase móvil	Longitud de onda	Flujo	Tiempo de retención
Ciprofloxacino base	Ác. acético 0,1M: acetonitrilo (80:20)	254 nm	1mL/min	3 min
Ciprofloxacino clorhidrato	Ác. acético 0,1M: acetonitrilo (80:20)	254 nm	1mL/min	3,2 min
Ceftazidima	Metanol: agua (70:30)	245 nm	1mL/min	4,3 min
Fluconazol	Metanol: agua (60:40)	268 nm	1mL/min	3,5 min

Estudios de liberación

Los ensayos de liberación se realizaron con el fin de evaluar la cinética del proceso para cada uno de los fármacos desde las muestras elaboradas y se diseñaron teniendo en cuenta las características de las muestras (cementos óseos o hidrogeles, laminados o fluidos). En el caso de las muestras procedentes de las mezclas de antibiótico y cemento óseo los ensayos se realizaron en condiciones estáticas y dinámicas. A continuación, se describen los detalles de los estudios de liberación de antibiótico en cada una de las situaciones.

Cinética de liberación del fármaco a partir del cemento óseo

Condiciones estáticas. Las muestras de ensayo se depositaron individualmente en tubos Pírex de 40 mL de capacidad y a continuación se incorporaron 10mL de una disolución tampón fosfato 160mM a pH=7,4. Los tubos se taparon con ayuda de un tapón de rosca y se mantuvieron en agitación constante en un baño termostatado (baño Unitronic Orbital

Selecta) a 37ºC. A tiempos prefijados se procedió a la toma de muestras y reposición del volumen tomado. Los tiempos seleccionados fueron 1, 3, 5, 7, 24, 32, 48 56, 72, y 168 horas después de la inmersión y posteriormente una vez a la semana hasta completar un período de 8 semanas (muestra final fue tomada 56 días después de la inmersión).

Condiciones dinámicas. Se procedió de modo similar al descrito en el ensayo anterior, pero se empleó un aparato de disolución de flujo continuo aceptado por la USP como método 4 en ensayos de disolución en flujo continuo (Sotax CE7®) seleccionando un flujo de trabajo de 12 mL/min que se mantuvo con recirculación durante 48 h. A las 0,25, 0,5, 0,75, 1, 1,5, 2, 3, 4, 5, 24, 26, 28 y 48 horas se realizó la toma de muestra de 1mL y su reposición.

Cinética de liberación del fármaco a partir de los hidrogeles

En el caso de los hidrogeles, se depositaron 2,12 g de muestra en el interior de una malla metálica. En el caso de las láminas de hidrogel, se recortaron piezas de 1 cm^2 de superficie. La preparación y desarrollo del ensayo es la misma que la indicada en el epígrafe *condiciones estáticas* de las muestras de cementos. En el caso de los hidrogeles, se obtuvieron muestras a las 0,5, 1, 1,5, 2,5, 3,5, 4, 27 y 52 horas tras la inmersión y posteriormente una vez al día hasta completar un periodo de 6 días desde el inicio del ensayo. Para el desarrollo del ensayo de las láminas, se obtuvieron muestras a las 0,16, 0,33, 0,66, 1, 1,5, 2, 3, 4, 24, 25,5 y 52 horas tras la inmersión y posteriormente una vez al día hasta completar un periodo de 6 días desde el inicio del ensayo.

Todos los ensayos se realizaron por triplicado.

Cinética de liberación

Para cada tiempo de toma de muestra, se determinó la cantidad de fármaco liberado (Mt) y se expresó en términos relativos a la cantidad máxima de fármaco liberado a tiempo infinito (M∞). Los valores experimentales así obtenidos se utilizaron para seleccionar el modelo más probable que describe la cinética de liberación del antibiótico desde el soporte que lo contiene. Los modelos evaluados para conocer la cinética de liberación del fármaco fueron los siguientes:

1. Modelo de orden 0 (ecuación 1):

$$\frac{M_t}{M_\infty} = k \cdot t \quad \text{ecuación 1}$$

Este modelo asume que la velocidad de liberación del fármaco es constante con el tiempo.

2. Modelo de Higuchi (ecuación 2):

$$\frac{M_t}{M\infty} = k \cdot t^{0.5} \quad \text{ecuación 2}$$

Este modelo asume que la velocidad de liberación es función de la raíz cuadrada del tiempo.

3. Modelo de Korsmeyer Peppas (ecuación 3):

$$\frac{M_t}{M_\infty} = k \cdot t^n \quad \text{ecuación 3}$$

En este modelo se considera el parámetro *n* que es un exponente difusional cuyo valor depende del mecanismo de liberación y de la geometría del sistema ensayado. Para los dispositivos cilíndricos (en el caso de los cementos óseos), cuando el valor de n≤0,45 el mecanismo de difusión obedece al proceso de difusión de Fick; en el caso de que este valor esté comprendido entre 0,45 y 0,89 (0,45 <n <0,89) el mecanismo de liberación es no Fickiano; si n=0,89 corresponde a un mecanismo de Caso II (relajante) y si n> 0,89 a un mecanismo super Caso II. Para los dispositivos esféricos (en el caso de los hidrogeles), cuando el valor de n≤0,43 el mecanismo de difusión obedece al proceso de difusión de Fick; en el caso de que este valor esté comprendido entre 0,43 y 0,85 (0,43 <n <0,85) el mecanismo de liberación es no Fickiano; si n=0,85 corresponde a un mecanismo de Caso II (relajante) y si n> 0,85 a un mecanismo super Caso II [31].

En todas las ecuaciones k representa la constante de velocidad de liberación del fármaco desde el soporte que lo contiene y en el caso de la ecuación 3 esta constante incorpora características estructurales y geométricas del dispositivo.

Evaluación de la bioactividad

La evaluación de la bioactividad de las muestras obtenidas por mezcla de los antibióticos y los soportes de liberación se desarrolló mediante estudios de simulación farmacocinética y de estudios microbiológicos.

1. *Estudios de simulación farmacocinética*

Se realizó una simulación de Monte Carlo para evaluar si la cantidad de fármaco liberado en biofase a diferentes tiempos superaba la concentración mínima inhibitoria (CMI) de las cepas de los microorganismos aislados con mayor frecuencia en las intervenciones quirúrgicas de artroplastia [14]. Para cada formulación se realizaron 1000 simulaciones utilizando el programa informático NONMEM versión VII [32]. La bioactividad se evaluó calculando para un tiempo determinado el porcentaje de pacientes cuya concentración de antibiótico en el lugar del implante sería superior a la CMI del microorganismo considerado.

El modelo farmacocinético empleado consta de dos compartimentos (figura 1); cemento cargado de antibiótico (C1) y espacio de la articulación (C2). Debido a que el drenaje local en las primeras 72 horas post-cirugía es muy elevado, durante este periodo se consideró despreciable la velocidad de distribución y de retorno (kd y kr respectivamente) del antibiótico desde el espacio de articulación (C2) a la circulación sistémica.

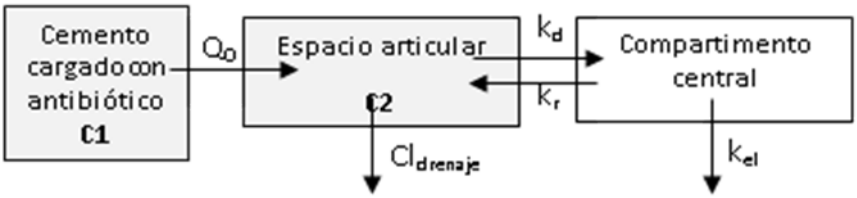


Figura 1.- Q_0: velocidad de liberación del antibiótico (mg/h); Cl drenaje: aclaramiento local de antibiótico debido al drenaje de la herida (mL/h); kd (h^{-1}): constante de velocidad de distribución desde el espacio articular al compartimento central; kr (h^{-1}): constante de velocidad de retorno del antibiótico desde el compartimento central al espacio articular; Kel (h^{-1}): constante de eliminación del antibiótico.

El aclaramiento local del antibiótico ($Cl_{drenaje}$) se calculó a partir de los volúmenes de exudado obtenidos tras la revisión de las historias clínicas de los pacientes intervenidos durante un año (año 2015) en el Hospital Dr. Peset de Valencia por artroplastia de rodilla (156 pacientes, durante 468 observaciones). Las ecuaciones utilizadas para obtener este parámetro fueron las siguientes:

$$Cl_{drenaje_t} = Cl_0 + k_0 \cdot t \quad \text{ecuación 4}$$

$$Cl_{drenaje_t} = Cl_0 \cdot e^{-k_1 \cdot t} \quad \text{ecuación 5}$$

$Cl_{drenaje_t}$ es el volumen exudado por unidad de tiempo correspondiente a un determinado tiempo post-intervención quirúrgica, t, Cl_0 es el volumen exudado por unidad de tiempo determinado en tiempo cero post-cirugía y k_0 y k_1 las constantes de velocidad de aclaramiento local de orden cero y de primer orden respectivamente.

Los parámetros utilizados para simular la concentración de fármaco en la biofase fueron los obtenidos en el modelo seleccionado para definir la cinética de elución del antibiótico (epígrafe anterior).

Por último, el volumen del espacio articular se tomó de la bibliografía (1,6 ± 1,1mL) [33].

2. Estudios microbiológicos

Se evaluó la inhibición de crecimiento bacteriano tras la incubación de las muestras en los medios de cultivo adecuados utilizando los métodos de recuento de las unidades formadoras de colonias (UFC) [34] y de evaluación de halo de inhibición [35]. En estos estudios se utilizaron las especies *S. aureus, S. epidermidis, P. aeruginosa* y *E. Coli* para ciprofloxacino y ceftazidima y *C. albicans* para fluconazol, que habían sido aisladas en infecciones articulares con anterioridad.

El recuento de UFC se realizó por inmersión e incubación de las muestras durante 24 horas en 10mL de caldo de cultivo Mueller Hinton que contenía una cantidad de microorganismo conocida ($150 \cdot 10^6$ UFC/mL). Con el fin de cuantificar las bacterias viables tras el periodo de incubación, el caldo se diluyó 1/10, se extendió 1 µL en una placa de agar y se incubó durante 24 horas a 37 ° C para permitir el crecimiento y el recuento de UFC.

Para la prueba de inhibición de halo, las muestras de estudio se depositaron en una placa Petri que contenía agar Mueller Hinton y 100 µL de caldo bacteriano. Las placas se incubaron durante 72 h a 37 °C [36]. La medición del halo de inhibición se realizó a las 24, 48 y 72 horas. Después de cada lectura, se retiraron las muestras de la placa Petri y se depositaron en otra placa Petri que contenía el inóculo bacteriano fresco.

En ambos métodos las pruebas se realizaron por duplicado.

Pruebas de citocompatibilidad

Con el fin de determinar la citocompatibilidad de los hidrogeles elaborados se realizaron ensayos de citotoxicidad utilizando cultivos celulares de fibroblastos embrionarios de ratón (NIH3T3 P15, ATCC®, Manassas, EE.UU.). La viabilidad celular se evaluó a través de la medida de

la actividad metabólica mitocondrial con 3-(4,5-dimetiltiazol-2-il)-2,5-difenil bromuro de tetrazolio (MTT) [37].

El ensayo de MTT permite examinar el efecto tóxico del material estudiado sobre las células, como resultado de la interacción entre la célula y la muestra evaluada. El ensayo se realizó en placas de 96 pocillos, en los que se sembraron 100 µL de una suspensión de células NIH3T3 P15 (10^5 células/mL). Las células se cultivaron en medio Eagle modificado por DMEM-Dulbecco (Aldrich® Sigma, St. Louis, EE.UU.), suplementado con antibióticos al 1% (penicilina / Estreptomicina de Gibco®, Waltham, EE.UU.) y PBS al 10% (suero bovino fetal de Gibco®). Una vez alcanzada la confluencia se sustituyó el medio de cultivo por 25, 50 o 75 µL de las muestras evaluadas (diluciones 1/10, 1/5 y 1/3 respectivamente) y se completó con 225, 200 o 175 µL, respectivamente, de DMEM exento de suero. Se incubaron en CO_2 al 5% a 37 ° C durante 24h o 48h. Transcurrido el periodo de incubación se retiró el medio, se añadieron 20 µL de colorante MTT (5µg/mL) y se mantuvo a 37ºC durante 3h. Por último, se procedió a la determinación colorimétrica de las placas, en las que la absorbancia se midió utilizando un espectrofotómetro (Synergy H1 monochromator-based, Biotek, Winooski, USA) seleccionando la longitud de onda de 570 nm.

Métodos estadísticos

Para realizar las comparaciones estadísticas entre valores de flujo o cantidad liberada a un determinado tiempo, se utilizó la prueba paramétrica (ANOVA) con un análisis post-hoc Scheffé en caso de que las varianzas fueran homogéneas o T3 de Dunnett cuando no lo fueron. La homogeneidad de las varianzas se evaluó con la prueba de Levene. Se ha empleado el programa IBM SPSS Statistics 20.

La selección del modelo cinético de liberación del fármaco se realizó utilizando el criterio de información de Akaike (AIC) proporcionado por el programa informático NONMEM versión VII.

Resultados y discusión

En la tabla 1 se relaciona la composición de las muestras ensayadas. La obtención de las muestras mediante el proceso manual puede provocar que las mismas difieran en sus pesos y

dimensiones y, en consecuencia, en la cantidad de antibiótico que incorporan. En ningún caso el coeficiente de variación de peso o dimensiones de las muestras superó el 9,5 %, variabilidad aceptable de acuerdo con la técnica de elaboración empleada.

Factores que condicionan la velocidad y la magnitud de liberación del antibiótico

1. Especie química

Las muestras utilizadas para evaluar la influencia de la especie química del fármaco sobre la velocidad de liberación del antibiótico desde el cemento óseo, se prepararon de forma manual utilizando como fármaco modelo el ciprofloxacino, en forma de sal y en forma de base, y el cemento óseo Lima CMT1®. La figura 2 representa las cantidades de ciprofloxacino (expresado en porcentaje respecto a la cantidad total de fármaco incorporada) liberado del cemento óseo Lima CMT1® (ciprofloxacino base, la muestra D, o ciprofloxacino clorhidrato, muestra B). La cantidad de antibiótico liberado cuando se utiliza en forma de base representó el 35% de la cantidad liberada cuando se utilizó el antibiótico en forma de clorhidrato. La mayor solubilidad de la sal (ciprofloxacino clorhidrato), resulta en una mayor elución de ciprofloxacino desde el cemento. Este resultado se puede justificar por el hecho de que una mayor hidrosolubilidad del fármaco haría más rápida la génesis de poros y canales, ayudando a la difusión posterior del fármaco.

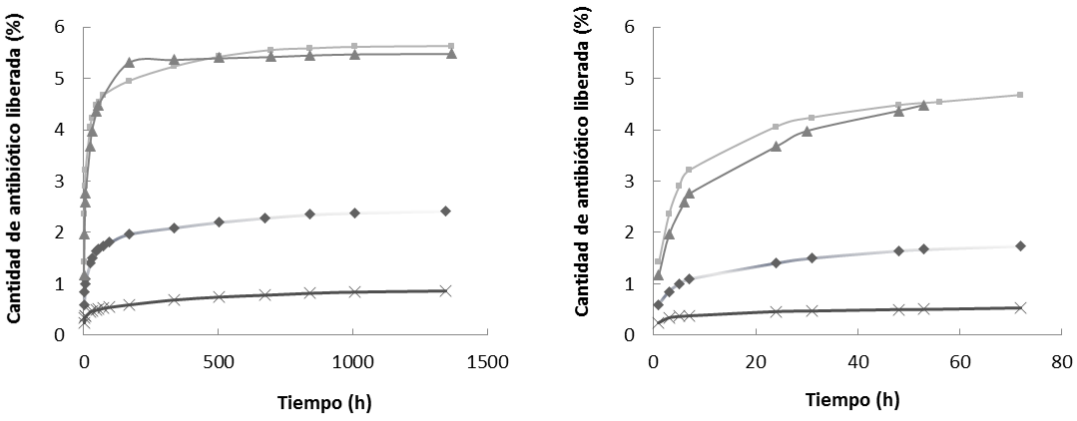


Figura 2.- Porcentaje de dosis de ciprofloxacino liberada a partir de las muestras B (♦) (ciprofloxacino clorhidrato, mezclado manual), D (×) (ciprofloxacino base, mezclado manual), F (■) (ciprofloxacino clorhidrato y vancomicina, mezclado manual) e I (▲) (Ciprofloxacino clorhidrato, mezclado mecánico). En todos los casos, se utilizó el cemento óseo Lima CMT 1®. En la parte izquierda de la figura se representan el total de muestras obtenidas y en la parte derecha se reproduce el perfil de liberación obtenido durante las primeras 80 h del ensayo.

2. *Tipo de mezclado: manual o mecánico*

La influencia del tipo de mezclado (manual o mecánico) sobre la velocidad de liberación del antibiótico y la cantidad de antibiótico liberada, se evaluó mediante la comparación de los resultados obtenidos entre las muestras A y H (Simplex® mezclado manual y mecánico respectivamente), entre las muestras B e I (Lima CMT1® mezclado manual y mecánico respectivamente), y entre las muestras C y J (PALACOS® mezclado manual y mecánico respectivamente). La figura 3 muestra el porcentaje de ciprofloxacino liberado a diferentes tiempos y la figura 4 la velocidad de liberación obtenida en cada tiempo de toma de muestra. Al comparar los dos procedimientos de obtención de las mezclas, no se encontraron diferencias estadísticamente significativas entre las mezclas formadas con los cementos óseos Simplex® y Palacos®, ni en la velocidad de liberación del fármaco ni en la cantidad de antibiótico liberada. Por el contrario, cuando se utilizó el cemento óseo Lima CMT1®, se observaron diferencias estadísticamente significativas hasta las 697 horas del ensayo en la cantidad liberada y para todos los tiempos de ensayo cuando se compararon las velocidades de elución.

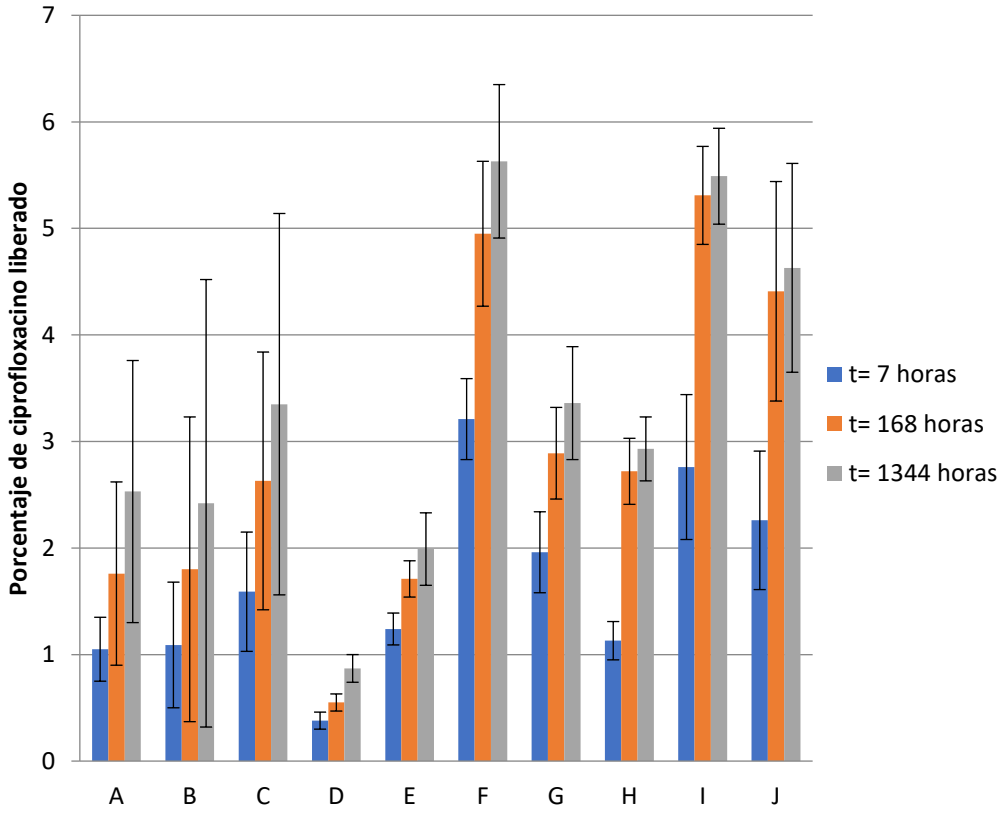


Figura 3.- Porcentaje de ciprofloxacino liberado a las 7, 168 y 1344 horas. A, B, C, H, I, y J (ciprofloxacino clorhidrato cargado en Simplex®, Lima CMT 1® y Palacos®, mezclado manual (A, B, C) y mecánico (H, I, J), muestras E, F y G (ciprofloxacino clorhidrato + vancomicina cargados en Simplex®, Lima CMT 1® y Palacos®, mezclado manual), y muestra D (ciprofloxacino base-cargado en Lima CMT 1®, mezclado manual).

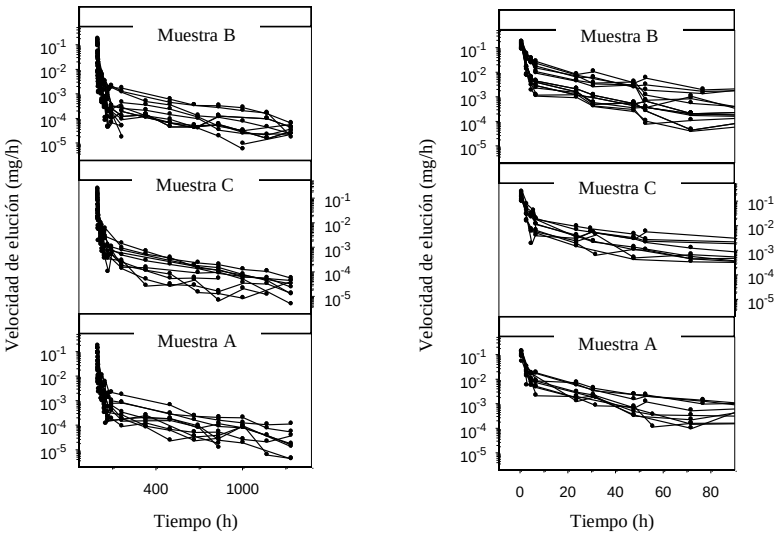
3. Marca comercial del cemento óseo

La figura 4 muestra en escala logarítmica, los valores individuales de la velocidad de elución de ciprofloxacino expresada en mg/h determinada para cada uno de los tiempos de muestra en los ensayos realizados con las muestras A, B y C (parte superior de la figura) (clorhidrato de ciprofloxacino mezclado de forma manual con los cementos óseos Simplex®, Lima CMT1® y Palacos® respectivamente) y con las muestras H, I y J (parte inferior de la figura) (clorhidrato de ciprofloxacino mezclado de forma mecánica con los cementos óseos Simplex®, Lima CMT1® y Palacos® respectivamente). Todas las muestras producen una velocidad de liberación elevada durante los primeros tiempos del ensayo. Sin embargo, a medida que se prolonga el tiempo del ensayo la velocidad de liberación se reduce hasta alcanzar un valor constante.

Los resultados obtenidos indican que cuando se utiliza el mezclado manual, la velocidad de liberación del fármaco a partir de cemento óseo Palacos® es ligeramente mayor que la obtenida a partir de los cementos óseos Simplex® y Lima CMT1®. No obstante, la prueba ANOVA reveló que las diferencias obtenidas no alcanzan significación estadística. Estos resultados difieren con los obtenidos en estudios anteriores que han demostrado que la velocidad de liberación de fármacos a partir de este cemento óseo es superior a la obtenida con otros cementos óseos comerciales probablemente debido a la mayor porosidad del cemento Palacos® en relación con las otras marcas comerciales estudiadas [38]. Cuando se utilizó el sistema de mezclado mecánico, la mayor velocidad de liberación de antibiótico se alcanzó con los cementos Lima CMT1 y Palacos®, aunque únicamente se obtuvieron diferencias estadísticamente significativas cuando se comparó la velocidad de liberación del antibiótico desde las mezclas obtenidas con los cementos Lima CMT1® y Simplex®.

A la vista de estos resultados se consideró que no existen diferencias en la magnitud de la liberación de ciprofloxacino debidas a la marca comercial del cemento empleado ni a la técnica de mezclado usada. Por ello, los ensayos posteriores se llevaron a cabo únicamente con preparaciones realizadas con el cemento Palacos ®.

MEZCLADO MANUAL. Muestra A: Simplex®; Muestra B: Lima®; Muestra C: Palacos®



MEZCLADO MECÁNICO. Muestra H: Simplex®; Muestra I: Lima®; Muestra J: Palacos®

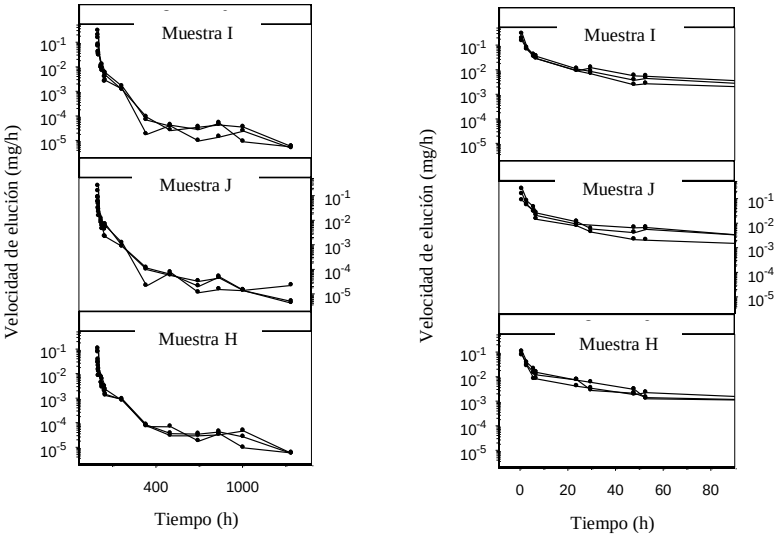


Figura 4.- Velocidad de liberación de ciprofloxacino (mg/h) a diferentes tiempos de toma de muestras. En las figuras de la derecha únicamente se representan los valores obtenidos hasta las 80 horas de ensayo. Mezclado manual: corresponde a las muestras A, B y C. Mezclado mecánico: corresponde a las muestras H, I y J.

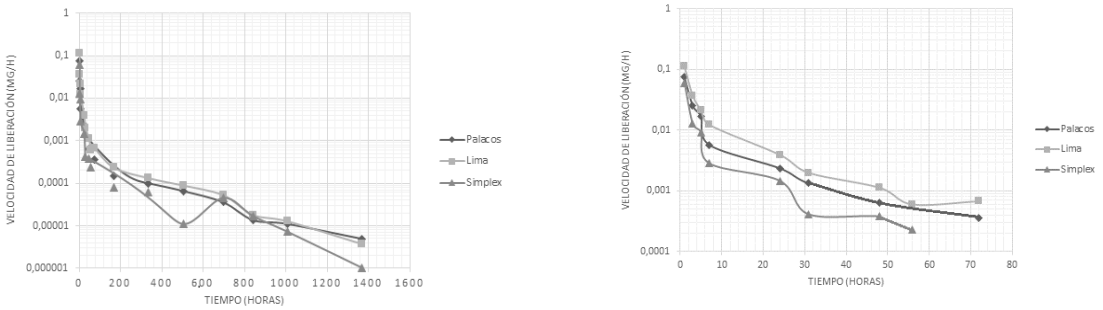
4. Elución de ciprofloxacino a partir de las mezclas ternarias

La figura 5 muestra la velocidad de liberación del ciprofloxacino y de la vancomicina a partir de las muestras estudiadas. En esta figura se observa que la fase inicial de liberación rápida de vancomicina es de menor duración y presenta una velocidad más elevada que la obtenida para el ciprofloxacino. Este fenómeno se puede atribuir a la mayor solubilidad acuosa de la vancomicina (100 mg/mL frente a 0,03 mg/mL del ciprofloxacino) e indica que la disolución de las partículas de vancomicina situadas sobre la superficie de la muestra se realiza con mayor velocidad facilitándose la liberación y posterior disolución de las partículas próximas a la superficie en un periodo de tiempo breve.

Las cantidades de ciprofloxacino liberadas a partir de las mezclas ternarias preparadas con el cemento más pososo (Lima CMT1®), son superiores a las cantidades de antibiótico liberadas a partir de mezclas binarias (ciprofloxacino y cemento) (232% superior) [39]. Estas diferencias permiten corroborar que el hecho de incorporar más de un fármaco a las mezclas con cemento óseo suficientemente poroso potencia la velocidad de liberación de ambos fármacos. En este caso la vancomicina incorporada en la mezcla, favorecería la formación de un mayor número de poros y canales que facilitarían la entrada de agua en la matriz y la posterior disolución del ciprofloxacino que está incluido en el interior de la misma. Por otra parte, la liberación de la vancomicina se produce a una velocidad elevada durante las primeras 24 horas de ensayo. Sin embargo, a partir de este momento la elución de este antibiótico cesa. Este hecho puede ser debido al elevado peso molecular de este antibiótico, en relación con el peso molecular del ciprofloxacino (1485 Dalton vs. 368 Dalton), el cual no permite que el fármaco atraviese los canales y poros que se forman, por lo que únicamente se disolvería la vancomicina adsorbida en la superficie.

Las cantidades de ciprofloxacino y vancomicina liberadas, así como la velocidad de liberación de los fármacos es mayor para las mezclas elaboradas con el cemento Lima CMT1® y Palacos®. Estos resultados son compatibles con la menor porosidad del cemento Simplex® indicada por Stryker®, laboratorio fabricante del producto [40].

CIPROFLOXACINO



VANCOMICINA

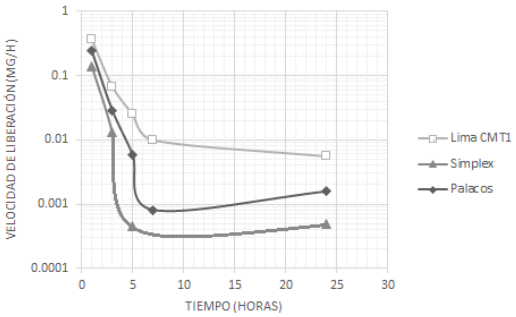


Figura 5.- Velocidades de elución de ciprofloxacino clorhidrato y vancomicina a partir de las mezclas ternarias obtenidas con los tres cementos óseos estudiados.

Evaluación de la liberación de ciprofloxacino, ceftazidima y fluconazol

Las figuras 6 y 7 muestran la cantidad y la velocidad de liberación de ciprofloxacino, ceftazidima y fluconazol a partir de las mezclas formadas en la proporción (antibiótico/cemento) utilizada en profilaxis antibiótica (1/40) y en tratamiento (4/40) (muestras K y L para ciprofloxacino, M y N para ceftazidima y O y P para fluconazol). En estas se puede observar que cuando el antibiótico se incorpora a las mezclas en mayor proporción se obtiene un mayor porcentaje de fármaco liberado y una mayor velocidad de liberación. En concreto, las cantidades de antibiótico liberadas a partir de las mezclas de antibiótico y cemento en la proporción 4/40 incrementan un 453%, 569%, y 648% para ceftazidima, ciprofloxacino y fluconazol respectivamente. Este incremento de la cantidad de fármaco liberado está relacionado con el peso molecular de la sustancia, siendo mayor cuanto menor es el peso molecular (ceftazidima: 632 Dalton, ciprofloxacino: 367 Dalton, fluconazol: 306 Dalton [41]). Los resultados obtenidos confirman que la estructura de los cementos óseos de PMMA es de baja porosidad por lo que los compuestos de bajo peso molecular difunden con mayor facilidad que los compuestos de mayor peso molecular. Este hecho, además de poner de manifiesto la importancia del peso

molecular del antibiótico sobre la capacidad de elución desde el cemento óseo, permite disponer de un criterio objetivo para seleccionar el antibiótico que debe incorporarse a los cementos óseos utilizados en cirugía ortopédica [42].

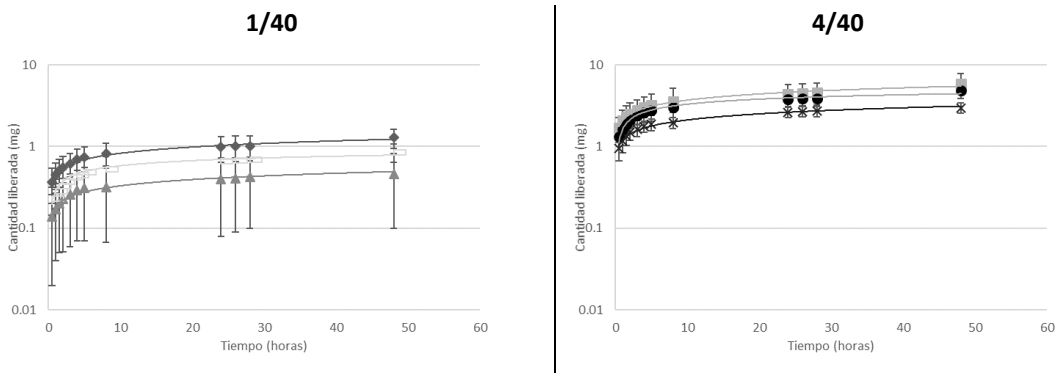


Figura 6.- Cantidad de antibiótico liberada vs. tiempo. Los símbolos representan los valores medios: (-) ciprofloxacino 1/40 -muestra K-; (●) ciprofloxacino 4/40 -muestra L-; (♦) ceftazidima 1/40 -muestra M-; (■) ceftazidima 4/40 -muestra N-; (▲) fluconazol 1/40 -muestra O-; (×) fluconazol 4/40 -muestra P-.

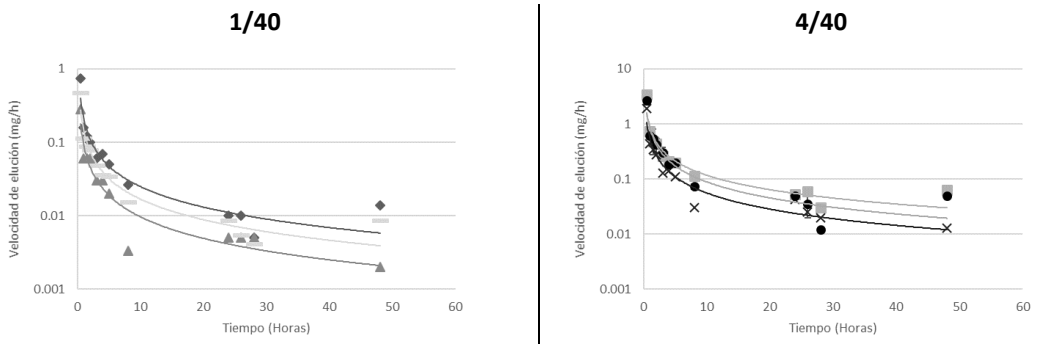


Figura 7.- Velocidad de liberación de antibiótico vs. tiempo. Los símbolos representan los valores medios: (-) ciprofloxacino 1/40; (●) ciprofloxacino 4/40; (♦) ceftazidima 1/40; (■) ceftazidima 4/40; (▲) fluconazol 1/40; (×) fluconazol 4/40.

La tabla 4 muestra el ajuste de los datos experimentales obtenidos con tres antibióticos ensayados en las dos proporciones de mezcla (1/40 y 4/40) a las cinéticas de orden 0, Higuchi y Korsmeyer-Peppas. El modelo de elección en todos los casos fue el de Korsmeyer-Peppas, ya que fue el que proporcionó un valor AIC inferior. En todos los casos, las predicciones del modelo son capaces de describir la tendencia de los datos experimentales. De acuerdo con el valor del coeficiente *n* de la ecuación Korsmeyer-Peppas obtenido (entre 0,242 y 0,254), el mecanismo de liberación del fármaco desde el cemento óseo se puede explicar mediante el proceso de

difusión de Fick en el que cantidad de antibiótico liberada es proporcional a la cantidad fármaco remanente en la matriz.

Tabla 4.- Valor de los parámetros y AIC obtenidos tras el ajustado de los datos experimentales a los diferentes modelos cinéticos. (RSE= error estándar; AIC=Akaike Information Criterion y k=constante de velocidad de liberación del antibiótico).

		Ceftazidima		**Fluconazol**		**Ciprofloxacino**	
Cinética	**Parámetros**	**1/40**	**4/40**	**1/40**	**4/40**	**1/40**	**4/40**
Orden 0	K (RSE%)	0.217 (46%)	0.25 (76%)	0.25 (-)	0.268 (146%)	0.181 (12%)	0.792 (1%)
	AIC	150.337	234.925	87.446	122.856	19.326	686.946
Higuchi	K (RSE%)	0.3 (34%)	0.293 (54%)	0.29 (228%)	0.306 (93%)	0.203 (11%)	1.23 (-)
	AIC	-326.798	-335.355	-336.97	-280.941	-621.623	183.267
Korsmeyer-Peppas	K (RSE%)	0.402 (29%)	0.384 (46%)	0.372 (48%)	0.396 (28%)	0.252 (12%)	1.76 (14%)
	n (RSE%)	0.244 (32%)	0.254 (145%)	0.251 (47%)	0.242 (95%)	0.264 (5%)	0.255 (9%)
	AIC	-717.731	-783.737	-596.022	-594.455	-1050.93	178.611

Evaluación de la bioactividad

1. Estudios de simulación farmacocinética

Las cantidades de antibiótico liberadas desde los cementos determinadas en los estudios *in-vitro* en combinación con los valores del aclaramiento local del fármaco, obtenidos en el estudio observacional, y con los valores del volumen de líquido en el espacio articular, tomados de la bibliografía, permitieron abordar el estudio de bioactividad mediante la realización de un ejercicio de simulación farmacocinética (figura 1).

La tabla 5 muestra el ajuste de los datos de volumen de exudado recogido durante las 72 horas post-intervención. Puede observarse que solo se ha podido obtener la variabilidad de los parámetros para el modelo de orden 0. Por consiguiente, atendiendo a este hecho y al valor del parámetro estadístico AIC se seleccionó la cinética de orden 0 para describir el aclaramiento local del fármaco en el lugar del implante.

Tabla 5.- Valores de los parámetros y AIC obtenidos tras el ajustado de los datos experimentales a los diferentes modelos cinéticos. (CV= coeficiente de variación; AIC=Akaike Information Criterion y k=constante de velocidad del antibiótico para cada cinética).

Aclaramiento a través del drenaje		
Cinética	**Parámetros**	**Valores**
Orden 0	k_0 (CV%)	$2.00 \cdot 10^{-4}$ (27.4%)
	Cl_0 (CV%)	$2.77 \cdot 10^{-2}$ (7.1%)
	AIC	-2963.25
Orden 1	k_1 (CV%)	$1.30 \cdot 10^{-2}$ (--)
	Cl_0 (CV%)	$3.01 \cdot 10^{-2}$ (--)
	AIC	-2958.81

A continuación, en la tabla 6, se muestra para cada muestra ensayada el porcentaje de pacientes en los que la concentración de antibiótico en biofase a las 72 horas post-intervención alcanza valores superiores a la CMI frente a *E. Coli, P. aeruginosa, S. epidermidis* y *S. aureus.*

Tabla 6.- Porcentaje de pacientes cuya concentración de fármaco en biofase a las 72h post-intervención es superior a la CMI de cada uno de los microorganismos seleccionados.

	Proporción	***E. Coli***	***P. aeruginosa***	***S. epidermidis***	***S. aureus***	***C. albicans***
Ciprofloxacino	**1/40**	24	6	2	1	-
	4/40	95	88	50	1	-
Ceftazidima	**1/40**	28	8	3	1	-
	4/40	97	92	52	1	-
Fluconazol	**1/40**	-	-	-	-	53
	4/40	-	-	-	-	99

La figura 8 muestra la evolución de las concentraciones simuladas en biofase durante las 80 horas posteriores a la intervención.

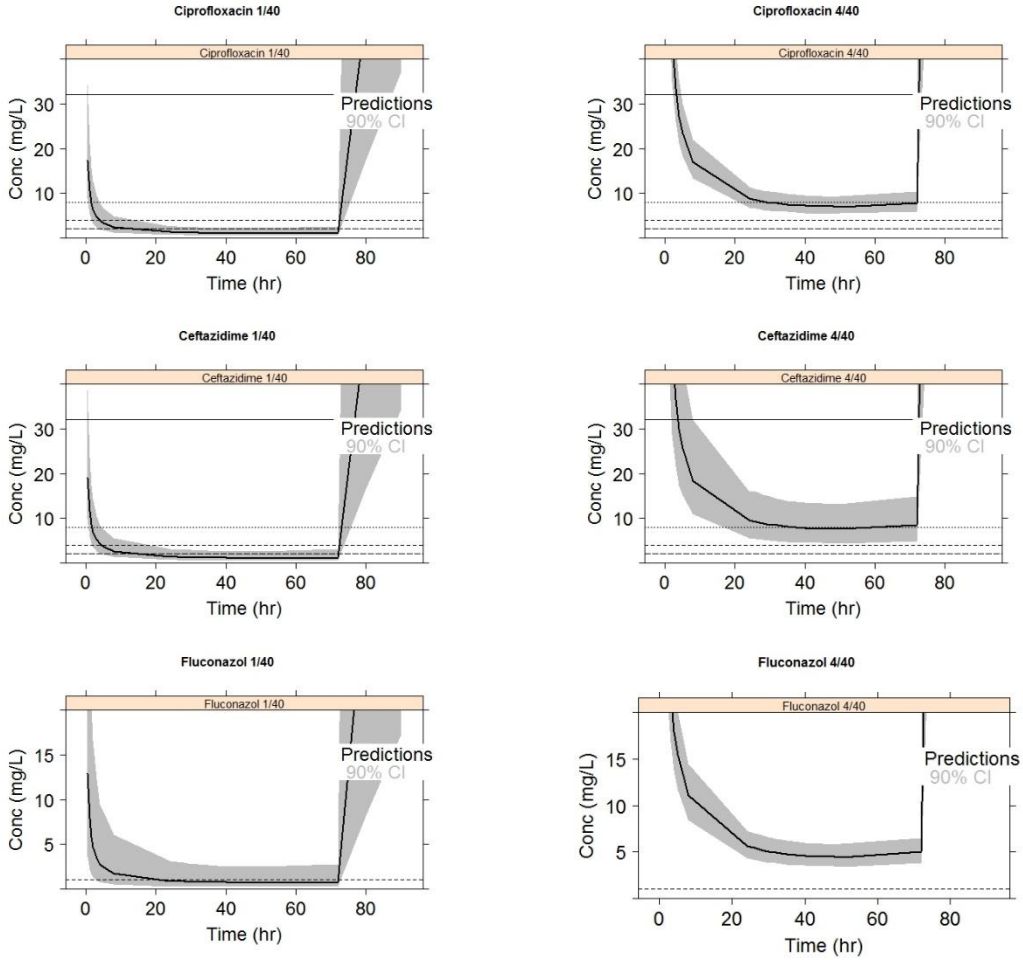


Figura 8.- Concentración de antibiótico simulada en biofase (eje de ordenadas) frente el tiempo post-intervención (eje de abscisas). Las CMI de los microorganismos seleccionados se marcan con líneas puntadas: CMI *S. aureus*=32mg/L, CMI *S. epidermidis*=8mg/L, CMI *E. Coli*=2mg/L, CMI *P. aeruginosa*=4mg/L y CMI *C. albicans*=1mg/L.

Como se aprecia en la figura 8, la concentración de antibiótico en biofase disminuye hasta las 72 horas post-intervención, momento en el que el drenaje externo se retira. A partir de este momento, el drenaje local se reduce y en consecuencia aumenta la concentración de fármaco en el lugar del implante. Cabe destacar que durante las primeras horas post-intervención las concentraciones de antibiótico en biofase serían superiores a las CMI de los microorganismos estudiados, a excepción de *S. aureus*, en un porcentaje de pacientes superior al 50% únicamente para las muestras que contienen la proporción antibiótico/cemento 4/40. De acuerdo con el ejercicio de simulación realizado, la bioactividad de ciprofloxacino, ceftazidima y fluconazol durante las 72 horas post-cirugía depende del microorganismo que produzca la infección, la proporción de antibiótico utilizado y especialmente el tiempo que transcurre desde la cirugía. Únicamente en el caso del fluconazol, utilizado en la proporción

antibiótico/cemento 4/40, se mantendría la concentración de fármaco en biofase por encima de la CMI durante las 72 horas post-cirugía para la totalidad de los pacientes.

2. Estudios microbiológicos

Con el fin de investigar el efecto antimicrobiano de las muestras cargadas con antibiótico se llevó a cabo el recuento de UFC. Esta prueba permite la cuantificación de las colonias bacterianas viables tras la incubación de las muestras en condiciones adecuadas. Únicamente hubo crecimiento microbiano de *P. aeruginosa* (100.000 y 10.000 UFC proliferaron cuando se utilizó ciprofloxacino 1/40 y 4/40 respectivamente; 1.000.000 y 100.000 UFC proliferaron cuando se utilizó ceftazidima 1/40 y 4/40 respectivamente) y *S epidermidis* (10.000 UFC proliferaron cuando se utilizó ciprofloxacino 1/40; 100.000 y 10.000 UFC proliferaron cuando se utilizó ceftazidima 1/40 y 4/40 respectivamente).

Asimismo, se realizó la prueba de evaluación del halo de inhibición de crecimiento bacteriano. La tabla 7 y la figura 9 muestran el halo de inhibición producido para cada uno de los microorganismos seleccionados y cada antibiótico a partir de las muestras estudiadas. El halo de inhibición fue mayor para la proporción antibiótico/cemento 4/40 ($p<0,05$) para todos los microorganismos, excepto para *P. aeruginosa*.

El recuento de UFC y el resultado de los halos de inhibición, mostraron algunas diferencias. Por una parte, *S. epidermidis* mostró crecimiento cuando se cultivó en medio líquido en ambas proporciones de ceftazidima/cemento y cuando se cultivó en medio líquido para la proporción de 1/40 de ciprofloxacino/cemento.

Por otra parte, ciprofloxacino y ceftazidima no fueron activos contra *P. aeruginosa* cuando se usó medio de cultivo líquido, pero sí lo fueron cuando se realizó la prueba del halo de inhibición. Este hecho podría ser debido a las propiedades de *P. aeruginosa,* ya que este microorganismo está provisto de una cápsula de alginato que es responsable de crear bio-películas en determinadas superficies [43, 44]. Por esta razón, durante las primeras horas de incubación de la bacteria en medio líquido el microorganismo podría tapizar los poros del cemento y con ello impedir la elución del antibiótico.

Tabla 7.- Halo de inhibición expresado en milímetros (mm) obtenidos tras el cultivo en medio Mueller-Hinton de las muestras ensayadas en placas Petri. R: resistente; DS: desviación estándar.

