QUANTITATIVE *IN VIVO* MICRODIALYSIS IN PHARMACOKINETIC STUDIES

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SUMMARY

Recent theoretical studies have yielded a more profound knowledge of the properties of recovery (the key parameter in quantitative microdialysis) and have put in evidence important limitations of the usual *in vivo* calibration methods used in quantitative microdialysis for pharmacokinetic studies. Recovery values obtained by using the more classical methods of calibration (the variation of flow rate perfusion method, the delivery and retrodialysis methods and the no net flux method) can only be used to accurately convert dialysate drug concentrations into extracellular concentrations, when the drug of interest is in the body under steady-state conditions. Therefore, these *in vivo* calibration procedures must not be used when the drug studied has to be administered using modalities of administration which do not provide steady-state concentrations (for example, intragastric, subcutaneous, intraperitoneal or intravenous bolus injections). The dynamic no net flux method (DNNF), however, can be considered the only *in vivo* calibration method useful in PK experiments developed under transient conditions, although this calibration procedure has several serious disadvantages. The new modified version of the ultraslow microdialysis (the MetaQuant technique) overcomes many of the limitations of both the classical calibration and the DNNF methods and, therefore, it could be considered a promising tool in pharmacokinetics.

KEYWORDS

Quantitative microdialysis; recovery; *in vivo* calibration; variation of perfusion flow rate method; ultraslow microdialysis; delivery method; retrodialysis method,; NNF method; DNNF method.

LIST OF ABBREVIATIONS

Cd, drug concentration in the dialysate; **Cecf**, drug concentration in the extracellular fluid; **Cin**, drug concentration in perfusate; **CSF**, cerebrospinal fluid; **DNNF**, dynamic no net flux method; **E**, extraction fraction, extraction efficiency or recovery; **ECF**, extracellular fluid; **MD**, microdialysis; **NNF**, no net flux; **PK**, pharmacokinetics; **Q**, perfusion flow rate

1. INTRODUCTION

The mathematical description of the time course of a drug (or its metabolite/s) levels in the biological media, after single or repeated administration to a living organism, is probably the most important objective of Pharmacokinetics (PK). This mathematical description has the fundamental goal to allow us to make accurate predictions of the pharmacological response.

To develop their mathematical models, PK has classically used serum or plasma samples, in spite of the knowledge that most drugs exert their effects not within the blood compartment, but in defined tissue sub-compartments in which drug receptors are located. This practice is based on the assumption that changes in serum or plasma drug concentrations are a true reflection of those occurring in the biophase. Although this methodology has provided, in general, satisfactory results for a large majority of drugs, there are notable exceptions. For example, it is now well established that drug penetration into certain types of tumors is variable and poor ***(1)***. Consequently, studies on the clinical efficacy of certain anticancer drugs have revealed a lack of correlation between serum drug concentrations and tumor exposure to the drug. Another paradigmatic example comes from the field of anti-infective drugs. Several studies have reported a high inter-tissue and inter-subject variability of antibiotic tissue distribution, with antibiotic tissue concentrations varying considerably from the corresponding plasma levels ***(2,3)***. These, and other examples, clearly suggest that direct concentration measurements at biophase might be more relevant in predicting clinical response than the estimation of response from plasma drug concentrations.

The use of drug concentrations at the level of the drug target to predict the pharmacological response has been a longstanding goal for the PK research. Microdialysis (MD) has allowed researchers to realize this old dream of pharmacologists.

To monitor drug concentrations in target tissues in animals and humans, several techniques (e.g. tissue biopsies, saliva and skin blister fluid sampling, imaging techniques) have been employed. But, in contrast to MD, which allows sequential sampling over time, traditional concentration measurements in bodily secretions or biopsy samples usually only yield a limited number of time points. Moreover, taking biopsies presents ethical limitations, and, more important, the resulting total concentrations in homogenized tissue usually lead to over- or under-estimation of actual drug tissue concentrations ***(4)***.

In vivo microdialysis (MD) can be considered the only method useful, at present, to accurately quantify the free extracellular levels of drugs in tissues of living animals. MD has become a common technique for monitoring changes in free concentrations of drugs and metabolites in various tissues. The use of MD in PK studies has increased considerably due, in part, to improvement of our theoretical knowledge of quantitative processes involved in MD and to the advances in the sensitivity and specificity of the analytical methodology. Numerous research studies incorporating MD have been published in the last few years. The applications of MD to PK research are presently numerous and include: blood and tissue pharmacokinetics, *in vivo* protein binding studies, drug and/or metabolite profiles in peripheral tissues, pharmacokinetics in discrete regions of the brain, studies of transport phenomena in the central nervous system, and so on.

The present chapter deals with the application of MD in PK studies. Since in this scientific field, knowledge of the free drug extracellular concentrations is crucial, it becomes imperative to have adequate calibration protocols that allow one to convert dialysate concentrations into extracellular concentrations. In the following, we describe and analyze the existing methods of probe calibration and the variables and factors affecting them, in the light of recent advances in our theoretical knowledge of mass transport processes implicated in MD.

1.1. Calibration of probes: a necessity in MD-PK studies

The purpose of PK is to study the time course of drug and/or metabolite concentrations in different tissues and excreta and to construct mathematical models suitable for interpreting such data. As commented above, without accurate measures of drug concentrations in tissues of interest it is not possible to carry out adequate predictions for clinical response. In this sense, the major concern in applying MD to PK studies is to accurately establish the absolute drug concentrations in the extracellular fluid (C_{ecf}) of the tissues analyzed. This problem arises from the fact that MD is a dynamic sampling technique where drug molecules diffuse across a semipermeable membrane in the presence of a concentration gradient (Fig. (**1**)).

Diffusing molecules are then swept away by a perfusion medium that is continuously pumped at a constant flow-rate (normally between 1 and 5 µl/min) through the MD probe. In this situation, the drug concentration in the microdialysate (C_d) is not that found in the extracellular fluid (ECF). Therefore, direct quantification of the drug in the dialysate samples yields drug concentration values clearly lower than those existing in the ECF surrounding the MD probe. The ratio between the drug concentration in the dialysate and the concentration of the same substance in the ECF distant to the probe is defined as relative recovery or, simply, “recovery” (E), and is expressed either as a ratio or