		Bacteria		Tiempo (días)		
				1	2	3
Ciprofloxacino	1/40	*S. aureus*	Halo de inhibición medio (mm)±DS	36,0±0,7	35,0±3,5	30,0±0
		S. epidermidis		24,0±0,7	15,0±1,4	R
		E. coli		50,0±1,4	46,0±4,2	42,0±2,2
		Ps. aeruginosa		45,0±0,7	35,0±0,7	38,0±3,5
	4/40	*S. aureus*		44,0±0	39,0±2,1	31,0±2,1
		S. epidermidis		34,0±1,4	30,0±2,1	26,0±0,7
		E. coli		60,0±7,77	50,0±1,4	45,0±1,4
		Ps. aeruginosa		53,0±1,4	40,0±0,7	39,0±0
Ceftazidima	1/40	*S. aureus*	Halo de inhibición medio (mm)±DS	22,0±0	18,5±4,9	R
		S. epidermidis		R	R	R
		E. coli		40,5±3,5	37,0±1,4	35,5±0,7
		Ps. aeruginosa		43,5±0,7	35,5±0,7	32,0±4,2
	4/40	*S. aureus*		33,0±0	29,0±1,4	27,5±0,7
		S. epidermidis		31,0±0,7	20,0±0,7	23,0±0
		E. coli		48,0±3,5	42,0±1,4	38,0±1,4
		Ps. aeruginosa		47,0±1,4	38,5±4,9	39,5±0,7

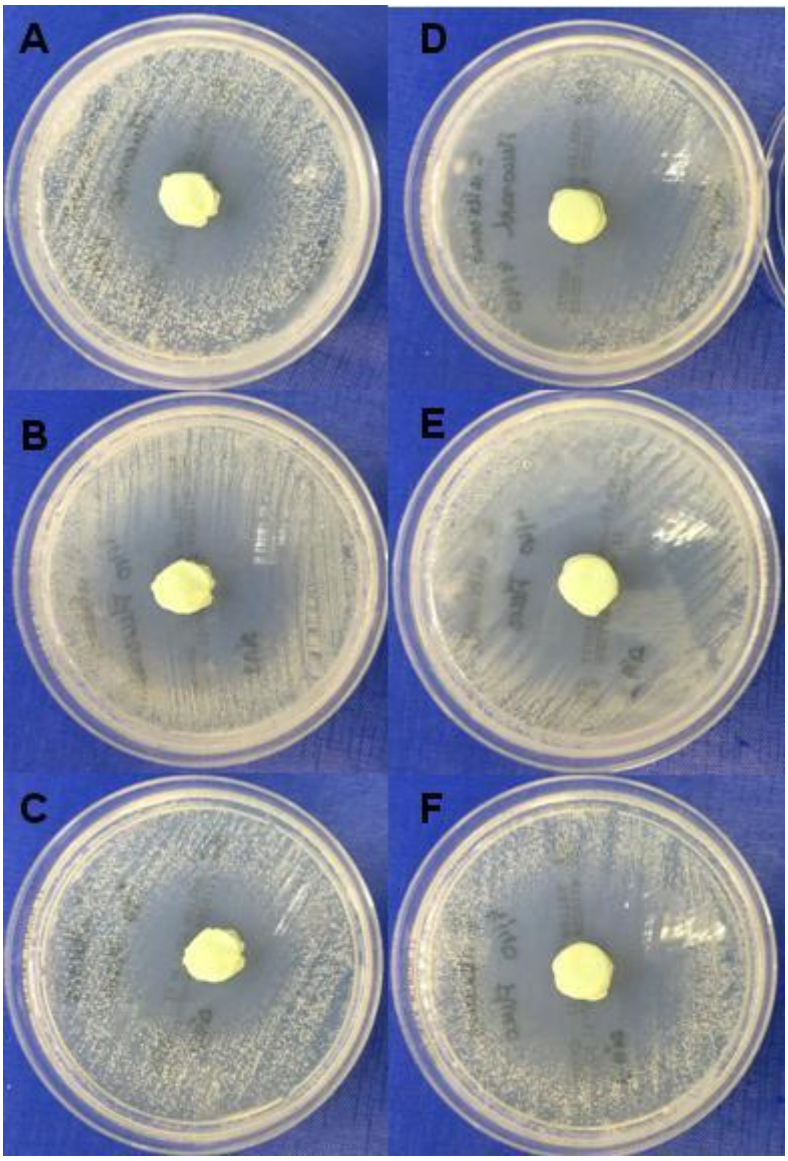


Figura 9.- Halo de inhibición de crecimiento bacteriano obtenido tras el cultivo de las muestras que contienen fluconazol 1/40 a las 24, 48 y 72 horas respectivamente (A, B y C) y fluconazol 4/40 a las 24, 48 y 72 horas respectivamente (D, E y F).

Diseño y evaluación de una nueva forma farmacéutica.

1. Evaluación de la liberación de antibiótico a partir de las láminas e hidrogeles

Las láminas ensayadas (1-16) (tabla 2) mostraron una liberación de antibiótico instantánea, independientemente de la proporción de polímero (quitosano), agente acidificante, antibiótico o proporción de antibiótico utilizados. También mostraron una liberación inmediata de fármaco las láminas 17-20 (tabla 2), que contienen gelatina de tipo B. Estos resultados motivaron estudiar la inlcusión de THPC en las muestras.

La figura 10 muestra la cantidad de ciprofloxacino liberada en función del tiempo a partir de las láminas 21, 22 y 23 que contienen un 12% de THPC. Al analizar la cantidad de antibiótico liberada en función de la carga antibiótica de las muestras, se observa que al aumentar la carga de ciprofloxacino (muestras con 0,5 mg/cm^2, 1 mg/cm^2 y 2 mg/cm^2) se produce una mayor liberación de antibiótico a los 6 días; no obstante, el incremento en la cantidad liberada no es proporcional a la carga de las láminas (cantidad liberada a partir de las muestras que contiene THPC 12% es de 1,78 mg, 2,59 mg y 3,18 mg para láminas cargadas con antibiótico con 0,5 mg/cm^2, 1 mg/cm^2 y 2 mg/cm^2, respectivamente). Esta ausencia de proporcionalidad podría atribuirse a que el reticulante se uniera con los grupos amina presentes en el ciprofloxacino y de esta manera, un incremento en la cantidad de ciprofloxacino favorecería la unión del reticulante con el fármaco y en consecuencia se reduciría la reticulación efectiva del agente reticulante (unión de los grupos hidroximetilo del THPC con los grupos amina del quitosano).

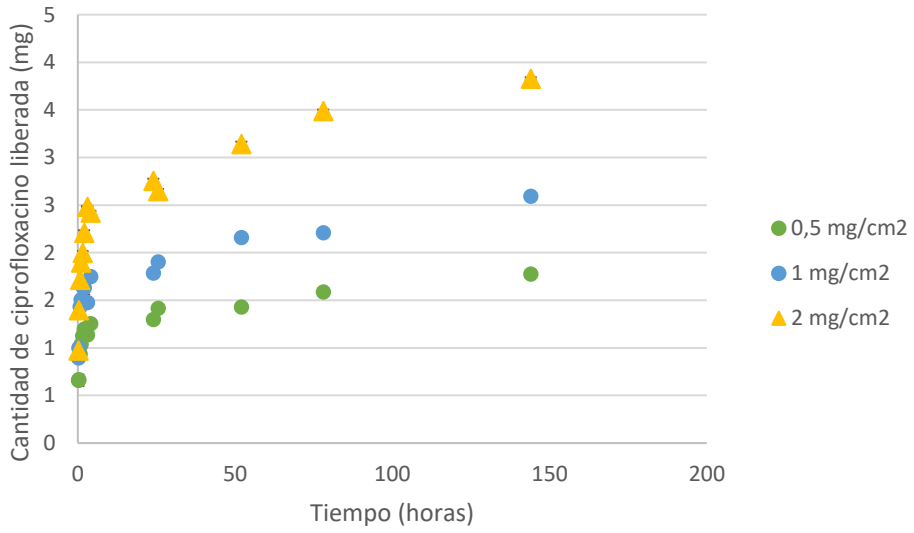


Figura 10.- Cantidad de ciprofloxacino liberado vs. tiempo a partir de las láminas estudiadas (muestras 21, 22 y 23) con 12% de THPC en función de la carga de antibiótico: 0.5, 1 y 2 mg/cm^2.

Debido a que tras la adición del agente reticulante las propiedades organolépticas de las muestras fueron óptimas el estudio se continuó utilizando la formulación en forma de hidrogel. Para ello, se seleccionó la mayor carga antibiótica de 2 mg/cm^2 con el objetivo de asegurar una correcta bioactividad. La influencia del agente reticulante sobre la liberación del antibiótico a partir de los hidrogeles se puede observar en las figuras 11 y 12 en las que se representa el porcentaje de ciprofloxacino liberado frente al tiempo de toma de muestra y la cantidad de

antibiótico liberada a los 6 días a partir de los hidrogeles que contienen una concentración de ciprofloxacino de 2mg/cm^2 en función de la proporción de reticulante que contiene la muestra (muestras 24, 25 y 26). Los resultados obtenidos indican que al aumentar el porcentaje del agente reticulante se reduce la cantidad de ciprofloxacino liberada de forma que la liberación de antibiótico se reduce en aproximadamente un 30% al duplicar la cantidad del agente reticulante (paso del 12 al 24% de THPC, o de 24 a 36% de THPC).

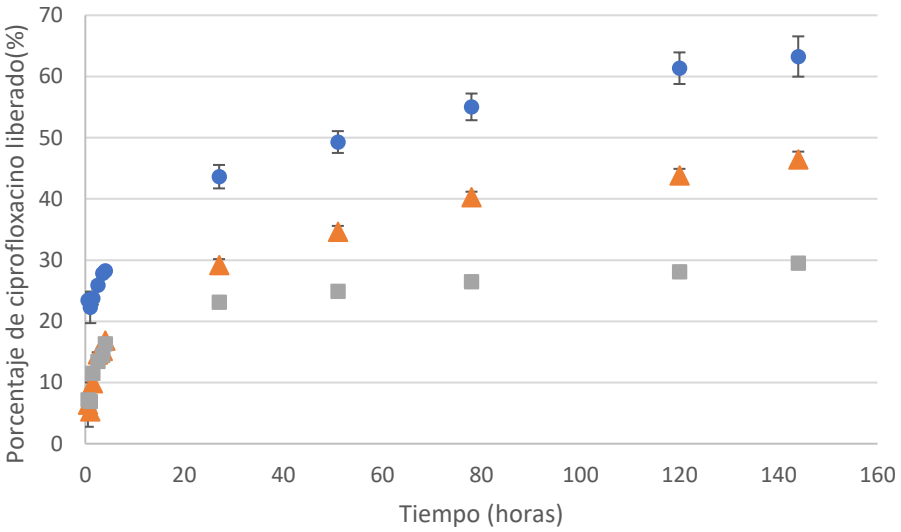


Figura 11.- Porcentaje de ciprofloxacino liberado vs. tiempo a partir de los hidrogeles que contienen 2mg/cm^2 de antibiótico: (●) THPC 12%; (▲) THPC 24% y (■) THPC 36%.

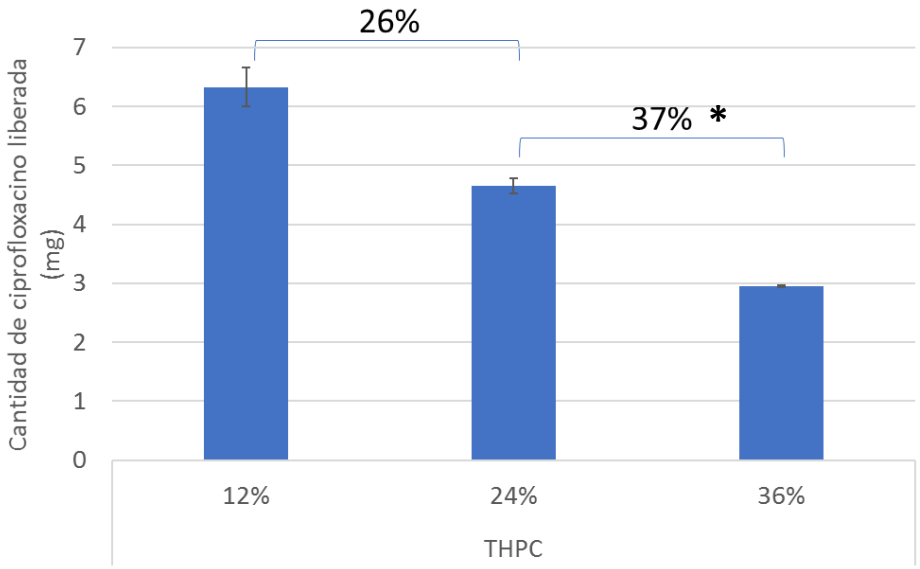


Figura 12.- Cantidad de ciprofloxacino liberada a partir de las láminas que contienen 2 mg/cm^2 de antibiótico vs. porcentaje de reticulante (THPC) que contiene la muestra. La medida corresponde a los 6 días. Asimismo, se indica el porcentaje de reducción de la cantidad de fármaco liberado entre las muestras evaluadas. *: diferencias estadísticamente significativas (p<0,05).

2. Evaluación de la bioactividad

El modelo propuesto para explicar la bioactividad local fue el expuesto en el apartado de material y métodos. La figura 13 muestra las concentraciones simuladas en el espacio articular durante las primeras 96 horas de las muestras 24, 25 y 26. Al igual que ocurría con los cementos óseos, la concentración en espacio articular disminuye hasta las 72 horas, momento en el cuál se retira el redon. En este caso, para cualquier proporción de reticulante, las concentraciones alcanzadas en biofase son muy superiores a las CMI de los microorganismo que más frecuentemente causan infección, por lo que garantizaría una eficacia adecuada.

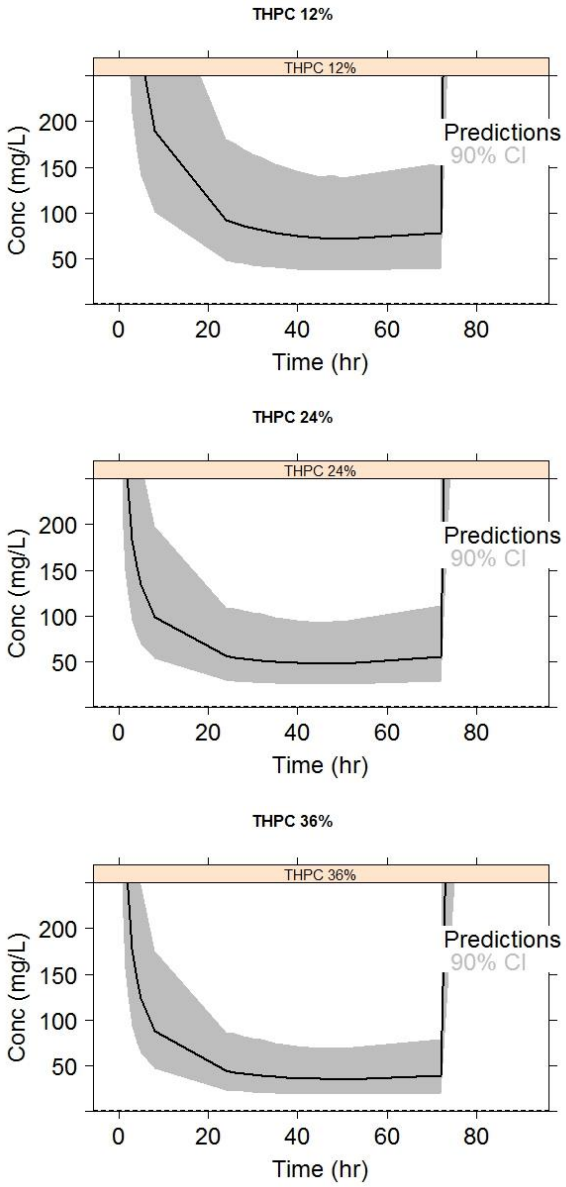


Figura 13.- Concentración de antibióticos simulada en el espacio articular para los hidrogeles que contienen 2mg/cm2 de antibiótico con THPC 12%, THPC 24% o THPC 36%.

Los resultados expuestos indican que esta forma farmaceutica sería util en la prevención de infecciones osteoarticulares. En estos casos, en los que los tres primeros días existe un gran volumen de drenaje a través del redón, las altas velocidades de liberación durante las primeras horas, proporcionarían una adecuada bioactividad eficaces contra los patógenos más comunes. Además, debido a su caracter biodegradable, la forma farmaceutica se implantaría en campo abierto durante la artroplastia y no sería necesario su retirada.

3. *Pruebas de citocompatibilidad*

La figura 14 muestra la viabilidad de los fibroblastos de ratón (NIH3T3) cultivados en presencia de las muestras ensayadas (muestras 24, 25 y 26). Los resultados obtenidos en este ensayo indican que tras 24 horas de incubación, la viabilidad de las células supera el 50%, para las tres concentraciones de reticulante, cuando la muestra se siembra en diluciones superiores a 1/5 (25 y 50 µL). Sin embargo, tras 48 horas de incubación, la viabilidad celular solo supera el 50% en la dilución 1/10. Estos resultados se deben interpretar con cautela, ya que se debe considerar la elevada dispersión de los datos, la densidad de fibroblastos en el lugar de aplicación de la forma farmacéutica *in vivo* probablemente sea mayor y, además, acompañada del drenaje correspondiente a la circulación sanguínea, minimizaría el efecto citotóxico del reticulante evaluado. No obstante, para continuar esta línea de trabajo, encaminada a diseñar una forma de administración de liberación local de antibióticos en cirugía ortopédica, se debería en primer lugar optimizar la cantidad de reticulante que debería incorporarse, ya que esta debería ser la mínima tras confirmar que se mantiene una liberación adecuada del fármaco en el lugar de administración. Los resultados obtenidos, indican que el reticulante utilizado en este estudio es citocompatible con fibroblastos de ratón NIH3T3 durante 48 horas si se utilizan un 12% del mismo para la reticulación del polímero. Una mayor cantidad de reticulante podría producir un deterioro de la función celular atribuible, tal como han indicado otros autores, a la formación de enlaces covalentes entre los grupos amina libre situados en la superficie celular y el agente reticulante THPC [24].

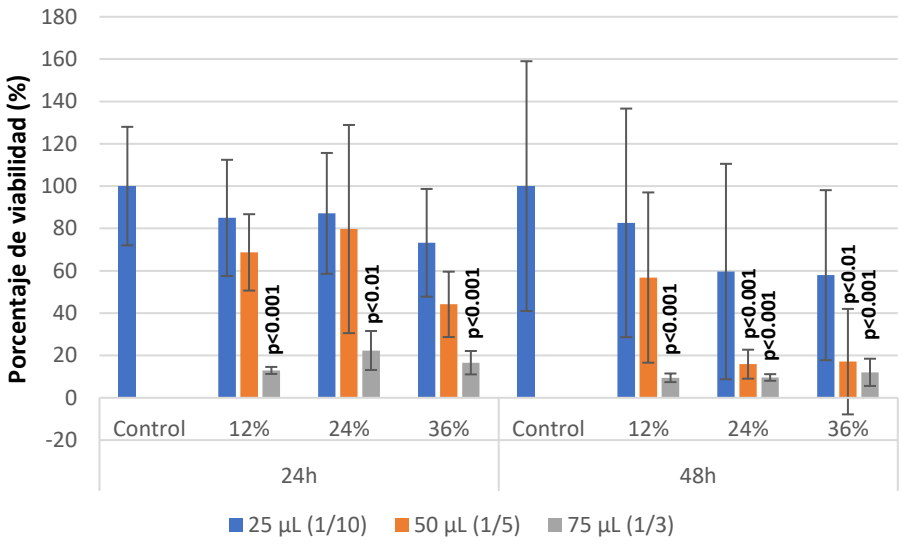


Figura 14.- Ensayo de viabilidad celular (MTT) tras 24 y 48 horas de incubación en presencia de hidrogel de quitosano que contiene agente reticulante (THPC) al 12%, 24% y 36%.

Conclusiones

El trabajo desarrollado ha permitido obtener las siguientes conclusiones.

1. La liberación de antibióticos desde las mezclas fármaco-cemento poliacrílico estudiados se produce de forma bifásica. La primera fase, de alta velocidad de elución, puede atribuirse a la disolución de las partículas de antibiótico adsorbidas a la superficie; la segunda fase, de una velocidad inferior, a la difusión del antibiótico desde el interior del cemento óseo hasta el medio.

2. La inclusión de ciprofloxacino en forma de base en el cemento óseo Lima CMT1®, proporciona una cantidad de fármaco eluido de un 35% respecto a la obtenida cuando el antibiótico se incorpora al cemento en forma de clorhidrato.

3. El mezclado mecánico asegura una menor variabilidad inter-lote y las mismas cantidades de antibiótico liberadas que las obtenidas con las mezclas elaboradas de forma manual. El cemento empleado condiciona la cantidad liberada, en el caso del ciprofloxacino, el cemento Simplex® libera con una velocidad inferior que los cementos Palacos® y Lima®.

4. La cantidad de ciprofloxacino liberada a partir de las mezclas con Lima CMT1® y vancomicina es superior a la que se obtiene a partir de las mezclas formadas por este mismo cementos óseo y ciprofloxacino, ya que la presencia de un segundo antibiótico en la muestra puede favorecer la formación de mayor número de poros y canales que facilitan la cesión de antibiótico al medio.

5. Los cementos óseos son sistemas adecuados para incorporar ciprofloxacino, ceftazidima y fluconazol en las proporciones utilizadas para profilaxis (1/40) y tratamiento (4/40). La bioactividad durante las 72 h post intervención quirúrgica de las mezclas está condicionada por la concentración mínima inhibitoria del microorganismo causante de la infección. Sin embargo, transcurrido este periodo de tiempo la concentración de antibiótico en biofase aumenta y es probable que supere la concentración mínima inhibitoria en la totalidad de los pacientes.

6. Las láminas poliméricas, tanto las elaboradas con el biopolímero quitosano como las elaboradas con las mezclas de quitosano y gelatina, proporcionan una liberación instántanea del antibiótico. Sin embargo, cuando se sustituye gelatina por cloruro de tetrakis (hidroximetil) fosfonio, la liberación del fármaco se realiza de forma sostenida

durante al menos seis días. No obstante, las características organolépticas de estos sistemas no fueron satisfactorias.

7. Los hidrogeles formulados con la mezcla de quitosano y cloruro de tetrakis (hidroximetil) fosfonio proporcionan una liberación sostenida de ciprofloxacino durante 6 días. Los hidrogeles reticulados con un 12% de cloruro de tetrakis (hidroximetil) fosfonio mantinen la viabilidad celular de fibroblastos de ratón durante 48 horas de ensayo y proporcionarían una adecuada bioactividad durante las primeras 96 horas frente a los patógenos más frecuentes.

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CAPÍTULO 1

Antibiotic-loaded Bone Cement as Prophylaxis in Total Joint Replacement

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Accepted.

ABSTRACT

One of its most serious complications of arthroplasty is associated with the development of infections, although its prevalence is between 0.5%-3%, in some cases can lead to death. Therefore, an important challenge in joint surgery is the prevention of infections when an arthroplasty is performed. The use of antibiotic-loaded cements could be a suitable tool due to its numerous advantages; the main advantage of the use of antibiotic loaded into bone cement derives directly from antibiotic release in the effect-site, allowing to achieving high concentrations at the site of action, and minimal or no systemic toxicity. This route of administration was first described by Buchholz and Engelbrecht. In the case of infection treatment, this method is an established method and its good results are confirmed. However, its role in infection prevention, and therefore the use of these systems in clinical practice, has proved controversial because of the uncertainty about the development of possible antibiotic resistance after prolonged exposure time, the effectiveness, cost of these systems, toxicity and loosening of mechanical properties. This review discusses all these topics, focusing on effectiveness and safety, antibiotic decision, cement type, mixing method, release kinetics and future perspectives. The final objective is to provide the orthopaedic surgeons right information in their clinical practice based on current evidence.

Keywords: Bone cement, arthroplasty, antibiotic, elution kinetics, orthopaedics, bioactivity.

INTRODUCTION

Total joint replacement is one of the most common and successful orthopaedic operations. The replacement is performed when there is irreversible damage in the joint, and in general, it is recommended in the elderly, in which bearable of the prosthesis is much smaller due to its low physical activity, reducing the possibility of failure. One of its most serious complications is associated with the development of infections, although its prevalence is between 0.5% and 3% (1), which in some cases can lead to death. In these cases, it is required high dose of antibiotics to reach effective concentrations at the implantation site. Nevertheless, high dose of antibiotics could cause toxicity. To prevent the genesis of complications associated to the development of infections, the inclusion of antibiotics into the bone cement intended for mechanical attachment of the prosthesis to bone tissue has been suggested. The main advantage of this use of antibiotics derives directly from antibiotic release in the effect-site, allowing to achieving high concentrations at the site of action, and minimal or no systemic toxicity (2, 3). Currently, polymethylmethacrylate (PMMA) is the most widely used bone cement material for loading antibiotics and represents the current standard as an antibiotic delivery vehicle in orthopaedic surgery.

This route of administration was first described by Buchholz and Engelbrecht (4). However, its role in infection prevention, and therefore the use of these systems in clinical practice, has proved controversial because of the uncertainty about the development of possible antibiotic resistance after prolonged exposure time, the effectiveness, cost of these systems, toxicity and loosening of mechanical properties (5-9). These aspects are reviewed in this document.

METHOD

A systematic review of the available literature was performed using the keyword terms “antibiotic loaded bone cement” and “arthroplasty”; there was no limit on the year of publication. The search was limited to English papers. The following databases were accessed on 9th June 2016: PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez/). In order to be considered eligible for inclusion, studies needed to focused on the prophylaxis of infection. Studies were excluded if: (1) outcomes of antibiotic-loaded bone cements (ALBC) use in primary TKA were not reported; (2) it was impossible to extrapolate or calculate the necessary data from the published results.

EVIDENCE OF EFFECTIVENESS AND SAFETY

The review of Kynaston-Pearson et al. shows that 8% of all primary hip replacement prosthesis implanted in 2011 and recorded by the National Joint Registry (NJR) had no readily available evidence relating to their safety or effectiveness (10). This has led to further research in this field. Nevertheless, it is very difficult because there is a high number of cement brands and prosthesis brands; for example, in UK in 1996 there were 62 components in the market and in 2011 there were 265 different implants (11). This fact added to the low number of patients included in the studies, makes that the studies cannot provide sufficient evidence.

The Food and Drug Administration (FDA) in 2003 authorized antibiotic-loaded into bone cement for second-stage reimplantation after infected arthroplasties. In contrast, the use of these delivery vehicles for prophylaxis in prosthesis surgery is an off-label use (12). Nevertheless, the use of antibiotic loaded bone cement is recommended by most authors for joint arthroplasty revisions and in primary implants, which are at higher risk of infection (7). Live audience polling at the 2009 American Association of Hip and Knee Surgeons Annual Meeting demonstrated that 37% of surgeons in attendance ‘‘always’’ used antibiotic loaded into bone cement for routine primary total knee arthroplasty, while 45% used it on a more selective basis for high-risk patients (13). In the United States, off-label use of antibiotic-impregnated polymethylmethacrylate for primary joint replacement is increasing and multiple antibiotic-containing polymethylmethacrylate products are commercially available. However, the use of antibiotic loaded bone cement in primary arthroplasty is controversial because its inclusion can reduce the mechanical properties of the cement and its uses would produce bacteria resistance.

Currently, a few clinical assays evaluate the efficacy of antibiotic loaded cement in primary revision arthroplasty; there are only two meta-analysis that evaluate their efficacy (Table 1).

Table 1.- Summary of meta-analysis results.

References	No of studies included (no of prosthesis)	Superficial infection rate		Deep infection rate	
		RR	IC (95%)	RR	IC (95%)
Parvizi et al. (8)	6 studies (24,661)	--	--	0.55	0.34-0.75
Wang et al. (9)	8 studies (6,381)	1.47	1.13-1.91	0.41	0.17-0.97

1. Parvizi et al.(14) evaluated the efficacy of gentamicin loaded cement in primary revision arthroplasty. A total of 21,444 knees arthroplasties impregnates with gentamicin or not were evaluated. Only one of the six studies evaluated by the authors reached the statistical significance in prophylaxis of infection. This paper concluded that the antibiotic loaded cement reduced about 50% the deep infection rate (from 2.3% to 1.3% when antibiotic loaded cement was used) with statistical significance in favour of antibiotic loaded into bone cement.
2. Wang et al. (15), evaluated the deep and superficial infection rate when antibiotic was incorporated into bone cements (3 studies with gentamicin included into Palacos, 1 with tobramycin included into Simplex P, 1 with cefuroxime included into Simplex P, 1 with erythromycin and colistine included into Simplex P and 2 with cefuroxime included into CMW) in primary revision arthroplasty. In this study, the authors stated that the meta-analysis reported by Parvizi et al. included some nonrandomized studies and their results should thus be treated with caution. Therefore, the inclusion criteria applied by Wang et al. were more restrictive and evaluated a total of 6,381 arthroplasties. The authors found statistically significant differences in deep infection rate but not in superficial infection rate. However, there were no statistically significant differences in aseptic loosening of prostheses (noninfectious loosening is defined as normal erythrocyte sedimentation rate, no pain and bacteriologic cultures to be negative) neither in clinical objectives (articular function evaluation).

In summary, attending to both meta-analysis results it can be considered that the antibiotic loaded bone cement would provide clinical profit in primary surgery, as prophylaxis, in order to prevent deep infection.

ANTIBIOTIC DECISION

Dose of Antibiotic

The dose of antibiotic to be used in arthroplasty is not completely established, it depends if it is going to be used as treatment or prophylaxis. In most cases, it appears that the dose is set according to its influence on the mechanical properties of the cement, rather than to its therapeutic efficacy. It is established that to pursuit therapeutic treatment, it is usually recommended to add 3.6 g of antibiotic to 40 g of acrylic cement in order to guarantee the correct drug levels (16, 17). Conversely, for a prophylactic effect, it appears to be sufficient with low dose of antibiotic. In this case it is recommended to use 1 g of antibiotic per 40 g of cement; the lower proportion of antibiotic is less likely to alter the mechanical properties of the cement (18).

Characteristics of the Antibiotic

Experience has shown that not all antibiotics satisfy the properties required to be incorporated into bone cements. At the moment, it is known that antibiotic election has to satisfy some criteria:

1. Stability at high temperature. The polymerization of PMMA increases the temperature of the cement mixture to 60ºC-80ºC (19). Furthermore, it should be ensured that the degradation products, derivate of high temperature exposure, are not toxic drugs.
2. Different authors have reported that the inclusion of liquid antibiotic shows higher amount of antibiotic eluted but a loosening of the mechanical properties (this cements do not satisfy the ISO normative 5833 -Annex E-) (20, 21). In this way, the antibiotic included in bone cement must be in solid form. Nevertheless, the mechanical properties influenced by each antibiotic in solid form must be studied in order to guarantee that the corresponding ISO normative is accomplished.
3. The antibiotic must be effective against most frequently microorganism that cause infection (wide antibacterial spectrum), specifically, methicillin-resistant Staphylococcus aureus (MRSA) (22) and gram negative aerobic bacillus (23).
4. Antibiotic elution from bone cement depends on the penetration of the surrounding media into the cement matrix, which is dictated by the wettability of the polymer, by the number and size of the pores in it and by antibiotic solubility (24). Then antibiotics are required high solubility in water (19).

5. Although antibiotic doses (1 g of antibiotic per 40 g of cement) are established to preserve mechanical integrity, the antibiotic doses are not equipotent. Thus, it is not the same 1 g of gentamicin (habitual intravenous dose is 240 mg /24 hours) with that 1 g of amoxicillin (common intravenous dose is 3000 mg/24 hours). For these reason another feature for the antibiotic inclusion is that it has to be effective at low doses.

However, the antibiotic elution requirements are unknown to date.

Single Antibiotic Incorporation

The most commonly mixed antibiotics are gentamicin, tobramycin, vancomycin and clindamycin. These antibiotics satisfy the commented criteria and are found on the market, such as ready-mixed. The two antibiotics ready-mixed more used are gentamicin and vancomycin. Ferraris et al. compared the commercial antibiotic-loaded bone cement (Palacos R + G®) and manually mixed (Palacos R® and Palacos LV® added with gentamicin) evaluating their antibacterial behavior based on inhibition zones. They concluded that commercial formulation produces an inhibition zone that is a bit larger (23% greater, $P<0.05$) and more regular than the manually mixed preparation. They attributed the differences to the lack of use of vacuum mixing techniques in manual mixtures (25). A limitation of this study is that the antibiotic powder employed in manual mixing is a commercial gentamicin sulphate, which is a mix of different substances; this limitation is present in many studies. Therefore it can be concluded that the manual addition of commercial antibiotics to PMMA-based bone cement produces inhibition zones that are moderately smaller and more irregular compared to commercial formulations of the same antibiotic-loaded bone cements.

Other antibiotics, under research, that have been mixed by some authors are ciprofloxacin (26), cefazolin (27), moxifloxacin (28), amoxicillin clavulanate (28) (table 2).

Table 2.- Summary of clinical studies of antibiotic into bone cement. N/S= unknown; ND=no differences; (1).- Axial Compression testing.

Study	Mixture	Antibiotic in cement (per 40g)	Cement type	Percentage released (time)	Bioactivity	Mechanical properties
Ferraris et al. 2010 (25)	Pre-mixed	Gentamicin 0.5 g	Palacos R®	N/S	Inh zone= 8.1 mm	N/S
	Manual	Gentamicin 0.5 g	Palacos R + G®	N/S	Inh zone= 10.0 mm	N/S
Neut et al. 2010 (49)	Pre-mixed	Gentamicin 0.5 g	Refobacin Palacos R	8.6 ± 0.6% (168 h)		N/S
		Gentamicin 0.5 g	Refobacin Bone Cement R	12.2 ± 0.8% (168 h)	A gentamicin-sensitive bacterium did not survive. Survival was independent of the level of burst release by the bone cement.	N/S
		Gentamicin 0.5 g	Palacos R + G	12.5 ± 3.6% (168 h)		N/S
		Gentamicin 0.5 g	SmartSet GHV	3.6 ± 0.4% (168 h)		N/S
Martínez-Moreno et al. 2015 (26)	Manual	Ciprofloxacin hydrochloride (1 g)	Simplex®	2.65% (1344 h)		N/S
			Lima®	2.42% (1344 h)		N/S
			Palacos®	3.50% (1344 h)	The concentrations of ciprofloxacin reachable in the implant would be higher than 0.1 µg/mL in 100% of patients, decreasing the coverage when higher concentrations are need.	N/S
		Ciprofloxacin base (1 g)	Lima®	0.75% (1344 h)		N/S
	Vacuum	Ciprofloxacin hydrochloride (1 g)	Simplex®	2.85% (1344 h)		N/S
			Lima®	5.40% (1344 h)		N/S
			Palacos®	4.63% (1344 h)		N/S
Paz E. et al 2015 (27)	Vacuum	Vaconmycin (1 g)	Palacos®	8.58% (672 h)	N/S	ND
		Vaconmycin (4 g)	Palacos®	2.89% (672 h)	N/S	ND
		Cefazolin (1 g)	Palacos®	27.14% (672 h)	N/S	ND
Gálvez-López R. et al 2014 (28)	Manually	Vancomycin (1 g)	CMW®	31.32% (720 h)	N/S	586.2 mPa
		Gentamycin (1 g)	CMW®	13.31% (720 h)	N/S	166.27 mPa
		Moxifloxacin (1 g)	CMW®	50.40% (720 h)	N/S	383 mPa
		Rifampicin (1 g)	CMW®	41.24% (720 h)	N/S	42 mPa
		Daptomycin (1 g)	CMW®	17.09% (720 h)	N/S	78.5 mPa
		Ertapenem (1 g)	CMW®	22.54% (720 h)	N/S	121 mPa
		Meropenem (1g)	CMW®	27.24% (720 h)	N/S	342 mPa

		Cefotaxime (1g)	CMW®	26.50% (720 h)	N/S	75.82 mPa
		Ampicilin (1g)	CMW®	0.99% (720 h)	N/S	N/S
		Cefepime (1 g)	CMW®	1.49% (720 h)	N/S	144 mPa
Hsu et al 2014 (29)	Manual	Daptomycn (0.5 g)	Osteobond ®	9.59%	All bone cements of the three daptomycin preparations (low, mid, and high) produced detectable bacterial inhibition on day 1. However, growth inhibition for all groups rapidly declined from day 2.	112.39 mPa
		Daptomycn (1 g)	Osteobond ®	15.25%		112.97 mPa
		Daptomycn (2 g)	Osteobond ®	20.64%		112.97 mPa
Snir et al. 2013 (30)	Manual	--	Smart Set GHV ® and CMW1®	--	--	2285 N (1)
	Manual	Linezolid (1 g)	Smart Set GHV ® and CMW1®	N/S	MRSA MIC=0.625 mcg/mL S. epidermidis MIC=0.312 mcg/mL	2552 N (1)
		Vancomycin (1 g)	Smart Set GHV ® and CMW1®	N/S	MRSA MIC=1.25 mcg/mL S. epidermidis MIC=1.25 mcg/mL	2344 N (1)
		Gentamicin (1 g)	Smart Set GHV ® and CMW1®	N/S	MRSA MIC=0.1 mcg/mL S. epidermidis MIC=7.81 mcg/mL	2301 N (1)
		Linezolid (1 g) + Vancomycin (1 g)	Smart Set GHV ® and CMW1®	N/S	MRSA MIC=0.625 mcg/mL S. epidermidis MIC=0.312 mcg/mL	2480 N (4)
		Linezolid (1 g) + Gentamicin (1 g)	Smart Set GHV ® and CMW1®	N/S	MRSA MIC=0.625 mcg/mL S. epidermidis MIC=0.312 mcg/mL	2513 N (1)
Van de Belt et al. 2001 (18)	Manual	Gentamicin (1 g)	CMW1 ®	3.52% (168 h)	Reduction on biofilm formation only before 6 h	N/S
			CMW3®	3.16% (168 h)	Reduction on biofilm formation from 24 to 72 h	N/S
			CMW Endurance®	3.40% (168 h)	Reduction on biofilm formation only after 48-72 h	N/S
			CMW2000 ®	2.64% (168 h)	Reduction on biofilm formation only after 48-72 h	N/S
		Gentamicin (0.82 g)	Palacos®	3.43% (168 h)	Reduction on biofilm formation only before 6 hours	N/S
			Palamed®	6.86% (168 h)	Reduction on biofilm formation only after 48-72 h	N/S
Cerretani et al.2002 (48)	Manual	Vancomycin (2 g)	CMW1	2.00% (840 h)	N/S	N/S
		Vancomycin (2 g)	Simplex P	1.69% (840 h)	N/S	N/S
		Vancomycin (2 g)	Palacos-R	1.94% (840 h)	N/S	N/S
		Vancomycin (2 g) + Imipenem-cilastatin (2 g)	CMW1	2.61% (840 h)	N/S	N/S
		Vancomycin (2 g) + Imipenem-	Simplex P	2.54% (840 h)	N/S	N/S

	cilastatin (2g)				
	Vancomycin (2g) + Imipenem-cilastatin (2g)	Palacos-R	2.91% (840 h)	N/S	N/S

Our research team, examined ciprofloxacin release from three trademarks of bone cements (Simplex®, Lima® and Palacos®) and its bioactivity using as variables, the mixing method, the chemical form of the antibiotic and the antibiotic combination. The antibiotic amount released in base form represents 35% of antibiotic amount released when hydrochloride form is incorporated. Moreover, the combination (vancomycin and ciprofloxacin) shows a stronger release (132%) than hydrochloride ciprofloxacin alone. Three cements tested show equal drug release profile ($P > 0.05$). A bioactivity simulation exercise showed that until 72 hours post-surgery, ciprofloxacin concentrations in the implant would be higher than 0.1 µg/mL in 100% of the patients. The limitations of this study is that no bending nor modulus strengths were calculated and the bioactivity was evaluated by means of a simulation exercise (26).

Paz E. et al studied the inclusion of vancomycin or cefazolin at prophylaxis doses (1 g of antibiotic per 40 g of bone cement) into bone cement Palacos R+G®; vancomycin and cefazolin release, fluid absorption, and mechanical properties were evaluated under physiological conditions. Cefazolin at 672 hours showed higher release (227.28 ±23.91 µg/mL) compared to vancomycin (71.86 ± 25.34 µg/mL) ($P<0.01$). However, the differences in release between both antibiotics was not so marked during the first 24 hours, being 44.26 ± 3.37 µg/mL and 32.46 ± 9.70 µg/mL for cefazolin and vancomycin respectively ($P=0.281$). The compressive strength of cements added of the two antibiotics without aging and after aging for 1 month in phosphate buffered saline (PBS) at 37ºC was calculated. All cements without aging showed no statically significant difference to the control cement ($P>0.01$). However cefazolin aged in PBS at 37ºC experienced significant reductions in compressive properties ($P<0.01$). The limitation of this study is that there is no data about bioactivity and therefore it cannot be assessed whether the differences are clinically significant (27).

Gálvez-López et al. evaluated different ALBC for elution kinetics, thermal stability, and mechanical properties. A 10% or 20% mixture (w/w) beads of medium viscosity bone cement (DePuy®) and vancomycin, gentamycin, daptomycin, moxifloxacin, rifampicin, cefotaxime, cefepime, ampicillin, meropenem, and ertapenem were evaluated. Elution kinetic profiles of all

antibiotics tested, with the exception of ampicillin and cefepime, demonstrated a triphasic pattern of release with a progressive increase in the first 24 h followed by a rapid decrease and a final phase with a low and steady decline through the rest of the experiment. In this general triphasic behavior, 3 particular behaviors of elution were identified depending on the antibiotics tested. Vancomycin, gentamycin, moxifloxacin, and rifampicin, loaded at 10% (w/w), demonstrated constant elution kinetics through the 30-day duration of the experiment. Daptomycin, meropenem, ertapenem, and cefotaxim although also having the triphasic pattern, showed a lower peak and a faster decrease of elution between days 3 and 30, but eluted concentrations remained above the minimum inhibitory concentration (MIC) of susceptible organisms, according to EUCAST clinical breakpoints. Finally, ampicillin and cefepime showed minimal elution with eluted concentrations being almost undetectable at day 4 and always below the MICs of susceptible organisms, according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints. The percentage eluted from each ALBC is shown in table 2. Presence of antibiotics did not affect the strength of ALBC with mean compression values greater than 70 mPa, except for rifampicin -loaded bone cement, for which the compression strength did not exceed 42.9 mPa (28). The limitation of this study is the measurement of antimicrobial properties; at the antibacterial activity was only measured at 30 minutes from the beginning of the assay.

Hsu et al. incorporated 0.5, 1 and 2 g of daptomicin (Cubicin®, the commercial antibiotic, that has more than 90% of pure antibiotic) per 40 g of PMMA; in this study, the authors showed that the mechanical strength is not compromised by daptomicin at any concentration, because all samples had a compressive strength higher than 100 MPa. The percentage of daptomcin eluted during 2 weeks was 9.59% ± 0.85%, 15.25% ± 0.69%, and 20.64% ± 20.33% from 0.5, 1 and 2 g of daptomicin, respectively. The bioactivity of the cements was also confirmed including MSSA, MRSA, S. Epidermidis, E. Faecalis, and E. Faecium. The authors concluded that the inclusion of commercial daptomycin at low dose in bone cement was satisfactory; both bioactivity and resistance tests were adequate (29).

Snir et al. studied 1 g of linezolid, vancomycin or gentamicin per 40 g included into PMMA (Smart Set GHV® and CMW1®). There were no differences between brands cements. The study showed that linezolid shows a minimum inhibitory concentration (MIC) of 0.625, 0.312, 1, 250 and 250 mcg/mL to Methicillin-resistant Staphylococcus aureus (MRSA), S. epidermidis, VRE (vancomycin-resistant enterococci), E. Coli and K. pneumoniae respectively. Vancomycin shows a MIC of 1.25, 1.25, 0.4, 125 and 125 mcg/mL to MRSA, S. epidermidis, VRE (vancomycin-resistant enterococci), E. Coli and K. pneumoniae respectively. Finally gentamicin shows a MIC

of 0.1, 7.81, 23.43, 1 and 0.625mcg/mL to MRSA, S. epidermidis, VRE (vancomycin-resistant enterococci), E. Coli and K. pneumoniae respectively. Table 3 shows the growth inhibitory time (GIT) of beads impregnated with antibiotics.

Table 3.- Growth inhibitory time of beads impregnated with antibiotics.

Antibiotic	MRSA	*S epidermidis*	VRE	*K pneumoniae*	*E coli*
Linezolid	21±0.75[a]	29±0.5[a]	15±4.6[a]	Resistant	Resistant
Gentamicin	Resistant	5±1.7	Resistant	10±1.73	16±2
Vancomycin	8±0.5	19±1.9	Resistant	Resistant	Resistant
Linezolid± gentamicin	>45[b]	38±0.95[b]	32[b]	>45	40±0.5
Linezolid±vancomycin	31±10[c]	>45[c]	17±1.15	Resistant	Resistant

MRSA, *methicillin-resistant Staphylococcus aureus*; VRE, *vancomycin-resistant enterococci.*

[a], These values are significantly longer (P,.01) compared with those of vancomycin for respective bacteria.

[b], These values are significantly longer (P,.01) compared with those of vancomycin, linezolid, or gentamicin alone for respective bacteria.

[c], These values are significantly longer (P,.01) compared with those of either vancomycin or linezolid alone for respective bacteria.

In conclusion, the authors showed that the GIT of linezolid was significantly longer than that of vancomycin and gentamicin for MRSA and S epidermidis. Axial compression test was performed to verify if the mechanical strength of PMMA was compromised because of the addition of antibiotics. The results revealed no reduction in the mechanical strength of PMMA beads (P>0.2) with the concentration of antibiotics used in this study (maximum 5% weight/weight antibiotic per PMMA packet). Both types of cements maintained similar mechanical properties. With this study, it can be said that linezolid is more effective than gentamicin and vancomycin against MRSA and S. epidermidis. Table 2 shows that the combination gentamicin plus linezolid or vancomycin plus linezolid, do not provide a greater bactericidal potency. It can be concluded that PMMA impregnated with linezolid has the potential to be efficacious in the prevention and treatment of bone and joint infections (30). Anguita-Alonso et al. (31) found that linezolid used at 3 different concentrations (2.5%, 5%, and 7.5% weight/weight) maintained excellent stability and elution after PMMA polymerization in vitro. The PMMA used was Simplex P® in the form of beads, and the indicator microorganism was Bacillus subtilis. They also reported that compared with other antibiotics (ie, cefazolin, ciprofloxacin, gatifloxacin, levofloxacin, and rifampicin), the elution of linezolid from PMMA was less affected by impregnated antibiotic concentration.

Another important aspect related with the antibiotic loaded into bone cement is if the exposure to antibiotic causes resistance; Corona et al. have seen in their study that the inclusion of gentamicin or tobramycin in cement spacers (4g. of antibiotic / 40 g of PMMA) seems to increase the gram-positive cocci resistance. They analyzed 113 chronic hip and knee prosthesis joint infection and observed that aminoglycoside-resistance in gram-positive cocci was significantly higher when aminoglycosides were incorporated in cement spacers respect to no use of it. Gentamicin resistance after previous aminoglycoside-cement spacers use was significantly higher (49.2% vs. 19.3%; P: 0.0001) as well as resistance to tobramycin (52.7% vs. 30.9%; P: 0.014) (32). There is little evidence of this aspect.

In conclusion, the commercial formulations produce a greater and more regular release of antibiotic from bone cement than the manually mixed preparations. One of the biggest issues of most of the studies is that the commercial form of the antibiotic, which comes with excipients in many occasions, is used. This fact may explain the differences between pre-mixed ALBC and manually mixed. Finally, currently there are a large number of combinations of bone cements with antibiotics, for which much remains to be elucidated and it cannot be concluded that a perfect unique combination exists, each one adapts to the requirements of the clinical condition.

Two antibiotics combination

It has been reported that the simultaneous incorporation of two antibiotics or more into bone cement resulted in higher rate of elution compared to one antibiotic loaded bone cement. When two antibiotics are incorporated, more voids and cracks are present in bone cements as the drugs are released, thus increasing the release of the remaining antibiotics. Moreover, authors have described a synergic effect between some antibiotics, i.e. it has been described synergistic effect between aminoglycosides and glycopeptides (19). A study about the optimal antibiotic combination for the antibiotics gentamicin, vancomycin and teicoplanin in cements showed that the combination of gentamicin and teicoplanin had a bactericidal activity more prolonged than gentamicin alone. Moreover, the synergic effect of teicoplanin and gentamicin had superior bactericidal activity compared to gentamicin and vancomycin (33, 34). Bertazzoni Minelli et al. compared gentamicin plus vancomycin spacers versus gentamicin alone spacers. The study showed that the combination was more effective than gentamicin alone (35). These results are coherent with those mentioned above.

To date, there is no ideal combination of antibiotic and cement that allow to cover all possible infections and therefore, the antibiotic election must be effective against most microorganisms that cause infection.

CEMENT TYPE.

Polymethylmethacrylate (PMMA) is the main component used in the fixation of joint prosthesis. It is prepared in the operating room, mixing the solid and liquid components. As bone cements have some disadvantages, these systems are fragile and produce necrosis due to exothermic reaction during the polymerization (36-39). There have been reports of thermal damage of cartilage and periosteum, leading to non-union of fractures and loosening of implants (38, 39).

Viscosity of the cement is very important in the mixing moment. Low viscosity promotes the mixing process; however, its mechanical strength is worse than that of high viscosity cements. Clinical outcomes of low viscosity bone cement demonstrate that they have higher risk of revision and loosening (40, 41).

The method that produces the loosening is unclear to date; Ayre et al. studied the mechanism that causes the aseptic loosening. In order to explain the aseptic loosening, two commercial high viscosity bone cements (Palacos® and Cemex Isoplastic®) were aged in an isotonic fluid at physiological temperatures. After 30 days ageing cements increased in weight of approximately 2% and the outermost layers of the cement were hydrolyzed. This study concluded that this molecular change and the plasticizing effect of water resulted in reduced mechanical and fatigue properties over time and therefore cement ageing contributes to the long-term failure of cemented joint replacements (42). This kind of studies are important to simulate the evolution of bone cement into the organism.

The addition of barium sulphate and zirconium oxide (for radiological detection) increases the risk of loosening (43). These radiopacifiers are hydrophilic and promote the hydrolysis of ester groups of methyl methacrylate (MMA) and PMMA. The previous study, suggests to employ hydrophobic radiopacifiers such as iodine-based ones, developed by Lewis et al. (44) in order to decrease the risk of loosening. Shearwood et al. studied the effect of barium sulphate agglomerates on mechanical characterisation of bone cement. They evaluated the effect of barium sulphate agglomeration on crack initiation processes in conventional, vacuum-mixed acrylic cement. The tendency of barium sulphate particles to agglomerate is

clearly evidenced to be detrimental to the fatigue performance of the cement (45). Gomoll et al. studied the effect of replacing barium sulphate microparticles that are usually present in commercial PMMA cements with barium sulphate nanoparticles. They concluded that the nanoparticulate substitution of radio-opacifiers substantially improved the in vitro mechanical properties of PMMA bone cement without changing the known chemical composition (46). Ultimately, the use of the hydrophilic radio-opacifiers damage the mechanical properties of bone cements, so there is more investigation required to find alternatives for the future.

Antibiotic elution from bone cement depends on cement composition and physicochemical characteristics of antibiotic. About gentamicin, Van de Belt et al. studied the formation of a Staphylococcus aureus biofilm on six gentamicin-loaded bone cements (CMW1®, CMW3®, CMW Endurance® and CMW2000® with 2.5% of gentamicin; Palacos® and Palamed® with 1.25% of gentamicin). None of gentamicin-loaded cements showed a reduction in biofilm formation relative to unloaded cements within 6 h after inoculation, whereas only gentamicin-loaded CMW1® and Palacos® reduced biofilm formation 24 h after inoculation. Alternatively, CMW Endurance®, CMW2000®, and Palamed® did not exhibit any initial reductions in biofilm formation, but effects started after 48, and 72 h, respectively. Biofilm reduction by gentamicin-loaded CMW3® lasted the longest from 24 to 72 h. Biofilm formation on all cements follows a similar pattern in time, but the gentamicin-loaded cements demonstrate different reductions of biofilm formation, that seems unrelated with the gentamicin-release kinetics from the cements, previously measured (table 2). The authors conclude that biofilm formation on bone cements is not only related to gentamicin release, but may also be dependent on other properties of the cement surface, such as its roughness (18). Scott et al., compared the bioactivity of the two most used aminoglycosides (tobramycin and gentamicin) from different cements (Palacos® and Simplex®), and showed that tobramycin incorporated into Simplex® has antibacterial activity against 98% of P. aeruginosa while gentamicin into Palacos® against 93% of the same bacteria ($P<0.001$). In this study, the authors compared the zone of inhibition of gentamicin and tobramycin loaded into bone cement at prophylaxis doses against 100 clinical isolates of P. aeruginosa collected from sputum, urine, ear… but none that has caused a prosthetic infection. Results are consistent with the type of antibiotic, because tobramycin is slightly more effective than gentamicin against P. aeruginosa (47). With respect to vancomycin, Cerretani et al., compared the 2 g of vancomycin elution from 40 g of CMW1®, Palacos-R® and Simplex-P® with a pharmacokinetic study. The authors performed a pharmacokinetic study in which evaluated the area under the concentration-time curve against time (AUC), which represents the amount of drug released and pharmacologically available; the half-life of release (t1/2); peak

concentration (Cmax); and time at which Cmax is obtained (Tmax). The cements released 2.00%, 1.94% and 1.69% of antibiotic incorporated after 35 days, respectively. Only t1/2 showed statistically significant differences between bone cements brands; having CMW1® a significantly longer release half-life. Although there are significant differences, the clinical implications that this may involve are not clarified; bioactivity studies are needed in order to extract the clinical impact of differences (48).

About the comparison of pre-mixed commercial ALBC, Neut et al. investigated differences in gentamicin release and the antibacterial efficacy of the eluent between four cement brands (Refobacin Palacos R®, Refobacin Bone Cement R®, Palacos R + G® and SmartSet GHV®). Table 2 shows the differences in the amount of antibiotic eluted and the bioactivity. Although the cements Refobacin Bone Cement R® and Palacos R + G® provided higher release of antibiotic, there was no colony growth in any cement sample during the one-week study, so it can be said that all commercial cements with gentamicin had adequate bioactivity during the first week (49).

MIXING METHOD

The mixing method characterizes the antibiotic elution. The best antibiotic elution is associated to high cement porosity. The problem of high porosity is the loss of mechanical properties (33, 50, 51). The presence of air trapped into cement, decrease its resistance. The vacuum mixing decreases the air trapped into cement from 25% to 1%. Therefore, this mixing method provides advantages: the resistance increases from 70 to 90 MPa and fatigue resistance rises from 10 to 30 MPa (51, 52). Nevertheless, the preparation under vacuum conditions causes a major reduction of bone cement and then a worse adhesion from bone cement-to bone is obtained (41, 53). There is a division of opinions according to the authors (54). Meyer J et al., (55) compared 6 commercial bone cements (Cemex Genta Gentamicin 1.0 g/40 g, Cobalt G-HV Gentamicin 0.5 g/40 g, Palacos R+G Gentamicin 0.5 g/40 g, Simplex P Tobramycin 1.0 g/40 g, SmartSet GMV Gentamicin 1.0 g/40 g and VersaBond AB Gentamicin 1.0 g/40 g) mixed at atmospheric pressure and under vacuum conditions. A standard Kirby-Bauer bioassay technique was subsequently used to quantify antibiotic elution from the products. The results from the study demonstrated that vacuum mixing produced lower antibiotic release from Cemex®, SmartSet® and Versabond® and increased release of antibiotic from Palacos®, Simplex® and Cobalt® (Fig. 1). According to these statements, the study concluded that the effect of vacuum-mixing on antibiotic elution is product-specific (55). Our research team, compared the manual

and vacuum mixing when ciprofloxacin hydrochloride was mixed with different bone cements (Simplex®, Palacos® and Lima CMT1®). When comparing the two mixing procedures, no statistically significant differences were found between vacuum and manual mixing with respect to the drug release rate from Simplex® and Palacos® bone cements. On the contrary when Lima CMT1® bone cement was used, significant differences were observed up to 697 hours. However, no statistically significant differences in the percentage of amount released were observed at subsequent testing times. This significant difference can be explained if the high variability of the manual batches tested is considered. It should be emphasized that variability of the percentage of drug released from the vacuum-mixed samples was much lower than that seen with manually-mixed ones. Ultimately, vacuum mixing reduces variability in the release profiles, but the influence on kinetic properties are product-specific.

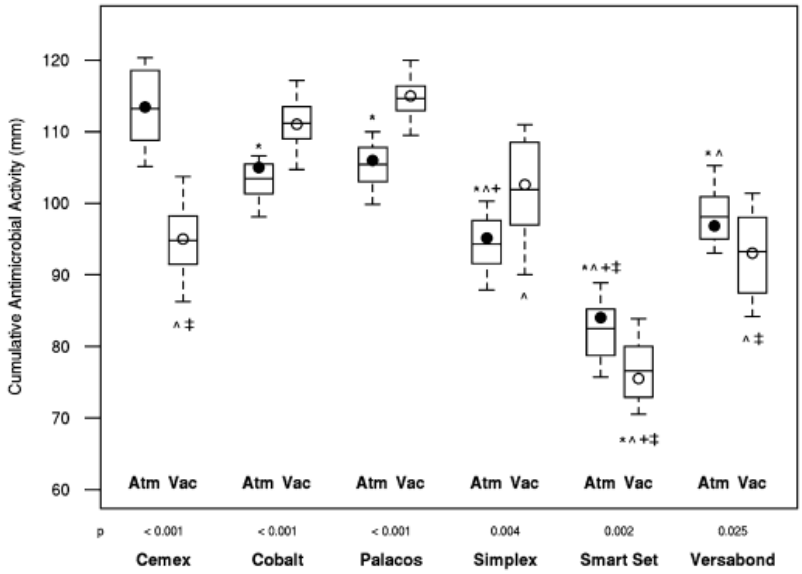

Figure 1.- Summary of use of vacuum mixing when Cemex®, SmartSet®, Versabond®, Palacos®, Simplex® and Cobalt® was used.

Some authors advocate for vacuum mixing, because the surgeons have less exposure to cement vapours; to date several studies have shown that exposure to vapours from bone cements provide undetectable plasma levels. Homlar et al. studied the effect of exposure to PMMA. Twenty healthy volunteers were exposed during the mixing of polymethylmethacrylate cement in a simulated operating room environment (this study was purposefully designed using non-laminar flow rooms, open bowl mixing technique, and without the use of personal exhaust hoods to simulate a worst case scenario exposure). Methyl methacrylate was not detected in any of experimental specimens (56).

Another important aspect is the mixing speed; Pithankuakul K. et al. evaluated the effect of the mixing speed of hand-mixing bone cement. In the study, the antibiotic-loaded bone cement used was Vancomycin-Palacos LV. The authors concluded that bone cement prepared with high-speed hand mixing and delayed antibiotic addition can increase vancomycin release (57).

As shown, antibiotic elution depends on many factors (cement characteristics, physicochemical properties of the antibiotic and mixing procedure, among others), and there is no absolute best option; but there is an optimal combination (antibiotic plus cement brand) for each microorganism. The continuing emergence of new commercially-available brands of ALBCs makes it important to establish which one will provide the most favorable antibiotic release, and consequently yields the best antibacterial efficacy.

RELEASE KINETICS.

Different authors have indicated that the inclusion of the antibiotic into bone cement provide high antibiotic level in first days, followed by a sustained release (19). There are different studies showing evidence that release can be produced during the first hours in some cases or for several weeks in others (58, 59). First the antibiotic is eluted from the cement surface and then from the cement inside. The fluids in contact penetrate into cement and dissolve the antibiotic. Then, the antibiotic dissolved is eluted from void and cracks of bone cements (60, 61). Various authors had stated that antibiotic elution from bone cement is conditioned by cement type and porosity, antibiotic molecular weight and physico-chemical properties, surface in contact with the liquid of the environment and amount of antibiotic incorporated (16, 20, 21, 62, 63). The problem is that the PMMA is a highly hydrophobic polymer, which limits the elution. For this reason, some antibiotics are only eluted during the first hours, that is, only antibiotic on surface is released (64). Only high solubility and low molecular weight antibiotics would be elute through voids and cracks. (16, 20, 21, 62, 63)

Since antibiotic dissolved from cement surface represents the highest amount released, cement surface in contact with fluids conditions efficacy. Moojen et al. and Bertzzoni et al. showed that the initial release is proportional to the rugosity and then to the surface (35, 65); while release in the following days is proportional to cement porosity. This statement is logical and it should always be extrapolated into clinical practice.

As stated above, currently, it has been approved the use of premixed antibiotic loaded bone cement. Only commercially available antibiotic- PMMA can be used for reconstruction of a previously septic total knee or total hip replacement. The antibiotic incorporation to bone cement by surgeon is not permitted and therefore the only antibiotics available are vancomycin, clindamycin, tobramycin and gentamicin. Meta-analysis previously referenced (15, 66) showed that the inclusion of antibiotic into bone cement demonstrated its efficacy in deep infection but not in superficial infection. This evidence was expected because the antibiotic released out of cement, would stay in the cement-bone interface. In any case, the antibiotic release from bone cement would be an effective system for deep infection, which is more complicated due to poor blood supply.

In summary, the PMMA highly hydrophobic polymer, limits the elution, and makes it dependent on features of the antibiotic and the surface in contact. Some authors discussed the possible systemic bioavailability of antibiotics from bone cement. Kendoff et al. evaluated the systemic bioavailability of antibiotics from bone cement after implantation determining the concentrations of gentamicin and vancomycin in plasma and urine of patients receiving a novel bone cement during one-stage revision in periprosthetic hip infections. The mean postoperative maximum gentamicin plasma concentration at 5.85 hours was 209.65 ng/mL. For vancomycin, a mean postoperative maximum plasma concentration of 134.64 ng/mL was determined at 20.03 hours. The authors concluded that it exists slow absorption of both antibiotics after release from the cement resulting in plasma concentrations well below toxic levels, that do not result in a critical systemic concentration potentially inducing bacterial resistance (67). In any case, ALBC are safe from the pharmacotherapeutical point of view, with a very low systemic absorption.

PERSPECTIVES AND CONCLUSIONS.

Currently, researchers are looking for ways to increase and improve these systems release. In this manner, there are studies where some substances are included into bone cement in order to improve the elution. As an example, it has been observed that vitamin E is a scavenger of free radical in the oxidative process. Moreover, its inclusion in bone cement reduces the temperature of the harden process (62 to 36 degrees C) and therefore, increases cytocompatibility. Up to 25% of vitamin E does not decrease the mechanical strength (68). Penalba Arias et al. studied the effect of bone cement loaded with daptomycin alone or in combination with gentamicin or PEG600 in the prevention of biofilm formation of S. epidermidis.

For comparison, PMMA loaded with gentamicin or vancomycin was tested. The study showed that vancomycin was superior to daptomycin and gentamicin inhibiting staphylococcal adherence in vitro. However, PMMA loaded with daptomycin combined with gentamicin or PEG600 completely inhibited S. epidermidis-biofilm formation (69).

It has been demonstrated that the inclusion of chitosan nanoparticles has activity against S. aureus y S. epidermidis, without decrease in mechanical strength compared to PMMA alone (70). The inclusion of this polysaccharide would have antimicrobial activity per se. These findings support the possibility of combining in cements this polymer with antibiotics. Another improvement is the inclusion of silver nanoparticles (71). When this metal is included in cements it is eluted and has antimicrobial activity against A. baumanii, P. aeruginosa, P. mirabilis y S. aureus, but its inclusion reduces the mechanical strength of cement (72).

Although there are still many variables to elucidate, antibiotic loaded bone cements are a successful alternative to decrease the infection rate. Many questions, like, which is the optimal dose, which patients would benefit of it or which is the optimal antibiotic-cement combination in order to eradicate microorganisms specifically, are still open. Nevertheless, there are a lot of ways for improving these delivery systems that can lead in the future to ALBC able to provide clinical profit in primary surgery, as prophylaxis, in order to prevent deep infection.

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CAPÍTULO 2

Study of the Influence of Bone Cement Type and Mixing Method on the Bioactivity and the Elution Kinetics of Ciprofloxacin

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Journal of arthroplasty. 2015;30(7):1243-9.

Abstract

The objectives of this in vitro study were to examine ciprofloxacin release from three trademarks of bone cements (Simplex®, Lima® and Palacos®) and its bioactivity using as variables, the mixing method, the chemical form of the antibiotic and the antibiotic combination

The antibiotic amount released in base form represents 35% of antibiotic amount released when hydrochloride form is incorporated. Moreover, the combination (vancomycin and ciprofloxacin) shows a stronger release (132%) than hydrochloride ciprofloxacin alone. Three cements show equal drug release profile ($p>0.05$).

A bioactivity simulation exercise showed that until 72 h post-surgery, ciprofloxacin concentrations in the implant would be higher than 0.1 µg/mL in 100% of the patients. After drain removal, it is expected that bioactivity would increase since drug clearance from implant would decrease.

Key words

Bone Cement, ciprofloxacin, elution kinetics, orthopedics, bioactivity, PMMA.

Introduction

Antibiotic loaded bone cement used in prosthesis fixing, is a local release form that minimizes the prevalence and the complications that the antibiotics would unleash when administered intravenously. This system, described by Buchholz and Engelbrecht(1), is a well-established tool in the prophylaxis(2,3) and treatment of orthopedic infections(4) in humans and animals(5), with meta-analyses indicating that its use reduces the infection rate(6). Polymethylmetacrylate – PMMA - is characterized by excellent biocompatibility with low intrinsic toxicity and inflammatory activation(7), but experience has shown that not all antibiotics have the properties necessary for their incorporation in this cement. In this context, aminoglycosides and glycopeptides (vancomycin) are known to be the two groups of antibiotics that satisfy the optimal criteria to be included in these cements (availability in powder form, wide antibacterial spectrum, bactericidity at low concentrations, elution from PMMA in high concentrations for prolonged periods, thermal stability, low or no risk of allergy or delayed hypersensitivity, low influence on the mechanical properties of the cement, and low serum protein binding)(8).

50% of surgical site infections (both superficial and deep) are caused by Staphylococcus aureus methicillin-resistant (MRSA); thus, staphylococcal species should be the primary target of antibiotic-loaded bone cement(9). Unfortunately, the increasing number of multi drug-resistant bacteria(10-13) limits the continued effectiveness of this tool. In addition, the prevalence of MRSA in many hospitals influences strategies for the treatment and prevention of prosthetic joint infections(14), leading to interest in incorporating alternative antibacterial agents into PMMA cement (10,14,15).

On the other hand, despite the wide use of antibiotics in orthopedic surgery for more than 30 years, the exact mechanism by which they are eluted from PMMA is still not fully understood (8). It seems to involve a biphasic profile, consisting of an initial rapid release of drug followed by a much slower sustained release. The following factors affect the release of antibiotics from bone cement: type and quantity of antibiotic (16,17); type and porosity of cement (18); surface characteristics (19); and how the cement has been prepared (20-23). Thus, to date only a few antibiotics have been satisfactorily incorporated into cements.

In this context, it would be desirable to incorporate new drugs into bone cements in order to increase coverage to infections caused by different organisms. In the present work, ciprofloxacin (1 g antibiotic / 40 g PMMA) was selected to be assayed. This synthetic fluoroquinolone is an antibacterial agent that can be administered safely and effectively to treat

most clinical isolates in infections associated with joint prostheses and chronic osteomyelitis. Additionally, ciprofloxacin possesses a broad spectrum against Gram positive and negative strains (24). However, there are few data concerning the ability of ciprofloxacin to elute from bone cement and to retain activity against resistant pathogens after elution (25,26).

In this study we set out to characterize the elution profile of ciprofloxacin from bone cements. The following variables were evaluated: source of drug (base and hydrochloride); cement composition (three brands); mixture method (manual and vacuum); and presence of a second antibiotic in the mixture. In addition, different equations were fit to release profiles in order to explain the release mechanism. Finally, bioactivity of the mixtures was evaluated by means of a simulation exercise.

Materials and methods

Ciprofloxacin hydrochloride, Ciprofloxacin base and Vancomycin hydrochloride were purchased from Aldrich (Madrid, Spain). Lima CMT1® bone cement was purchased from Lima Implantes (Barcelona, España), and Palacos® and Simplex® from Ibersugical (Valencia, España). Each cement was provided as two separate components: a powder mixture and a liquid component. The composition of the cements is shown in Table 1, according to the information provided by the manufacturers.

Table 1. Composition of the different acrylic bone cements, as provided by the manufacturer.

	LIMA CMT 1	SIMPLEX	PALACOS
Solid Component (40g)	Methyl methacrylate 87.6% Benzoyl peroxide 2.4% Barium Sulphate 10%	Methyl methacrylate-styrene copolymer 30g Polymethyle methacrylate 6g Barium Sulphate E.P. 4g	Poly(methylacrylate. methyl methacrylate) 33.8 g Zirconium Dioxide 6.0 g Benzoyl peroxide 0.2 g Colorant E141 0.008 g
Liquid component (20mL)	Methyl methacrylate 84.4% Buthylmethacrylate 13.2% N, N-dimethyl pare toluidine 2.4% Hydroquinone 20ppm	Methyl methacrylate 19.5mL N, N-dimethyl pare toluidine 0.5mL Hydroquinone, USP 1.5 mg	Methyl methacrylate 18.4 g N,N-dimethyl-p-toluidine 0.4 g Hydroquinone Colorant E141 0.005 g
Viscosity	STANDARD	MEDIUM	HIGH

Palamix uno®, the vacuum mixing system employed, was supplied by Heraeus Medical GmbH (Madrid, España).

Buffered saline solution pH 7.4 was prepared as described in the US Pharmacopoeia: disodium hydrogen phosphate anhydrous 2.38 g, mono-potassium hydrogen phosphate anhydrous 0.19 g and sodium chloride 8 g per 1 L of purified water. All reagents were analytical grade (Panreac).

Antibiotic-loaded bone cement cylinders were prepared as follows: 1 g of the drug was added to 40 g of solid component of the cement, and, after mixing the powder, the liquid component was added following the manufacturer's instructions. Cylinders of antibiotic bone cement were made for each batch in a standardized fashion according to the ISO normative 5833 (Annex E). Samples were prepared using Teflon molds in which they were kept for 1 hour until completely hardened into a cylinder/disk shape. Each specimen was carefully weighed and measured and the theoretical amount of loaded ciprofloxacin calculated. This value was used for calculating the exact percentage released from each sample.

When two antibiotics were incorporated into the cement the total amount of antibiotic in the mixture was 1g (50% each one).

Samples were immersed in a water bath in 10 ml phosphate buffer saline pH 7.4 at 37ºC and stirred for 8 weeks. Samples were taken 1, 3, 5, 7, 24, 32, 48 56, 72, and 168 hours after immersion, and subsequently once a week for a period of 8 weeks (final sample was taken 56 days after immersion). Three samples per batch were tested. Antibiotic homogeneity distribution within batches was indirectly evaluated by means of the statistical analysis of the percentage of the total antibiotic released from the samples assayed. The phosphate buffer was replaced every time a sample was taken in order to maintain the sink condition (defined as the volume of medium at least three times that required in order to form a saturated solution of drug substance). All samples taken were frozen at -20ºC until analyzed. Table 2 summarizes the test conditions (a total of 61 samples were processed).

Table 2. Samples assayed in each condition tested.

Mixture	Antibiotic	Bone Cement	Sample
Manual	Ciprofloxacin hydrochloride	Simplex®	A
		Lima®	B
		Palacos®	C
	Ciprofloxacin base	Lima®	D
	Ciprofloxacin hydrochloride and vancomycin	Simplex®	E
		Lima®	F
		Palacos®	G
Vacuum	Ciprofloxacin hydrochloride	Simplex®	H
		Lima®	I
		Palacos®	J

Ciprofloxacin concentration was assayed by HPLC, using a Perkin Elmer® Series 200 equipped with a Waters 484® UV detector (□=254 nm). The mobile phase consisted of Acetic Acid solution 0.1 M: Acetonitrile (80:20) and was filtered through a 0.45 µm membrane filter before use. The mobile phase was eluted at a flow rate of 1 ml/min. The column was a Kromasil® C-18 with a pore size of 5.0 µm, measuring 150 mm (length) x 4.6 mm (diameter)(27).

The elution rate at each time interval (mg/h) was obtained by dividing the total quantity of antibiotic released in each interval by the elution time (in hours). The elution rates and the total amount of antibiotic released (expressed as a percentage) at each time point were compared using one-way analysis of variance (ANOVA).

Zero order (equation 1), First order (equation 2), Higuchi (equation 3) and Korsmeyer-Peppas (equation 4) equations were fit to data to characterize elution parameters and the mechanism of release of ciprofloxacin from bone cement:

$$Q_t = k_0 t \qquad \text{(Equation 1)}$$

$$Q_t = Q_0 \cdot e^{-k1t} \qquad \text{(Equation 2)}$$

$$\frac{Q_t}{Q\infty} = K_h \cdot t^{0.5} \qquad \text{(Equation 3)}$$

$$\frac{Q_t}{Q_\infty} = K_k t^n \qquad \text{(Equation 4)}$$

where t is time, Q_t is the amount of drug released at time t, Q_0 is the initial amount of drug in the specimen, Q_∞ is the amount of drug released at time ∞, n is the release exponent and K_0, K_1, K_h and K_k are the ciprofloxacin elution rate constants of each of the kinetics.

The surface morphology and internal structure of the samples were characterized using a scanning electron microscope (SEM, S-4100 Hitachi, Madrid, Spain). The samples were mounted on an aluminum stub using double-sided tape. They were made electrically conductive by coating with gold-palladium under vacuum. The SEM picture was taken at an excitation voltage of 20 kV. For the internal structure evaluation samples were fractured and the broken surfaces sputter-coated with gold and a layer of palladium for examination at 20.0 kV.

The elutions rate from different cements were used to simulate biophase concentration for 100 patients using NONMEM version VII. Simulations were performed for the three days post-surgery, using the clearance of synovial liquid values previously reported (20.42±11.3 mL/h for the first day; 9.33±11.02 mL/h for the second day and 4.11±2.95 mL/h for the third day) (28) and considering that the distribution of the antibiotic from the location of the implant to the systemic circulation is negligible. Bioactivity was evaluated using MIC distributions for P. aeruginosa (MIC= 0.25-1 ug/mL), S. aureus (MIC= 0.12 to 0.5 ug/mL) and E. coli (MIC= 0.016-0.004 ug/mL) (29) and calculating the percentage of patients whose levels of antibiotic at the site of the implant would be higher than the MIC.

Results

The variation coefficient of total amount of ciprofloxacin released within a batch was lower than 10 %. These results were considered as representative of homogeneous distribution of the drug into the samples assayed.

The influence of the chemical form was evaluated in samples B, D and F. These samples were prepared manually with Lima CMT1® bone cement. In Figure 1 are represented the amounts of ciprofloxacin (expressed as a percentage) released from the Lima CMT1® bone cement in which it was incorporated alone, as base (ciprofloxacin, sample D) or salt (ciprofloxacin hydrochloride, sample B); sample F corresponds to ciprofloxacin hydrochloride in a binary mixture with vancomycin. The amount of antibiotic released when used as a base

represented 35% of the amount released when the antibiotic was incorporated in its hydrochloride form. Moreover, the combination of vancomycin and ciprofloxacin led to a higher amount of ciprofloxacin being released; 132% the amount released from the salt form.

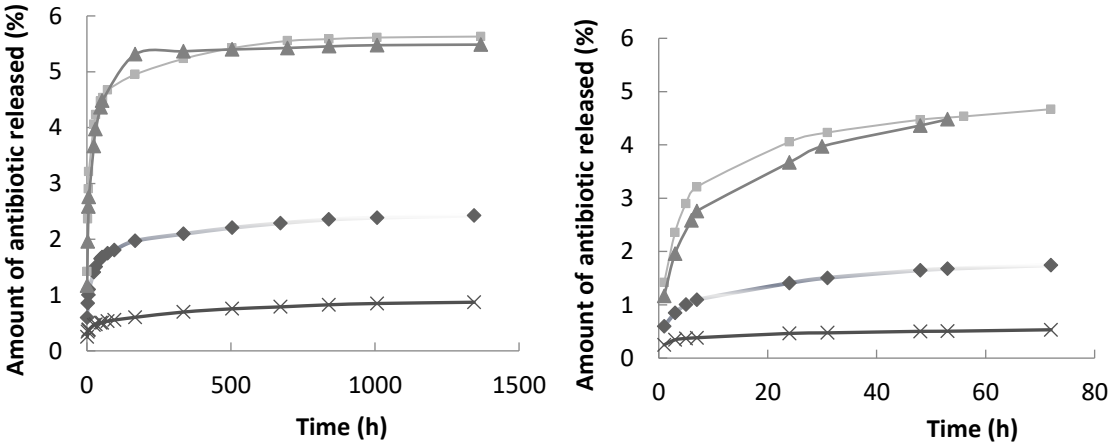

Figure 1. Percentage of ciprofloxacin released from specimens B (♦) (Ciprofloxacin hydrochloride, manual mixing), D(×) (ciprofloxacin base, manual mixing),F(■) (Ciprofloxacin hydrochloride and vancomycin, manual mixing) and I(▲)(Ciprofloxacin hydrochloride, vacuum mixing). Lima CMT 1® cement was used in all cases.

Figure 2 shows the elution rate of ciprofloxacin in mg/h at different time points, plotted on a logarithmic scale, corresponding to samples A, B and C (ciprofloxacin hydrochloride-hand mixing) and samples H, I and J (ciprofloxacin hydrochloride-vacuum mixing). All samples produced high early release rates, followed by a lower sustained release. Statistical analysis with ANOVA revealed no significant differences among the percentage of total antibiotic released from samples A, B and C, indicating that the type of cement used did not modify the amount of drug released when mixing was performed manually. On the other hand, in vacuum-prepared samples significant differences were obtained in the total amount of antibiotic released between samples H (Simplex®) and I (Lima CMT1®).

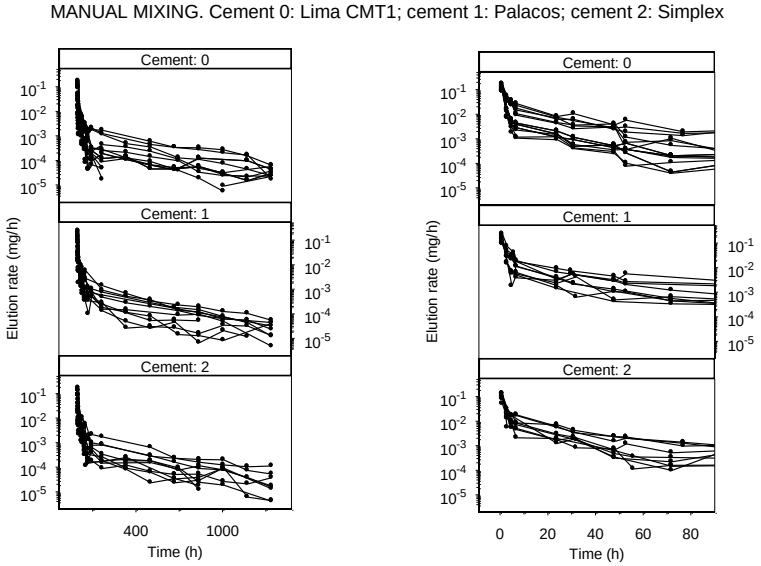

Figure 2. Ciprofloxacin elution rate (mg/h) at different time points plotted on a logarithmic scale. Manual mixing: Samples A, B and C. Vacuum mixing: Samples H, I and J.

The influence of the mixing procedure on elution rate was evaluated by comparing the results obtained between samples A and H (Simplex®, manual and vacuum mixing), between

samples B and I (Lima CMT1®, manual and vacuum mixing), and between samples C and J (Palacos® manual and vacuum mixing). Statistical differences were obtained only in Lima CMT1®. The differences observed referred to percentages released up to 697 hours and log elution rates at all time-points (hand and vacuum Lima CMT1®). Figure 3 shows the percentage of ciprofloxacin released from each sample assayed.

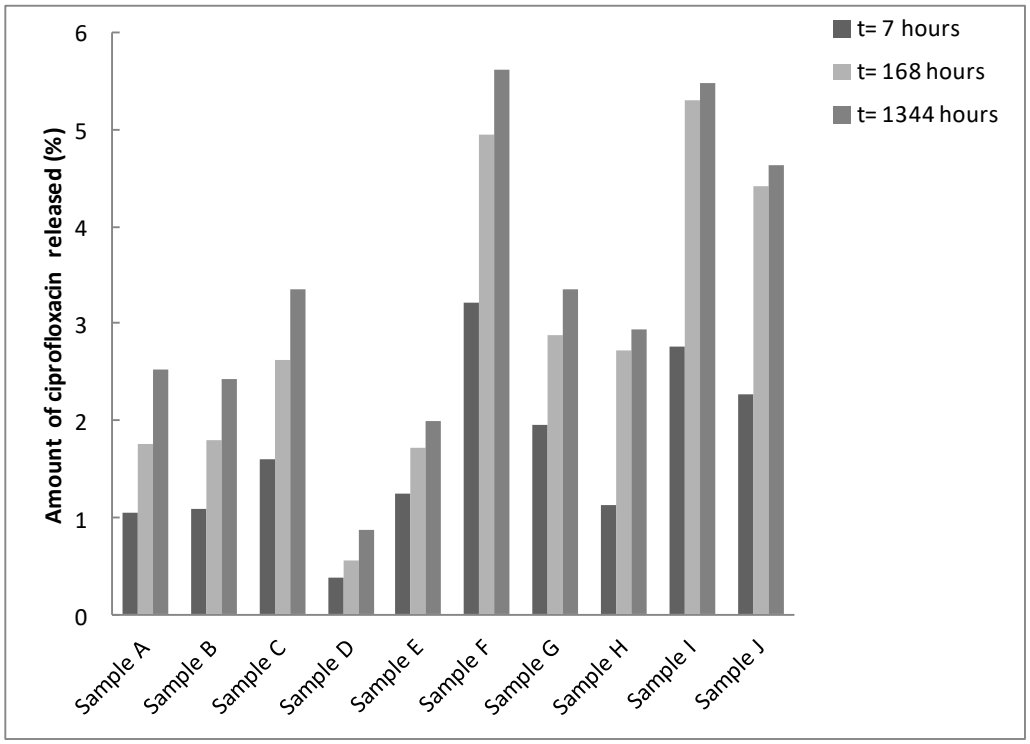

Figure 3. Percentage of ciprofloxacin released from the ten samples at 7, 168 and 1344 hours. Samples A, B, C, H, I, and J (ciprofloxacin hydrochloride-loaded Simplex®, Lima CMT 1® and Palacos® bone cement, hand and vacuum mixing), samples E, F and G (ciprofloxacin hydrochloride- and vancomycin-loaded Simplex®, Lima CMT 1® and Palacos® bone cement hand mixing), and from samples D (ciprofloxacin base-loaded Lima CMT 1® bone cement).

Table 3 provides the parameter values and statistical AIC (Akaike information criterion) figures obtained after fitting the tested models (equations 1-4) to elution data. Korsmeyer-Peppas was selected among the models assayed.

Table 3. Parameter values and statistical AIC obtained after fitting the different kinetic equations to data. (R= Correlation Coefficient; SS=sum of squares; AIC= *Akaike* Information Criterion and K_0, K_1, $K_h$and K_k are the ciprofloxacin elution rate constants for each kinetics.

Kinetic	Parameters	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F	Sample G	Sample H	Sample I	Sample J
Zero order	K0 (mg/h)	0.0012	0.0011	0.0015	0.0004	0.0006	0.002	0.0012	0.0014	0.0022	0.0019
	R	0.868	0.782	0.831	0.906	0.709	0.691	0.705	0.7230	0.680	0.682
	SS	1.555	2.010	3.167	0.122	1.123	12.897	4.152	4.873	15.966	11.7124
	AIC	11.51	15.86	22.44	-31.8	5.85	44.91	26.776	27.76	45.56	40.91
First order	K1 (h^{-1})	0.0417	0.1198	0.0996	0.161	0.2354	0.1738	0.1904	0.0432	0.1005	0.0923
	R	0.808	0.837	0.846	0.6811	0.887	0.912	0.904	0.948	0.926	0.934
	SS	2.19	1.547	2.913	0.366	0.483	4.153	1.501	1.052	4.245	2.810
	AIC	17.36	11.42	21.10	-13.10	-7.66	26.7801	10.497	4.77	25.686	19.50
Higuchi	k_h	0.0881	0.0929	0.1186	0.0306	0.0781	0.222	0.1315	0.1114	0.2135	0.1793
	SS	7.797	9.870	16.603	0.978	10.670	75.802	26.903	12.614	61.217	42.165
	AIC	36.914	40.92	46.95	1.63	39.88	71.25	54.68	40.02	63.72	58.12
Korsmeyer-Peppas	k_k	**0.3001**	**0.3472**	**0.3508**	**0.0795**	**0.2953**	**0.4433**	**0.4474**	**0.3198**	**0.4018**	**0.3861**
	N	**0.1704**	**0.154**	**0.1508**	**0.1659**	**0.104**	**0.1234**	**0.1207**	**0.1723**	**0.1403**	**0.1439**
	R	**0.993**	**0.982**	**0.986**	**0.998**	**0.950**	**0.944**	**0.948**	**0.951**	**0.934**	**0.933**
	SS	**0.091**	**0.186**	**0.289**	**0.003**	**0.221**	**2.685**	**0.842**	**0.991**	**3.776**	**2.828**
	AIC	**-36.78**	**-24.57**	**-15.86**	**-94.75**	**-20.16**	**19.80**	**1.25**	**3.86**	**23.92**	**19.59**

Scanning Electron Microscopy (SEM) (Figure 4) revealed the higher level of porosity of Lima CMT® and Palacos® vs. Simplex®.

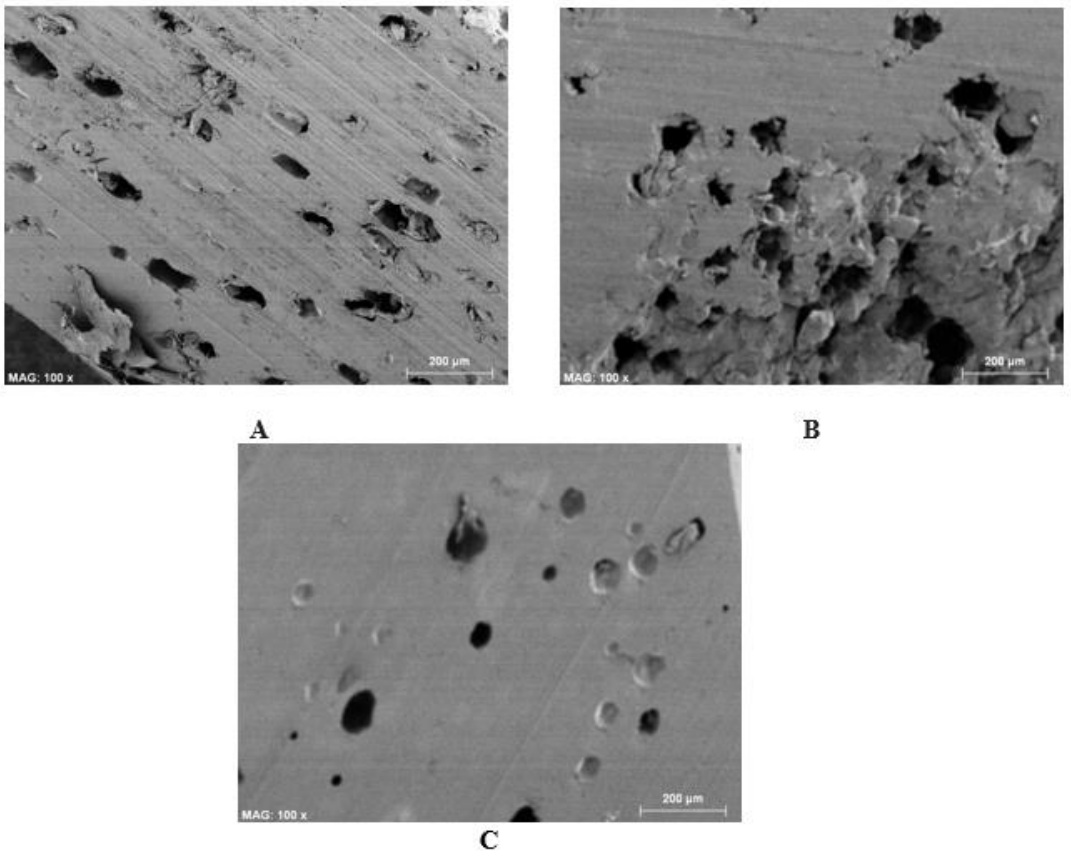

Figure 4. Cement internal structure under SEM: A (Lima), B (Palacos) and C (Simplex).

Since no differences in release were observed among cement type, mean values were used for simulating levels of antibiotic in the location of the implant. Figure 5 evidence that all simulated patients data would reach ciprofloxacin concentrations higher than 0.1 mcg/mL for the first three days post-surgery. According to this simulation study, the first day of elution, the coverage would be complete, decreasing slightly for following days.

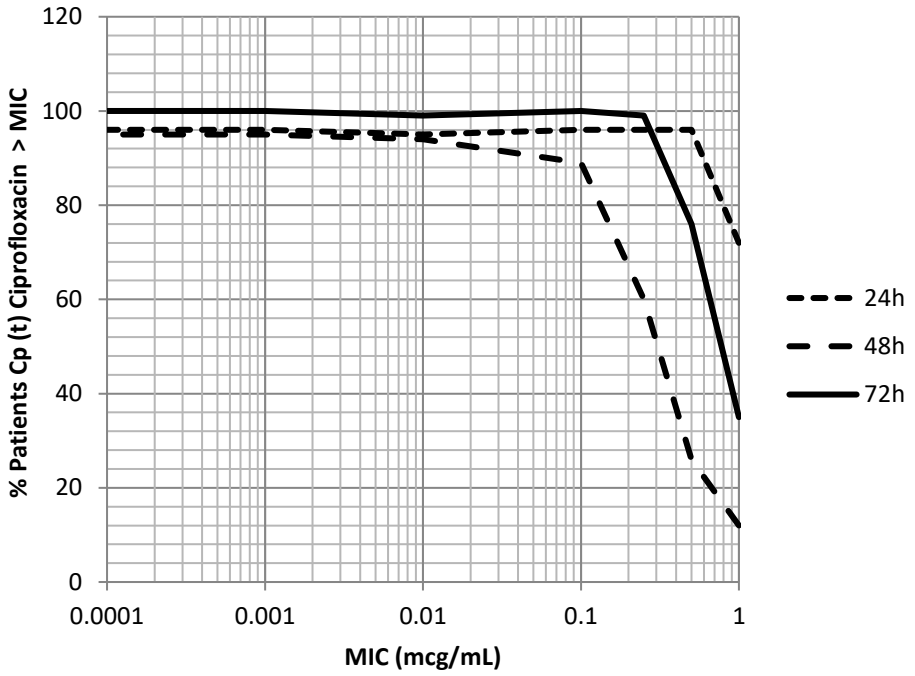

Figure 5. Percentage of patients, predicted with the simulation exercise, for which antibiotic level at the site of the implant would be higher than MIC values in the range 0.0001-1 mcg/mL.

Discussion

The use of bone cement combined with antibiotics is based on the principle that the antibiotic will be gradually released from the cement over time. The elution mechanism is still not fully understood, though it is known to be affected by different factors.

Ciprofloxacin is a broad-spectrum bactericidal quinolone that is effective against major infection-causing microorganisms (24). Previous studies have evaluated the use of other antibiotics, while there are only a few reports concerning ciprofloxacin (25,26,30), whose use could expand the possibilities available. A comparison of the elution kinetics of ciprofloxacin loaded in different acrylic bone cements and the influence of the mixing procedure on kinetic properties have not been reported previously and are the subject of this study.

PMMA is a highly hydrophobic polymer, and is thus impervious to drug diffusion (31). The release of ciprofloxacin from samples assayed can be explained by the Van De Belt theory (32), as during the first hours of the experiment (24h) only ciprofloxacin molecules located in the superficial layers were released. Once these molecules are in solution, the release of antibiotic is reduced and it depends on the penetration of the surrounding media into the cement matrix, which is dictated by the wettability of the polymer and by the number and size

of the pores in it. The principal limitation of these systems is the low proportion of antibiotic in relation to bone cement and the high variability of different samples, which leads to heterogeneous release profiles.

As shown in figures 1 and 3, the greater elution of the ciprofloxacin is obtained when the drug is incorporated to the bone cement as hydrochloride as a result of the varying solubility of the two forms of the antibiotic, since the salt form (ciprofloxacin hydrochloride) is four times more soluble than the base form (ciprofloxacin). As stated before, the drug release mechanism in these cases depends on the dissolution of drug particles adsorbed onto the matrix of the cement, and thus on the solubility of the antibiotic.

As can be seen in figures 1 and 3, binary mixtures achieved the highest release of all the samples evaluated, which may have been due to the solubility of vancomycin, which is three times that of ciprofloxacin hydrochloride. This suggests that, when vancomycin dissolves, more voids and cracks are present in bone cements, thus increasing the release of ciprofloxacin.

In terms of the effect of the type of bone cement on drug release, results obtained represent that antibiotic release from Palacos® bone cement is slightly higher than from Simplex® and Lima CMT1®. However, the ANOVA test revealed not significant differences. Despite previous studies have demonstrated that the highest elution rate was achieved with Palacos® (33), results reported in this study indicate that although Palacos® has superior porosity (Figure 4), elution kinetics from Palacos®, is not significantly different from the other cements studied. When the vacuum mixing system was used, the highest level of antibiotic release was achieved with Lima CMT1® and Palacos®, although only release from Lima CMT1® and Simplex® were statistically different. The superior drug release from Lima CMT1® and Palacos® can be attributed to the greater porosity of Palacos® and Lima CMT1® (figure 4).

When comparing the two mixing procedures, no statistically significant differences were found between vacuum and manual mixing with respect to the drug release rate from Simplex® and Palacos® bone cement. On the contrary when LIMA CMT1® bone cement was used, significant differences were observed up to 697 hours. However, no statistically significant differences in the percentage of amount released were observed at subsequent testing times. This significant difference can be explained if the high variability of the manual batches tested is considered. It should be emphasized that variability of the percentage of drug released from the vacuum-mixed samples was much lower than that seen with manually-mixed ones.

Table 3 shows that the Korsmeyer-Peppas model is the best equation for describing ciprofloxacin elution from all the samples assayed. In the Korsmeyer-Peppas equation, the

parameter n describes the type of release from bone cement. In this case, all the samples have a value under 0.5, which means that the drug release mechanism is a Fickian diffusion (the amount of antibiotic released is proportional to the amount remaining in the dosage form). The results obtained are in accordance with the nature of the cement, as it is a non-erodible and non-swellable matrix.

In general, low dose antibiotic-impregnated bone cements release less than 10% of the dose in most cases. Previous papers showed that around 3% of gentamicin (31,34) and vancomycin (18) were released. These values are very similar to the ones of our study, 2.5 to 5.5%, depending on the conditions. Only antibiotic combinations or additives inclusion improve this elution and increase the percentage of dose released to 10% (8). Some studies have shown that these amounts are sufficient to improve the deep infection rate (6).

The simulation study performed evidence that the concentrations of ciprofloxacin reachable in the implant would be higher than 0.1 □g/mL in 100% of patients (figure 5), decreasing the coverage when higher concentrations are need. In the first three days, E. Coli is the microorganism that would be covered by all cement specimens, unlike P. aeruginosa, S. aureus, would depend on the type of cement and specially on microorganism sensitivity. After the third day post-surgery the clearance attributed to local drainage dramatically decreases and consequently it is expected that local bioactivity would increase.

According to the results, the bioactivity of ciprofloxacin in the first three days post-surgery would depend on the sensitivity of the microorganism, increasing substantially after drain removal, usually at 72 hours.

Despite the differences on elution among different brands and batches these would appear to lack of clinical relevance, because the burst effect in the first moments and the decrease of external drainage in the third day post-surgery, would ensure bactericidal action of ciprofloxacin.

In conclusion, ciprofloxacin is suitable for incorporating into bone cements, as its release mechanism responds to Fickian diffusion principles, filtering through voids and cracks. Ultimately, hydrochloride ciprofloxacin has a better release profile than base ciprofloxacin. All the bone cement brands assayed behave similarly and vacuum mixing ensures lower variability and higher amounts of antibiotic released.

Limitations of the study

While this study furthered our understanding of elution of antibiotics from PMMA, it has several limitations. First, the work was done completely in vitro, and to have a larger impact it would have to be reproduced in an animal model or clinical setting. Second, there are many types of PMMA currently available and this study tested just three. Third, our in vitro testing was done under static conditions that did not include fluid flow or other stresses to which the beads may be exposed to in vivo. Lastly, the bioactivity of samples has been evaluated through a simulation exercise taking into account that the surface of impregnated cement in contact with extracellular body fluid could be equivalent to the surface of the samples assayed using clearance of synovial liquid values from literature and considering that the distribution of the antibiotic from the location of the implant to the systemic circulation is negligible. Consequently, the amount of antibiotic released to the medium could not be exactly the same.

On the other side, one aspect that strengthens our results is that all antibiotic released would be kept in place, and consequently would reach high local concentration, in deep infection.

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CAPÍTULO 3

Evaluación de la bioactividad de ciprofloxacino y vancomicina incorporados en cementos óseos poliacrílicos.

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Anales De La Real Academia Nacional De Farmacia. Vol. 81, Nº 3 (2015), pp. 239-246

Resumen

La inclusión de antibióticos en el cemento óseo destinado a la fijación mecánica de las prótesis constituye un sistema de liberación local de antibiótico que permite minimizar la prevalencia y la gravedad de las reacciones adversas que pueden desencadenar los fármacos cuando éstos se administran por vía sistémica.

El objetivo del trabajo es estudiar el mecanismo y cinética de liberación in vitro de ciprofloxacino y vancomicina incorporados en diferentes cementos óseos comerciales y evaluar la bioactividad mediante un ejercicio de simulación farmacocinética.

Se prepararon mezclas de los cementos de estudio con ciprofloxacino clorhidrato y vancomicina (40:0,5:0,5). Los estudios de liberación se realizaron en agitación continua en solución salina de tampón fosfatos, pH=7,4, durante dos meses a 37ºC. El análisis estadístico de las cantidades de antibiótico liberadas acumuladas y las velocidades de elución se realizó mediante ANOVA. Con el fin estudiar la bioactividad, se realizó una simulación de Monte Carlo.

La cantidad total liberada de ciprofloxacino en un periodo de 8 semanas fue de 0,29±0,06mg desde los cementos Palacos®, 0,44±0,06mg LimaCMT1® y 0,18±0,04mg Simplex®.

La cantidad total de vancomicina liberada en 24 horas fue de 0,34±0,17mg desde el cemento Palacos®, 0,68±0,16mg LimaCMT1® y 0,17±0,02mg Simplex®. Transcurrido este tiempo la liberación cesó.

El estudio de simulación, muestra que durante las primeras 72 horas, la cobertura antibiótica dependería tanto del cemento elegido como de la sensibilidad del microorganismo y el tiempo postquirúrgico. En tiempos posteriores, es de prever que la bioactividad local aumente.

Abstract

Antibiotic loaded bone cement used in prosthesis fixing, is a local release form that minimizes the prevalence and the complications that the antibiotics would unleash when administered intravenously.

The aim of this work is to study in vitro release kinetics of ciprofloxacin and vancomycin loaded in different commercial cements and evaluate the bioactivity through a simulation exercise pharmacokinetics

Samples were prepared with commercial bone cement and ciprofloxacin and vancomycin hydrochloride (40:0,5:0,5). Release study were carried out under stirring in phosphate buffer, pH=7,4, for two months at 37ºC. The antibiotic amount and elution rate, were compared using ANOVA. In order to study the bioactivity, Monte Carlo simulation was performed.

The ciprofloxacin released from samples for 8 weeks was 0.29±0.06 mg from the Palacos® cements, 0.44±0.06 mg from LimaCMT1® and 0.18±0.04 mg from Simplex®.

The vancomycin released for 24 hours was 0.34±0.17 mg from Palacos® cement, 0.68±0.16 mg from LimaCMT1® and 0.17±0.02 mg from Simplex®. After this time the release stopped.

The simulation study shows that during the first 72 hours, the antibiotic coverage would depends on the bone cement, the sensitivity of the microorganism and postoperative day. At subsequence times, it is expected that local bioactivity increases.

Palabras clave: Cementos óseos, ciprofloxacino, vancomicina, cinética de elución, bioactividad, traumatología, PMMA.

Key words: Bone Cement, ciprofloxacin, vancomycin, elution kinetics, bioactivity, orthopedics, PMMA.

Introducción

La artroplastia de cadera/rodilla consiste en la cirugía ortopédica que reemplaza de forma total o parcial la articulación por un implante artificial llamado prótesis en aquellos casos en los que el daño de la articulación es irreversible. Una de las complicaciones más grave se asocia al desarrollo de alguna infección, que aunque presente prevalencia entre el 0,5% y el 3%, en algunos casos puede ser de gravedad elevada y conduce al fracaso de la intervención, incluso en algunos casos puede desencadenar la muerte del paciente. Para prevenir la génesis de complicaciones asociadas al desarrollo de infecciones, se ha propuesto, desde hace algún tiempo, la inclusión de antibióticos en el cemento óseo destinado a la fijación mecánica de las prótesis, ya que los sistemas de liberación local de antibiótico facilitan el aprovechamiento del fármaco, a la vez que reducen la prevalencia y gravedad de las reacciones adversas que pueden desencadenar los fármacos cuando éstos se administran por vía sistémica (1).

La combinación de antibióticos con los cementos poliacrílicos fue descrita por primera vez por Buchholz y Engelbrecht (2). Los numerosos y variados trabajos de investigación publicados en este contexto son contradictorios en cuanto a su capacidad de protección en la prevención de infecciones, debido a la incertidumbre existente sobre el posible desarrollo de resistencias a los antibióticos tras una exposición prolongada a bajas dosis de antibiótico, la eficacia y el coste de este sistema de vehiculización. A pesar de ello, la evidencia clínica indica que el uso de cementos cargados con antibióticos reduce significativamente el riesgo de infección (3); por ello, en la práctica clínica habitual se utilizan, aunque la cinética y el mecanismo de liberación de la mayoría de los antibióticos interpuestos en la matriz acrílica siguen siendo aspectos desconocidos. Las variables que influyen en el proceso de liberación del antibiótico desde el cemento son múltiples, entre ellas destacan cantidad y tipo de antibiótico incorporado al cemento (4, 5). En este sentido, resaltar que la velocidad de liberación del antibiótico desde el cemento (cantidad/tiempo) es mayor cuando se incorpora en forma líquida. Sin embargo, la utilización de formas líquidas en esta práctica clínica está limitada debido a su influencia negativa sobre las propiedades mecánicas de los cementos. Por el contrario, los fármacos en estado sólido tienen un efecto insignificante sobre la estabilidad mecánica de cemento óseo, siempre y cuando la proporción antibiótico/cemento se mantenga por debajo del 10%. La dosis de antibiótico a utilizar no queda totalmente establecida, varía según sea para el tratamiento o para la profilaxis; en el caso de perseguir el tratamiento terapéutico, se suele aconsejar adicionar 4 gramos de antibiótico a 40 gramos de cemento acrílico. Por el contrario, para conseguir un efecto profiláctico se recomienda utilizar dosis menores a 1 g de antibiótico por 40 g de cemento. Otro factor importante a tener en cuenta es el tipo y porosidad del cemento óseo y forma de

preparación de la mezcla (6-9), ya que la porosidad del polímero facilita el acceso de los fluidos de disolución a la matriz del polímero y, en consecuencia, la liberación de los antibióticos a partir del cemento. Por otra parte, la porosidad está relacionada, en gran medida, con el mayor o menor volumen de aire atrapado durante la manipulación, mezclado y amasado de la muestra. De ahí que las cantidades de antibiótico liberadas desde el cemento puedan diferir según se empleen productos comerciales de cemento óseo impregnado de antibiótico premezclados o, por el contrario, se utilicen las preparaciones mezcladas de forma manual en el momento previo a la intervención quirúrgica.

Se han comercializado cementos poliacrílicos, de uso en artroplastias, cargados con antibióticos aminoglicósidos, en particular gentamicina y tobramicina, y con antibióticos glucopéptidos (10), que han demostrado su utilidad clínica en términos de eficacia y seguridad del tratamiento. Sin embargo, el problema de su uso es que el número de cepas multirresistentes (10, 11), con capacidad de adherirse sobre el cemento, colonizándolo tras largos periodos de implantación se ha incrementado recientemente. De hecho, en el momento actual el incremento de resistencias de Staphylococcus aureus hacia los aminoglucósidos condiciona la eficacia terapéutica de este grupo de antibióticos. Se trata de una realidad preocupante, ya que el 30% de las infecciones de origen quirúrgico son causadas por la cepa Staphylococcus aureus resistente a meticilina (SARM), lo que determina las estrategias para el tratamiento y prevención de las infecciones en las prótesis articulares (12).

En este contexto, se ha considerado oportuno estudiar la cinética de liberación de nuevos antibióticos incorporados a distintos cementos óseos comerciales y de esta forma obtener información relevante orientada a facilitar la selección del fármaco más adecuado en términos de eficacia y seguridad, ampliando así la disponibilidad de tratamientos utilizados en cirugía ortopédica.

El ciprofloxacino es una fluoroquinolona efectiva frente a microorganismos Gram-positivos y Gram-negativos. La vancomicina es un glicopéptido sumamente efectivo frente a bacterias Gram-positivas. Ambos antibióticos se presentan en estado sólido, son estables a la temperatura de fraguado de los cementos y no alteran las características mecánicas de éstos, por lo que incorporados en cementos poliacrílicos son candidatos para su utilización en cirugía ortopédica (13).

El objetivo del trabajo que se presenta es estudiar el mecanismo y cinética de liberación in vitro de ciprofloxacino y vancomicina incorporados en proporciones profilácticas en

diferentes cementos óseos comerciales y evaluar la bioactividad potencial de las mezclas mediante estudios de simulación farmacocinética.

Material y métodos

El ciprofloxacino clorhidrato y la vancomicina han sido suministrados por Guinama (Valencia, España). De acuerdo con las especificaciones del proveedor ambos antibióticos cumplían las especificaciones marcadas por la Farmacopea Europea. Los cementos poliacrílicos Palacos® y Simplex® fueron adquiridos en Ibersugical (Valencia, España) y LimaCMT1® en Lima Implantes (Barcelona, España).

Las cantidades de antibiótico incorporadas al cemento se seleccionaron de acuerdo con las recomendaciones realizadas por diferentes autores (14-16) con la finalidad de alcanzar un efecto antibiótico profiláctico (1 g de antibiótico por 40 g de cemento).

Se preparó un lote de los cementos acrílicos Palacos®, Simplex® y LimaCMT1® con ciprofloxacino clorhidrato y vancomicina (40:0,5:0,5) siguiendo las instrucciones proporcionadas por los fabricantes. Las mezclas obtenidas se introdujeron en moldes de teflón siguiendo la normativa ISO 5833-Anexo E, y se dejaron endurecer durante 24 horas. Previamente al ensayo de liberación, cada muestra se caracterizó en cuanto a peso, diámetro y espesor.

Los estudios de liberación del antibiótico se realizaron en un total de 9 muestras, 3 por cada cemento, manteniéndolas en condiciones de agitación continua en 10 mL de solución salina de tampón fosfatos, pH=7,4, durante 8 semanas en baño termostático a 37ºC. A intervalos de tiempo preestablecidos, la totalidad de la solución tampón fue recogida y reemplazada por 10mL de tampón fosfato salino pH=7,4. Este proceso permite garantizar las condiciones sumidero, es decir que la concentración del fármaco en el medio nunca supere el 20% de su hidrosolubilidad. Las muestras experimentales extraídas a cada tiempo de muestreo se guardaron en una cámara frigorífica a 5ºC hasta el momento de su cuantificación.

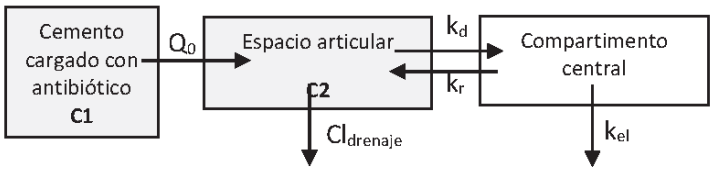
Para la determinación de ciprofloxacino se desarrolló y validó un método analítico por cromatografía líquida de alta resolución (HPLC) con detección UV (λ=254nm). Para ello, se utilizó como fase estacionaria una columna Kromasil® C18 (150x4,6 mm) y como fase móvil una mezcla acetonitrilo y ácido acético 0,1M en proporción volumétrica 20:80 (V/V). La exactitud y precisión del método analítico se evaluaron con la determinación del error relativo y el coeficiente de variación intra-muestra (inferior al 2,4% y al 1,5%, respectivamente, en el ámbito de

concentraciones estudiadas). La determinación de vancomicina se realizó mediante inmunoanálisis de micropartículas quimioluminiscentes (ARCHITECT i1000SR) (17).

Las cantidades liberadas acumuladas, la velocidad de liberación y el logaritmo de la velocidad de liberación a partir de las muestras de ciprofloxacino combinado con vancomicina se evaluaron mediante la prueba estadística ANOVA.

Para evaluar la bioactividad de los antibióticos en las muestras estudiadas se realizaron simulaciones de Monte Carlo. Se simularon para 100 pacientes las concentraciones de antibiótico en la zona local del implante a las 24, 48 y 72 h utilizando el programa informático NONMEM versión VII. Para ello, se utilizaron los parámetros característicos del proceso de liberación de los antibióticos obtenidos en el estudio in vitro y los parámetros fisiológicos de volumen articular (1,6±1,1mL) (18) y aclaramiento local del fármaco, atribuido al drenaje de la herida a las 24, 48 y 72 h post-implante (20,42±11,3 mL/h; 9,33±11,02 mL/h y 4,11±2,95 mL/h respectivamente) (19).

El modelo farmacocinético aplicado para realizar la simulación de concentraciones de antimicrobiano en biofase (Figura 1) consta de dos compartimentos; cemento cargado con el antibiótico (C1) y espacio articular (C2). La liberación del antibiótico se realiza desde el compartimento C1 mediante una cinética de orden cero regida por la constante de velocidad Q0 (mg/h). A su vez, el fármaco se elimina desde el compartimento C2 mediante una cinética de primer orden regida por la velocidad de drenaje de la herida (mL/h). Puesto que durante las 72 horas posteriores a la intervención el drenaje es muy elevado, se ha considerado despreciable la distribución (kd) y retorno del antibiótico (kr) desde el espacio articular (C2) a la circulación sistémica y viceversa. Por ello, para obtener las concentraciones simuladas en el lugar del implante (C2) sólo se han considerado los compartimentos sombreados de la figura 1.



Q_0: velocidad de liberación del antibiótico desde el cemento (mg/h); $Cl_{drenaje}$: aclaramiento local del fármaco debido al drenaje de la herida (mL/h); k_d (h^{-1}): constante de distribución del antibiótico desde el lugar de implante al torrente circulatorio; k_r (h^{-1}): constante de retorno del antibiótico desde el torrente circulatorio al lugar de implante; kel (h^{-1}): constante de velocidad de eliminación del fármaco

Figura 1. Modelo farmacocinético utilizado para calcular las concentraciones de antimicrobiano en el lugar de implante de la prótesis.

La evaluación de la bioactividad de las mezclas a los tiempos seleccionados se ha realizado utilizando los valores de CMI de S. aureus Meticilin resistente (CMI= 0,5-2 mcg/mL) (20) y S. Coagulasa Negativos –SCN- (CMI= 0,25-1 mcg/mL) (20), gérmenes sobre los que la vancomicina muestra actividad, y de S. aureus (CMI= 0,12-0.5 mcg/mL), Pseudomonas aeruginosa (CMI= 0,25-1mcg/mL) y E. Coli (CMI= 0,004-0,016 mcg/mL) (21), gérmenes sensibles al ciprofloxacino. Estos microorganismos han demostrado ser los responsables del 70% de las infecciones articulares desarrolladas en nuestro entorno (22). Se calculó para cada tiempo (24, 48 y 72 h) el porcentaje de pacientes cuya concentración de antibiótico en el lugar del implante (C2) sería superior a la CMI seleccionada.

Resultados

En la Tabla 1 se muestran las cantidades de ciprofloxacino clorhidrato liberadas acumuladas durante 2 meses y las cantidades de vancomicina liberadas acumuladas hasta las 72 horas. Las cantidades de ciprofloxacino y de vancomicina liberadas desde las mezclas de los antibióticos y el cemento LimaCMT1® fueron superiores a las cantidades de antibióticos liberadas desde el resto de mezclas estudiadas ($p<0,05$).

Tabla 1. Media y desviación estándar de la cantidad de ciprofloxacino y vancomicina (mg) liberados a los tiempos indicados desde los diferentes cementos poliacrílicos ensayados.

	Ciprofloxacino HCl						**Vancomicina**					
	Simplex®		**LimaCMT1®**		**Palacos®**		**Simplex®**		**LimaCMT1®**		**Palacos®**	
Tiempo (h)	**MEDIA (mg)**	**SD**	**MEDIA (mg)**	**SD**	**MEDIA (mg)**	**SD**	**MEDIA (mg)**	**SD**	**MEDIA (mg)**	**SD**	**MEDIA (mg)**	**SD**
24	0.134	0.018	0.318	0.032	0.209	0.045	0.174	0.021	0.659	0.141	0.339	0.166
48	0.144	0.019	0.351	0.044	0.229	0.047	-	-	0.677	0.161	-	-
72	0.144	0.022	0.367	0.050	0.235	0.047	-	-	0.681	0.162	-	-
1344	0.177	0.043	0.442	0.061	0.289	0.052	-	-	-	-	-	-

En la Figura 2 se representa la evolución de la velocidad de liberación de los antibióticos a partir de los cementos estudiados durante el desarrollo del ensayo. Los ensayos realizados con el ciprofloxacino muestran dos etapas; en la primera (primeras 48 h) la velocidad de liberación

es rápida, y en la segunda la velocidad de liberación del antibiótico disminuye hasta que se mantiene en un valor constante. Por el contrario, los resultados obtenidos con vancomicina únicamente muestran una etapa, ya que durante las primeras 48 h del ensayo el antibiótico se libera rápidamente, pero en tiempos posteriores la velocidad de liberación del antibiótico cesa. La comparación estadística del logaritmo de la velocidad de liberación de ciprofloxacino, obtenida para cada uno de los cementos estudiados, puso de manifiesto la existencia de diferencias estadísticamente significativas en la primera etapa del estudio (primeras 48 h) a favor de LimaCMT1®, no existiendo diferencias estadísticamente significativas en tiempos posteriores a las 48 h del ensayo en los tres cementos óseos estudiados.

CIPROFLOXACINO **VANCOMICINA**

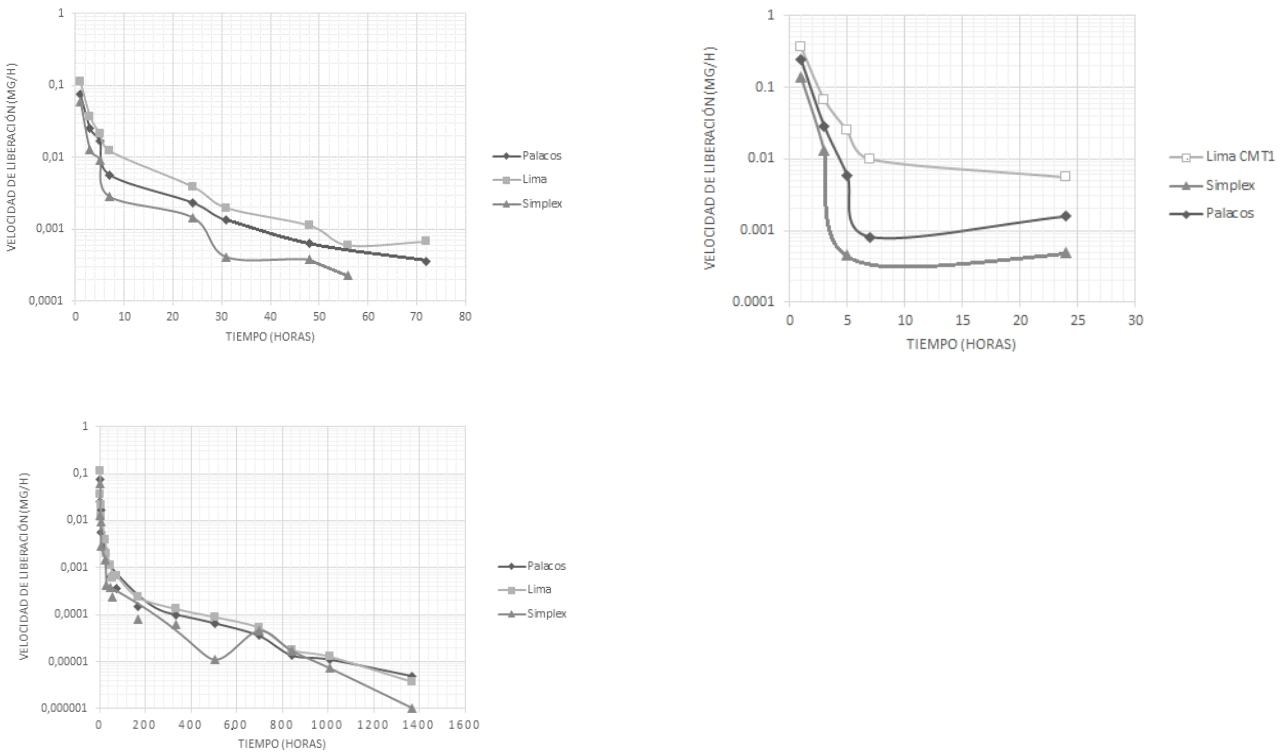


Figura 2. Velocidades de elución de ciprofloxacino clorhidrato y vancomicina desde los cementos poliacrílicos estudiados.

Mediante los ejercicios de simulación de Monte Carlo se ha obtenido una población simulada de 100 pacientes de una edad media de 69,17±14,28 años y de un peso medio de 74,18±14,47 kg. Las concentraciones simuladas de ciprofloxacino y vancomicina en el lugar del

implante (apartado de material y método) indican que transcurridas 24 horas de la intervención, en más del 90% de los pacientes serían superiores a 0,1 y 0,2 mcg/mL, de ciprofloxacino y vancomicina respectivamente (Figura 3).

CIPROFLOXACINO CLORHIDRATO **VANCOMICINA**

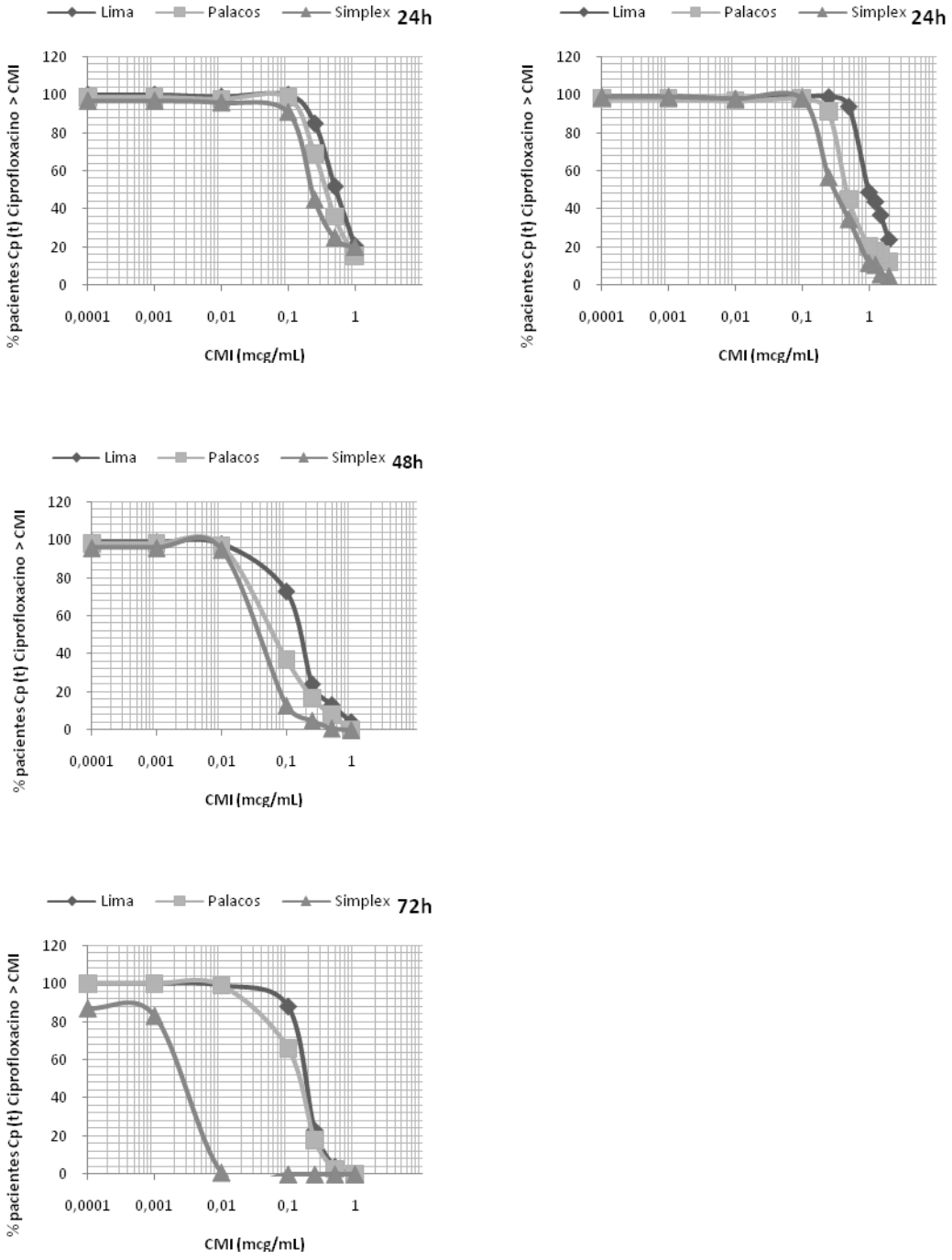


Figura 3. Evaluación de la bioactividad de las muestras estudiadas. En el eje de ordenadas se representa el porcentaje de pacientes cuya concentración de antibiótico en el lugar del implante, transcurridas 24, 48 y 72 h de la intervención para el ciprofloxacino y transcurridas 24 h de la intervención para la vancomicina, sería igual o superior a la CMI indicada en el eje de abscisas.

Discusión

La inclusión de antibióticos directamente en cementos poliacrílicos usados para la fijación de prótesis óseas representa un sistema de liberación modificada de fármacos. Estos sistemas facilitan el acceso del antibiótico a la biofase y por ello aportan principalmente dos ventajas: el mayor aprovechamiento del fármaco en el lugar de acción y una disminución de reacciones adversas. La liberación modificada de fármacos a partir de sistemas matriciales exhibe un patrón de comportamiento común compuesto por dos etapas; una, de liberación inicial rápida, que por lo general ocurre durante las primeras 24-48 horas, y otra de liberación lenta, en tiempos posteriores a las 48 horas, en la que el fármaco se libera a una velocidad más lenta. La liberación inicial está fundamentalmente determinada por la cantidad de fármaco que queda adsorbido en la superficie de la matriz y la difusión por los poros y canales que componen su estructura interna, los cuales se llenan con el medio de incubación durante las primeras horas de ensayo. En la fase posterior, de liberación lenta, en la que la velocidad de liberación y la cantidad de fármaco liberada es inferior, contribuyen los procesos de difusión a través de los poros y canales del sistema matricial, los cuales se forman como producto del proceso de fabricación o por la modificación de la estructura matricial, como consecuencia de la disolución de componentes hidrosolubles de su composición.

La fase inicial, de velocidad de liberación elevada, es de menor duración para la vancomicina y presenta una velocidad más elevada que la obtenida para el ciprofloxacino. Este fenómeno se puede atribuir a la mayor solubilidad acuosa de la vancomicina (100 mg/mL frente a 30 mg/L del ciprofloxacino). Este hecho indica que la disolución de las partículas de vancomicina situadas sobre la superficie se realiza con mayor velocidad facilitándose la liberación y posterior disolución de las partículas próximas a la superficie en un periodo de tiempo breve.

Las cantidades de ciprofloxacino liberadas a partir de las mezclas evaluadas en este estudio son superiores a las cantidades de antibiótico liberadas en un estudio previo realizado en nuestro grupo de investigación en el que se evaluó la liberación del ciprofloxacino a partir de mezclas simples, constituidas por el antibiótico y los diferentes cementos óseos (225% superior en las mezclas combinadas con el cemento LimaCMT1®, un 183% con el cemento Palacos® y un 126% con el cemento Simplex®)(23). Estas diferencias permiten corroborar que el hecho de incorporar más de un fármaco a los cementos óseos potencia en gran medida la velocidad de liberación de ambos. En este caso la vancomicina incorporada en la mezcla, favorece la

formación de un mayor número de poros y canales que facilitan la entrada de agua en la matriz y la posterior disolución del ciprofloxacino desde su interior.

Las cantidades de antibiótico liberadas así como la evolución temporal de la velocidad de liberación es mayor para las mezclas elaboradas con el cemento LimaCMT1® (Figuras 2 y 3). Estos resultados son compatibles con la menor porosidad del cemento Simplex® indicada por Stryker®, laboratorio fabricante del producto (24).

Las cantidades de antibiótico liberadas desde los cementos estudiados en combinación con los valores del aclaramiento local del fármaco, atribuido mayoritariamente al drenaje de la herida, y los valores del volumen de líquido en el espacio articular permitieron abordar el estudio de bioactividad simulada. Como se observa en la Figura 3, a las 24 horas post intervención quirúrgica las concentraciones de ciprofloxacino predichas en el lugar del implante son superiores a 0,1 mcg/mL con los tres cementos estudiados y las concentraciones de vancomicina superiores a 0,5, 0,3 y 0,1 mcg/mL en el caso de LimaCMT1®, Palacos® y Simplex®, respectivamente, en el total de la población simulada. Estos resultados indican que es de prever que la cobertura local con los antibióticos estudiados sea eficaz para patógenos sensibles a concentraciones inferiores a las indicadas. Transcurridas 48 h de la intervención, en el total de la población simulada, la concentración de ciprofloxacino en el lugar del implante se reduciría una décima parte; a las 72 h post-implante la concentración en el lugar del implante incrementaría y alcanzaría un valor equivalente al obtenido a las 24 h excepto en el caso del cemento Simplex®. En el caso de la vancomicina, a partir de las 24 horas posteriores a la intervención quirúrgica, la liberación del antibiótico desde los cementos Palacos® y Simplex® es nula, y a efectos prácticos también despreciable desde el cemento LimaCMT1®.

Las oscilaciones de concentración de ciprofloxacino durante los primeros días de la intervención quirúrgica, están relacionadas con la fluctuación y la variabilidad del drenaje de la herida quirúrgica producida durante las 72 horas posteriores a la intervención, ya que en este corto periodo de tiempo el drenaje local se reduce hasta alcanzar valores que representan entorno el 25% del valor inicial (20,42±11,3 mL/h; 9,33±11,02 mL/h y 4,11±2,95 mL/h respectivamente) (19). En general, el drenaje externo de la herida se retira a partir del tercer día de la intervención quirúrgica. A partir de este momento, la evolución temporal de la concentración de antibiótico en el lugar del implante estará condicionada por el proceso de distribución y retorno del fármaco a la circulación sistémica. Por ello, es de prever que a partir del tercer día post-intervención quirúrgica la concentración de fármaco en el lugar del implante

de la prótesis alcance valores más elevados, aumentando si cabe la cobertura antibiótica obtenida durante los primeros días.

En resumen, el estudio realizado pone de manifiesto que la bioactividad de las muestras es dependiente del cemento acrílico, el día postquirúrgico, el microorganismo causante y su sensibilidad. Si se analiza la prevalencia de infecciones diagnosticadas en nuestro entorno se observa que S. aureus es el principal agente causante de las infecciones protésicas, ya que se ha aislado en un 30% del total de infecciones, mientras que otros microorganismos, entre ellos S. Coagulasa Negativos y Pseudomonas aeruginos y E. Coli se han aislado en un porcentaje inferior, 14% los dos primeros y 12% el último (22). Teniendo en cuenta esta situación y considerando que el ámbito de valores de la CMI de ciprofloxacino para S. aureus Meticilin resistente está comprendido entre 0,5 y 2 mcg/mL únicamente se alcanzarían concentraciones efectivas en el lugar de acción para cepas sensibles al ciprofloxacino a concentraciones inferiores a 1 mcg/mL. La bioactividad de vancomicina durante las 24 horas posteriores a la intervención quirúrgica es superior a la de ciprofloxacino. Sin embargo, puede ser nula para tiempos posteriores ya que la cantidad de vancomicina retenida en el interior de la matriz no se libera al medio. Durante las primeras 72 horas posteriores a la intervención quirúrgica la velocidad de liberación del ciprofloxacino incorporado al cemento LimaCMT1® es superior, lo que indica que éste reúne una capacidad cinética ligeramente superior a los otros cementos ensayados. En tiempos posteriores la bioactividad del ciprofloxacino incorporado en los tres cementos estudiados es similar.

Conclusiones

Los cementos poliacrílicos cargados con ciprofloxacino y vancomicina son sistemas de liberación modificada que asegurarían durante las primeras 72 h bioactividad frente algunos microorganismos, pero no garantizan una cobertura completa para todos los posibles agentes causantes. Ante una situación de mayor riesgo sería conveniente seleccionar el cemento LimaCMT1®, por las propiedades cinéticas ligeramente favorables en relación a los otros cementos estudiados, así como utilizar la combinación de antibióticos que facilite la velocidad de liberación del antibiótico desde la matriz. Por último, es deseable seguir investigando y profundizando en estos estudios con la finalidad de disponer de información que ayude a optimizar la incorporación de antibióticos a los cementos óseos para facilitar la selección de mezclas que aseguren una bioactividad elevada, fundamentalmente, durante los primeros tres días post implante de la prótesis articular.

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CAPÍTULO 4

Bioactivity of ceftazidime and fluconazole included in polymethyl methacrylate bone cement for use in arthroplasty.

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Journal of Arthroplasty. DOI: 10.1016/j.arth.2017.04.057

Accepted

Abstract

Background.- The microorganisms that most frequently cause prosthetic joint infection are methicillin-resistant Staphylococcus aureus and gram-negative aerobic bacillus. Studies have documented the efficacy of mixing antibiotics with polymethyl methacrylate (PMMA), but that of antifungal drugs has not received much attention. The objective of this in-vitro study was to characterize the elution profile and bioactivity of ceftazidime and fluconazole when incorporated into bone cement in proportions intended for prophylaxis and treatment of bone infections.

Methods.- Antibiotic-loaded bone cement cylinders in a proportion of 1:40 and 4:40 (grams of antibiotic:grams of cement) were assayed. Drug delivery was investigated in a flow-through dissolution apparatus (SotaxCE7®). In order to assess bioactivity, antibiotic concentrations were simulated in the joint space of 1000 patients. Antibacterial properties were evaluated by counting colony forming units and the inhibition-halo test.

Results.- The ratio of released ceftazidime and fluconazole was 453 and 648% higher when used for treatment proportions than prophylaxis proportions. A bioactivity simulation exercise showed that the efficacy of ceftazidime/fluconazole determined as the amount of drug released at the active site in the first three days post-surgery would depended on the sensitivity of the microorganism and would increase substantially after drain removal. The microbiology study showed that *P.aeruginosa* biofilm formation could be a problem when ceftazidime was used in treatment or prophylaxis proportions.

Conclusion.- Our in-vitro findings suggest that ceftazidime and fluconazole can be added into PMMA for the prevention/treatment of infections associated to joint surgery. Their efficacy depends on the sensitivity of the microorganism causing the infection.

Key words

Bone cement, arthroplasty, ceftazidime, fluconazole, elution kinetics, orthopedics, bioactivity, PMMA.

Introduction

One of the most serious complications in arthroplasty is the development of infections (prevalence between 1% and 2.5%) [1], which lead to death in some cases. In case of bone infection, high doses of antibiotics are required to achieve effective concentrations at the implantation site. However, high doses of intravenous or oral antibiotics can cause toxicity. Antibiotic-loaded acrylic bone cement, described by Buchholz and Engelbrecht [2], is a well-established tool used in prophylaxis [3, 4] and treatment of orthopedic infections [5] in humans and animals [6]. Polymethylmetacrylate – PMMA - is characterized by excellent biocompatibility, with low intrinsic toxicity and inflammatory activation [7], but experience has shown that not all antibiotics have the appropriate properties to be incorporated into this cement. Aminoglycosides and glycopeptides are the two groups of antibiotics that satisfy the optimal criteria for inclusion in this cement, namely, availability in powder form, wide antibacterial spectrum, bactericidity at low concentrations, elution from PMMA in high concentrations for prolonged periods, thermal stability, low or no risk of allergy or delayed hypersensitivity, little influence on the mechanical properties of the cement, and low serum protein binding [8]. In fact, some antibiotics, such as gentamicin and vancomycin, are available premixed within bone cement, ready for use [8].

Nevertheless, the increased prevalence of multi drug-resistant bacteria [9-12] limits the continued efficacy of the aforementioned mixtures. It has been established that the microorganisms that most frequently cause prosthetic joint infection are methicillin-resistant Staphylococcus aureus (MRSA) [13] and gram negative aerobic bacillus [14]. Around 80% of fungal infections of bone prostheses are produced by Candida spp. Altough some studies have documented the efficacy of mixing antibiotics with PMMA, the efficacy of antifungal drugs mixed with PMMA has received little attention [15].

In this study we set out to characterize the elution profile of ceftazidime and fluconazole in bone cement. Cephalosporins are the most commonly used antibiotics to treat osteoarticular infections due to their broad spectrum of activity; among other actions, they disrupt the synthesis of the peptidoglycan layer of the bacterial cell wall. Ceftazidime is a third-generation cephalosporin used to treat infections produced by Gram-positive and Gram-negative bacteria, and acts against Pseudomonas. Fluconazole is an antifungal agent of the triazole group used to treat infections produced by C. albicans. Fluconazole inhibits the cytochrome P450 enzyme14α-demethylase, which prevents the formation of ergosterol, thus increasing membrane permeability and cell destruction.

Prophylaxis and treatment proportions (1:40 and 4:40 grams of drug:grams of cement, respectively) were assayed. Different equations were applied to the profiles of release in order to explain the delivery mechanism. Finally, the bioactivity of the mixtures was evaluated by means of two methods, a simulation exercise and a microbiology analysis.

Materials and methods

1. Materials

Fluconazole (Diflucan®) and ceftazidime (Fortam®) were purchased from Vinci Farma, S.A. and Glaxosmithkline, S.A. respectively, and Palacos® bone cement was purchased from Ibersugical (Valencia, Spain). The bone cement was provided as two separate components: a powder mixture and a liquid. The composition of the bone cement is shown in Table 1, according to the information provided by the manufacturer.

Table 1.- Composition of the acrylic bone cement, as provided by the manufacturer.

	Solid Component (40g)	Liquid component (20mL)	Viscosity
Palacos®	Poly(methylacrylate. methyl methacrylate) 33.8 g Zirconium Dioxide 6.0 g Benzoyl peroxide 0.2 g Colorant E141 0.008 g	Methyl methacrylate 18.4 g N,N-dimethyl-p-toluidine 0.4 g Hydroquinone Colorant E141 0.005 g	High

A buffered saline solution pH 7.4 was prepared as described in the US Pharmacopoeia: disodium hydrogen phosphate anhydrous 2.38 g, mono-potassium hydrogen phosphate anhydrous 0.19 g and sodium chloride 8 g per 1 L of purified water. All reagents were analytical grade (Panreac – Barcelona, Spain-).

Methanol gradient grade for liquid chromatography, Mueller-Hinton agar plates and standard bacterial broth were purchased from Sigma-Aldrich (Madrid, Spain). The latters were prepared according to the manufacturer's instructions.

2. Methods

Six batches per antibiotic (three for each gram of antibiotic:grams of cement proportion assayed, 1:40 and 4:40) with five samples per batch were prepared. A total of 60 antibiotic-loaded bone cement cylinders were prepared as follows: 1g or 4g of the drug, to reproduce the ratio antibiotic:cement used in prophylaxis or treatment in clinical practice respectively, was added to 40 g of the powder component of the cement, and, after mixing the powder, the liquid component was added following the manufacturer's instructions. Cylinders of antibiotic bone cement were made for each batch in a standard fashion according to the ISO normative 5833 (Annex E): samples were poured into teflon moulds in which they were kept for 1 hour until completely hardened into a cylinder/disk shape. Each specimen was carefully weighed and measured and the theoretical amount of loaded antibiotic calculated.

2.1. Drug release assays

Samples were introduced into flow-through dissolution equipment (Sotax CE7®) with 100 mL of phosphate buffer saline (pH 7.4) at 37ºC recirculating at a rate of 12mL/min for 48 hours. Samples were taken 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 24, 26, 28 and 48 hours after immersion. These sampling times were selected because it was expected that the release of antibiotic would be constant for the first 24 hours. Antibiotic homogeneity distribution within batches was indirectly evaluated by statistical analysis of the percentage of the total antibiotic released from the assayed samples. 100mL of phosphate buffer ensures that the sink condition is satisfied (defined as at least three times the volume of medium required to produce a saturated solution of drug substance). All samples were frozen at -20ºC until analysis.

Fluconazole and ceftazidime concentrations were assayed by high performance liquid chromatography (HPLC) using a Perkin Elmer® Series 200 UV detector (λ=268 nm and 245nm, respectively). The mobile phase consisted of methanol:water (60:40 and 70:30 V/V respectively) and was filtered through a 0.45 µm membrane filter before use. The mobile phase was eluted at a flow rate of 1 mL/min in both cases. The column was a Kromasil® C-18 with a pore size of 5.0 µm, measuring 150 mm (length) x 4.6 mm (diameter) [16, 17].

2.2. Kinetical analysis and biosimulation

The elution rate at each time interval (mg/h) was obtained by dividing the total quantity of antibiotic released in each interval by the elution time (in hours).

The kinetics analysis of the release of antibiotic was performed sequentially. First, various kinetic models were fitted to the cumulative amount of antibiotic released for each proportion. In a second step, kinetic parameters of the model selected and physiological parameters (volumen from redon for 72 h from surgery obtained from a group 156 patients) were used for the biosimulation (see appendix for details about both procedures).

2.3. Microbiology analysis

Antibacterial properties of ceftazidime for S. aureus, S. epidermidis, P. aeruginosa and E. Coli and fluconazole for C. albicans were evaluated by means of a colony forming unit (CFU) count [18] and inhibition halo test [19].

CFU count was performed by dipping both, samples and commercial cements alone (as a control), in a standard bacterial broth containing approximately 150•106 CFU/mL and incubating them for 24 h at 37 °C. In order to quantify the bacteria proliferated in the culture broth, the broth was serially diluted, spread on an agar plate and incubated overnight at 37 °C to allow the growth and counting of CFU.

For the inhibition halo test, samples were placed in contact with an agar plate (Mueller Hinton agar) uniformly covered with a bacterial broth (previously prepared following a standard procedure) and incubated overnight at 37 °C, as described in [20]. Afterwards, the inhibition zone was observed and measured. For all bone cements, the inhibition halo was repeated up to 3 days: at the end of the first incubation the samples were removed and placed in a new agar plate containing fresh bacterial inoculum.

All antibacterial tests were performed in duplicate.

2.4. Statistical analysis

Elution rates and the total amount of antibiotic released (expressed as a fraction of loaded amount) at each time point were compared using one-way analysis of variance (ANOVA).

The inhibition halo test data were compared using one-way analysis of variance (ANOVA).

In both cases, statistical significance was set at $p<0.05$.

Results

1. Release characterization and statistical analysis

Figure 1 shows the amount of each drug released from Palacos® bone cement loaded in the proportions studied. The amount of fluconazole released at 48 hours was approximately half of that of ceftazidime. The amount of ceftazidime released from treatment to profilaxis ratio systems was 453% higher, whereas fluconazole release increased 648% when increasing drug into cement.

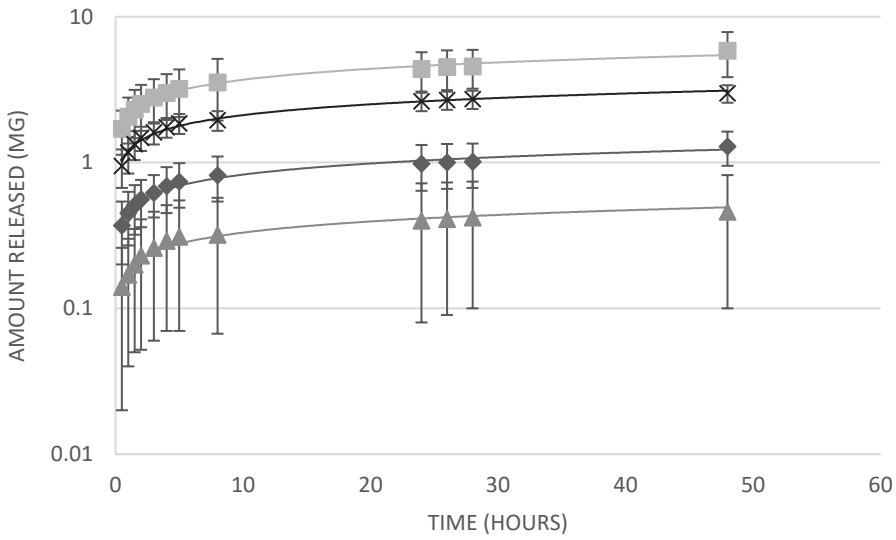

Figure 1.- Amount released vs. time profile of antibiotic loaded into bone cement. Symbols represent the mean observed values with their corresponding SD: (♦) ceftazidime 1:40, (■) ceftazidime 4:40, (▲) fluconazole 1:40, (×) fluconazole 4:40.

Figure 2 shows the elution rate of ceftazidime and fluconazole in mg/h at different time intervals, plotted on a logarithmic scale. All samples produced high early release rates followed by a lower sustained release.

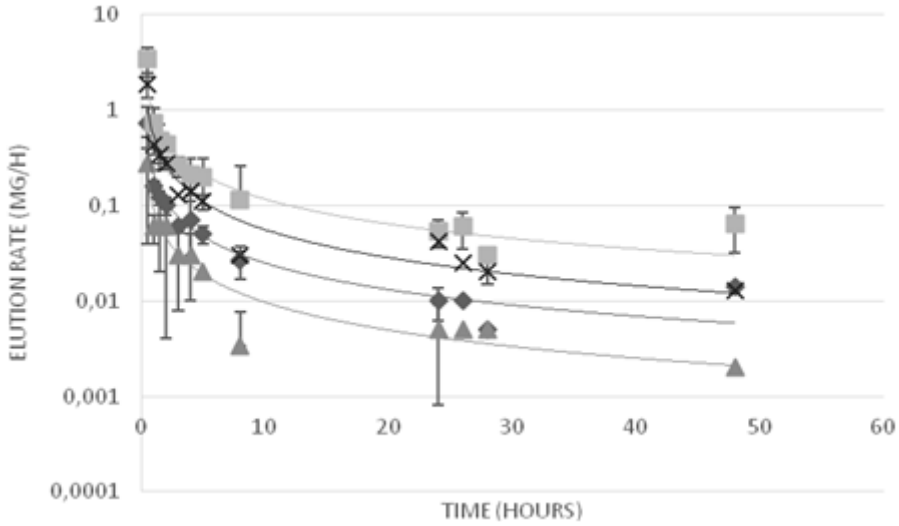

Figure 2.- Elution rate vs. time profile of antibiotic from bone cement. Symbols represent the mean observed values with their corresponding SD: (♦) ceftazidime 1:40, (■) ceftazidime 4:40, (▲) fluconazole 1:40, (×) fluconazole 4:40.

Comparison of the amounts released and release rate between the different antibiotics and proportions showed statistically significant differences.

2. Drug release models

Table 2 shows parameter values and statistical AIC obtained after fitting the different kinetic equations to data.

Table 2. Parameter values and statistical AIC obtained after fitting the different kinetic equations. RSE= Relative Standard Error inter-sample; AIC= *Akaike* Information Criterion, *k*=the antibiotic elution rate constants for each kinetics).

Antibiotic elution from bone cement					
		Ceftazidime		**Fluconazole**	
Kinetic	**Parameters**	**1/40**	**4/40**	**1/40**	**4/40**
Zero order	k (RSE%)	0.217 (46%)	0.25 (76%)	0.25 (-)	0.268 (146%)
	AIC	150.337	234.925	87.446	122.856
Higuchi	k (RSE%)	0.3 (34%)	0.293 (54%)	0.29 (228%)	0.306 (93%)
	AIC	-326.798	-335.355	-336.97	-280.941
Korsmeyer-Peppas	k (RSE%)	**0.402 (29%)**	**0.384 (46%)**	**0.372 (48%)**	**0.396 (28%)**
	n (RSE%)	**0.244 (32%)**	**0.254 (145%)**	**0.251 (47%)**	**0.242 (95%)**
	AIC	**-717.731**	**-783.737**	**-596.022**	**-594.455**

Clearance from redon		
Kinetic	**Parameters**	**Values**
Zero order	k_0 (RSE%)	$2.00 \cdot 10^{-4}$ (27.4%)
	Cl_0 (RSE%)	$2.77 \cdot 10^{-2}$ (7.1%)
	AIC	-2963.25
First order	k_1 (RSE%)	$1.30 \cdot 10^{-2}$ (--)
	Cl_0 (RSE%)	$3.01 \cdot 10^{-2}$ (--)
	AIC	-2958.81

Korsmeyer-Peppas was selected among the models assayed to characterize the elution of antibiotic from bone cement. The model predictions were able to describe the experimental data release profiles for all formulations, as represented in figure 3.

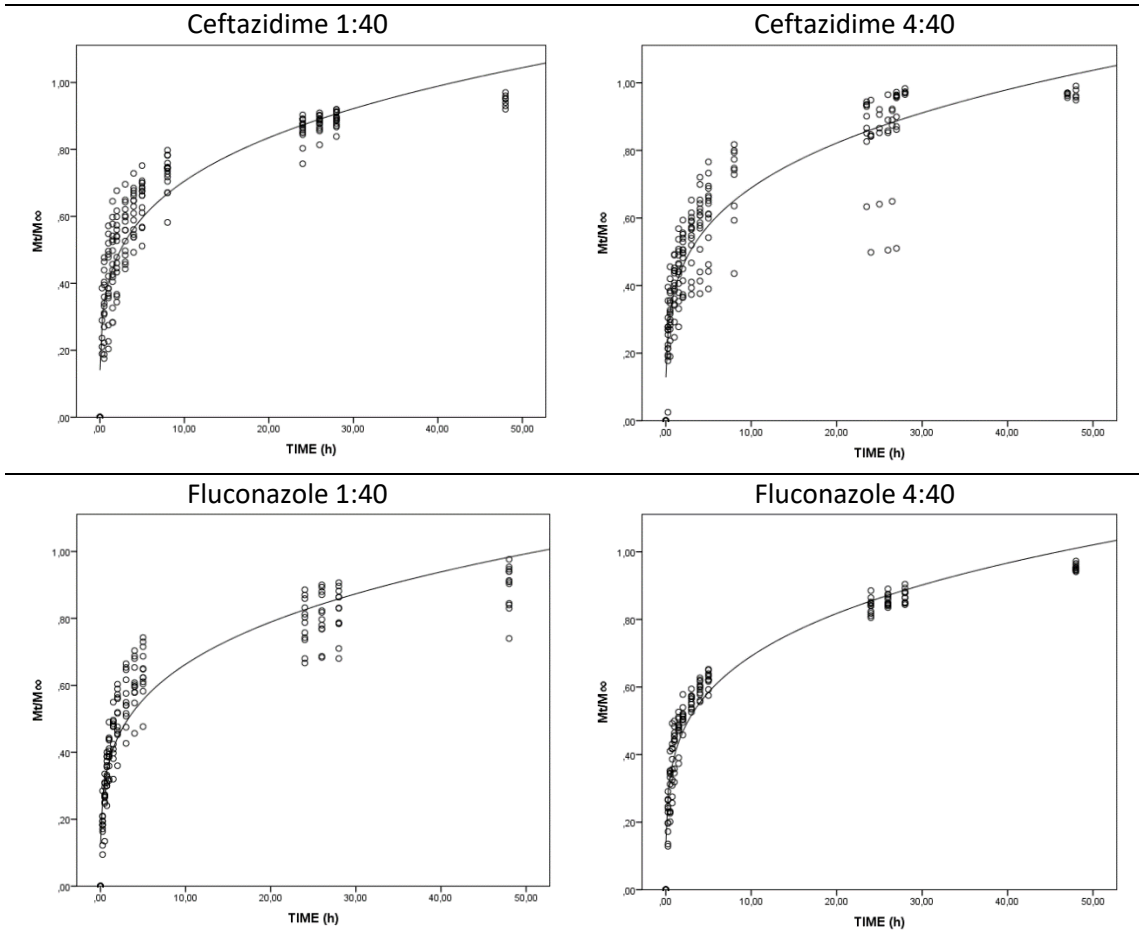

Figure 3.- Cumulative fraction of antibiotic released vs. time profiles after fitting data to the Korsmeyer-Peppas equation.

Exponent n in the Korsmeyer-Peppas equation had values between 0.242 and 0.254 in all the samples. These values, under 0.45, represent that the drug release mechanism is a Fickian diffusion (the amount of antibiotic released is proportional to the amount remaining in the system).

3. Bioactivity model

The model proposed to fit the data, outlined in figure 4, had been previously applied to describe the bioactivity of ciprofloxacin loaded into bone cement [21].

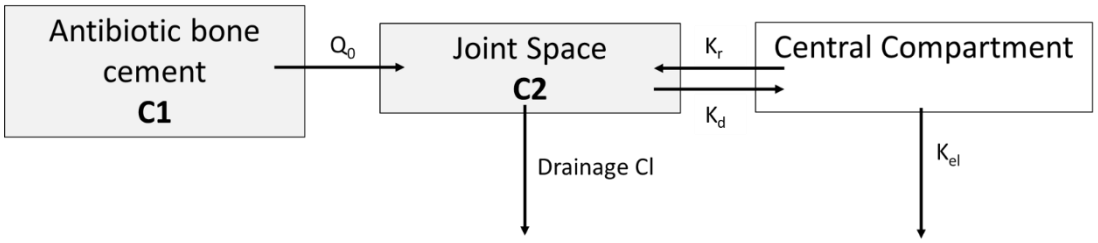

Figure 4.- Q_0: antibiotic release from the cement (mg/h); Drainage Cl: Local drug clearance due to wound drainage (mL/h); Kd (h^{-1}) antibiotic distribution rate constant from the joint space to the central compartment; Kr (h^{-1}): antibiotic return from the central compartment to joint space rate constant; Kel (h^{-1}): antibiotic elimination rate constant.

Figure 5 shows the simulated concentrations of antiobiotics in the implantation site after surgery. For the simulation exercise, drug delivery from cement was calculated according to the Korsmeyer-Peppas model and clearance of redon was described with a zero order kinetics (table 2).

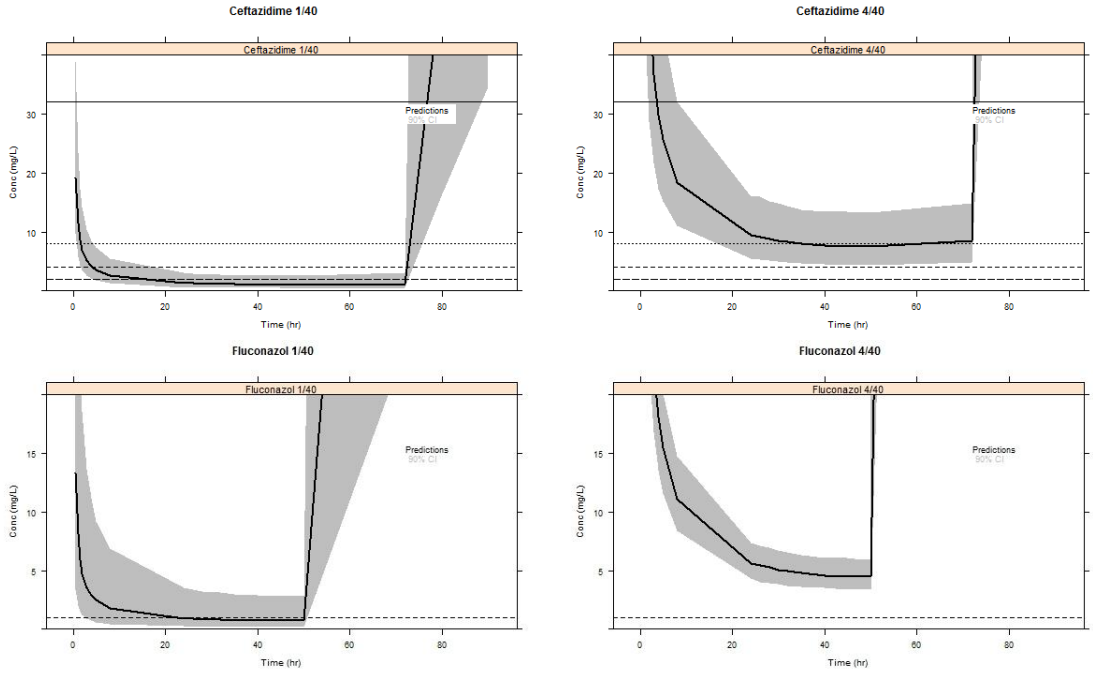

Figure 5.- Concentration of antibiotic simulated in joint space. MIC *S. aureus*=32mg/L, MIC *S.epidermidis*=8mg/L, MIC *E. Coli*=2mg/L, MIC *P. aeruginosa*=4mg/L and MIC *C. albicans*=1mg/L.

As can be seen in all the figures, the concentration decreased progressively until 72 hours, at which point the redon was removed. At this point, concentrations above MIC of E. Coli,

P. aeruginosa, S. epidermidis and S. aureus would be detected in 28%, 8%, 3% and 1% of patients, respectively in the case of ceftazidime 1:40 and in 97%, 92%, 52% and 1% of patients respectively in the case of ceftazidime 4:40. Concerning fluconazole, 53% and 99% of patients would have concentrations higher than MIC of C. albicans when 1:40 and 4:40 proportions of fluconazole would be used, respectively.

4. Microbiology analysis

In order to investigate the antimicrobial effect of the antibiotic loaded into the bone cement, a CFU count was carried out. This is a quantitative test that allows the quantification of proliferated bacterial colonies. In neither case growths was observed, except for P. aeruginosa and S. epidermidis (a CFU count of 1,000,000 and 100,000 of P. aeruginosa was registered when 1:40 and 4:40 proportions of ceftazidime were used, respectively, while a CFU of 100,000 and 10,000 of S. epidermidis was registered when ceftazidime 1:40 and 4:40 were used, respectively).

In order to investigate further the antibacterial effect of the antibiotic loaded into bone cement, the inhibition halo test was also performed. Table 3 and figure 6 show the inhibition halo produced by each antibiotic and proportion, for each of the microorganisms. In all cases, the microorganisms were sensitive to the antibiotic, except in the case of S.aureus to ceftazidime 1:40 at 72 hours and S. epidermidis to ceftazidime 1:40 at all time points. The inhibition halos for the proportions of 1:40 and 4:40 were statistically different for all microorganisms except for P. aeruginosa.

Table 3. Diameter of inhibition halo test for ceftazidime expressed in millimeters (mm) measured after 1, 2 and 3 days of incubation.

		Ceftazidime proportion							
		1:40				**4:40**			
Bacteria		*S. aureus*	*S. epidermidis*	*E. coli*	*Ps. aeruginosa*	*S. aureus*	*S. epidermidis*	*E. coli*	*Ps. aeruginosa*
		Mean inhibition halo (mm)							
Incubation (Days)	1	22	R	40.5	43.5	33	31	48	47
	2	18.5	R	37	35.5	29	20	42	38.5
	3	R	R	35.5	32	27.5	23	38	39.5

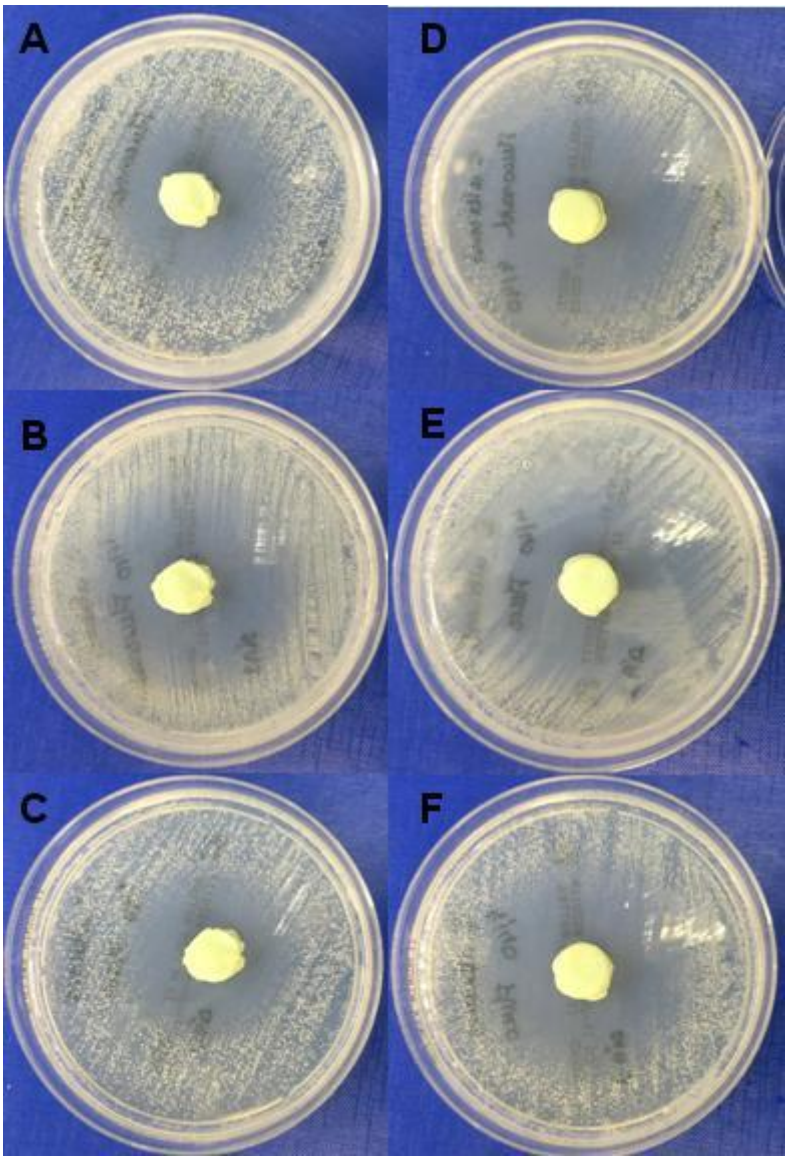

Figure 6.- Results of inhibition halo test expressed in millimetres (mm) for 1:40 fluconazole at 24h (A), 1:40 fluconazole at 48h (B), 1:40 fluconazole at 72 h (C), 4:40 fluconazole at 24h (D), 4:40 fluconazole at 48h (E), and 4:40 fluconazole at 72h (F).

Discussion

The use of bone cement containing antibiotics is based on the principle that the antibiotic will be released gradually from the cement over time and will therefore prevent bacterial proliferation. In order to eradicate infection in the bone and joints, it is essential to maintain a therapeutic concentration of antibiotic at the implantation site for an extended period of time. Currently, there are ready-to-use bone cements containing antibiotics on the market, but only some antibiotics are available, such as gentamicin and vancomycin. The increasing number of multidrug-resistant bacteria and fungal infections limits the future efficacy

of this tool and calls for the need to incorporate alternative agents into PMMA cement [9, 12]. Ceftazidime and fluconazole are known to exert a potent effect against P. aeruginosa and C. albicans respectively.

As shown in figures 1 and 2, the release profiles of antibiotics loaded into bone cement is biphasic. The release of antibiotic from the samples assayed can be explained by the Van De Belt theory [22], as only antibiotic molecules located in the superficial layers were released during the first hours of the experiment (24h). Once these molecules are dissolved, the release of antibiotic is reduced and depends on the penetration of the surrounding media into the cement matrix, which is dictated by the wettability of the polymer and by the number and size of the pores in it. The principal limitation of these systems is the low proportion of antibiotic in relation to solid bone cement components. This fact, in addition to the difference in solid particle sizes among the antibiotic and cement components, results in heterogeneous mixing, and therefore there is high variability of distribution of the antibiotic among samples, which leads to heterogeneous release profiles.

In both proportions, ceftazidime was released in a higher percentage than fluconazole. Although ceftazidime has a higher molecular weight than fluconazole (632 daltons vs. 306 daltons [23]), its solubility is also higher (396mg/L vs. 1mg/L), and our data suggest that its release is principally a surface phenomenon, as over 70% of the total amount released did it in the first 24 hours. Many factors mediate the release of antibiotic from polyacrylic cements. In this case, the data are consistent because, although the molecule of ceftazidime is approximately twice the size of that of fluconazole, it is much more soluble, which allows the rapid dissolution of the antibiotic on the surface into media.

In figures 1 and 2 it can also be observed that a higher proportion of antibiotic resulted in a greater release of antibiotic, but not of the same magnitude for both drugs; the release of ceftazidime increased 453% and that of fluconazole 648%, a difference that may have been due to the molecular weight of each one. As the concentration of drug increases, the voids created by the release of the drug facilitate the release of the drug trapped deeper inside. Our results confirm that PMMA is not a highly porous structure, and that compounds of a low molecular weight, as well as having higher diffusion coefficients, able to diffuse through more narrow spaces, are consequently released more readily than compounds of a higher molecular weight. These results highlight the relevance of the molecular weight of the compound selected for incorporation within bone cement [22].

The amounts of antibiotic released from the cements, in combination with the values of local clearance redon (attributable mainly to the wound drain), and that of liquid volume in the joint space, allowed to simulate the bioactivity of the cements. Figure 5 shows that the amount of antibiotic in the biophase would decrease up until 72 hours, at which point the redon would be removed and the amount of antibiotic in biophase increased. At 72 hours, in the best of cases, less than 30% of patients would reach the MIC of the reference microorganisms in the case of ceftazidime in a proportion 1:40. On the other hand, at 72 hours, more than 50% of patients would reach the MIC for all microorganisms except S. aureus when ceftazidime in a proportion of 4:40 would be used. In the case of fluconazole, around 50% and 99% of patients would reach the MIC of C. albicans when in proportions of 1:40 and 4:40, respectively.

In summary, according to our simulations, the bioactivity of fluconazole and ceftazidime depends on the microorganism that produces the infection, the proportion of antibiotic used, and above all the time passed since surgery, being highest during the first 24 hours. In the case of ceftazidime, the antibiotic proportion used is of importance during the first 72 hours, after which time it ceases to be relevant. Regarding fluconazole in a proportion of 4:40, all patients should be covered against C. albicans for the period simulated.

The release of ceftazidime and fluconazole from PMMA to agar indicated that polymerization of the PMMA did not adversely affect the action of the antibiotic and antifungal drugs. This finding is in line with previously reported studies. The CFU count and inhibition halos for both antibiotics (table 3 and figure 5) revealed some differences. Only S. epidermidis and P. aeruginosa grew when the cement was placed in a liquid-growing medium. The inhibition halo test revealed that the former was resistant to ceftazidime in a proportion of 1:40 in all samples. This is logical, since ceftazidime is not the most effective cephalosporin against S. epidermidis. On the other hand, ceftazidime was not active against P. aeruginosa when liquid-growing medium was used, but was active when halo of inhibition was measured. This could have been due to the properties of P. aeruginosa, as this microorganism has an alginate capsule that creates biofilms [24, 25]. For this reason, in the first few hours during which the bacteria was in liquid medium, it could have upholstered the pores of the cement, preventing elution of the antibiotic.

Our present study has some limitations. First, there are many types of PMMA currently available and we have tested just one, as it is the most used in our hospital. Second, we evaluated the bioactivity of samples through a simulation exercise based on the assumption that the surface of impregnated cement in contact with extracellular body fluid would be equivalent

to the surface of the samples assayed and assuming that the distribution of the antibiotic from the location of the implant to the systemic circulation would be negligible. Consequently, the amount of antibiotic released into the medium would not have been exactly the same. On the other hand, our results are strengthened by the fact that all the antibiotic released would have remained in the same place, and consequently would have reached a high local concentration.

Conclusions

The findings of the present study show that ceftazidime and fluconazole can be successfully incorporated within self-polymerizing PMMA. The bioactivity of ceftazidime at all proportions and fluconazole in a proportion of 1:40 depend on the sensitivity of the microorganism in the first three days post-surgery, increasing substantially after drain removal, usually at 72 hours. In the case of fluconazole 4:40, all patients should be protected against C. albicans for the duration of the post-surgery period. Our microbiology study revealed biofilm formation in the case of P. aeruginosa, which represents a problem for treatment and prophylaxis against this microorganism. Further clinical studies are essential in order to test the efficacy of these drug-delivery systems before their widespread use as prophylaxis and treatment of prosthetic join infections.

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Appendix

Drug release models

The in vitro fraction release profiles (Ft) for each formulation, calculated as the ratio of the absolute cumulative amounts of drug released at time t (Mt) to infinite time (M∞), were used to test the following models:

The zero-order kinetics model (equation 1):

$$Ft = \frac{M_t}{M_\infty} = k \cdot t \text{ equation 1}$$

where k is the zero order release constant. This model assumes that drug release is constant.

The Higuchi model (equation 2):

$$Ft = \frac{M_t}{M_\infty} = k \cdot t^{0.5} \text{ equation 2}$$

where k represents the release rate constant reflecting the design variables of the system.

The Korsmeyer-Peppas model (equation 3):

$$Ft = \frac{M_t}{M_\infty} = k \cdot t^n \text{ equation 3}$$

where k represents a rate constant incorporating structural and geometric characteristics of the device, and n is the release exponent. For cylindrical devices, $0.45 \leq n$ corresponds to a Fickian diffusion mechanism, $0.45 < n < 0.89$ to non-Fickian transport, $n = 0.89$ to Case II (relaxational) transport, and $n > 0.89$ to super case II transport. With this equation, the first 60% of a release curve should be used to calculate n, the exponent that explains the mechanism of release [26].

The models were selected based on the precision of parameter estimates, goodness-of-fit plots, and the minimum value of objective function [−2 log(likelihood): −2LL] provided by NONMEM program [27]. Because some of the models compared were not nested, −2LL was not used directly for comparative purposes, and the Akaike information criteria (AIC), computed as −2LL+2Np, where Np is the number of the parameters in the model, was used instead. The model with the lowest value of AIC was selected, as the precision of model parameters and data description were adequate.

Bioactivity models

In order to assess bioactivity, a Monte Carlo simulation was performed. Antibiotic concentrations in the joint space (for each antibiotic and proportion) of 1000 patients were simulated using NONMEM version 7.3. The percentage of patients whose levels of antibiotic at the site of the implant would be higher than the MIC was calculated.

Potential bioactivity was evaluated using the minimum inhibitory concentration (MIC) distributions of microorganisms sensitive to ceftazidime as a reference: P. aeruginosa (MIC= 4ug/mL), S. aureus (MIC= 32 ug/mL), S. epidermidis (MIC= 8 ug/mL) and E. Coli (MIC=8 ug/mL). C. albicans (MIC=1ug/mL) was used for fluconazole calculation.

The pharmacokinetic model used for simulating biophase antimicrobial concentrations (Figure 4) consists of two compartments: cement loaded with antibiotic (C1) and joint space (C2). Due to the fact that clearance by drainage is very high during the first 72h post-surgery, the distribution (Kd) from the joint space (C2) into the systemic circulation and return (Kr) were considered negligible for calculations. Volume of joint space, 1.6 ± 1,1mL, was obtained from literature [28]. Elution rate of the antibiotic was calculated from the in vitro study. A 1-year retrospective revision of volume obtained from redon in the first 72 hours after knee arthroplasty was carried out to assess clearance from the joint space in Dr. Peset hospital -Valencia, Spain-. 468 data were collected from 156 patients.

Clearance due to the redon in the first 72 hours was modelled by fitting zero and first order equations to data (equation 4 and 5 respectively):

$$Cl_t = Cl_0 + k_0 \cdot t \quad \text{equation 4}$$

$$Cl_t = Cl_0 \cdot e^{-k_1 t} \quad \text{equation 5}$$

where t is time, Cl_t is the clearance at time t, Cl_0 is the initial clearance, and k_0 and k_1 are the zero and first order constants.

In order to ensure the validity of the method, median and standard errors of the parameters of the final model were estimated using the nonparametric bootstrap technique within Perl-speaks-NONMEM (PSN ©). 200 replicates of the data were generated by the bootstrap method to obtain the median and 95% percentile of parameters and fixed- and

random-effect parameters. The bias of each parameter was calculated by computing the difference between the median value derived from the bootstrap and the final parameter estimate.

CAPÍTULO 5

Chitosan matrices to improve the antibiotic loaded into bone cement treatment in Total Knee Arthroplasty

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Journal of Arthroplasty.

Submitted

Abstract

Development of a pharmaceutical form for the superficial infections related with arthroplasties would be helpful for clinical practice. In this context, we set out to evaluate the ciprofloxacin and gentamicin elution from systems based on chitosan. For this proposal, various formulations (films and semisolid hydrogels) containing chitosan alone (2%) or in combination with gelatin (6%) or tetrakis (hydroxymethyl) phosphonium chloride (THPC) in different proportions (12% of THPC for films and 12%, 24% or 36% for hydrogel) were prepared. Moreover, different antibiotic doses were assayed (0.5mg/cm^2, 1mg/cm^2 and 2mg/cm^2). Apart from antibiotics release, the bioactivity exercise and the cytocompatibility was analyzed for the formulations that provided the best elution kinetics.

All samples containing chitosan or chitosan-gelatin released ciprofloxacin or gentamicin at very high rates. Samples containing ciprofloxacin into THPC-chitosan showed a biphasic release during the 6 days delivery assay. The amount of ciprofloxacin released at 6 days from THPC-chitosan films containing 12% of THPC was 1.78mg, 2.59mg and 3.18mg for antibiotic loaded at 0.5mg/cm^2, 1mg/cm2, and 2mg/cm^2, respectively. On the other hand, from the hydrogel containing 2mg/cm^2 of ciprofloxacin, the percentage of ciprofloxacin released was 63.26%, 46.47% and 29.50% for 12%-THPC, 24%-THPC and 36%-THPC, respectively.

THPC is suitable as crosslinker for chitosan when ciprofloxacin is incorporated showing a sustained release during 6 days. The release system of 12%-THPC-chitosan with 2mg/cm^2 of ciprofloxacin showed that 100% of patient would be covered during 72 hours post-surgery. The concentration of 12%-THPC did not show cytotoxicity in NIH3T3 mouse fibroblasts after 48 hours of assay.

Introduction

Total joint replacement is one of the most common and successful orthopaedic operations. One of its most serious complications is associated with the development of infections, although its prevalence is between 0.5% and 3% [1], in some cases can lead to death. Currently, a few clinical assays evaluate the efficacy of antibiotic loaded cement in primary revision arthroplasty; there are only two meta-analysis that evaluate their efficacy: Parvizi et al. [2] evaluated the efficacy of gentamicin loaded cement in primary revision arthroplasty. The authors concluded that the antibiotic loaded cement reduced about 50% the deep infection rate (from 2.3% to 1.3% when antibiotic loaded cement was used) with statistical significance in favour of antibiotic loaded into bone cement. Wang et al. [3], evaluated the deep and superficial infection rate when antibiotic (gentamicin, tobramycin, cefuroxime, erythromycin or colistine) was incorporated into bone cements in primary revision arthroplasty. The authors found statistically significant differences in deep infection rate but not in superficial infection rate.

Therefore, the development of a pharmaceutical dosage form that would provide an initial high rate of delivery of the antibiotic after surgery would be helpful for the prophylaxis and treatment of superficial infections related with arthroplasties. For this purpose, a polymeric material that could be used as antibiotic vehicles are the hydrogels, as semisolid matrices or films. In this paper, chitosan and gelatin were selected as reference polymers. Chitosan is well known for its numerous and interesting biological properties. Indeed, this polysaccharide is a biocompatible, bioresorbable and bioactive biopolymer [4]. Chitosan is a cationic polymer, hydrophilic, biocompatible, and can be crosslinked with natural and synthetic polyanions, such as gelatin [5] and tetrakis (hydroxymethyl) phosphonium chloride (THPC) [6], respectively. Gelatin is a promising biopolymer for the preparation of cellular supports due to its biocompatibility, biodegradability and its similarity with the extracellular matrix of the tissues of the skin, cartilage and bone [7]. Moreover, it presents low immunogenicity, and could increase cell adhesion, proliferation and differentiation, because in its composition it may contain the amino acid sequence of RGD (arginine-glycine-aspartic acid). This sequence of tripeptides is found in the adhesion proteins of the extracellular matrix (ECM), mainly in fibronectin, which, when interacting with the integrins of the cell membrane, facilitates the adhesion of the cells and trigger the biological responses [8]. On the other hand, tetrakis (hydroxymethyl) phosphonium chloride (THPC) is also a good crosslinker for chitosan, because allows the covalent bound with the amino groups of chitosan. It is a relatively inexpensive, water-soluble organophosphorus compound made up of four hydroxymethyl groups linked to an electronegative phosphorus atom. Chung et al. [6] have proposed this molecule as a crosslinking

agent for protein based materials. The authors, characterized the THPC-amine reaction and demonstrated the use of THPC in tuning hydrogel properties and showed its cytocompatibility.

Ciprofloxacin, a synthetic fluoroquinolone, is an antibacterial agent that can be administered safely and effectively to treat most clinical isolates in infections associated with joint prostheses and chronic osteomyelitis [9]. Gentamicin, an aminoglycoside, is an antibiotic used to treat several kinds of bacterial infections because it is active against a wide range of bacterial infections, mostly Gram-negative bacteria. Gentamicin is used in the treatment of bone and soft tissues infections [10].

In this context, first we set out to evaluate ciprofloxacin and gentamicin elution from chitosan formulations. In a second step ciprofloxacin was incorporated into chitosan films added with gelatin. Thirdly, film or semisolid hydrogel formulations with chitosan and THPC were evaluated as delivery systems and their cytocompatibility was analyzed.

Material and methods

Ciprofloxacin hydrochloride, gentamicin, chitosan medium molecular weight (MPM, GD = 81%, PM = 190-310 kDa) and gelatin from bovine skin (type B), were purchased from Sigma-Aldrich (Madrid, Spain). Tetrakis-(hydroxymethyl)-phosphonium chloride was acquired from Tokyo Chemical Industry.

Buffered saline solution pH 7.4 was prepared as described in the US Pharmacopoeia: disodium hydrogen phosphate anhydrous 2.38 g, mono-potassium hydrogen phosphate anhydrous 0.19 g and sodium chloride 8 g per 1 L of purified water. All reagents were analytical grade (Panreac –Barcelona, Spain-).

In the first step, the film forming solutions were prepared using chitosan alone as polymer, and the influence of the acidifier agent (acetic acid or lactic acid) as well as the antibiotic (ciprofloxacin or gentamicin) and its proportion in the mixture was studied. Secondly, gelatin and THPC were tested as crosslinkers. Lately, formulation as film or as hydrogel was evaluated.

Table 1 shows the samples prepared at each step. In order to obtain the different samples, a solution of chitosan was prepared by dissolving the polymer in an aqueous solution of the acidifier. For each hydrogel, once the appropriate amount of antibiotic was dissolved, different crosslinker concentrations in solution were added. This mixture was vortexed for about

10 seconds. Finally, the hydrogels were placed in petri dishes and desiccated at 40ºC for 48 hours. Three samples per condition were prepared.

Table 1. Samples prepared and evaluated.

		Samples																							
	Proportion	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**
Drug	**Ciprofloxacin**	X	X	X	X	X	X	X	X									X	X	X	X	X	X	X	X	X	X
	Gentamicin									X	X	X	X	X	X	X	X										
Drug concentration	**0.5 mg/cm²**	X		X		X		X		X		X		X		X		X	X			X					
	1 mg/cm²																						X				
	2 mg/cm²		X		X		X		X		X		X		X		X			X	X			X	X	X	X
Acidifying	**Acetic acid 0.5%**	X	X			X	X			X	X			X	X							X	X	X	X	X	X
	Lactic acid 1%			X	X			X	X			X	X			X	X	X	X	X	X						
Polymer	**Chitosan 1.5%**	X	X	X	X					X	X	X	X					X		X							
	Chitosan 2%					X	X	X	X					X	X	X	X		X		X	X	X	X	X	X	X
Crosslinker	**Gelatin 6·%**																	X	X	X	X						
	THPC 12%																					X	X	X	X		
	THPC 24%																									X	
	THPC 36%																										X
Pharmaceutical Form	**Film**	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X			
	Gel																								X	X	X

To perform release studies, 5 pieces of 1cm^2 of the film were immersed into 10 ml phosphate buffer saline pH 7.4 at 37ºC for 6 days. Samples were taken 0.16, 0.33, 0.66, 1, 1.5, 2, 3, 4, 24, 25.5, 52, 78 and 144 hours after immersion. In the case of hydrogel, 2.12 g were introduced into dialysis membranes and were immersed in 10 ml phosphate buffer saline pH 7.4 at 37ºC for 6 days. Samples were taken 0.5, 1, 1.5, 2.5, 3.5, 4, 27, 52, 78, 120 and 144 hours after immersion. In both cases, the phosphate buffer was replaced every time a sample was taken in order to maintain the sink conditions.

Ciprofloxacin concentration was assayed by HPLC, using a Perkin Elmer® Series 200 equipped with an UV detector (λ=254 nm). The mobile phase consisted of Acetic Acid solution 0.1 M: Acetonitrile (80:20) and was filtered through a 0.45 µm membrane filter before use. The mobile phase was eluted at a flow rate of 1 ml/min. The column was a Kromasil® C-18 with a pore size of 5.0 µm, measuring 150 mm (length) x 4.6 mm (diameter) [11]. Gentamicin determination was performed by immunoassay of chemiluminescent microparticles (ARCHITECT i1000SR).

In order to compare formulations, the total amount of antibiotic released and the percentage of antibiotic released at each time point were compared using one-way analysis of variance (ANOVA). As a post hoc test, Scheffe's test, was used.

To evaluate the bioactivity of hydrogels a simulation of the amount of drug into biophase as a function of time was carried out. For this proposal, first the kinetics of the release of antibiotic was characterised [12]. The *in vitro* fraction release profiles (Ft) for each formulation, calculated as the ratio of the absolute cumulative amounts of drug released at time t (Mt) to infinite time (M∞), were used to test the following models:

The zero-order kinetics model (equation 1):

$$\frac{M_t}{M_\infty} = k \cdot t \quad \text{equation 1}$$

where k is the zero order release constant. This model assumes that drug release is constant.

The Higuchi model (equation 2):

$$\frac{M_t}{M\infty} = k \cdot t^{0.5} \quad \text{equation 2}$$

where k represents the release rate constant reflecting the design variables of the system.

The Korsmeyer-Peppas model (equation 3):

$$\frac{M_t}{M_\infty} = k \cdot t^n$$ equation 3

where k represents a rate constant incorporating structural and geometric characteristics of the device, and n is the release exponent. For spherical devices, n≤0.43 corresponds to a Fickian diffusion mechanism, 0.43<n<0.85 to non-Fickian transport, n=0.85 to Case II (relaxational) transport, and n>0.85 to super case II transport. With this equation, the first 60% of a release curve should be used to calculate n, the exponent that explains the mechanism of release [13].

The models were selected based on the precision of parameter estimates, goodness-of-fit plots, and the minimum value of objective function [−2 log(likelihood): −2LL] provided by NONMEM. Because some of the models compared were not nested, −2LL was not used directly for comparative purposes, and the Akaike information criteria (AIC), computed as −2LL+2Np, where Np is the number of the parameters in the model, was used instead. The model with the lowest value of AIC was selected, as the precision of model parameters and data description were adequate.

Finally, to assess the bioactivity, a Monte Carlo simulation was performed. Antibiotic concentrations in the joint space (for each crosslinker proportion) of 1000 patients were simulated using NONMEM version 7.3. The pharmacokinetic model used for simulating biophase antimicrobial concentrations, which was described in previous work [14], consists of two compartments: hydrogel loaded with antibiotic and joint space.

Potential bioactivity was evaluated using the minimum inhibitory concentration (MIC) distributions of microorganisms sensitive to ciprofloxacin as a reference: *Pseudomonas aeruginosa* (MIC = 0.25–1 µg/mL), *S. aureus* (MIC = 0.12–0.5 µg/mL) *and Escherichia coli* (MIC =0.016–0.004 µg/mL) [15]. The percentage of patients whose levels of antibiotic at the site of the implant were higher than the MIC was calculated.

In order to determine the cytocompatibility of the elaborated hydrogels, cytotoxicity assays were performed using mouse fibroblast cells (NIH3T3 P15, ATCC®, Manassas, USA). Cell viability was assessed by measuring mitochondrial metabolic activity with 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) [16]. The MTT assay allows to examine the toxic effect of the material studied on the cells, as a result of the interaction between the cell and the evaluated sample. The assay was performed in 96-well plates, in which

100 µL of a suspension of NIH3T3 P15 cells ($1.11*10^5$ cells / mL) were seeded. Cells were cultured in DMEM-Dulbecco modified Eagle's medium (Aldrich® Sigma, St. Louis, USA), supplemented with 1% antibiotics (Gibco® penicillin / Streptomycin, Waltham, USA) and 10% FBS (fetal bovine serum from Gibco®). After reaching confluence, the culture medium was replaced with 25, 50 or 75 µL of the samples and supplemented with 225, 200 or 175 µL respectively, of serum-free DMEM (dilutions 1/10, 1/5 and 1/3 respectively). They were incubated in 5% CO2 at 37° C for 24h or 48h. After the incubation period the medium was removed, 20 µL of MTT dye (5 µg / mL) was added and maintained at 37° C for 3 h. Finally, the absorbance of the purple, blue formazan dye was measured spectrophotometrically in a microplate reader at 570 nm (Synergy H1 monochromator-based, Biotek, Winooski, USA).

Results and discussion

The modulation of the drug release from biopolymer systems is currently one of the main challenge for biomedicine. Chitosan, a biocompatible, bioerodible and bioactive biopolymer [4], has the perfect characteristics to be used as a carrier material for controlled release systems. The use of crosslinkers to delay the drug release from chitosan polymers has been reported previously, since the high chitosan' hydrophilicity allows a very fast hydration and an instantaneous release. In this study, all samples from 1 to 16 (table 1) resulted an instant release regardless of the proportion of chitosan, acidifying agent or antibiotic ratio included.

The gelatin type B addition to chitosan (samples from 17 to 20 in table 1), did not produce a delay in the release either. In this study, since first sampling time, all antibiotic was released and therefore no increase in release during the 6 days after was observed. These results motivated to study the inclusion of THPC as a crosslinker in the samples.

The inclusion of THPC was based in the paper from Chung et al. [6]. These results confirm that THPC is an appropriate crosslinker. The release of ciprofloxacin from samples that contained chitosan-THPC showed a biphasic release; during the first hours, all the ciprofloxacin that was present in the superficial layers was released. Once these molecules were in solution, rate of release of antibiotic was reduced and it depends on the penetration of the surrounding aqueous media into the polymer network [17].

Figure 1 shows the profile of amount of ciprofloxacin released vs. time from samples 21, 22 and 23 containing 12% THPC. Analysing the amount of antibiotic released as a function of the amount of antibiotic loaded into the samples, a higher loading of ciprofloxacin resulted in a

greater release at 6 days; however, the increase in the amount released is not proportional to the charge of the samples (amount released for 12% THPC is 1.78 mg, 2.59 mg and 3.18 mg for antibiotic loaded with 0.5 mg / cm^2, 1 mg / cm2, and 2 mg / cm^2, respectively). This less than proportional increase of release with drug concentration could be due to binding of the crosslinker to the amino groups of ciprofloxacin. In this way, an increase in the dose of ciprofloxacin would result in increased binding of the cross-linker to the drug and a decrease in the effective crosslinking (the effective crosslinking is the bonding of the hydroxymethyl groups of the THPC with the amine groups of the chitosan).

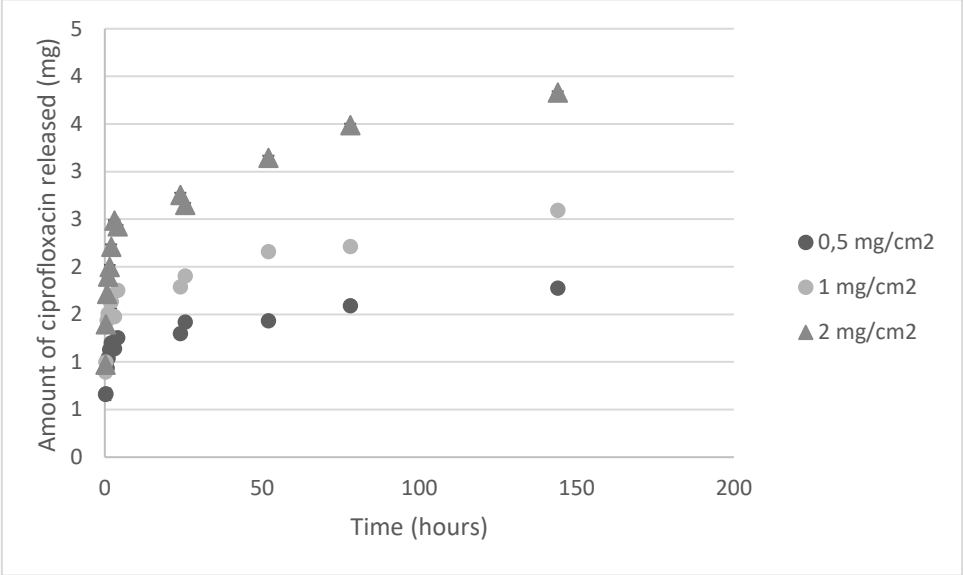

Figure 1.- Amount released vs. time profile of ciprofloxacin loaded into 12% THPC-chitosan films loaded with 0.5, 1 and 2 mg/cm^2 respectively.

Although the release of the drug from the films was satisfactory, the organoleptic properties of the formulations following the addition of the crosslinker were not. Due to this problem it was decided to change the formulation to an hydrogel form. The highest antibiotic load (2 mg/cm^2) was selected in order to ensure high drug concentration in biophase. In these conditions, the influence of the crosslinking ratio on the delivery properties and biocompatibility of the formulation was evaluated. Figure 2 shows the profile of percentage of ciprofloxacin released *vs* time when the antibiotic was loaded on 12, 24 or 36% of THPC on hydrogel (samples 24 to 26). In these cases, the increase of percentage of THPC (a greater degree of crosslinking) resulted in a minor amount of ciprofloxacin released. The total amount released at the end of the assay was 6.33mg, 4.65mg and 2.95 mg for 12% THPC, 24% THPC and 36% THPC respectively.

The amount released decreased a 26% when 24% THPC is used respect to 12% THPC and a 37% when 36% THPC is used respect to 24%. This behaviour was expected and it can be said that the release of antibiotic is reduced by about 30% when doubling the amount of THPC. The comparison of the amounts released and percentage released from film and hydrogel among the different antibiotics proportions and THPC proportions showed statistically significant differences (p<0,05).

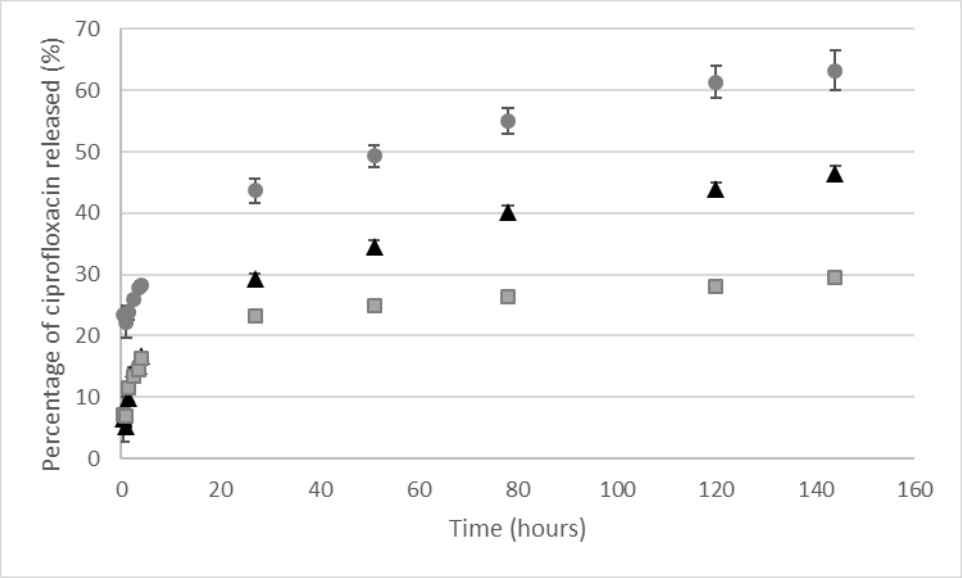

Figure 2.- Percentage of ciprofloxacin released vs. time profile when loaded into THPC-chitosan gel. Symbols represent: (●) THPC 12%; (▲) THPC 24% and (■) THPC 36%.

Table 2 shows parameter values and statistical AIC obtained after fitting the data from samples 24, 25 and 26 to different kinetic equations. Korsmeyer-Peppas was selected among the models assayed to characterize the elution of antibiotic from hydrogels. According to the value of the exponent *n* obtained in the Korsmeyer-Peppas equation - between 0.20 and 0.33 - all the samples had a value under 0.43, which means that the drug release mechanism is a Fickian diffusion (the amount of antibiotic released is proportional to the amount remaining in the release system).

Table 2. Parameter values and statistical AIC obtained after fitting the different kinetic equations to experimental data (samples 24, 25 and 26). (RSE= Relative Standard Error inter-sample; AIC= Akaike Information Criterion and k are the antibiotic elution rate constants for each kinetics).

Ciprofloxacin elution from hydrogel				
Kinetic	**Parameters**	**12% THPC**	**24% THPC**	**36% THPC**
Zero order	K (RSE%)	1.04 (0.5)	0.37 (22.6)	0.41 (0.6)
	AIC	202.02	121.21	126.64
Higuchi	K (RSE%)	1.39 (0.5)	0.64(5.1)	0.61 (0.7)
	AIC	96.81	14.21	25.71
Korsmeyer-Peppas	K (RSE%)	2.30 (0.7)	0.91 (0.5)	0.98 (0.7)
	n (RSE%)	0.20 (1.8)	0.33 (0.5)	0.23 (0.5)
	AIC	-48.96	-25.69	-59.52

The model proposed to explain local bioactivity, had been previously applied to describe the bioactivity of ciprofloxacin loaded into bone cement [12, 14]. Figure 3 shows the concentrations simulated in the biophase. As can be seen, the concentration decreased until 72 hours, at which point the redon was removed. Nevertheless, after this time point, concentrations would be above MIC of *E. Coli, P. aeruginosa* and *S. aureus* in 100% of patients ensuring adequate effectiveness.

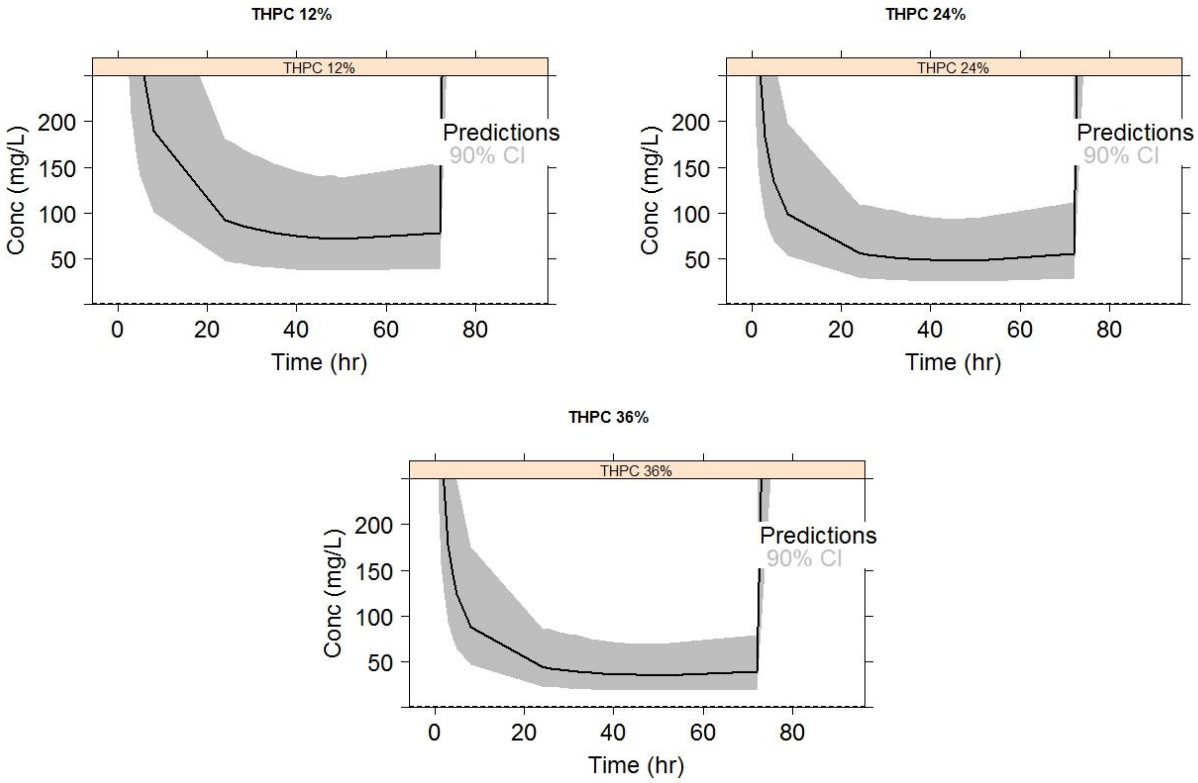

Figure 3.- Concentration of antibiotic simulated in joint space.

Figure 4 shows the viability of mouse fibroblasts after exposure to the formulations compared with the control. In this assay, the dilution 1/10 and 1/5 did not show statistically significant differences respect to the control regardless of the proportion of THPC at 24h. On the other hand, the 1/3 dilution, showed statistically significant differences with a decrease in cell viability around 90% at 24 hours. After 48 hours of incubation, no statistically significant difference with controls were observed when 1/10 dilution was used for all ratios and when the dilution of 1/5 was used for the ratio of 12%. These results indicate that the concentration of 12% THPC when used at a 1/10 dilution in the medium did not cause cytotoxicity in NIH3T3 mouse fibroblasts after 48 hours of assay. Chung et al. described that free amines located on the cell surface are susceptible to crosslinking with THPC (and all covalent crosslinkers that target primary amines) and may affect cell function. This fact could explain the drop in cell viability when the croslinker is used at very high ratios.

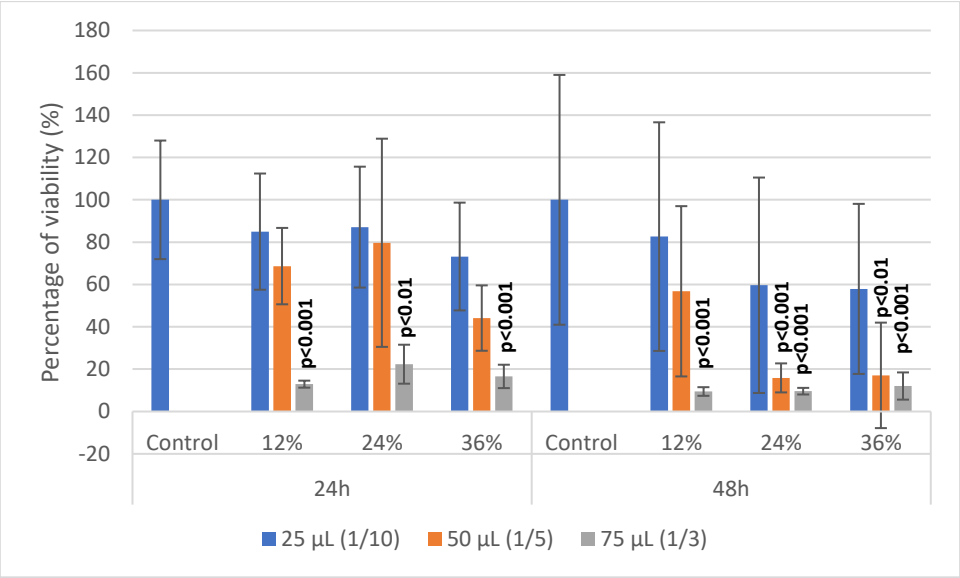

Figure 4.- MTT assay results showing cell proliferation after 24 and 48 hours of incubation in the presence of hydrogel cross-linked with 12%, 24% and 36% of THPC. Symbol * represents statistically significant differences with respect to control.

The use of chitosan semisolid hydrogel as wound healing accelerators has been reported, and their effects are due to the enhancement of inflammatory cell and fibroblasts function, which promotes tissue granulation and organization [18, 19]. The problem with chitosan is that due to its great water solubility, the release of the drug is almost instantaneous. In this case, the use of THPC has been adequate in order to extend the release of antibiotic during the subsequent six days and ensuring a correct bioactivity during the 72 hours post-surgery (the most important moment for the prevention of infections).

The pharmaceutical form developed could be very useful to be implanted on the opened field during surgery interventions for replacement in order to protect locally against superficial infection associated with arthroplasty. Moreover, this pharmaceutical form would not have to be removed as it is fully biodegradable. In these cases, approximately during the three days after the surgery, when there is a redon that applies vacuum in order to eliminate the substances of waste, the high release of the antibiotic would provide high local concentrations effective to protect against the most common pathogens.

Conclusions

The hydrogels prepared containing ciprofloxacin are promising for the prophylaxis of superficial infections associated to joint replacement. THPC is suitable as crosslinker for chitosan when ciprofloxacin is incorporated as the antibiotic showed a sustained release during the 6 days after the immersion. The release system of 12% THPC-chitosan with 2mg/cm^2 of ciprofloxacin would provide a correct bioactivity since a very high ratio of patients would be covered during the 72 hours post-surgery. The concentration of 12% THPC when using a 1/10 dilution (12% THPC-chitosan / culture medium) did not show cytotoxicity in NIH3T3 mouse fibroblasts after 48 hours of assay.

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ANEXO

Antibiotic-loaded Bone Cement as Prophylaxis in Total Joint Replacement

Orthopaedic Surgery - Decision on Manuscript ID OS-2016-10-0205

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Sincerely,
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Antibiotic-loaded Bone Cement as Prophylaxis in Total Joint Replacement

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Running title: Antibiotic Loaded Into Bone Cement in Prophylaxis.

Disclosure: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

ABSTRACT

One of its most serious complications of arthroplasty is associated with the development of infections, although its prevalence is between 0.5%-3%, in some cases can lead to death. Therefore, an important challenge in joint surgery is the prevention of infections when an arthroplasty is performed. The use of antibiotic-loaded cements could be a suitable tool due to its numerous advantages; the main advantage of the use of antibiotic loaded into bone cement derives directly from antibiotic release in the effect-site, allowing to achieving high concentrations at the site of action, and minimal or no systemic toxicity. This route of administration was first described by Buchholz and Engelbrecht. In the case of infection treatment, this method is an established method and its good results are confirmed. However, its role in infection prevention, and therefore the use of these systems in clinical practice, has proved controversial because of the uncertainty about the development of possible antibiotic resistance after prolonged exposure time, the effectiveness, cost of these systems, toxicity and loosening of mechanical properties. This review discusses all these topics, focusing on effectiveness and safety, antibiotic decision, cement type, mixing method, release kinetics and future perspectives. The final objective is to provide the orthopaedic surgeons right information in their clinical practice based on current evidence.

Key words: Bone cement; Arthroplasty; Antibiotic; Elution kinetics; Orthopaedics; Bioactivity.

Flow chart showing the content of the article.

Introduction

Total joint replacement is one of the most common and successful orthopaedic operations. The replacement is performed when there is irreversible damage in the joint, and in general, it is recommended in the elderly, in which bearable of the prosthesis is much smaller due to its low physical activity, reducing the possibility of failure. One of its most serious complications is associated with the development of infections, although its prevalence is between 0.5% and 3% (1), which in some cases can lead to death. In these cases, it is required high dose of antibiotics to reach effective concentrations at the implantation site. Nevertheless, high dose of antibiotics could cause toxicity. To prevent the genesis of complications associated to the development of infections, the inclusion of antibiotics into the bone cement intended for mechanical attachment of the prosthesis to bone tissue has been suggested. The main advantage of this use of antibiotics derives directly from antibiotic release in the effect-site, allowing to achieving high concentrations at the site of action, and minimal or no systemic toxicity (2, 3). Currently, polymethylmethacrylate (PMMA) is the most widely used bone cement material for loading antibiotics and represents the current standard as an antibiotic delivery vehicle in orthopaedic surgery.

This route of administration was first described by Buchholz and Engelbrecht (4). However, its role in infection prevention, and therefore the use of these systems in clinical practice, has proved controversial because of the uncertainty about the development of possible antibiotic resistance after prolonged exposure time, the effectiveness, cost of these systems, toxicity and loosening of mechanical properties (5-9). These aspects are reviewed in this document.

Method

A systematic review of the available literature was performed using the keyword terms "antibiotic loaded bone cement" and "arthroplasty"; there was no limit on the year of publication. The search was limited to English papers. The following databases were accessed on 9th June 2016: PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez/). In order to be considered eligible for inclusion, studies needed to focused on the prophylaxis of infection. Studies were excluded if: (1) outcomes of antibiotic-loaded bone cements (ALBC) use in primary TKA were not reported; (2) it was impossible to extrapolate or calculate the necessary data from the published results.

Evidence of Effectiveness and Safety

The review of Kynaston-Pearson et al. shows that 8% of all primary hip replacement prosthesis implanted in 2011 and recorded by the National Joint Registry (NJR) had no readily available evidence relating to their safety or effectiveness (10). This has led to further research in this field. Nevertheless, it is very difficult because there is a high number of cement brands and prosthesis brands; for example, in UK in 1996 there were 62 components in the market and in 2011 there were 265 different implants (11). This fact added to the low number of patients included in the studies, makes that the studies cannot provide sufficient evidence.

The Food and Drug Administration (FDA) in 2003 authorized antibiotic-loaded into bone cement for second-stage reimplantation after infected arthroplasties. In contrast, the use of these delivery vehicles for prophylaxis in prosthesis surgery is an off-label use (12). Nevertheless, the use of antibiotic loaded bone cement is recommended by most authors for joint arthroplasty revisions and in primary implants, which are at higher risk of

infection (7). Live audience polling at the 2009 American Association of Hip and Knee Surgeons Annual Meeting demonstrated that 37% of surgeons in attendance ‘‘always’’ used antibiotic loaded into bone cement for routine primary total knee arthroplasty, while 45% used it on a more selective basis for high-risk patients (13). In the United States, off-label use of antibiotic-impregnated polymethylmethacrylate for primary joint replacement is increasing and multiple antibiotic-containing polymethylmethacrylate products are commercially available. However, the use of antibiotic loaded bone cement in primary arthroplasty is controversial because its inclusion can reduce the mechanical properties of the cement and its uses would produce bacteria resistance.

Currently, a few clinical assays evaluate the efficacy of antibiotic loaded cement in primary revision arthroplasty; there are only two meta-analysis that evaluate their efficacy (Table 1).

1. Parvizi et al.(14) evaluated the efficacy of gentamicin loaded cement in primary revision arthroplasty. A total of 21,444 knees arthroplasties impregnates with gentamicin or not were evaluated. Only one of the six studies evaluated by the authors reached the statistical significance in prophylaxis of infection. This paper concluded that the antibiotic loaded cement reduced about 50% the deep infection rate (from 2.3% to 1.3% when antibiotic loaded cement was used) with statistical significance in favour of antibiotic loaded into bone cement.
2. Wang et al. (15), evaluated the deep and superficial infection rate when antibiotic was incorporated into bone cements (3 studies with gentamicin included into Palacos, 1 with tobramycin included into Simplex P, 1 with cefuroxime included into Simplex P, 1 with erythromycin and colistine included into Simplex P and 2 with cefuroxime included into CMW) in primary revision arthroplasty. In this study, the authors stated that the meta-analysis reported by Parvizi et al.

included some nonrandomized studies and their results should thus be treated with caution. Therefore, the inclusion criteria applied by Wang et al. were more restrictive and evaluated a total of 6,381 arthroplasties. The authors found statistically significant differences in deep infection rate but not in superficial infection rate. However, there were no statistically significant differences in aseptic loosening of prostheses (noninfectious loosening is defined as normal erythrocyte sedimentation rate, no pain and bacteriologic cultures to be negative) neither in clinical objectives (articular function evaluation).

In summary, attending to both meta-analysis results it can be considered that the antibiotic loaded bone cement would provide clinical profit in primary surgery, as prophylaxis, in order to prevent deep infection.

Antibiotic Decision

Dose of Antibiotic

The dose of antibiotic to be used in arthroplasty is not completely established, it depends if it is going to be used as treatment or prophylaxis. In most cases, it appears that the dose is set according to its influence on the mechanical properties of the cement, rather than to its therapeutic efficacy. It is established that to pursuit therapeutic treatment, it is usually recommended to add 3.6 g of antibiotic to 40 g of acrylic cement in order to guarantee the correct drug levels (16, 17). Conversely, for a prophylactic effect, it appears to be sufficient with low dose of antibiotic. In this case it is recommended to use 1 g of antibiotic per 40 g of cement; the lower proportion of antibiotic is less likely to alter the mechanical properties of the cement (18).

Characteristics of the Antibiotic

Experience has shown that not all antibiotics satisfy the properties required to be incorporated into bone cements. At the moment, it is known that antibiotic election has to satisfy some criteria:

- ①Stability at high temperature. The polymerization of PMMA increases the temperature of the cement mixture to 60ºC-80ºC (19). Furthermore, it should be ensured that the degradation products, derivate of high temperature exposure, are not toxic drugs.
- ②Different authors have reported that the inclusion of liquid antibiotic shows higher amount of antibiotic eluted but a loosening of the mechanical properties (this cements do not satisfy the ISO normative 5833 -Annex E-) (20, 21). In this way, the antibiotic included in bone cement must be in solid form. Nevertheless, the mechanical properties influenced by each antibiotic in solid form must be studied in order to guarantee that the corresponding ISO normative is accomplished.
- ③The antibiotic must be effective against most frequently microorganism that cause infection (wide antibacterial spectrum), specifically, methicillin-resistant *Staphylococcus aureus* (MRSA) (22) and gram negative aerobic bacillus (23).
- ④Antibiotic elution from bone cement depends on the penetration of the surrounding media into the cement matrix, which is dictated by the wettability of the polymer, by the number and size of the pores in it and by antibiotic solubility (24). Then antibiotics are required high solubility in water (19).
- ⑤Although antibiotic doses (1 g of antibiotic per 40 g of cement) are established to preserve mechanical integrity, the antibiotic doses are not equipotent. Thus, it is not the same 1 g of gentamicin (habitual intravenous dose

is 240 mg /24 hours) with that 1 g of amoxicillin (common intravenous dose is 3000 mg/24 hours). For these reason another feature for the antibiotic inclusion is that it has to be effective at low doses.

However, the antibiotic elution requirements are unknown to date.

Single Antibiotic Incorporation

The most commonly mixed antibiotics are gentamicin, tobramycin, vancomycin and clindamycin. These antibiotics satisfy the commented criteria and are found on the market, such as ready-mixed. The two antibiotics ready-mixed more used are gentamicin and vancomycin. Ferraris et al. compared the commercial antibiotic-loaded bone cement (Palacos R + G®) and manually mixed (Palacos R® and Palacos LV® added with gentamicin) evaluating their antibacterial behavior based on inhibition zones. They concluded that commercial formulation produces an inhibition zone that is a bit larger (23% greater, $P<0.05$) and more regular than the manually mixed preparation. They attributed the differences to the lack of use of vacuum mixing techniques in manual mixtures (25). A limitation of this study is that the antibiotic powder employed in manual mixing is a commercial gentamicin sulphate, which is a mix of different substances; this limitation is present in many studies. Therefore it can be concluded that the manual addition of commercial antibiotics to PMMA-based bone cement produces inhibition zones that are moderately smaller and more irregular compared to commercial formulations of the same antibiotic-loaded bone cements.

Other antibiotics, under research, that have been mixed by some authors are ciprofloxacin (26), cefazolin (27), moxifloxacin (28), amoxicillin clavulanate (28) (table 2). Our research team, examined ciprofloxacin release from three trademarks of bone cements

(Simplex®, Lima® and Palacos®) and its bioactivity using as variables, the mixing method, the chemical form of the antibiotic and the antibiotic combination. The antibiotic amount released in base form represents 35% of antibiotic amount released when hydrochloride form is incorporated. Moreover, the combination (vancomycin and ciprofloxacin) shows a stronger release (132%) than hydrochloride ciprofloxacin alone. Three cements tested show equal drug release profile ($P > 0.05$). A bioactivity simulation exercise showed that until 72 hours post-surgery, ciprofloxacin concentrations in the implant would be higher than 0.1 μg/mL in 100% of the patients. The limitations of this study is that no bending nor modulus strengths were calculated and the bioactivity was evaluated by means of a simulation exercise (26).

Paz E. et al studied the inclusion of vancomycin or cefazolin at prophylaxis doses (1 g of antibiotic per 40 g of bone cement) into bone cement Palacos R+G®; vancomycin and cefazolin release, fluid absorption, and mechanical properties were evaluated under physiological conditions. Cefazolin at 672 hours showed higher release (227.28 ±23.91 μg/mL) compared to vancomycin (71.86 ± 25.34 μg/mL) ($P<0.01$). However, the differences in release between both antibiotics was not so marked during the first 24 hours, being 44.26 ± 3.37 μg/mL and 32.46 ± 9.70 μg/mL for cefazolin and vancomycin respectively ($P=0.281$). The compressive strength of cements added of the two antibiotics without aging and after aging for 1 month in phosphate buffered saline (PBS) at 37ºC was calculated. All cements without aging showed no statically significant difference to the control cement ($P>0.01$). However cefazolin aged in PBS at 37ºC experienced significant reductions in compressive properties ($P<0.01$). The limitation of this study is that there is no data about bioactivity and therefore it can not be assessed whether the differences are clinically significant (27).

Gálvez-López et al. evaluated different ALBC for elution kinetics, thermal stability, and mechanical properties. A 10% or 20% mixture (w/w) beads of medium viscosity bone cement (DePuy®) and vancomycin, gentamycin, daptomycin, moxifloxacin, rifampicin, cefotaxime, cefepime, ampicillin, meropenem, and ertapenem were evaluated. Elution kinetic profiles of all antibiotics tested, with the exception of ampicillin and cefepime, demonstrated a triphasic pattern of release with a progressive increase in the first 24 h followed by a rapid decrease and a final phase with a low and steady decline through the rest of the experiment. In this general triphasic behaviour, 3 particular behaviours of elution were identified depending on the antibiotics tested. Vancomycin, gentamycin, moxifloxacin, and rifampicin, loaded at 10% (w/w), demonstrated constant elution kinetics through the 30-day duration of the experiment. Daptomycin, meropenem, ertapenem, and cefotaxim although also having the triphasic pattern, showed a lower peak and a faster decrease of elution between days 3 and 30, but eluted concentrations remained above the minimum inhibitory concentration (MIC) of susceptible organisms, according to EUCAST clinical breakpoints. Finally, ampicillin and cefepime showed minimal elution with eluted concentrations being almost undetectable at day 4 and always below the MICs of susceptible organisms, according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints. The percentage eluted from each ALBC is shown in table 2. Presence of antibiotics did not affect the strength of ALBC with mean compression values greater than 70 mPa, except for rifampicin -loaded bone cement, for which the compression strength did not exceed 42.9 mPa (28). The limitation of this study is the measurement of antimicrobial properties; at the antibacterial activity was only measured at 30 minutes from the beginning of the assay.

Hsu et al. incorporated 0.5, 1 and 2 g of daptomicin (Cubicin®, the commercial antibiotic, that has more than 90% of pure antibiotic) per 40 g of PMMA; in this study, the authors

showed that the mechanical strength is not compromised by daptomicin at any concentration, because all samples had a compressive strength higher than 100 MPa. The percentage of daptomcin eluted during 2 weeks was 9.59% ± 0.85%, 15.25% ± 0.69%, and 20.64% ± 20.33% from 0.5, 1 and 2 g of daptomicin, respectively. The bioactivity of the cements was also confirmed including MSSA, MRSA, *S. Epidermidis*, *E. Faecalis, and E. Faecium*. The authors concluded that the inclusion of commercial daptomycin at low dose in bone cement was satisfactory; both bioactivity and resistance tests were adequate (29).

Snir et al. studied 1 g of linezolid, vancomycin or gentamicin per 40 g included into PMMA (Smart Set GHV® and CMW1®). There were no differences between brands cements. The study showed that linezolid shows a minimum inhibitory concentration (MIC) of 0.625, 0.312, 1, 250 and 250 mcg/mL to Methicillin-resistant Staphylococcus aureus (MRSA), *S. epidermidis*, VRE (vancomycin-resistant enterococci), *E. Coli* and *K. pneumoniae* respectively. Vancomycin shows a MIC of 1.25, 1.25, 0.4, 125 and 125 mcg/mL to MRSA, *S. epidermidis*, VRE (vancomycin-resistant enterococci), *E. Coli* and *K. pneumoniae* respectively. Finally gentamicin shows a MIC of 0.1, 7.81, 23.43, 1 and 0.625mcg/mL to MRSA, *S. epidermidis*, VRE (vancomycin-resistant enterococci), *E. Coli* and *K. pneumoniae* respectively. Table 3 shows the growth inhibitory time (GIT) of beads impregnated with antibiotics. In conclusion, the authors showed that the GIT of linezolid was significantly longer than that of vancomycin and gentamicin for MRSA and S epidermidis. Axial compression test was performed to verify if the mechanical strength of PMMA was compromised because of the addition of antibiotics. The results revealed no reduction in the mechanical strength of PMMA beads (P>0.2) with the concentration of antibiotics used in this study (maximum 5% weight/weight antibiotic per PMMA packet). Both types of cements maintained similar mechanical properties. With this study,

it can be said that linezolid is more effective than gentamicin and vancomycin against MRSA and S. epidermidis. Table 2 shows that the combination gentamicin plus linezolid or vancomycin plus linezolid, do not provide a greater bactericidal potency. It can be concluded that PMMA impregnated with linezolid has the potential to be efficacious in the prevention and treatment of bone and joint infections (30). Anguita-Alonso et al. (31) found that linezolid used at 3 different concentrations (2.5%, 5%, and 7.5% weight/weight) maintained excellent stability and elution after PMMA polymerization in vitro. The PMMA used was Simplex P ® in the form of beads, and the indicator microorganism was Bacillus subtilis. They also reported that compared with other antibiotics (ie, cefazolin, ciprofloxacin, gatifloxacin, levofloxacin, and rifampicin), the elution of linezolid from PMMA was less affected by impregnated antibiotic concentration.

Another important aspect related with the antibiotic loaded into bone cement is if the exposure to antibiotic causes resistance; Corona et al. have seen in their study that the inclusion of gentamicin or tobramycin in cement spacers (4g. of antibiotic / 40 g of PMMA) seems to increase the gram positive cocci resistance. They analysed 113 chronic hip and knee prosthesis joint infection and observed that aminoglycoside-resistance in gram-positive cocci was significantly higher when aminoglycosides were incorporated in cement spacers respect to no use of it. Gentamicin resistance after previous aminoglycoside-cement spacers use was significantly higher (49.2% vs. 19.3%; *P*: 0.0001) as well as resistance to tobramycin (52.7% vs. 30.9%; *P*: 0.014) (32). There is little evidence of this aspect.

In conclusion, the commercial formulations produce a greater and more regular release of antibiotic from bone cement than the manually mixed preparations. One of the biggest issues of most of the studies is that the commercial form of the antibiotic, which comes

with excipients in many occasions, is used. This fact may explain the differences between pre-mixed ALBC and manually mixed. Finally, currently there are a large number of combinations of bone cements with antibiotics, for which much remains to be elucidated and it cannot be concluded that a perfect unique combination exists, each one adapts to the requirements of the clinical condition.

Two Antibiotics Combination

It has been reported that the simultaneous incorporation of two antibiotics or more into bone cement resulted in higher rate of elution compared to one antibiotic loaded bone cement. When two antibiotics are incorporated, more voids and cracks are present in bone cements as the drugs are released, thus increasing the release of the remaining antibiotics. Moreover, authors have described a synergic effect between some antibiotics, i.e. it has been described synergistic effect between aminoglycosides and glycopeptides (19). A study about the optimal antibiotic combination for the antibiotics gentamicin, vancomycin and teicoplanin in cements showed that the combination of gentamicin and teicoplanin had a bactericidal activity more prolonged than gentamicin alone. Moreover, the synergic effect of teicoplanin and gentamicin had superior bactericidal activity compared to gentamicin and vancomycin (33, 34). Bertazzoni Minelli et al. compared gentamicin plus vancomycin spacers versus gentamicin alone spacers. The study showed that the combination was more effective than gentamicin alone (35). These results are coherent with those mentioned above.

To date, there is no ideal combination of antibiotic and cement that allow to cover all possible infections and therefore, the antibiotic election must be effective against most microorganisms that cause infection.

Cement Type

Polymethylmethacrylate (PMMA) is the main component used in the fixation of joint prosthesis. It is prepared in the operating room, mixing the solid and liquid components. As bone cements have some disadvantages, these systems are fragile and produce necrosis due to exothermic reaction during the polymerisation (36-39). There have been reports of thermal damage of cartilage and periosteum, leading to non-union of fractures and loosening of implants (38, 39).

Viscosity of the cement is very important in the mixing moment. Low viscosity promotes the mixing process; however, its mechanical strength is worse than that of high viscosity cements. Clinical outcomes of low viscosity bone cement demonstrate that they have higher risk of revision and loosening (40, 41).

The method that produces the loosening is unclear to date; Ayre et al. studied the mechanism that causes the aseptic loosening. In order to explain the aseptic loosening, two commercial high viscosity bone cements (Palacos® and Cemex Isoplastic®) were aged in an isotonic fluid at physiological temperatures. After 30 days ageing cements increased in weight of approximately 2% and the outermost layers of the cement were hydrolysed. This study concluded that this molecular change and the plasticising effect of water resulted in reduced mechanical and fatigue properties over time and therefore cement ageing contributes to the long-term failure of cemented joint replacements (42). This kind of studies are important to simulate the evolution of bone cement into the organism.

The addition of barium sulphate and zirconium oxide (for radiological detection) increases the risk of loosening (43). These radiopacifiers are hydrophilic and promote the

hydrolysis of ester groups of methyl methacrylate (MMA) and PMMA. The previous study, suggests to employ hydrophobic radiopacifiers such as iodine-based ones, developed by Lewis et al. (44) in order to decrease the risk of loosening. Shearwood et al. studied the effect of barium sulphate agglomerates on mechanical characterisation of bone cement. They evaluated the effect of barium sulphate agglomeration on crack initiation processes in conventional, vacuum-mixed acrylic cement. The tendency of barium sulphate particles to agglomerate is clearly evidenced to be detrimental to the fatigue performance of the cement (45). Gomoll et al. studied the effect of replacing barium sulphate microparticles that are usually present in commercial PMMA cements with barium sulphate nanoparticles. They concluded that the nanoparticulate substitution of radio-opacifiers substantially improved the in vitro mechanical properties of PMMA bone cement without changing the known chemical composition (46). Ultimately, the use of the hydrophilic radio-opacifiers damage the mechanical properties of bone cements, so there is more investigation required to find alternatives for the future.

Antibiotic elution from bone cement depends on cement composition and physicochemical characteristics of antibiotic. About gentamicin, Van de Belt et al. studied the formation of a Staphylococcus aureus biofilm on six gentamicin-loaded bone cements (CMW1®, CMW3®, CMW Endurance® and CMW2000® with 2.5% of gentamicin; Palacos® and Palamed® with 1.25% of gentamicin). None of gentamicin-loaded cements showed a reduction in biofilm formation relative to unloaded cements within 6 h after inoculation, whereas only gentamicin-loaded CMW1® and Palacos® reduced biofilm formation 24 h after inoculation. Alternatively, CMW Endurance®, CMW2000®, and Palamed® did not exhibit any initial reductions in biofilm formation, but effects started after 48, and 72 h, respectively. Biofilm reduction by gentamicin-loaded CMW3® lasted the longest from 24 to 72 h. Biofilm formation on all cements follows a similar pattern in

time, but the gentamicin-loaded cements demonstrate different reductions of biofilm formation, that seems unrelated with the gentamicin-release kinetics from the cements, previously measured (table 2). The authors conclude that biofilm formation on bone cements is not only related to gentamicin release, but may also be dependent on other properties of the cement surface, such as its roughness (18). Scott et al., compared the bioactivity of the two most used aminoglycosides (tobramycin and gentamicin) from different cements (Palacos® and Simplex®), and showed that tobramycin incorporated into Simplex® has antibacterial activity against 98% of *P. aeruginosa* while gentamicin into Palacos® against 93% of the same bacteria (P<0.001). In this study, the authors compared the zone of inhibition of gentamicin and tobramycin loaded into bone cement at prophylaxis doses against 100 clinical isolates of *P. aeruginosa* collected from sputum, urine, ear… but none that has caused a prosthetic infection. Results are consistent with the type of antibiotic, because tobramycin is slightly more effective than gentamicin against *P. aeruginosa* (47). With respect to vancomycin, Cerretani et al., compared the 2 g of vancomycin elution from 40 g of CMW1®, Palacos-R® and Simplex-P® with a pharmacokinetic study. The authors performed a pharmacokinetic study in which evaluated the area under the concentration-time curve against time (AUC), which represents the amount of drug released and pharmacologically available; the half-life of release (t1/2); peak concentration (Cmax); and time at which Cmax is obtained (Tmax). The cements released 2.00%, 1.94% and 1.69% of antibiotic incorporated after 35 days, respectively. Only t1/2 showed statistically significant differences between bone cements brands; having CMW1® a significantly longer release half-life. Although there are significant differences, the clinical implications that this may involve are not clarified; bioactivity studies are needed in order to extract the clinical impact of differences (48).

About the comparison of pre-mixed commercial ALBC, Neut et al. investigated differences in gentamicin release and the antibacterial efficacy of the eluent between four cement brands (Refobacin Palacos R®, Refobacin Bone Cement R®, Palacos R + G® and SmartSet GHV®). Table 2 shows the differences in the amount of antibiotic eluted and the bioactivity. Although the cements Refobacin Bone Cement R® and Palacos R + G® provided higher release of antibiotic, there was no colony growth in any cement sample during the one week study, so it can be said that all commercial cements with gentamicin had adequate bioactivity during the first week (49).

Mixing Method

The mixing method characterizes the antibiotic elution. The best antibiotic elution is associated to high cement porosity. The problem of high porosity is the loss of mechanical properties (33, 50, 51). The presence of air trapped into cement, decrease its resistance. The vacuum mixing decreases the air trapped into cement from 25% to 1%. Therefore, this mixing method provides advantages: the resistance increases from 70 to 90 MPa and fatigue resistance rises from 10 to 30 MPa (51, 52). Nevertheless, the preparation under vacuum conditions causes a major reduction of bone cement and then a worse adhesion from bone cement-to bone is obtained (41, 53). There is a division of opinions according to the authors (54). Meyer J et al., (55) compared 6 commercial bone cements (Cemex Genta Gentamicin 1.0 g/40 g, Cobalt G-HV Gentamicin 0.5 g/40 g, Palacos R+G Gentamicin 0.5 g/40 g, Simplex P Tobramycin 1.0 g/40 g, SmartSet GMV Gentamicin 1.0 g/40 g and VersaBond AB Gentamicin 1.0 g/40 g) mixed at atmospheric pressure and under vacuum conditions. A standard Kirby-Bauer bioassay technique was subsequently used to quantify antibiotic elution from the products. The results from the study

demonstrated that vacuum mixing produced lower antibiotic release from Cemex®, SmartSet® and Versabond® and increased release of antibiotic from Palacos®, Simplex® and Cobalt® (Fig. 1). According to these statements, the study concluded that the effect of vacuum-mixing on antibiotic elution is product-specific (55). Our research team, compared the manual and vacuum mixing when ciprofloxacin hydrochloride was mixed with different bone cements (Simplex®, Palacos® and Lima CMT1®). When comparing the two mixing procedures, no statistically significant differences were found between vacuum and manual mixing with respect to the drug release rate from Simplex® and Palacos® bone cements. On the contrary when Lima CMT1® bone cement was used, significant differences were observed up to 697 hours. However, no statistically significant differences in the percentage of amount released were observed at subsequent testing times. This significant difference can be explained if the high variability of the manual batches tested is considered. It should be emphasized that variability of the percentage of drug released from the vacuum-mixed samples was much lower than that seen with manually-mixed ones. Ultimately, vacuum mixing reduces variability in the release profiles, but the influence on kinetic properties are product-specific.

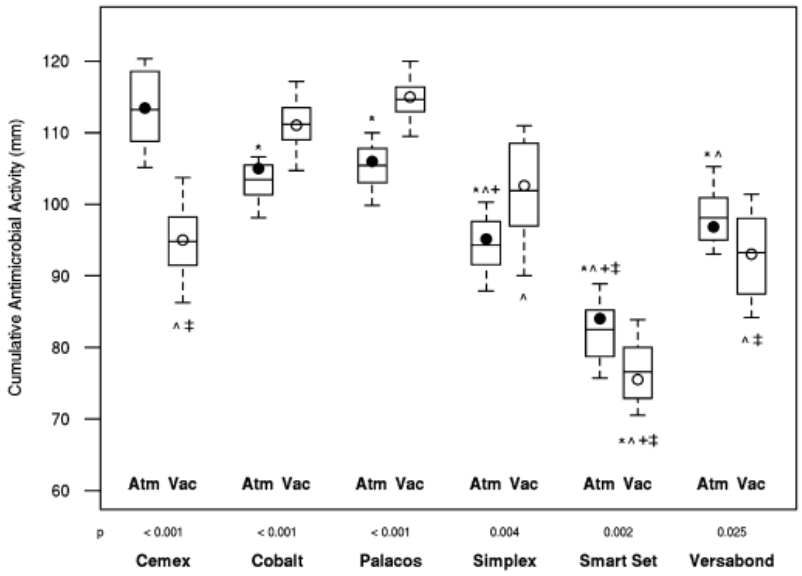

Figure 1.- Summary of use of vacuum mixing when Cemex®, SmartSet®, Versabond®, Palacos®, Simplex® and Cobalt® was used.

Some authors advocate for vacuum mixing, because the surgeons have less exposure to cement vapours; to date several studies have shown that exposure to vapours from bone cements provide undetectable plasma levels. Homlar et al. studied the effect of exposure to PMMA. Twenty healthy volunteers were exposed during the mixing of polymethylmethacrylate cement in a simulated operating room environment (this study was purposefully designed using non-laminar flow rooms, open bowl mixing technique, and without the use of personal exhaust hoods to simulate a worst case scenario exposure). Methyl methacrylate was not detected in any of experimental specimens (56).

Another important aspect is the mixing speed; Pithankuakul K. et al. evaluated the effect of the mixing speed of hand-mixing bone cement. In the study, the antibiotic-loaded bone cement used was Vancomycin-Palacos LV. The authors concluded that bone cement prepared with high-speed hand mixing and delayed antibiotic addition can increase vancomycin release (57).

As shown, antibiotic elution depends on many factors (cement characteristics, physicochemical properties of the antibiotic and mixing procedure, among others), and there is no absolute best option; but there is an optimal combination (antibiotic plus cement brand) for each microorganism. The continuing emergence of new commercially-available brands of ALBCs makes it important to establish which one will provide the most favorable antibiotic release, and consequently yields the best antibacterial efficacy.

Release Kinetics

Different authors have indicated that the inclusion of the antibiotic into bone cement provide high antibiotic level in first days, followed by a sustained release (19). There are different studies showing evidence that release can be produced during the first hours in some cases or for several weeks in others (58, 59). First the antibiotic is eluted from the cement surface and then from the cement inside. The fluids in contact penetrate into cement and dissolve the antibiotic. Then, the antibiotic dissolved is eluted from void and cracks of bone cements (60, 61). Various authors had stated that antibiotic elution from bone cement is conditioned by cement type and porosity, antibiotic molecular weight and physico-chemical properties, surface in contact with the liquid of the environment and amount of antibiotic incorporated (16, 20, 21, 62, 63). The problem is that the PMMA is a highly hydrophobic polymer, which limits the elution. For this reason, some antibiotics are only eluted during the first hours, that is, only antibiotic on surface is released (64). Only high solubility and low molecular weight antibiotics would be elute through voids and cracks. (16, 20, 21, 62, 63)

Since antibiotic dissolved from cement surface represents the highest amount released, cement surface in contact with fluids conditions efficacy. Moojen et al. and Bertzzoni et

al. showed that the initial release is proportional to the rugosity and then to the surface (35, 65); while release in the following days is proportional to cement porosity. This statement is logical and it should always be extrapolated into clinical practice.

As stated above, currently, it has been approved the use of premixed antibiotic loaded bone cement. Only commercially available antibiotic- PMMA can be used for reconstruction of a previously septic total knee or total hip replacement. The antibiotic incorporation to bone cement by surgeon is not permitted and therefore the only antibiotics available are vancomycin, clindamycin, tobramycin and gentamicin. Meta-analysis previously referenced (15, 66) showed that the inclusion of antibiotic into bone cement demonstrated its efficacy in deep infection but not in superficial infection. This evidence was expected because the antibiotic released out of cement, would stay in the cement-bone interface. In any case, the antibiotic release from bone cement would be an effective system for deep infection, which is more complicated due to poor blood supply.

In summary, the PMMA highly hydrophobic polymer, limits the elution, and makes it dependent on features of the antibiotic and the surface in contact. Some authors discussed the possible systemic bioavailability of antibiotics from bone cement. Kendoff et al. evaluated the systemic bioavailability of antibiotics from bone cement after implantation determining the concentrations of gentamicin and vancomycin in plasma and urine of patients receiving a novel bone cement during one-stage revision in periprosthetic hip infections. The mean postoperative maximum gentamicin plasma concentration at 5.85 hours was 209.65 ng/mL. For vancomycin, a mean postoperative maximum plasma concentration of 134.64 ng/mL was determined at 20.03 hours. The authors concluded that it exists slow absorption of both antibiotics after release from the cement resulting in plasma concentrations well below toxic levels, that do not result in a critical systemic

concentration potentially inducing bacterial resistance (67). In any case, ALBC are safe from the pharmacotherapeutical point of view, with a very low systemic absorption.

Perspectives and Conclusions

Currently, researchers are looking for ways to increase and improve these systems release. In this manner, there are studies where some substances are included into bone cement in order to improve the elution. As an example, it has been observed that vitamin E is a scavenger of free radical in the oxidative process. Moreover, its inclusion in bone cement reduces the temperature of the harden process (62 to 36 degrees C) and therefore, increases cytocompatibility. Up to 25% of vitamin E does not decrease the mechanical strength (68). Penalba Arias et al. studied the effect of bone cement loaded with daptomycin alone or in combination with gentamicin or PEG600 in the prevention of biofilm formation of S. epidermidis. For comparison, PMMA loaded with gentamicin or vancomycin was tested. The study showed that vancomycin was superior to daptomycin and gentamicin inhibiting staphylococcal adherence in vitro. However, PMMA loaded with daptomycin combined with gentamicin or PEG600 completely inhibited S. epidermidis-biofilm formation (69).

It has been demonstrated that the inclusion of chitosan nanoparticles has activity against *S. aureus* y *S. epidermidis*, without decrease in mechanical strength compared to PMMA alone (70). The inclusion of this polysaccharide would have antimicrobial activity per se. These findings support the possibility of combining in cements this polymer with antibiotics. Another improvement is the inclusion of silver nanoparticles (71). When this metal is included in cements it is eluted and has antimicrobial activity against *A.*

baumanii, P. aeruginosa, P. mirabilis y *S. aureus*, but its inclusion reduces the mechanical strength of cement (72).

Although there are still many variables to elucidate, antibiotic loaded bone cements are a successful alternative to decrease the infection rate. Many questions, like, which is the optimal dose, which patients would benefit of it or which is the optimal antibiotic-cement combination in order to eradicate microorganisms specifically, are still open. Nevertheless, there are a lot of ways for improving these delivery systems that can lead in the future to ALBC able to provide clinical profit in primary surgery, as prophylaxis, in order to prevent deep infection.

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Table 1 Summary of meta-analysis results.

Study	No. of studies included (no. of prosthesis)	Superficial infection rate		Deep infection rate	
		RR	IC (95%)	RR	IC (95%)
Parvizi et al.(8)	6 studies (24,661)	--	--	0.55	0.34-0.75
Wang et al. (9)	8 studies (6,381)	1.47	1.13-1.91	0.41	0.17-0.97

Table 2 Summary of clinical studies of antibiotic into bone cement.

Study	Mixture	Antibiotic in cement (per 40g)	Cement type	Percentage released (time)	Bioactivity	Mechanical properties
Ferraris et al. 2010 (25)	Pre-mixed	Gentamicin 0.5 g	Palacos R®	N/S	Inh zone= 8.1 mm	N/S
	Manual	Gentamicin 0.5 g	Palacos R + G®	N/S	Inh zone= 10.0 mm	N/S
Neut et al. 2010 (49)	Pre-mixed	Gentamicin 0.5 g	Refobacin Palacos R	8.6 ± 0.6% (168 h)	A gentamicin-sensitive bacterium did not survive. Survival was independent of the level of burst release by the bone cement.	N/S
		Gentamicin 0.5 g	Refobacin Bone Cement R	12.2 ± 0.8% (168 h)		N/S
		Gentamicin 0.5 g	Palacos R + G	12.5 ± 3.6% (168 h)		N/S
		Gentamicin 0.5 g	SmartSet GHV	3.6 ± 0.4% (168 h)		N/S
Martínez-Moreno et al. 2015 (26)	Manual	Ciprofloxacin hydrochloride (1 g)	Simplex®	2.65% (1344 h)		N/S
			Lima®	2.42% (1344 h)		N/S
			Palacos®	3.50% (1344 h)	The concentrations of ciprofloxacin reachable in the implant would be higher than 0.1 μg/mL in 100% of patients, decreasing the coverage when higher concentrations are need.	N/S
		Ciprofloxacin base (1 g)	Lima®	0.75% (1344 h)		N/S
	Vacuum	Ciprofloxacin hydrochloride (1 g)	Simplex®	2.85% (1344 h)		N/S
			Lima®	5.40% (1344 h)		N/S
			Palacos®	4.63% (1344 h)		N/S
Paz E. et al 2015 (27)	Vacuum	Vaconmycin (1 g)	Palacos®	8.58% (672 h)	N/S	ND
		Vaconmycin (4 g)	Palacos®	2.89% (672 h)	N/S	ND
		Cefazolin (1 g)	Palacos®	27.14% (672 h)	N/S	ND
Gálvez-López R. et al 2014 (28)	Manually	Vancomycin (1 g)	CMW®	31.32% (720 h)	N/S	586.2 mPa
		Gentamycin (1 g)	CMW®	13.31% (720 h)	N/S	166.27 mPa
		Moxifloxacin (1 g)	CMW®	50.40% (720 h)	N/S	383 mPa
		Rifampicin (1 g)	CMW®	41.24% (720 h)	N/S	42 mPa
		Daptomycin (1 g)	CMW®	17.09% (720 h)	N/S	78.5 mPa
		Ertapenem (1 g)	CMW®	22.54% (720 h)	N/S	121 mPa
		Meropenem (1g)	CMW®	27.24% (720 h)	N/S	342 mPa
		Cefotaxime (1g)	CMW®	26.50% (720 h)	N/S	75.82 mPa

		Ampicilin (1g)	CMW®	0.99% (720 h)	N/S	N/S
		Cefepime (1 g)	CMW®	1.49% (720 h)	N/S	144 mPa
Hsu et al 2014 (29)	Manual	Daptomycn (0.5 g)	Osteobond ®	9.59%	All bone cements of the three daptomycin preparations (low, mid, and high) produced detectable bacterial inhibition on day 1. However, growth inhibition for all groups rapidly declined from day 2. ,	112.39 mPa
		Daptomycn (1 g)	Osteobond ®	15.25%		112.97 mPa
		Daptomycn (2 g)	Osteobond ®	20.64%		112.97 mPa
Snir et al. 2013 (30)	Manual	--	Smart Set GHV ® and CMW1®	--	--	2285 N (1)
	Manual	Linezolid (1 g)	Smart Set GHV ® and CMW1®	N/S	MRSA MIC=0.625 mcg/mL S. epidermidis MIC=0.312 mcg/mL	2552 N (1)
		Vancomycin (1 g)	Smart Set GHV ® and CMW1®	N/S	MRSA MIC=1.25 mcg/mL S. epidermidis MIC=1.25 mcg/mL	2344 N (1)
		Gentamicin (1 g)	Smart Set GHV ® and CMW1®	N/S	MRSA MIC=0.1 mcg/mL S. epidermidis MIC=7.81 mcg/mL	2301 N (1)
		Linezolid (1 g) + Vancomycin (1 g)	Smart Set GHV ® and CMW1®	N/S	MRSA MIC=0.625 mcg/mL S. epidermidis MIC=0.312 mcg/mL	2480 N (4)
		Linezolid (1 g) + Gentamicin (1 g)	Smart Set GHV ® and CMW1®	N/S	MRSA MIC=0.625 mcg/mL S. epidermidis MIC=0.312 mcg/mL	2513 N (1)
Van de Belt et al. 2001 (18)	Manual	Gentamicin (1 g)	CMW1 ®	3.52% (168 h)	Reduction on biofilm formation only before 6 h	N/S
			CMW3®	3.16% (168 h)	Reduction on biofilm formation from 24 to 72 h	N/S
			CMW Endurance®	3.40% (168 h)	Reduction on biofilm formation only after 48-72 h	N/S
			CMW2000 ®	2.64% (168 h)	Reduction on biofilm formation only after 48-72 h	N/S
		Gentamicin (0.82 g)	Palacos®	3.43% (168 h)	Reduction on biofilm formation only before 6 hours	N/S
			Palamed®	6.86% (168 h)	Reduction on biofilm formation only after 48-72 h	N/S
Cerretani et al.2002 (48)	Manual	Vancomycin (2 g)	CMW1	2.00% (840 h)	N/S	N/S
		Vancomycin (2 g)	Simplex P	1.69% (840 h)	N/S	N/S
		Vancomycin (2 g)	Palacos-R	1.94% (840 h)	N/S	N/S
		Vancomycin (2 g) + Imipenem-cilastatin (2 g)	CMW1	2.61% (840 h)	N/S	N/S
		Vancomycin (2 g) + Imipenem-cilastatin (2g)	Simplex P	2.54% (840 h)	N/S	N/S
		Vancomycin (2g) +	Palacos-R	2.91% (840 h)	N/S	N/S

	Imipenem-cilastatin (2g)	

N/S, unknown; ND, no differences;

(1).- Axial Compression testing.

Table 3 Growth inhibitory time of beads impregnated with antibiotics.

Antibiotic	MRSA	*S epidermidis*	VRE	*K pneumoniae*	*E coli*
Linezolid	21±0.75[a]	29±0.5[a]	15±4.6[a]	Resistant	Resistant
Gentamicin	Resistant	5±1.7	Resistant	10±1.73	16±2
Vancomycin	8±0.5	19±1.9	Resistant	Resistant	Resistant
Linezolid± gentamicin	>45[b]	38±0.95[b]	32[b]	>45	40±0.5
Linezolid±vancomycin	31±10[c]	>45[c]	17±1.15	Resistant	Resistant

MRSA, *methicillin-resistant Staphylococcus aureus*; VRE, *vancomycin-resistant enterococci.*

[a], These values are significantly longer (P,.01) compared with those of vancomycin for respective bacteria.

[b], These values are significantly longer (P,.01) compared with those of vancomycin, linezolid, or gentamicin alone for respective bacteria.

[c], These values are significantly longer (P,.01) compared with those of either vancomycin or linezolid alone for respective bacteria.

Study of the Influence of Bone Cement Type and Mixing Method on the Bioactivity and the Elution Kinetics of Ciprofloxacin

The Journal of Arthroplasty 30 (2015) 1243–1249

Contents lists available at ScienceDirect

The Journal of Arthroplasty

journal homepage: www.arthroplastyjournal.org

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ARTICLE INFO

Article history:
Received 9 October 2014
Received in revised form 20 January 2015
Accepted 11 February 2015

Keywords:
bone cement
ciprofloxacin
elution kinetics
orthopedics
bioactivity
PMMA

ABSTRACT

The objectives of this study were to examine ciprofloxacin release from three trademarks of bone cements (Simplex®, Lima® and Palacos®) and its bioactivity using as variables, the mixing method, the chemical form of the antibiotic and the antibiotic combination. The antibiotic amount released in base form represents 35% of antibiotic amount released when hydrochloride form is incorporated. Moreover, the combination (vancomycin and ciprofloxacin) shows a stronger release (132%) than hydrochloride ciprofloxacin alone. Three cements show equal drug release profile ($P > 0.05$). A bioactivity simulation exercise showed that until 72 hours post-surgery, ciprofloxacin concentrations in the implant would be higher than 0.1 μg/mL in 100% of the patients. After drain removal, it is expected that bioactivity would increase since drug clearance from implant would decrease.

Antibiotic-loaded acrylic bone cement, described by Buchholz and Engelbrecht [1], is a well-established tool in the prophylaxis [2,3] and treatment of orthopedic infections [4] in humans and animals [5], with meta-analyses indicating that its use reduces the infection rate [6]. Polymethylmetacrylate – PMMA – is characterized by excellent biocompatibility with low intrinsic toxicity and inflammatory activation [7], but experience has shown that not all antibiotics have the properties necessary for their incorporation in this cement. In this context, aminoglycosides and glycopeptides (vancomycin) are known to be the two groups of antibiotics that satisfy the optimal criteria to be included in these cements (availability in powder form, wide antibacterial spectrum, bactericidity at low concentrations, elution from PMMA in high concentrations for prolonged periods, thermal stability, low or no risk of allergy or delayed hypersensitivity, low influence on the mechanical properties of the cement, and low serum protein binding) [8].

50% of surgical site infections (both superficial and deep) are caused by *Staphylococcus aureus* methicillin-resistant (MRSA); thus, staphylococcal species should be the primary target of antibiotic-loaded bone cement [9]. Unfortunately, the increasing number of multidrug-resistant bacteria [10–13] limits the continued effectiveness of this tool. In addition, the prevalence of MRSA in many hospitals influences strategies for the treatment and prevention of prosthetic joint infections [14], leading to interest in incorporating alternative antibacterial agents into PMMA cement [10,14,15].

On the other hand, despite the wide use of antibiotics in orthopedic surgery for more than 30 years, the exact mechanism by which they are eluted from PMMA is still not fully understood [8]. It seems to involve a biphasic profile, consisting of an initial rapid release of drug followed by a much slower sustained release. The following factors affect the release of antibiotics from bone cement: type and quantity of antibiotic [16,17]; type and porosity of cement [18]; surface characteristics [19]; and how the cement has been prepared [20–23]. Thus, to date only a few antibiotics have been satisfactorily incorporated into cements.

In this context, it would be desirable to incorporate new drugs into bone cements in order to increase coverage to infections caused by different organisms. In the present work, ciprofloxacin (1 g antibiotic/40 g PMMA) was selected to be assayed. This synthetic fluoroquinolone is an antibacterial agent that can be administered safely and effectively to treat most clinical isolates in infections associated with joint prostheses and chronic osteomyelitis. Additionally, ciprofloxacin possesses a broad spectrum against Gram positive and negative strains [24]. However, there are few data concerning the ability of ciprofloxacin to elute from bone cement and to retain activity against resistant pathogens after elution [25,26].

No author associated with this paper has disclosed any potential or pertinent conflicts which may be perceived to have impending conflict with this work. For full disclosure statements refer to http://dx.doi.org/10.1016/j.arth.2015.02.016.

Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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http://dx.doi.org/10.1016/j.arth.2015.02.016
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In this study we set out to characterize the elution profile of ciprofloxacin from bone cements. The following variables were evaluated: source of drug (base and hydrochloride); cement composition (three brands); mixture method (manual and vacuum); and presence of a second antibiotic in the mixture. In addition, different equations were fit to release profiles in order to explain the release mechanism. Finally, bioactivity of the mixtures was evaluated by means of a simulation exercise.

Materials and Methods

Ciprofloxacin hydrochloride, ciprofloxacin base and vancomycin hydrochloride were purchased from Aldrich (Madrid, Spain). Lima CMT1® bone cement was purchased from Lima Implantes (Barcelona, Spain), and Palacos® and Simplex® from Ibersugical (Valencia, Spain). Each cement was provided as two separate components: a powder mixture and a liquid component. The composition of the cements is shown in Table 1, according to the information provided by the manufacturers.

Palamix uno®, the vacuum mixing system employed, was supplied by Heraeus Medical GmbH (Madrid, Spain).

Buffered saline solution pH 7.4 was prepared as described in the US Pharmacopoeia: disodium hydrogen phosphate anhydrous 2.38 g, mono-potassium hydrogen phosphate anhydrous 0.19 g and sodium chloride 8 g per 1 L of purified water. All reagents were analytical grade (Panreac).

Antibiotic-loaded bone cement cylinders were prepared as follows: 1 g of the drug was added to 40 g of solid component of the cement, and, after mixing the powder, the liquid component was added following the manufacturer's instructions. Cylinders of antibiotic bone cement were made for each batch in a standardized fashion according to the ISO normative 5833 (Annex E). Samples were prepared using Teflon molds in which they were kept for 1 hour until completely hardened into a cylinder/disk shape. Each specimen was carefully weighed and measured and the theoretical amount of loaded ciprofloxacin calculated. This value was used for calculating the exact percentage released from each sample.

When two antibiotics were incorporated into the cement the total amount of antibiotic in the mixture was 1 g (50% each one).

Samples were immersed in a water bath in 10 ml phosphate buffer saline pH 7.4 at 37 °C and stirred for 8 weeks. Samples were taken 1, 3, 5, 7, 24, 32, 48 56, 72, and 168 hours after immersion, and subsequently once a week for a period of 8 weeks (final sample was taken 56 days after immersion). Three samples per batch were tested. Antibiotic homogeneity distribution within batches was indirectly evaluated by means of the statistical analysis of the percentage of the total antibiotic released from the samples assayed. The phosphate buffer was replaced every time a sample was taken in order to maintain the sink condition (defined as the volume of medium at least three times that required in order to form a saturated solution of drug substance). All samples taken were frozen at −20 °C until analyzed. Table 2 summarizes the test conditions (a total of 61 samples were processed).

Ciprofloxacin concentration was assayed by HPLC, using a Perkin Elmer® Series 200 equipped with a Waters 484® UV detector (λ = 254 nm). The mobile phase consisted of acetic acid solution 0.1 M: acetonitrile (80:20) and was filtered through a 0.45 μm membrane filter before use. The mobile phase was eluted at a flow rate of 1 ml/min. The column was a Kromasil® C-18 with a pore size of 5.0 μm, measuring 150 mm (length) × 4.6 mm (diameter) [27].

Table 2
Samples Assayed in Each Condition Tested.

Mixture	Antibiotic	Bone Cement	Sample
Manual	Ciprofloxacin hydrochloride	Simplex®	A
		Lima®	B
		Palacos®	C
	Ciprofloxacin base	Lima®	D
	Ciprofloxacin hydrochloride and vancomycin	Simplex®	E
		Lima®	F
		Palacos®	G
Vacuum	Ciprofloxacin hydrochloride	Simplex®	H
		Lima®	I
		Palacos®	J

The elution rate at each time interval (mg/h) was obtained by dividing the total quantity of antibiotic released in each interval by the elution time (in hours). The elution rates and the total amount of antibiotic released (expressed as a percentage) at each time point were compared using one-way analysis of variance (ANOVA).

Zero order (Eq. (1)), first order (Eq. (2)), Higuchi (Eq. (3)) and Korsmeyer–Peppas (Eq. (4)) equations were fit to data to characterize elution parameters and the mechanism of release of ciprofloxacin from bone cement:

$$Q_t = k_0 t \tag{1}$$

$$Q_t = Q_0 \cdot e^{-k1\,t} \tag{2}$$

$$\frac{Q_t}{Q\infty} = K_h \cdot t^{0.5} \tag{3}$$

$$\frac{Q_t}{Q_\infty} = K_k t^n \tag{4}$$

where t is time, Q_t is the amount of drug released at time t, Q_0 is the initial amount of drug in the specimen, Q_∞ is the amount of drug released at time ∞, n is the release exponent and K_0, K_1, K_h and K_k are the ciprofloxacin elution rate constants of each of the kinetics.

Turface morphology and internal structure of the samples were characterized using a scanning electron microscope (SEM, S-4100 Hitachi, Madrid, Spain). The samples were mounted on an aluminum stub using double-sided tape. They were made electrically conductive by coating with gold-palladium under vacuum. The SEM picture was taken at an excitation voltage of 20 kV. For the internal structure evaluation samples were fractured and the broken surfaces sputter-coated with gold and a layer of palladium for examination at 20.0 kV.

The elution rate from different cements was used to simulate biophase concentration for 100 patients using NONMEM version VII.

Table 1
Composition of the Different Acrylic Bone Cements, As Provided by the Manufacturer.

	Lima CMT 1	Simplex	Palacos
Solid component (40 g)	Methyl methacrylate 87.6% Benzoyl peroxide 2.4% Barium sulfate 10%	Methyl methacrylate-styrene copolymer 30 g Polymethyle methacrylate 6 g Barium sulfate E.P. 4 g	Poly(methylacrylate. methyl methacrylate) 33.8 g Zirconium dioxide 6.0 g Benzoyl peroxide 0.2 g Colorant E141 0.008 g
Liquid component (20 mL)	Methyl methacrylate 84.4% Buthylmethacrylate 13.2% N, N-dimethyl pare toluidine 2.4% Hydroquinone 20 ppm	Methyl methacrylate 19.5 mL N, N-dimethyl pare toluidine 0.5 mL Hydroquinone, USP 1.5 mg	Methyl methacrylate 18.4 g N, N-dimethyl-p-toluidine 0.4 g Hydroquinone Colorant E141 0.005 g
Viscosity	Standard	Medium	High

Simulations were performed for the three days post-surgery, using the clearance of synovial liquid values previously reported (20.42 ± 11.3 mL/h for the first day; 9.33 ± 11.02 mL/h for the second day and 4.11 ± 2.95 mL/h for the third day) [28] and considering that the distribution of the antibiotic from the location of the implant to the systemic circulation is negligible. Bioactivity was evaluated using MIC distributions for *Pseudomonas aeruginosa* (MIC = 0.25–1 μg/mL), *S. aureus* (MIC = 0.12–0.5 μg/mL) and *Escherichia coli* (MIC = 0.016–0.004 μg/mL) [29] and calculating the percentage of patients whose levels of antibiotic at the site of the implant would be higher than the MIC.

Results

The variation coefficient of total amount of ciprofloxacin released within a batch was lower than 10%. These results were considered as representative of homogeneous distribution of the drug into the samples assayed.

The influence of the chemical form was evaluated in samples B, D and F. These samples were prepared manually with Lima CMT1® bone cement. In Fig. 1 are represented the amounts of ciprofloxacin (expressed as a percentage) released from the Lima CMT1® bone cement in which it was incorporated alone, as base (ciprofloxacin, sample D) or salt (ciprofloxacin hydrochloride, sample B); sample F corresponds to ciprofloxacin hydrochloride in a binary mixture with vancomycin. The amount of antibiotic released when used as a base represented 35% of the amount released when the antibiotic was incorporated in its hydrochloride form. Moreover, the combination of vancomycin and ciprofloxacin led to a higher amount of ciprofloxacin being released; 132% the amount released from the salt form.

Fig. 2 shows the elution rate of ciprofloxacin in mg/h at different time points, plotted on a logarithmic scale, corresponding to samples A, B and C (ciprofloxacin hydrochloride-hand mixing) and samples H, I and J (ciprofloxacin hydrochloride-vacuum mixing). All samples produced high early release rates, followed by a lower sustained release. Statistical analysis with ANOVA revealed no significant differences among the percentage of total antibiotic released from samples A, B and C, indicating that the type of cement used did not modify the amount of drug released when mixing was performed manually. On the other hand, in vacuum-prepared samples significant differences were obtained in the total amount of antibiotic released between samples H (Simplex®) and I (Lima CMT1®).

The influence of the mixing procedure on elution rate was evaluated by comparing the results obtained between samples A and H (Simplex®, manual and vacuum mixing), between samples B and I (Lima CMT1®, manual and vacuum mixing), and between samples C and J (Palacos® manual and vacuum mixing). Statistical differences were obtained only in Lima CMT1®. The differences observed referred to percentages released up to 697 hours and log elution rates at all time-points (hand and vacuum Lima CMT1®). Fig. 3 shows the percentage of ciprofloxacin released from each sample assayed.

Table 3 provides the parameter values and statistical AIC (Akaike information criterion) figures obtained after fitting the tested models (Eqs. (1)–(4)) to elution data. Korsmeyer–Peppas was selected among the models assayed.

Scanning electron microscopy (SEM) (Fig. 4) revealed the higher level of porosity of Lima CMT® and Palacos® vs. Simplex®.

Since no differences in release were observed among cement type, mean values were used for simulating levels of antibiotic in the location of the implant. Fig. 5 evidences that all simulated patient data would reach ciprofloxacin concentrations higher than 0.1 μg/mL for the first three days post-surgery. According to this simulation study, the first day of elution, the coverage would be complete, decreasing slightly for following days.

Discussion

The use of bone cement combined with antibiotics is based on the principle that the antibiotic will be gradually released from the cement over time. The elution mechanism is still not fully understood, though it is known to be affected by different factors.

Ciprofloxacin is a broad-spectrum bactericidal quinolone that is effective against major infection-causing microorganisms [24]. Previous studies have evaluated the use of other antibiotics, while there are only a few reports concerning ciprofloxacin [25,26,30], whose use could expand the possibilities available. A comparison of the elution kinetics of ciprofloxacin loaded in different acrylic bone cements and the influence of the mixing procedure on kinetic properties have not been reported previously and are the subject of this study.

PMMA is a highly hydrophobic polymer, and is thus impervious to drug diffusion [31]. The release of ciprofloxacin from samples assayed can be explained by the Van De Belt theory [32], as during the first hours of the experiment (24 hours) only ciprofloxacin molecules located in the superficial layers were released. Once these molecules are in solution, the release of antibiotic is reduced and it depends on the penetration of the surrounding media into the cement matrix, which is dictated by the wettability of the polymer and by the number and size of the pores in it. The principal limitation of these systems is the low proportion of antibiotic in relation to bone cement and the high variability of different samples, which leads to heterogeneous release profiles.

As shown in Figs. 1 and 3, the greater elution of the ciprofloxacin is obtained when the drug is incorporated to the bone cement as hydrochloride as a result of the varying solubility of the two forms of the antibiotic, since the salt form (ciprofloxacin hydrochloride) is four times more soluble than the base form (ciprofloxacin). As stated before,

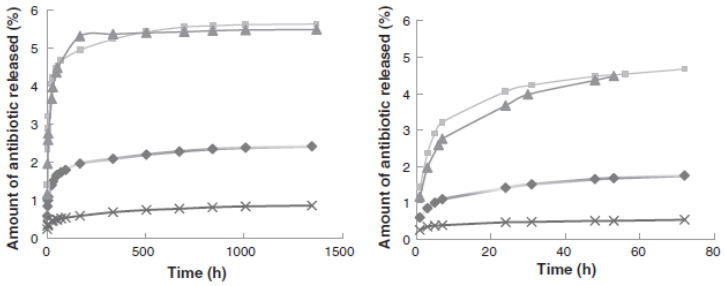

Fig. 1. Percentage of ciprofloxacin released from specimens B (♦) (ciprofloxacin hydrochloride, manual mixing), D (×) (ciprofloxacin base, manual mixing), F (■) (ciprofloxacin hydrochloride and vancomycin, manual mixing) and I (▲) (ciprofloxacin hydrochloride, vacuum mixing). Lima CMT 1® cement was used in all cases.

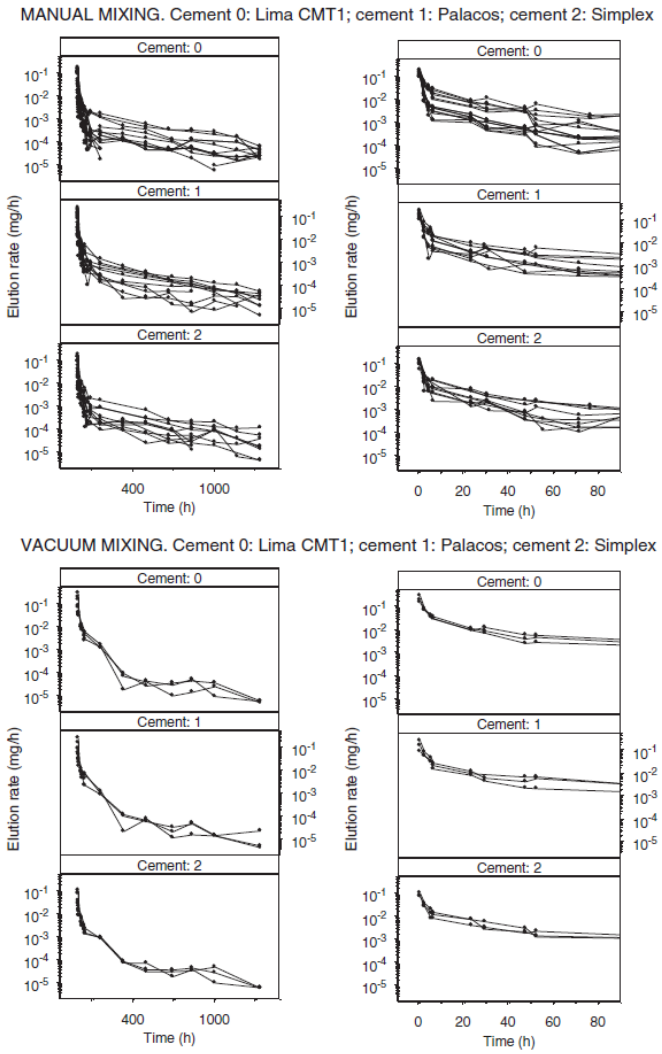

Fig. 2. Ciprofloxacin elution rate (mg/h) at different time points plotted on a logarithmic scale. Manual mixing: Samples A, B and C. Vacuum mixing: Samples H, I and J.

the drug release mechanism in these cases depends on the dissolution of drug particles adsorbed onto the matrix of the cement, and thus on the solubility of the antibiotic.

As can be seen in Figs. 1 and 3, binary mixtures achieved the highest release of all the samples evaluated, which may have been due to the solubility of vancomycin, which is three times that of ciprofloxacin hydrochloride. This suggests that, when vancomycin dissolves, more voids and cracks are present in bone cements, thus increasing the release of ciprofloxacin.

In terms of the effect of the type of bone cement on drug release, results obtained represent that antibiotic release from Palacos® bone cement is slightly higher than from Simplex® and Lima CMT1®. However, the ANOVA test revealed no significant differences. Despite previous studies have demonstrated that the highest elution rate was achieved with Palacos® [33], results reported in this study indicate that although Palacos® has superior porosity (Fig. 4), elution kinetics from Palacos®, are not significantly different from the other cements studied. When the vacuum mixing system was used, the highest level

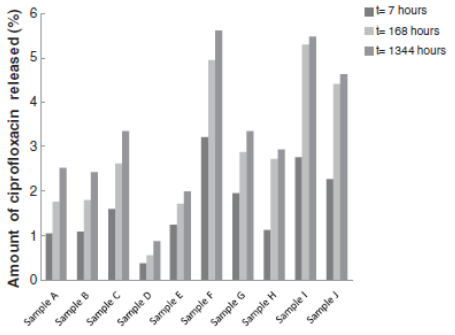

Fig. 3. Percentage of ciprofloxacin released from the ten samples at 7, 168 and 1 344 hours. Samples A, B, C, H, I, and J (ciprofloxacin hydrochloride-loaded Simplex®, Lima CMT 1® and Palacos® bone cement, hand and vacuum mixing), samples E, F and G (ciprofloxacin hydrochloride- and vancomycin-loaded Simplex®, Lima CMT 1® and Palacos® bone cement hand mixing), and from samples D (ciprofloxacin base-loaded Lima CMT 1® bone cement).

of antibiotic release was achieved with Lima CMT1® and Palacos®, although only release from Lima CMT1® and Simplex® was statistically different. The superior drug release from Lima CMT1® and Palacos® can be attributed to the greater porosity of Palacos® and Lima CMT1® (Fig. 4).

When comparing the two mixing procedures, no statistically significant differences were found between vacuum and manual mixing with respect to the drug release rate from Simplex® and Palacos® bone cement. On the contrary when LIMA CMT1® bone cement was used, significant differences were observed up to 697 hours. However, no statistically significant differences in the percentage of amount released were observed at subsequent testing times. This significant difference can be explained if the high variability of the manual batches tested is considered. It should be emphasized that variability of the percentage of drug released from the vacuum-mixed samples was much lower than that seen with manually-mixed ones.

Table 3 shows that the Korsmeyer–Peppas model is the best equation for describing ciprofloxacin elution from all the samples assayed. In the Korsmeyer–Peppas equation, the parameter n describes the type of release from bone cement. In this case, all the samples have a value under 0.5, which means that the drug release mechanism is a Fickian diffusion (the amount of antibiotic released is proportional to the amount remaining in the dosage form). The results obtained are in accordance with the nature of the cement, as it is a non-erodible and non-swellable matrix.

In general, low dose antibiotic-impregnated bone cements release less than 10% of the dose in most cases. Previous papers showed that around 3% of gentamicin [31,34] and vancomycin [18] were released. These values are very similar to the ones of our study, 2.5–5.5%, depending on the conditions. Only antibiotic combinations or additives inclusion improve this elution and increase the percentage of dose released to 10% [8]. Some studies have shown that these amounts are sufficient to improve the deep infection rate [6].

The simulation study performed evidence that the concentrations of ciprofloxacin reachable in the implant would be higher than 0.1 μg/mL in 100% of patients (Fig. 5), decreasing the coverage when higher concentrations are need. In the first three days, *E. coli* is the microorganism that would be covered by all cement specimens, unlike *P. aeruginosa*, *S. aureus*, would depend on the type of cement and especially on microorganism sensitivity. After the third day post-surgery the clearance attributed to local drainage dramatically decreases and consequently it is expected that local bioactivity would increase.

According to the results, the bioactivity of ciprofloxacin in the first three days post-surgery would depend on the sensitivity of the microorganism, increasing substantially after drain removal, usually at 72 hours.

Despite the differences on elution among different brands and batches these would appear to lack clinical relevance, because the burst effect in the first moments and the decrease of external drainage in the third day post-surgery, would ensure bactericidal action of ciprofloxacin.

In conclusion, ciprofloxacin is suitable for incorporating into bone cements, as its release mechanism responds to Fickian diffusion principles, filtering through voids and cracks. Ultimately, hydrochloride ciprofloxacin has a better release profile than base ciprofloxacin. All the bone cement brands assayed behave similarly and vacuum mixing ensures lower variability and higher amounts of antibiotic released.

Limitations of the Study

While this study furthered our understanding of elution of antibiotics from PMMA, it has several limitations. First, the work was done completely in vitro, and to have a larger impact it would have to be reproduced in an animal model or clinical setting. Second, there are many types of PMMA currently available and this study tested just three. Third, our in vitro testing was done under static conditions that did not include fluid flow or other stresses to which the beads may be exposed to in vivo. Lastly, the bioactivity of samples has been evaluated through a simulation exercise taking into account that the surface of impregnated cement in contact with extracellular body fluid could be

Table 3
Parameter Values and Statistical AIC Obtained After Fitting the Different Kinetic Equations to Data.

Kinetic	Parameters	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F	Sample G	Sample H	Sample I	Sample J
Zero order	K0 (mg/h)	0.0012	0.0011	0.0015	0.0004	0.0006	0.002	0.0012	0.0014	0.0022	0.0019
	R	0.868	0.782	0.831	0.906	0.709	0.691	0.705	0.7230	0.680	0.682
	SS	1.555	2.010	3.167	0.122	1.123	12.897	4.152	4.873	15.966	11.7124
	AIC	11.51	15.86	22.44	−31.8	5.85	44.91	26.776	27.76	45.56	40.91
First order	K1 (h^{-1})	0.0417	0.1198	0.0996	0.161	0.2354	0.1738	0.1904	0.0432	0.1005	0.0923
	R	0.808	0.837	0.846	0.6811	0.887	0.912	0.904	0.948	0.926	0.934
	SS	2.19	1.547	2.913	0.366	0.483	4.153	1.501	1.052	4.245	2.810
	AIC	17.36	11.42	21.10	−13.10	−7.66	26.7801	10.497	4.77	25.686	19.50
Higuchi	k_h	0.0881	0.0929	0.1186	0.0306	0.0781	0.222	0.1315	0.1114	0.2135	0.1793
	SS	7.797	9.870	16.603	0.978	10.670	75.802	26.903	12.614	61.217	42.165
	AIC	36.914	40.92	46.95	1.63	39.88	71.25	54.68	40.02	63.72	58.12
Korsmeyer–Peppas	k_k	**0.3001**	**0.3472**	**0.3508**	**0.0795**	**0.2953**	**0.4433**	**0.4474**	**0.3198**	**0.4018**	**0.3861**
	N	**0.1704**	**0.154**	**0.1508**	**0.1659**	**0.104**	**0.1234**	**0.1207**	**0.1723**	**0.1403**	**0.1439**
	R	**0.993**	**0.982**	**0.986**	**0.998**	**0.950**	**0.944**	**0.948**	**0.951**	**0.934**	**0.933**
	SS	**0.091**	**0.186**	**0.289**	**0.003**	**0.221**	**2.685**	**0.842**	**0.991**	**3.776**	**2.828**
	AIC	**−36.78**	**−24.57**	**−15.86**	**−94.75**	**−20.16**	**19.80**	**1.25**	**3.86**	**23.92**	**19.59**

R = correlation coefficient; SS = sum of squares; AIC = Akaike information criterion and K_0, K_1, K_h and K_k are the ciprofloxacin elution rate constants for each kinetics.

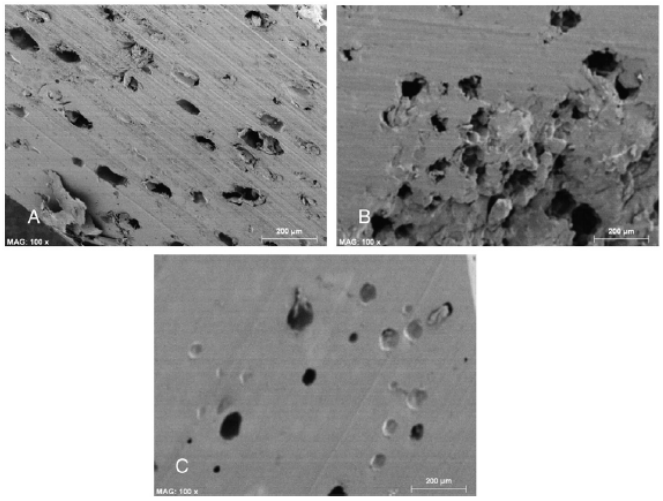

Fig. 4. Cement internal structure under SEM: A (Lima), B (Palacos) and C (Simplex).

equivalent to the surface of the samples assayed using clearance of synovial liquid values from literature and considering that the distribution of the antibiotic from the location of the implant to the systemic circulation is negligible. Consequently, the amount of antibiotic released to the medium could not be exactly the same. On the other side, one aspect that strengthens our results is that all antibiotic released would be kept in place, and consequently would reach high local concentration, in deep infection.

Acknowledgment

The authors thank Octavio Díez (Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Valencia, Spain; Instituto de Reconocimiento Molecular y Desarrollo Tecnológico, Centro Mixto Universidad Politécnica de Valencia-Universidad de Valencia, Spain) and José Luis Rodrigo (Servicio de Traumatología, Hospital Universitario Doctor Peset de Valencia, Spain).

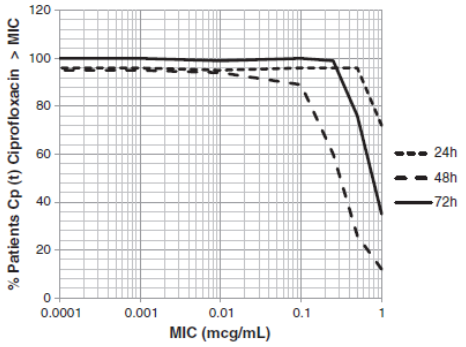

Fig. 5. Percentage of patients, predicted with the simulation exercise, for which antibiotic level at the site of the implant would be higher than MIC values in the range 0.0001–1 μg/mL.

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Evaluación de la bioactividad de ciprofloxacino y vancomicina incorporados en cementos óseos poliacrílicos.

ANALES DE LA REAL ACADEMIA NACIONAL DE FARMACIA
ISSN (Online) 1697-4298
Article
analesranf.com

Evaluation of ciprofloxacin and vancomycin bioactivity loaded in bone cements

Title in Spanish: *Evaluación de la bioactividad de ciprofloxacino y vancomicina incorporados en cementos óseos poliacrílicos*

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ABSTRACT: Antibiotic loaded bone cement used in prosthesis fixing, is a local release form that minimizes the prevalence and the complications that the antibiotics would unleash when administered intravenously. The aim of this work is to study in vitro release kinetics of ciprofloxacin and vancomycin loaded in different commercial cements and evaluate the bioactivity through a simulation exercise pharmacokinetics. Samples were prepared with commercial bone cement and ciprofloxacin and vancomycin hydrochloride (40:0,5:0,5). Release study were carried out under stirring in phosphate buffer, pH=7,4, for two months at 37ºC. The antibiotic amount and elution rate, were compared using ANOVA. In order to study the bioactivity, Monte Carlo simulation was performed. The ciprofloxacin released from samples for 8 weeks was 0.29±0.06 mg from the Palacos® cements, 0.44±0.06 mg from LimaCMT1® and 0.18±0.04 mg from Simplex®. The vancomycin released for 24 hours was 0.34±0.17 mg from Palacos® cement, 0.68±0.16 mg from LimaCMT1® and 0.17±0.02 mg from Simplex®. After this time the release stopped. The simulation study shows that during the first 72 hours, the antibiotic coverage would depends on the bone cement, the sensitivity of the microorganism and postoperative day. At subsequence times, it is expected that local bioactivity increases.

RESUMEN: La inclusión de antibióticos en el cemento óseo destinado a la fijación mecánica de las prótesis constituye un sistema de liberación local de antibiótico que permite minimizar la prevalencia y la gravedad de las reacciones adversas que pueden desencadenar los fármacos cuando éstos se administran por vía sistémica. El objetivo del trabajo es estudiar el mecanismo y cinética de liberación *in vitro* de ciprofloxacino y vancomicina incorporados en diferentes cementos óseos comerciales y evaluar la bioactividad mediante un ejercicio de simulación farmacocinética. Se prepararon mezclas de los cementos de estudio con ciprofloxacino clorhidrato y vancomicina (40:0,5:0,5). Los estudios de liberación se realizaron en agitación continua en solución salina de tampón fosfatos, pH=7,4, durante dos meses a 37ºC. El análisis estadístico de las cantidades de antibiótico liberadas acumuladas y las velocidades de elución se realizó mediante ANOVA. Con el fin estudiar la bioactividad, se realizó una simulación de Monte Carlo. La cantidad total liberada de ciprofloxacino en un periodo de 8 semanas fue de 0,29±0,06mg desde los cementos Palacos®, 0,44±0,06mg LimaCMT1® y 0,18±0,04mg Simplex®. La cantidad total de vancomicina liberada en 24 horas fue de 0,34±0,17mg desde el cemento Palacos®, 0,68±0,16mg LimaCMT1® y 0,17±0,02mg Simplex®. Transcurrido este tiempo la liberación cesó. El estudio de simulación, muestra que durante las primeras 72 horas, la cobertura antibiótica dependería tanto del cemento elegido como de la sensibilidad del microorganismo y el tiempo postquirúrgico. En tiempos posteriores, es de prever que la bioactividad local aumente.

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Received: Mars 12, 2015 Accepted: July 16, 2015
An Real Acad Farm Vol. 81, Nº 3 (2015), pp. 239-246
Language of Manuscript: Spanish

1. INTRODUCCIÓN

La artroplastia de cadera/rodilla consiste en la cirugía ortopédica que reemplaza de forma total o parcial la articulación por un implante artificial llamado prótesis en aquellos casos en los que el daño de la articulación es irreversible. Una de las complicaciones más grave se asocia al desarrollo de alguna infección, que aunque presente prevalencia entre el 0,5% y el 3%, en algunos casos puede ser de gravedad elevada y conduce al fracaso de la intervención, incluso en algunos casos puede desencadenar la muerte del paciente. Para prevenir la génesis de complicaciones asociadas al desarrollo de infecciones, se ha propuesto, desde hace algún tiempo, la inclusión de antibióticos en el cemento óseo destinado a la

fijación mecánica de las prótesis, ya que los sistemas de liberación local de antibiótico facilitan el aprovechamiento del fármaco, a la vez que reducen la prevalencia y gravedad de las reacciones adversas que pueden desencadenar los fármacos cuando éstos se administran por vía sistémica (1).

La combinación de antibióticos con los cementos poliacrílicos fue descrita por primera vez por Buchholz y Engelbrecht (2). Los numerosos y variados trabajos de investigación publicados en este contexto son contradictorios en cuanto a su capacidad de protección en la prevención de infecciones, debido a la incertidumbre existente sobre el posible desarrollo de resistencias a los antibióticos tras una exposición prolongada a bajas dosis de antibiótico, la eficacia y el coste de este sistema de vehiculización. A pesar de ello, la evidencia clínica indica que el uso de cementos cargados con antibióticos reduce significativamente el riesgo de infección (3); por ello, en la práctica clínica habitual se utilizan, aunque la cinética y el mecanismo de liberación de la mayoría de los antibióticos interpuestos en la matriz acrílica siguen siendo aspectos desconocidos. Las variables que influyen en el proceso de liberación del antibiótico desde el cemento son múltiples, entre ellas destacan cantidad y tipo de antibiótico incorporado al cemento (4, 5). En este sentido, resaltar que la velocidad de liberación del antibiótico desde el cemento (cantidad/tiempo) es mayor cuando se incorpora en forma líquida. Sin embargo, la utilización de formas líquidas en esta práctica clínica está limitada debido a su influencia negativa sobre las propiedades mecánicas de los cementos. Por el contrario, los fármacos en estado sólido tienen un efecto insignificante sobre la estabilidad mecánica de cemento óseo, siempre y cuando la proporción antibiótico/cemento se mantenga por debajo del 10%. La dosis de antibiótico a utilizar no queda totalmente establecida, varía según sea para el tratamiento o para la profilaxis; en el caso de perseguir el tratamiento terapéutico, se suele aconsejar adicionar 4 gramos de antibiótico a 40 gramos de cemento acrílico. Por el contrario, para conseguir un efecto profiláctico se recomienda utilizar dosis menores a 1 g de antibiótico por 40 g de cemento. Otro factor importante a tener en cuenta es el tipo y porosidad del cemento óseo y forma de preparación de la mezcla(6-9), ya que la porosidad del polímero facilita el acceso de los fluidos de disolución a la matriz del polímero y, en consecuencia, la liberación de los antibióticos a partir del cemento. Por otra parte, la porosidad está relacionada, en gran medida, con el mayor o menor volumen de aire atrapado durante la manipulación, mezclado y amasado de la muestra. De ahí que las cantidades de antibiótico liberadas desde el cemento puedan diferir según se empleen productos comerciales de cemento óseo impregnado de antibiótico premezclados o, por el contrario, se utilicen las preparaciones mezcladas de forma manual en el momento previo a la intervención quirúrgica.

Se han comercializado cementos poliacrílicos, de uso en artroplastias, cargados con antibióticos aminoglicósidos, en particular gentamicina y tobramicina, y con antibióticos glucopéptidos (10), que han demostrado su utilidad clínica en términos de eficacia y seguridad del tratamiento. Sin embargo, el problema de su uso es que el número de cepas multirresistentes (10, 11), con capacidad de adherirse sobre el cemento, colonizándolo tras largos periodos de implantación se ha incrementado recientemente. De hecho, en el momento actual el incremento de resistencias de *Staphylococcus aureus* hacia los aminoglucósidos condiciona la eficacia terapéutica de este grupo de antibióticos. Se trata de una realidad preocupante, ya que el 30% de las infecciones de origen quirúrgico son causadas por la cepa *Staphylococcus aureus resistente a meticilina* (SARM), lo que determina las estrategias para el tratamiento y prevención de las infecciones en las prótesis articulares (12).

En este contexto, se ha considerado oportuno estudiar la cinética de liberación de nuevos antibióticos incorporados a distintos cementos óseos comerciales y de esta forma obtener información relevante orientada a facilitar la selección del fármaco más adecuado en términos de eficacia y seguridad, ampliando así la disponibilidad de tratamientos utilizados en cirugía ortopédica.

El ciprofloxacino es una fluoroquinolona efectiva frente a microorganismos Gram-positivos y Gram-negativos. La vancomicina es un glicopéptido sumamente efectivo frente a bacterias Gram-positivas. Ambos antibióticos se presentan en estado sólido, son estables a la temperatura de fraguado de los cementos y no alteran las características mecánicas de éstos, por lo que incorporados en cementos poliacrílicos son candidatos para su utilización en cirugía ortopédica (13).

El objetivo del trabajo que se presenta es estudiar el mecanismo y cinética de liberación *in vitro* de ciprofloxacino y vancomicina incorporados en proporciones profilácticas en diferentes cementos óseos comerciales y evaluar la bioactividad potencial de las mezclas mediante estudios de simulación farmacocinética.

2. MATERIAL Y MÉTODOS

El ciprofloxacino clorhidrato y la vancomicina han sido suministrados por Guinama (Valencia, España). De acuerdo con las especificaciones del proveedor ambos antibióticos cumplían las especificaciones marcadas por la Farmacopea Europea. Los cementos poliacrílicos Palacos® y Simplex® fueron adquiridos en Ibersugical (Valencia, España) y LimaCMT1® en Lima Implantes (Barcelona, España).

Las cantidades de antibiótico incorporadas al cemento se seleccionaron de acuerdo con las recomendaciones realizadas por diferentes autores (14-16) con la finalidad de alcanzar un efecto antibiótico profiláctico (1 g de antibiótico por 40 g de cemento).

Se preparó un lote de los cementos acrílicos Palacos®, Simplex® y LimaCMT1® con ciprofloxacino clorhidrato y vancomicina (40:0,5:0,5) siguiendo las instrucciones proporcionadas por los fabricantes. Las mezclas obtenidas

se introdujeron en moldes de teflón siguiendo la normativa ISO 5833-Anexo E, y se dejaron endurecer durante 24 horas. Previamente al ensayo de liberación, cada muestra se caracterizó en cuanto a peso, diámetro y espesor.

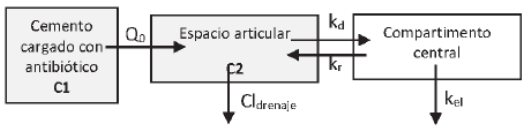
Los estudios de liberación del antibiótico se realizaron en un total de 9 muestras, 3 por cada cemento, manteniéndolas en condiciones de agitación continua en 10 mL de solución salina de tampón fosfatos, pH=7,4, durante 8 semanas en baño termostático a 37ºC. A intervalos de tiempo preestablecidos, la totalidad de la solución tampón fue recogida y reemplazada por 10mL de tampón fosfato salino pH=7,4. Este proceso permite garantizar las condiciones sumidero, es decir que la concentración del fármaco en el medio nunca supere el 20% de su hidrosolubilidad. Las muestras experimentales extraídas a cada tiempo de muestreo se guardaron en una cámara frigorífica a 5ºC hasta el momento de su cuantificación.

Para la determinación de ciprofloxacino se desarrolló y validó un método analítico por cromatografía líquida de alta resolución (HPLC) con detección UV (λ=254nm). Para ello, se utilizó como fase estacionaria una columna Kromasil® C18 (150x4,6 mm) y como fase móvil una mezcla acetonitrilo y ácido acético 0,1M en proporción volumétrica 20:80 (V/V). La exactitud y precisión del método analítico se evaluaron con la determinación del error relativo y el coeficiente de variación intra-muestra (inferior al 2,4% y al 1,5%, respectivamente, en el ámbito de concentraciones estudiadas). La determinación de vancomicina se realizó mediante inmunoanálisis de micropartículas quimioluminiscentes (ARCHITECT i1000SR) (17).

Las cantidades liberadas acumuladas, la velocidad de liberación y el logaritmo de la velocidad de liberación a partir de las muestras de ciprofloxacino combinado con vancomicina se evaluaron mediante la prueba estadística ANOVA.

Para evaluar la bioactividad de los antibióticos en las muestras estudiadas se realizaron simulaciones de Monte Carlo. Se simularon para 100 pacientes las concentraciones de antibiótico en la zona local del implante a las 24, 48 y 72 h utilizando el programa informático NONMEM versión VII. Para ello, se utilizaron los parámetros característicos del proceso de liberación de los antibióticos obtenidos en el estudio *in vitro* y los parámetros fisiológicos de volumen articular (1,6±1,1mL) (18) y aclaramiento local del fármaco, atribuido al drenaje de la herida a las 24, 48 y 72 h post-implante (20,42±11,3 mL/h; 9,33±11,02 mL/h y 4,11±2,95 mL/h respectivamente) (19).

El modelo farmacocinético aplicado para realizar la simulación de concentraciones de antimicrobiano en biofase (Figura 1) consta de dos compartimentos; cemento cargado con el antibiótico (C1) y espacio articular (C2). La liberación del antibiótico se realiza desde el compartimento C1 mediante una cinética de orden cero regida por la constante de velocidad Q_0 (mg/h). A su vez, el fármaco se elimina desde el compartimento C2 mediante una cinética de primer orden regida por la velocidad de drenaje de la herida (mL/h). Puesto que durante las 72 horas posteriores a la intervención el drenaje es muy elevado, se ha considerado despreciable la distribución (kd) y retorno del antibiótico (kr) desde el espacio articular (C2) a la circulación sistémica y viceversa. Por ello, para obtener las concentraciones simuladas en el lugar del implante (C2) sólo se han considerado los compartimentos sombreados de la Figura 1.



Q_0: velocidad de liberación del antibiótico desde el cemento (mg/h); $Cl_{drenaje}$: aclaramiento local del fármaco debido al drenaje de la herida (mL/h); k_d (h^{-1}): constante de distribución del antibiótico desde el lugar de implante al torrente circulatorio; k_r (h^{-1}): constante de retorno del antibiótico desde el torrente circulatorio al lugar de implante; kel (h^{-1}): constante de velocidad de eliminación del fármaco

Figura 1. Modelo farmacocinético utilizado para calcular las concentraciones de antimicrobiano en el lugar de implante de la prótesis.

La evaluación de la bioactividad de las mezclas a los tiempos seleccionados se ha realizado utilizando los valores de concentración mínima inhibitoria (CMI) de *S. aureus Meticilin resistente* (CMI= 0,5-2 mcg/mL)(20) y *S. Coagulasa Negativos –SCN-* (CMI= 0,25-1 mcg/mL) (20), gérmenes sobre los que la vancomicina muestra actividad, y de *S. aureus* (CMI= 0,12-0.5 mcg/mL), *Pseudomonas aeruginosa* (CMI= 0,25-1mcg/mL) y *E. Coli* (CMI= 0,004-0,016 mcg/mL) (21), gérmenes sensibles al ciprofloxacino. Estos microorganismos han demostrado ser los responsables del 70% de las infecciones articulares desarrolladas en nuestro entorno (22). Se calculó para cada tiempo (24, 48 y 72 h) el porcentaje de pacientes cuya concentración de antibiótico en el lugar del implante (C2) sería superior a la CMI seleccionada.

3. RESULTADOS

En la Tabla 1 se muestran las cantidades de ciprofloxacino clorhidrato liberadas acumuladas durante 2 meses y las cantidades de vancomicina liberadas acumuladas hasta las 72 horas. Las cantidades de ciprofloxacino y de vancomicina liberadas desde las mezclas de los antibióticos y el cemento LimaCMT1® fueron superiores a las cantidades de antibióticos liberadas desde el resto de mezclas estudiadas ($p<0,05$).

Tabla 1. Media y desviación estándar de la cantidad de ciprofloxacino y vancomicina (mg) liberados a los tiempos indicados desde los diferentes cementos poliacrílicos ensayados.

	Ciprofloxacino HCl						Vancomicina					
	Simplex®		LimaCMT1®		Palacos®		Simplex®		LimaCMT1®		Palacos®	
Tiempo (h)	MEDIA (mg)	SD	MEDIA (mg)	SD	MEDIA (mg)	SD	MEDIA (mg)	SD	MEDIA (mg)	SD	MEDIA (mg)	SD
24	0.134	0.018	0.318	0.032	0.209	0.045	0.174	0.021	0.659	0.141	0.339	0.166
48	0.144	0.019	0.351	0.044	0.229	0.047	-	-	0.677	0.161	-	-
72	0.144	0.022	0.367	0.050	0.235	0.047	-	-	0.681	0.162	-	-
1344	0.177	0.043	0.442	0.061	0.289	0.052	-	-	-	-	-	-

En la Figura 2 se representa la evolución de la velocidad de liberación de los antibióticos a partir de los cementos estudiados durante el desarrollo del ensayo. Los ensayos realizados con el ciprofloxacino muestran dos etapas; en la primera (primeras 48 h) la velocidad de liberación es rápida, y en la segunda la velocidad de liberación del antibiótico disminuye hasta que se mantiene en un valor constante. Por el contrario, los resultados obtenidos con vancomicina únicamente muestran una etapa, ya que durante las primeras 48 h del ensayo el antibiótico se libera rápidamente, pero en tiempos posteriores la velocidad de liberación del antibiótico cesa. La comparación estadística del logaritmo de la velocidad de liberación de ciprofloxacino, obtenida para cada uno de los cementos estudiados, puso de manifiesto la existencia de diferencias estadísticamente significativas en la primera etapa del estudio (primeras 48 h) a favor de LimaCMT1®, no existiendo diferencias estadísticamente significativas en tiempos posteriores a las 48 h del ensayo en los tres cementos óseos estudiados.

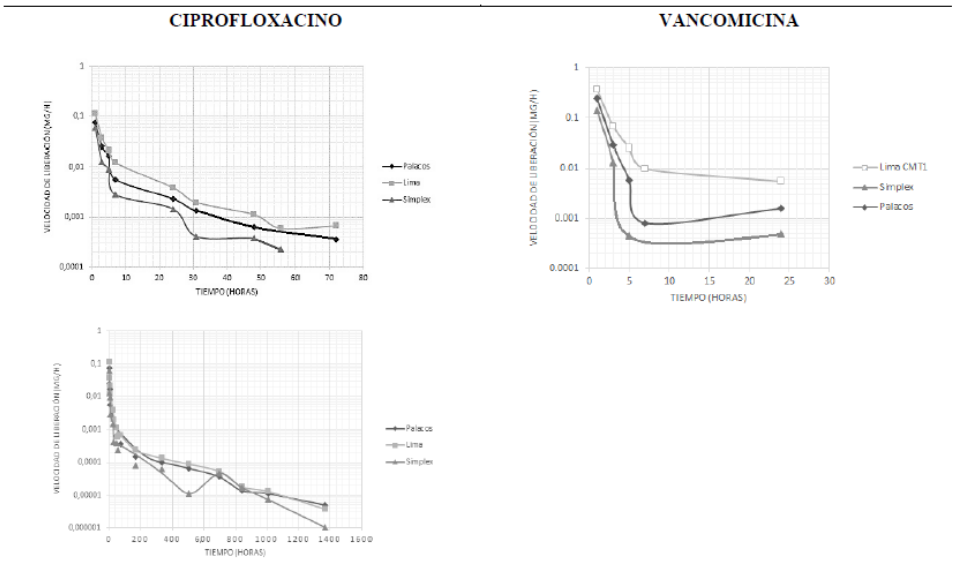


Figura 2. Velocidades de elución de ciprofloxacino clorhidrato y vancomicina desde los cementos poliacrílicos estudiados.

Mediante los ejercicios de simulación de Monte Carlo se ha obtenido una población simulada de 100 pacientes de una edad media de 69,17±14,28 años y de un peso medio de 74,18±14,47 kg. Las concentraciones simuladas de ciprofloxacino y vancomicina en el lugar del implante (apartado de material y método) indican que transcurridas 24 horas de la intervención, en más del 90% de los pacientes serían superiores a 0,1 y 0,2 mcg/mL, de ciprofloxacino y vancomicina respectivamente (Figura 3).

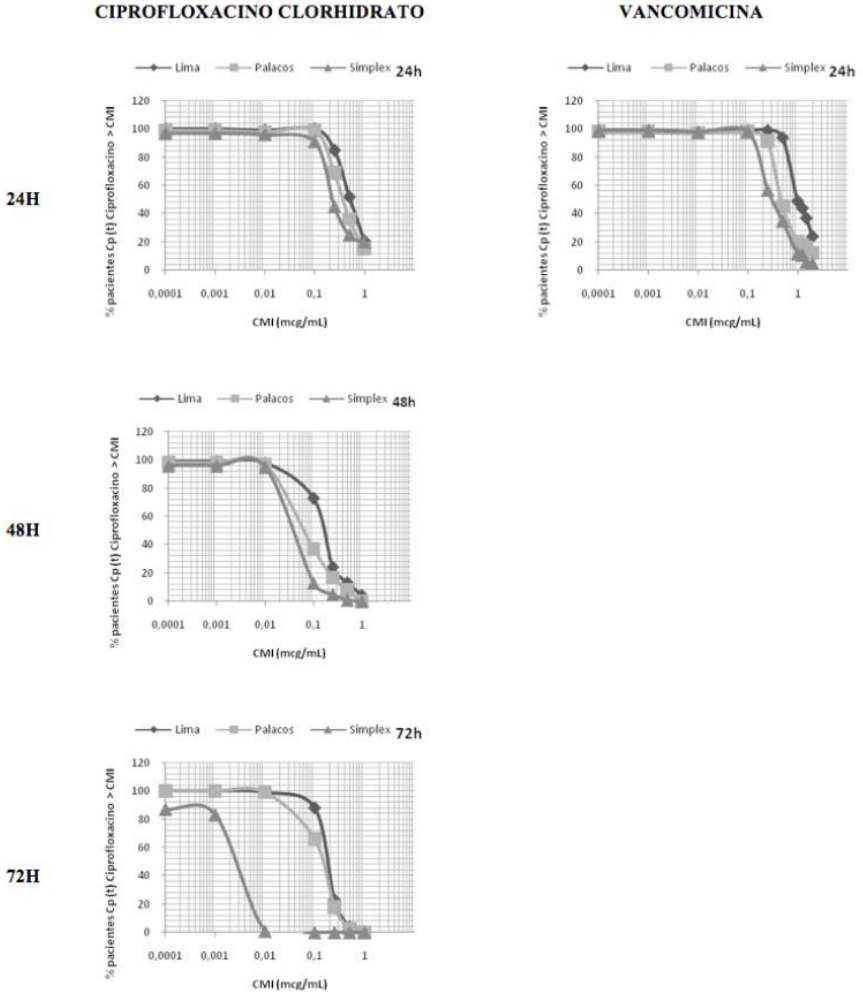


Figura 3. Evaluación de la bioactividad de las muestras estudiadas. En el eje de ordenadas se representa el porcentaje de pacientes cuya concentración de antibiótico en el lugar del implante, transcurridas 24, 48 y 72 h de la intervención para el ciprofloxacino y transcurridas 24 h de la intervención para la vancomicina, sería igual o superior a la concentración mínima inhibitoria (CMI) indicada en el eje de abscisas.

4. DISCUSIÓN

La inclusión de antibióticos directamente en cementos poliacrílicos usados para la fijación de prótesis óseas

representa un sistema de liberación modificada de fármacos. Estos sistemas facilitan el acceso del antibiótico a la biofase y por ello aportan principalmente dos ventajas: el mayor aprovechamiento del fármaco en el lugar de acción y una disminución de reacciones adversas. La liberación modificada de fármacos a partir de sistemas matriciales exhibe un patrón de comportamiento común compuesto por dos etapas; una, de liberación inicial rápida, que por lo general ocurre durante las primeras 24-48 horas, y otra de liberación lenta, en tiempos posteriores a las 48 horas, en la que el fármaco se libera a una velocidad más lenta. La liberación inicial está fundamentalmente determinada por la cantidad de fármaco que queda adsorbido en la superficie de la matriz y la difusión por los poros y canales que componen su estructura interna, los cuales se llenan con el medio de incubación durante las primeras horas de ensayo. En la fase posterior, de liberación lenta, en la que la velocidad de liberación y la cantidad de fármaco liberada es inferior, contribuyen los procesos de difusión a través de los poros y canales del sistema matricial, los cuales se forman como producto del proceso de fabricación o por la modificación de la estructura matricial, como consecuencia de la disolución de componentes hidrosolubles de su composición.

La fase inicial, de velocidad de liberación elevada, es de menor duración para la vancomicina y presenta una velocidad más elevada que la obtenida para el ciprofloxacino. Este fenómeno se puede atribuir a la mayor solubilidad acuosa de la vancomicina (100 mg/mL frente a 30 mg/L del ciprofloxacino). Este hecho indica que la disolución de las partículas de vancomicina situadas sobre la superficie se realiza con mayor velocidad facilitándose la liberación y posterior disolución de las partículas próximas a la superficie en un periodo de tiempo breve.

Las cantidades de ciprofloxacino liberadas a partir de las mezclas evaluadas en este estudio son superiores a las cantidades de antibiótico liberadas en un estudio previo realizado en nuestro grupo de investigación en el que se evaluó la liberación del ciprofloxacino a partir de mezclas simples, constituidas por el antibiótico y los diferentes cementos óseos (225% superior en las mezclas combinadas con el cemento LimaCMT1®, un 183% con el cemento Palacos® y un 126% con el cemento Simplex®)(23). Estas diferencias permiten corroborar que el hecho de incorporar más de un fármaco a los cementos óseos potencia en gran medida la velocidad de liberación de ambos. En este caso la vancomicina incorporada en la mezcla, favorece la formación de un mayor número de poros y canales que facilitan la entrada de agua en la matriz y la posterior disolución del ciprofloxacino desde su interior.

Las cantidades de antibiótico liberadas así como la evolución temporal de la velocidad de liberación es mayor para las mezclas elaboradas con el cemento LimaCMT1® (Figuras 2 y 3). Estos resultados son compatibles con la menor porosidad del cemento Simplex® indicada por Stryker®, laboratorio fabricante del producto (24).

Las cantidades de antibiótico liberadas desde los cementos estudiados en combinación con los valores del aclaramiento local del fármaco, atribuido mayoritariamente al drenaje de la herida, y los valores del volumen de líquido en el espacio articular permitieron abordar el estudio de bioactividad simulada. Como se observa en la Figura 3, a las 24 horas post intervención quirúrgica las concentraciones de ciprofloxacino predichas en el lugar del implante son superiores a 0,1 mcg/mL con los tres cementos estudiados y las concentraciones de vancomicina superiores a 0,5, 0,3 y 0,1 mcg/mL en el caso de LimaCMT1®, Palacos® y Simplex®, respectivamente, en el total de la población simulada. Estos resultados indican que es de prever que la cobertura local con los antibióticos estudiados sea eficaz para patógenos sensibles a concentraciones inferiores a las indicadas. Transcurridas 48 h de la intervención, en el total de la población simulada, la concentración de ciprofloxacino en el lugar del implante se reduciría una décima parte; a las 72 h post-implante la concentración en el lugar del implante incrementaría y alcanzaría un valor equivalente al obtenido a las 24 h excepto en el caso del cemento Simplex®. En el caso de la vancomicina, a partir de las 24 horas posteriores a la intervención quirúrgica, la liberación del antibiótico desde los cementos Palacos® y Simplex® es nula, y a efectos prácticos también despreciable desde el cemento LimaCMT1®.

Las oscilaciones de concentración de ciprofloxacino durante los primeros días de la intervención quirúrgica, están relacionadas con la fluctuación y la variabilidad del drenaje de la herida quirúrgica producida durante las 72 horas posteriores a la intervención, ya que en este corto periodo de tiempo el drenaje local se reduce hasta alcanzar valores que representan entorno el 25% del valor inicial (20,42±11,3 mL/h; 9,33±11,02 mL/h y 4,11±2,95 mL/h respectivamente) (19). En general, el drenaje externo de la herida se retira a partir del tercer día de la intervención quirúrgica. A partir de este momento, la evolución temporal de la concentración de antibiótico en el lugar del implante estará condicionada por el proceso de distribución y retorno del fármaco a la circulación sistémica. Por ello, es de prever que a partir del tercer día post-intervención quirúrgica la concentración de fármaco en el lugar del implante de la prótesis alcance valores más elevados, aumentando si cabe la cobertura antibiótica obtenida durante los primeros días.

En resumen, el estudio realizado pone de manifiesto que la bioactividad de las muestras es dependiente del cemento acrílico, el día postquirúrgico, el microorganismo causante y su sensibilidad. Si se analiza la prevalencia de infecciones diagnosticadas en nuestro entorno se observa que *S. aureus* es el principal agente causante de las infecciones protésicas, ya que se ha aislado en un 30% del total de infecciones, mientras que otros microorganismos, entre ellos *S. Coagulasa Negativos y Pseudomonas aeruginos y E. Coli* se han aislado en un porcentaje inferior, 14% los dos primeros y 12% el último (22). Teniendo en cuenta esta situación y considerando que el ámbito de valores de la CMI de ciprofloxacino para *S.*

aureus Meticilin resistente está comprendido entre 0,5 y 2 mcg/mL únicamente se alcanzarían concentraciones efectivas en el lugar de acción para cepas sensibles al ciprofloxacino a concentraciones inferiores a 1 mcg/mL. La bioactividad de vancomicina durante las 24 horas posteriores a la intervención quirúrgica es superior a la de ciprofloxacino. Sin embargo, puede ser nula para tiempos posteriores ya que la cantidad de vancomicina retenida en el interior de la matriz no se libera al medio. Durante las primeras 72 horas posteriores a la intervención quirúrgica la velocidad de liberación del ciprofloxacino incorporado al cemento LimaCMT1® es superior, lo que indica que éste reúne una capacidad cinética ligeramente superior a los otros cementos ensayados. En tiempos posteriores la bioactividad del ciprofloxacino incorporado en los tres cementos estudiados es similar.

5. CONCLUSIONES

Los cementos poliacrílicos cargados con ciprofloxacino y vancomicina son sistemas de liberación modificada que asegurarían durante las primeras 72 h bioactividad frente algunos microorganismos, pero no garantizan una cobertura completa para todos los posibles agentes causantes. Ante una situación de mayor riesgo sería conveniente seleccionar el cemento LimaCMT1®, por las propiedades cinéticas ligeramente favorables en relación a los otros cementos estudiados, así como utilizar la combinación de antibióticos que facilite la velocidad de liberación del antibiótico desde la matriz. Por último, es deseable seguir investigando y profundizando en estos estudios con la finalidad de disponer de información que ayude a optimizar la incorporación de antibióticos a los cementos óseos para facilitar la selección de mezclas que aseguren una bioactividad elevada, fundamentalmente, durante los primeros tres días post implante de la prótesis articular.

6. CONFLICTO DE INTERESES

Los autores declaran no tener conflicto de intereses

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Bioactivity of Ceftazidime and Fluconazole Included in Polymethyl Methacrylate Bone Cement for Use in Arthroplasty

The Journal of Arthroplasty xxx (2017) 1–8

Contents lists available at ScienceDirect

The Journal of Arthroplasty

journal homepage: www.arthroplastyjournal.org

Bioactivity of Ceftazidime and Fluconazole Included in Polymethyl Methacrylate Bone Cement for Use in Arthroplasty

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ARTICLE INFO

Article history:
Received 1 September 2016
Received in revised form
11 April 2017
Accepted 25 April 2017
Available online xxx

Keywords:
bone cement
arthroplasty
ceftazidime
fluconazole
elution kinetics
bioactivity

ABSTRACT

Background: The microorganisms that most frequently cause prosthetic joint infection are methicillin-resistant *Staphylococcus aureus* and gram-negative aerobic bacillus. Studies have documented the efficacy of mixing antibiotics with polymethyl methacrylate, but that of antifungal drugs has not received much attention. The objective of this in vitro study was to characterize the elution profile and bioactivity of ceftazidime and fluconazole when incorporated into bone cement in proportions intended for prophylaxis and treatment of bone infections.

Methods: Antibiotic-loaded bone cement cylinders in a proportion of 1:40 and 4:40 (ratio of grams of antibiotic to grams of cement) were assayed. Drug delivery was investigated in a flow-through dissolution apparatus (SotaxCE7). To assess bioactivity, antibiotic concentrations were simulated in the joint space of 1000 patients. Antibacterial properties were evaluated by counting colony forming units and the inhibition-halo test.

Results: The ratio of released ceftazidime and fluconazole was 453% and 648%, respectively, higher when used for treatment proportions than prophylaxis proportions. A bioactivity simulation exercise showed that the efficacy of ceftazidime/fluconazole determined as the amount of drug is released at the active site in the first 3 days after surgery would depend on the sensitivity of the microorganism and would increase substantially after drain removal. The microbiology study showed that biofilm formation by *Pseudomonas aeruginosa* could be a problem when ceftazidime was used in treatment or prophylaxis proportions.

Conclusion: Our in vitro findings suggest that ceftazidime and fluconazole can be added into polymethyl methacrylate for the prevention/treatment of infections associated to joint surgery. Their efficacy depends on the sensitivity of the microorganism causing the infection.

One of the most serious complications in arthroplasty is the development of infections (prevalence between 1% and 2.5%) [1], which lead to death in some cases. In case of bone infection, high doses of antibiotics are required to achieve effective concentrations at the implantation site. However, high doses of intravenous or oral antibiotics can cause toxicity. Antibiotic-loaded acrylic bone cement, described by Buchholz and Engelbrecht [2], is a well-established tool used in prophylaxis [3,4] and treatment of orthopedic infections [5] in humans and animals [6]. Polymethyl methacrylate (PMMA) is characterized by excellent biocompatibility, with low intrinsic toxicity and inflammatory activation [7], but experience has shown that not all antibiotics have the appropriate properties to be incorporated into this cement. Aminoglycosides and glycopeptides are the 2 groups of antibiotics that satisfy the optimal criteria for inclusion in this cement,

This research did not receive any specific grant from any funding agency in the public, commercial, or non-profit sectors.

No author associated with this paper has disclosed any potential or pertinent conflicts which may be perceived to have impending conflict with this work. For full disclosure statements refer to http://dx.doi.org/10.1016/j.arth.2017.04.057.

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http://dx.doi.org/10.1016/j.arth.2017.04.057
0883-5403/

namely, availability in powder form, wide antibacterial spectrum, bactericidity at low concentrations, elution from PMMA in high concentrations for prolonged periods, thermal stability, low or no risk of allergy or delayed hypersensitivity, little influence on the mechanical properties of the cement, and low serum protein binding [8]. In fact, some antibiotics, such as gentamicin and vancomycin, are available premixed within bone cement, ready for use [8].

Nevertheless, the increased prevalence of multidrug-resistant bacteria [9–12] limits the continued efficacy of the aforementioned mixtures. It has been established that the microorganisms that most frequently cause prosthetic joint infection are methicillin-resistant *Staphylococcus aureus* [13] and gram-negative aerobic bacillus [14]. Around 80% of fungal infections of bone prostheses are produced by *Candida* spp. Although some studies have documented the efficacy of mixing antibiotics with PMMA, the efficacy of antifungal drugs mixed with PMMA has received little attention [15].

In this study, we set out to characterize the elution profile of ceftazidime and fluconazole in bone cement. Cephalosporins are the most commonly used antibiotics to treat osteoarticular infections because of their broad spectrum of activity; among other actions, they disrupt the synthesis of the peptidoglycan layer of the bacterial cell wall. Ceftazidime is a third-generation cephalosporin used to treat infections produced by gram-positive and gram-negative bacteria and acts against *Pseudomonas*. Fluconazole is an antifungal agent of the triazole group used to treat infections produced by *Candida albicans*. Fluconazole inhibits the cytochrome P450 enzyme 14α-demethylase, which prevents the formation of ergosterol, thus increasing membrane permeability and cell destruction.

Prophylaxis and treatment proportions (1:40 and 4:40, respectively, ratio of grams of drug to grams of cement) were assayed. Different equations were applied to the profiles of release to explain the delivery mechanism. Finally, the bioactivity of the mixtures was evaluated by means of 2 methods, a simulation exercise and a microbiology analysis.

Materials and Methods

Materials

Fluconazole (Diflucan) and ceftazidime (Fortam) were purchased from Vinci Farma, SA and GlaxoSmithKline, SA, respectively, and Palacos bone cement was purchased from Ibersugical (Valencia, Spain). The bone cement was provided as 2 separate components: powder mixture and liquid. The composition of the bone cement is shown in Table 1, according to the information provided by the manufacturer.

A buffered saline solution pH 7.4 was prepared as described in the US Pharmacopoeia: disodium hydrogen phosphate anhydrous 2.38 g, monopotassium hydrogen phosphate anhydrous 0.19 g, and sodium chloride 8 g per 1 L of purified water. All reagents were analytical grade (Panreac, Barcelona, Spain).

Methanol gradient grade for liquid chromatography, Mueller-Hinton agar plates, and standard bacterial broth were purchased from Sigma-Aldrich (Madrid, Spain). The plates and broth were prepared according to the manufacturer's instructions.

Table 1
Composition of the Acrylic Bone Cement, as Provided by the Manufacturer.

	Solid Component (40 g)	Liquid Component (20 mL)	Viscosity
Palacos	Poly(methylacrylate–methyl methacrylate), 33.8 g Zirconium dioxide, 6.0 g Benzoyl peroxide, 0.2 g Colorant E141, 0.008 g	Methyl methacrylate, 18.4 g *N,N*-dimethyl-*p*-toluidine, 0.4 g Hydroquinone, Colorant E141, 0.005 g	High

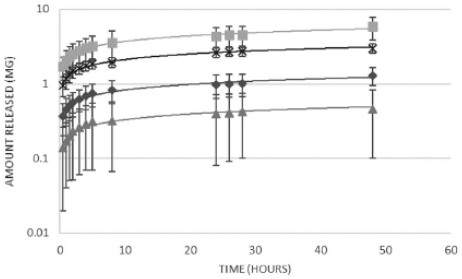

Fig. 1. Amount released vs time profile of antibiotic loaded into bone cement. Symbols represent the mean observed values with their corresponding standard deviation (SD): (◆) ceftazidime 1:40, (■) ceftazidime 4:40, (▲) fluconazole 1:40, and (×) fluconazole 4:40.

Methods

Six batches per antibiotic (3 for each proportion assayed, 1:40 and 4:40, ratio of grams of antibiotic to grams of cement) with 5 samples per batch were prepared. A total of 60 antibiotic-loaded bone cement cylinders were prepared as follows: 1 or 4 g of the drug, to reproduce the ratio of antibiotic-to-cement used in prophylaxis or treatment in clinical practice, respectively, was added to 40 g of the powder component of the cement, and after mixing the powder, the liquid component was added following the manufacturer's instructions. Cylinders of antibiotic bone cement were made for each batch in a standard fashion according to the International Organization for Standardization normative 5833 (Annex E): samples were poured into Teflon moulds (created by US for elution test) in which they were kept for 1 hour until completely hardened into a cylinder/disk shape. Each specimen was carefully weighed and measured and the theoretical amount of loaded antibiotic calculated.

Drug Release Assays

Samples were introduced into flow-through dissolution equipment (Sotax CE7; Teknokroma, Barcelona, Spain) with 100 mL of phosphate buffer saline (pH 7.4) at 37°C recirculating at a rate of 12 mL/min for 48 hours. Samples were taken 0.25, 0.5, 1, 1.5, 2, 3, 4, 5,

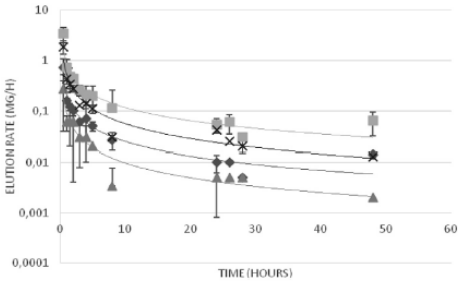

Fig. 2. Elution rate vs time profile of antibiotic from bone cement. Symbols represent the mean observed values with their corresponding SD: (◆) ceftazidime 1:40, (■) ceftazidime 4:40, (▲) fluconazole 1:40, and (×) fluconazole 4:40.

Table 2
Parameter Values and Statistical AIC Obtained After Fitting the Different Kinetic Equations.

Antibiotic Elution From Bone Cement					
Kinetic	Parameters	Ceftazidime		Fluconazole	
		1/40	4/40	1/40	4/40
Zero order	k (RSE%)	0.217 (46)	0.25 (76)	0.25 (—)	0.268 (146)
	AIC	150.337	234.925	87.446	122.856
Higuchi	k (RSE%)	0.3 (34)	0.293 (54)	0.29 (228)	0.306 (93)
	AIC	−326.798	−335.355	−336.97	−280.941
Korsmeyer-Peppas	k (RSE%)	**0.402 (29)**	**0.384 (46)**	**0.372 (48)**	**0.396 (28)**
	n (RSE%)	**0.244 (32)**	**0.254 (145)**	**0.251 (47)**	**0.242 (95)**
	AIC	**−717.731**	**−783.737**	**−596.022**	**−594.455**

Clearance From Redon		
Kinetics	Parameters	Values (%)
Zero order	k_0 (RSE%)	$\mathbf{2.00 \cdot 10^{-4}}$ **(27.4)**
	Cl_0 (RSE%)	$\mathbf{2.77 \cdot 10^{-2}}$ **(7.1)**
	AIC	**−2963.25**
First order	k_1 (RSE%)	$\mathbf{1.30 \cdot 10^{-2}}$ (—)
	Cl_0 (RSE%)	$\mathbf{3.01 \cdot 10^{-2}}$ (—)
	AIC	**−2958.81**

Values in bold indicate the selected kinetics.
AIC, Akaike Information Criterion; *k*, the antibiotic elution rate constants for each kinetic; RSE, relative standard error intersample.

24, 26, 28, and 48 hours after immersion. These sampling times were selected because it was expected that the release of antibiotic would be constant for the first 24 hours. Antibiotic homogeneity distribution within batches was indirectly evaluated by statistical analysis of the percentage of the total antibiotic released from the assayed samples. Of the phosphate buffer, 100 mL ensures that the sink condition is satisfied (defined as at least 3 times the volume of medium required to produce a saturated solution of drug substance). All samples were frozen at −20°C until analysis.

Fluconazole and ceftazidime concentrations were assayed by high-performance liquid chromatography using a PerkinElmer Series 200 UV detector (λ = 268 and 245 nm, respectively); the mobile phase consisted of methanol:water (60:40 and 70:30 v/v, respectively) and was filtered through a 0.45-μm membrane filter before use. The mobile phase was eluted at a flow rate of 1 mL/min in both cases. The column was a Kromasil C-18 with a pore size of 5.0 μm, measuring 150 mm (length) × 4.6 mm (diameter) [16,17].

Kinetic Analysis and Biosimulation

The elution rate at each time interval (mg/h) was obtained by dividing the total quantity of antibiotic released in each interval by the elution time (in hours).

The kinetic analysis of the release of antibiotic was performed sequentially. First, various kinetic models were fitted to the cumulative amount of antibiotic released for each proportion. In a second step, kinetic parameters of the model selected and physiological parameters (volumen from redon for 72 hours from surgery obtained from a group 156 patients) were used for the biosimulation (see Appendix for details about both procedures).

Microbiology Analysis

Antibacterial properties of ceftazidime for *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Escherichia coli* and fluconazole for *C. albicans* were evaluated by means of a colony-forming unit (CFU) count [18] and inhibition halo test [19].

CFU count was performed by dipping both, samples and commercial cements alone (as a control), in a standard bacterial broth containing approximately 150 × 10^6 CFU/mL and incubating them for 24 hours at 37°C. To quantify the bacteria proliferated in the culture broth, the broth was serially diluted, spread on an agar plate, and incubated overnight at 37°C to allow the growth and counting of CFU.

For the inhibition halo test, samples were placed in contact with an agar plate (Mueller-Hinton agar) uniformly covered with bacterial broth (previously prepared following a standard procedure) and incubated overnight at 37°C, as described in the study by Vitale-Brovarone et al [20]. Afterward, the inhibition zone was observed and measured. For all bone cements, the inhibition halo was repeated up to 3 days: at the end of the first incubation, the samples were removed and placed in a new agar plate containing fresh bacterial inoculum.

All antibacterial tests were performed in duplicate.

Statistical Analysis

Elution rates and the total amount of antibiotic released (expressed as a fraction of loaded amount) at each time point were compared using 1-way analysis of variance. The inhibition halo test data were compared using 1-way analysis of variance. In both cases, statistical significance was set at $P < .05$.

Results

Release Characterization and Statistical Analysis

Figure 1 shows the amount of each drug released from Palacos bone cement loaded in the proportions studied. The amount of fluconazole released at 48 hours was approximately half of that of ceftazidime. The amount of ceftazidime released from treatment to prophylaxis ratio systems was 453% higher, whereas fluconazole release increased 648% when increasing drug into cement.

Figure 2 shows the elution rate of ceftazidime and fluconazole in milligrams per hour at different time intervals, plotted on a logarithmic scale. All samples produced high early-release rates followed by a lower sustained release.

Comparison of the amounts released and release rate between the different antibiotics and proportions showed statistically significant differences.

Drug Release Models

Table 2 shows parameter values and statistical Akaike Information Criterion obtained after fitting the different kinetic equations to data. Korsmeyer-Peppas model was selected among the models assayed to characterize the elution of antibiotic from bone cement. The model predictions were able to describe the experimental data release profiles for all formulations, as represented in Figure 3.

Exponent *n* in the Korsmeyer-Peppas equation had values between 0.242 and 0.254 in all the samples. These values, under 0.45, represent that the drug-release mechanism is a Fickian diffusion (the amount of antibiotic released is proportional to the amount remaining in the system).

Bioactivity Models

The model proposed to fit the data, outlined in Figure 4, had been previously applied to describe the bioactivity of ciprofloxacin loaded into bone cement [21].

Figure 5 shows the simulated concentrations of antibiotics in the implantation site after surgery. For the simulation exercise, drug delivery from cement was calculated according to the Korsmeyer-Peppas model, and clearance of redon was described with a zero-order kinetics (Table 2).

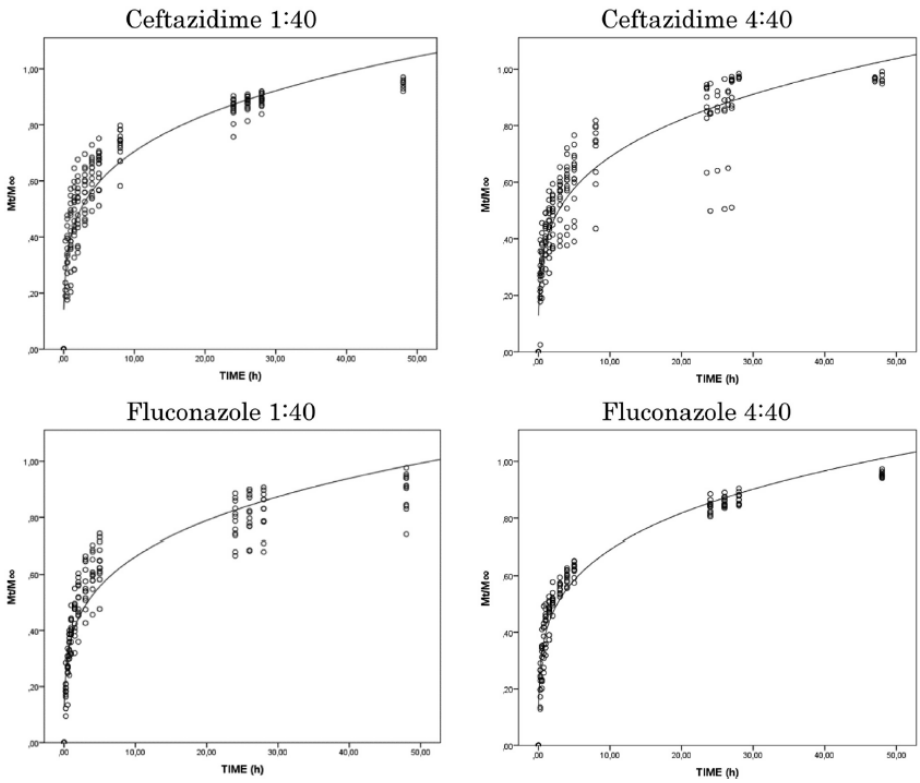

Fig. 3. Cumulative fraction of antibiotic released vs time profiles after fitting data to the Korsmeyer-Peppas equation.

As can be seen in all the figures, the concentration decreased progressively until 72 hours, at which point the redon was removed. At this point, concentrations above minimum inhibitory concentration (MIC) of *E. coli, P. aeruginosa, S. epidermidis*, and *S. aureus* would be detected in 28%, 8%, 3%, and 1% of patients, respectively, in the case of ceftazidime 1:40 and in 97%, 92%, 52%, and 1% of patients, respectively, in the case of ceftazidime 4:40. Concerning fluconazole, 53% and 99% of patients would have concentrations higher than MIC of *C. albicans* when 1:40 and 4:40 proportions of fluconazole would be used, respectively.

Microbiology Analysis

To investigate the antimicrobial effect of the antibiotic loaded into the bone cement, a CFU count was carried out. This is a quantitative test that allows the quantification of proliferated bacterial colonies. In neither case growth was observed, except for *P. aeruginosa* and *S. epidermidis* (a CFU count of 1,000,000 and 100,000 of *P. aeruginosa* was registered when 1:40 and 4:40 proportions of ceftazidime were used, respectively, whereas a CFU of 100,000 and 10,000 of *S. epidermidis* was registered when

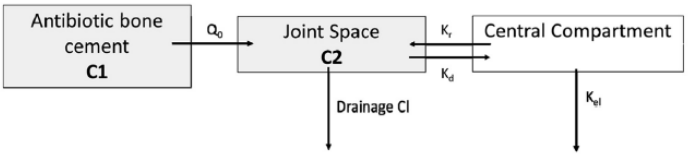

Fig. 4. Q_0: Antibiotic release from the cement (mg/h); drainage Cl: local drug clearance owing to wound drainage (mL/h); Kd (h^{-1}) antibiotic distribution rate constant from the joint space to the central compartment; Kr (h^{-1}): antibiotic return from the central compartment to joint space rate constant; and Kel (h^{-1}): antibiotic elimination rate constant.

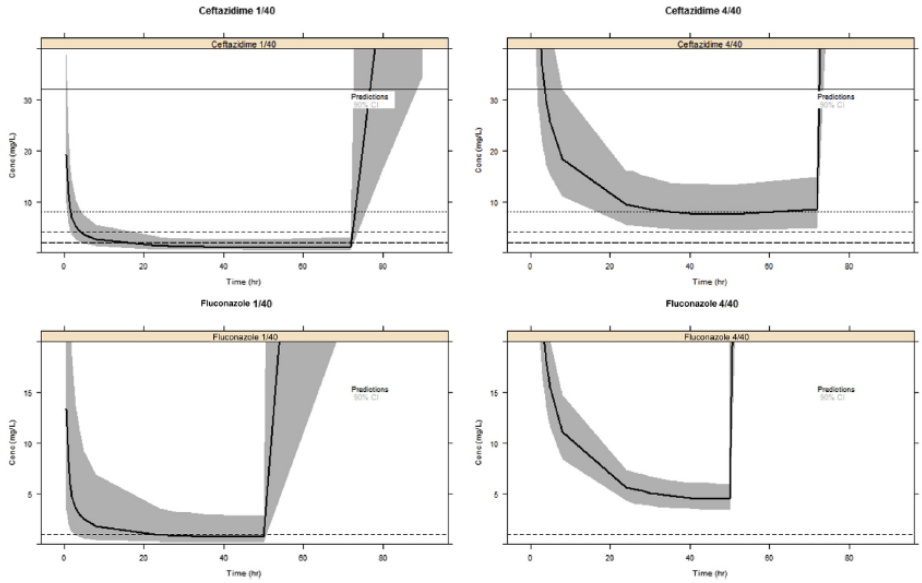

Fig. 5. Concentration of antibiotic simulated in joint space. Minimum inhibitory concentration (MIC) *Staphylococcus aureus* = 32 mg/L, MIC *Staphylococcus epidermidis* = 8 mg/L, MIC *Escherichia coli* = 2 mg/L, MIC *Pseudomonas aeruginosa* = 4 mg/L, and MIC *Candida albicans* = 1 mg/L.

ceftazidime proportions of 1:40 and 4:40, respectively, were used).

To investigate further the antibacterial effect of the antibiotic loaded into bone cement, the inhibition halo test was also performed. Table 3 and Figure 6 show the inhibition halo produced by each antibiotic and proportion, for each of the microorganisms. In all cases, the microorganisms were sensitive to the antibiotic, except in the case of *S. aureus* to ceftazidime 1:40 at 72 hours and *S. epidermidis* to ceftazidime 1:40 at all time points. The inhibition halos for the proportions of 1:40 and 4:40 were statistically different for all microorganisms except for *P. aeruginosa*.

Discussion

The use of bone cement containing antibiotics is based on the principle that the antibiotic will be released gradually from the cement over time and will therefore prevent bacterial proliferation. To eradicate infection in the bone and joints, it is essential to maintain a therapeutic concentration of antibiotic at the implantation site for an extended period. Currently, there are ready-to-use bone cements containing antibiotics on the market, but only some antibiotics are available, such as gentamicin and vancomycin. The increasing number of multidrug-resistant bacteria and fungal infections limits the future efficacy of this tool and calls for the need to incorporate alternative agents into PMMA cement [9,12]. Ceftazidime and fluconazole are known to exert a potent effect against *P. aeruginosa* and *C. albicans*, respectively.

As shown in Figures 1 and 2, the release profiles of antibiotics loaded into bone cement is biphasic. The release of antibiotic from the samples assayed can be explained by the theory by van de Belt et al [22], as only antibiotic molecules located in the superficial layers were released during the first hours of the experiment (24 hours). Once these molecules are dissolved, the release of antibiotic is reduced and depends on the penetration of the surrounding media into the cement matrix, which is dictated by the wettability of the polymer and by the number and size of the pores in it. The principal limitation of these systems is the low proportion of antibiotic in relation to solid bone cement components. This fact, in addition to the difference in solid particle sizes among the antibiotic and cement components, results in heterogeneous mixing,

Table 3
Diameter of Inhibition Halo Test for Ceftazidime Expressed in Millimeters Measured After 1, 2, and 3 d of Incubation.

Bacteria	Ceftazidime Proportion							
	1:40				4:40			
	S. aureus	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. epidermidis*	*E. coli*	*P. aeruginosa*
	Mean Inhibition Halo (mm)							
Incubation (d)								
1	22	R	40.5	43.5	33	31	48	47
2	18.5	R	37	35.5	29	20	42	38.5
3	R	R	35.5	32	27.5	23	38	39.5

E. coli, *Escherichia coli*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *S. aureus*, *Staphylococcus aureus*; *S. epidermidis*, *Staphylococcus epidermidis*.

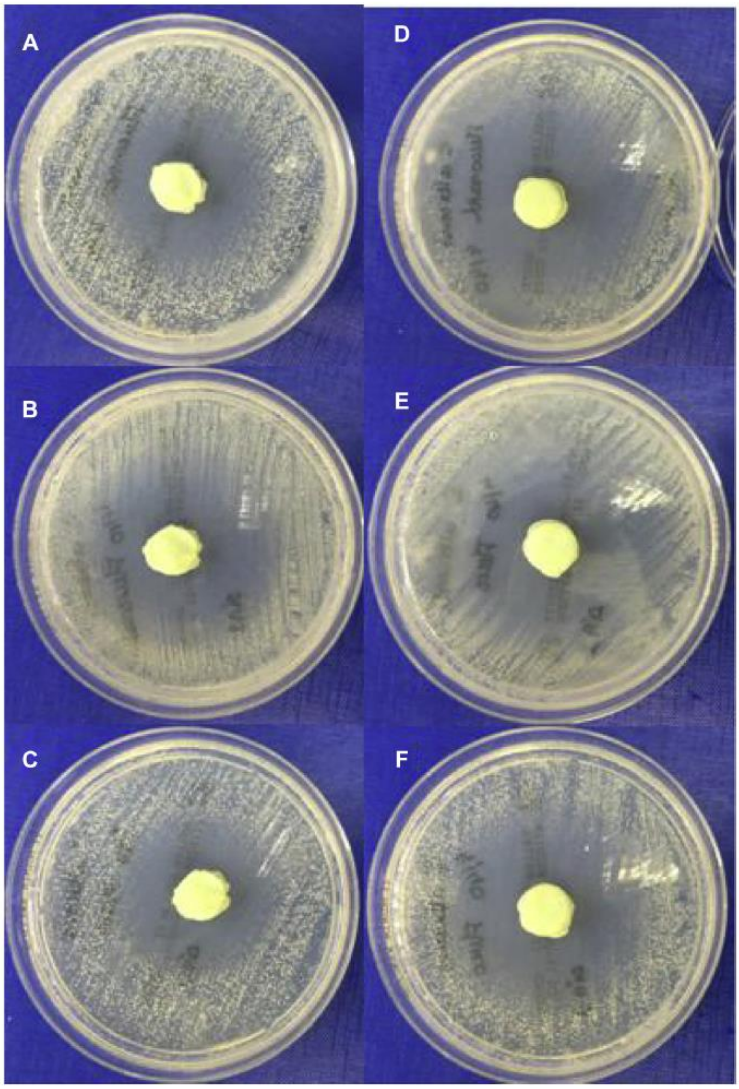

Fig. 6. Results of inhibition halo test expressed in millimeters for 1:40 fluconazole at 24 hours (A), 1:40 fluconazole at 48 hours (B), 1:40 fluconazole at 72 hours (C), 4:40 fluconazole at 24 hours (D), 4:40 fluconazole at 48 hours (E), and 4:40 fluconazole at 72 hours (F).

and therefore, there is high variability of distribution of the antibiotic among samples, which leads to heterogeneous release profiles.

In both proportions, ceftazidime was released in a higher percentage than fluconazole. Although ceftazidime has a higher molecular weight than fluconazole (632 vs 306 daltons [23]), its solubility is also higher (396 vs 1 mg/L), and our data suggest that its release is principally a surface phenomenon, as over 70% of the total amount released did it in the first 24 hours. Many factors mediate the release of antibiotic from polyacrylic cements. In this case, the data are consistent because, although the molecule of ceftazidime is approximately twice the size of that of fluconazole, it

is much more soluble, which allows the rapid dissolution of the antibiotic on the surface into media.

In Figures 1 and 2, it can also be observed that a higher proportion of antibiotic resulted in a greater release of antibiotic, but not of the same magnitude for both drugs; the release of ceftazidime increased 453% and that of fluconazole 648%, a difference that may have been due to the molecular weight of each one. As the concentration of drug increases, the voids created by the release of the drug facilitate the release of the drug trapped deeper inside. Our results confirm that PMMA is not a highly porous structure, and that compounds of low molecular weight, as well as having higher diffusion coefficients, able to diffuse through more narrow spaces, are consequently released more readily than compounds of higher molecular weight. These results highlight the relevance of the molecular weight of the compound selected for incorporation within bone cement [22].

The amount of antibiotic released from the cements, in combination with the values of local clearance redon (attributable mainly to the wound drain), and that of liquid volume in the joint space allowed to simulate the bioactivity of the cements. Figure 5 shows that the amount of antibiotic in the biophase would decrease up until 72 hours, at which point the redon would be removed and the amount of antibiotic in biophase increased. At 72 hours, in the best of cases, <30% of patients would reach the MIC of the reference microorganisms in the case of ceftazidime in a proportion of 1:40. On the other hand, at 72 hours, >50% of patients would reach the MIC for all microorganisms except *S. aureus* when ceftazidime in a proportion of 4:40 would be used. In the case of fluconazole, around 50% and 99% of patients would reach the MIC of *C. albicans* when in proportions of 1:40 and 4:40, respectively.

In summary, according to our simulations, the bioactivity of fluconazole and ceftazidime depends on the microorganism that produces the infection, the proportion of antibiotic used, and above all the time passed since surgery, being highest during the first 24 hours. In the case of ceftazidime, the antibiotic proportion used is of importance during the first 72 hours, after which time it ceases to be relevant. Regarding fluconazole in a proportion of 4:40, all patients should be covered against *C. albicans* for the period simulated.

The release of ceftazidime and fluconazole from PMMA to agar indicated that polymerization of the PMMA did not adversely affect the action of the antibiotic and antifungal drugs. This finding is in line with previously reported studies. The CFU count and inhibition halos for both antibiotics (Table 3 and Fig. 5) revealed some differences. Only *S. epidermidis* and *P. aeruginosa* grew when the cement was placed in a liquid-growing medium. The inhibition halo test revealed that the former was resistant to ceftazidime in a proportion of 1:40 in all samples. This is logical, since ceftazidime is not the most effective cephalosporin against *S. epidermidis*. On the other hand, ceftazidime was not active against *P. aeruginosa* when liquid-growing medium was used, but was active when halo of inhibition was measured. This could have been due to the properties of *P. aeruginosa*, as this microorganism has an alginate capsule that creates biofilms [24,25]. For this reason, in the first few hours during which the bacteria was in liquid medium, it could have upholstered the pores of the cement, preventing elution of the antibiotic.

Our present study has some limitations. First, there are many types of PMMA currently available, and we have tested just one, as it is the most used in our hospital. Second, we evaluated the bioactivity of samples through a simulation exercise based on the assumption that the surface of impregnated cement in contact with extracellular body fluid would be equivalent to the surface of the samples assayed and assuming that the distribution of the antibiotic from the location of the implant to the systemic circulation would be negligible. Consequently, the amount of antibiotic released into the medium would not have been exactly the same. On the other hand, our results are strengthened by the fact that all the antibiotic released would have remained in the same place and consequently would have reached a high local concentration.

Conclusions

The findings of the present study show that ceftazidime and fluconazole can be successfully incorporated within self-polymerizing PMMA. The bioactivity of ceftazidime at all proportions and fluconazole in a proportion of 1:40 depend on the sensitivity of the microorganism in the first 3 days after surgery, increasing substantially after drain removal, usually at 72 hours. In the case of fluconazole 4:40 proportion, all patients should be protected against *C. albicans* for the duration of the postsurgery period. Our microbiology study revealed biofilm formation in the case of *P. aeruginosa*, which represents a problem for treatment and prophylaxis against this microorganism. Further clinical studies are essential to test the efficacy of these drug-delivery systems before their widespread use as prophylaxis and treatment of prosthetic joint infections.

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Appendix

Drug Release Models

The in vitro fraction release profiles (Ft) for each formulation, calculated as the ratio of the absolute cumulative amounts of drug released at time t (M_t) to infinite time (M_∞), were used to test the following models:

The zero-order kinetics model (Equation 1):

$$Ft = \frac{M_t}{M_\infty} = k \cdot t \quad (1)$$

where k is the zero-order release constant. This model assumes that drug release is constant.

The Higuchi model (Equation 2):

$$Ft = \frac{M_t}{M_\infty} = k \cdot t^{0.5} \quad (2)$$

where k represents the release rate constant reflecting the design variables of the system.

The Korsmeyer-Peppas model (Equation 3):

$$Ft = \frac{M_t}{M_\infty} = k \cdot t^n \quad (3)$$

where k represents a rate constant incorporating structural and geometric characteristics of the device, and n is the release exponent. For cylindrical devices, $0.45 \leq n$ corresponds to a Fickian diffusion mechanism, $0.45 < n < 0.89$ to non-Fickian transport, $n = 0.89$ to case II (relaxational) transport, and $n > 0.89$ to super case II transport. With this equation, the first 60% of a release curve should be used to calculate n, the exponent that explains the mechanism of release [26].

The models were selected based on the precision of parameter estimates, goodness-of-fit plots, and the minimum value of objective function (−2 log [likelihood]: −2LL) provided by the NONMEM program (ICON plc, Republic of Ireland) [27]. Because some of the models compared were not nested, −2LL was not used directly for comparative purposes, and the Akaike information criteria, computed as −2LL + 2Np, where Np is the number of the parameters in the model, was used instead. The model with the lowest value of Akaike Information Criterion was selected, as the precision of model parameters and data description were adequate.

Bioactivity Models

To assess bioactivity, a Monte Carlo simulation was performed. Antibiotic concentrations in the joint space (for each antibiotic and proportion) of 1000 patients were simulated using NONMEM, version 7.3. The percentage of patients whose levels of antibiotic at the site of the implant would be higher than the MIC was calculated.

Potential bioactivity was evaluated using the minimum inhibitory concentration (MIC) distributions of microorganisms sensitive to ceftazidime as a reference: *Pseudomonas aeruginosa* (MIC = 4 μg/mL), *Staphylococcus aureus* (MIC = 32 μg/mL), *Staphylococcus epidermidis* (MIC = 8 μg/mL) and *Escherichia coli* (MIC = 8 μg/mL). *Candida albicans* (MIC = 1 μg/mL) was used for fluconazole calculation.

The pharmacokinetic model used for simulating biophase antimicrobial concentrations (Fig. 4) consists of 2 compartments: cement loaded with antibiotic (C1) and joint space (C2). Owing to the fact that clearance by drainage is very high during the first 72 hours post-surgery, the distribution (K_d) from the joint space (C2) into the systemic circulation and return (K_r) were considered negligible for calculations. Volume of joint space, 1.6 ± 1.1 mL, was obtained from literature [28]. Elution rate of the antibiotic was calculated from the in vitro study. A 1-year retrospective revision of volume obtained from redon in the first 72 hours after knee arthroplasty was carried out to assess clearance from the joint space in Dr Peset hospital, Valencia, Spain; 468 data were collected from 156 patients.

Clearance owing to the redon in the first 72 hours was modeled by fitting zero- and first-order equations to data (Equations 4 and 5, respectively):

$$Cl_t = Cl_0 + k_0 \cdot t \quad (4)$$

$$Cl_t = Cl_0 \cdot e^{-k_1 t} \quad (5)$$

where t is time, Cl_t is the clearance at time t, Cl_0 is the initial clearance, and k_0 and k_1 are the zero- and first-order constants.

To ensure the validity of the method, median and standard errors of the parameters of the final model were estimated using the nonparametric bootstrap technique within Perl-speaks-NONMEM (PSN). Two hundred replicates of the data were generated by the bootstrap method to obtain the median and 95% percentile of parameters and fixed- and random-effect parameters. The bias of each parameter was calculated by computing the difference between the median value derived from the bootstrap and the final parameter estimate.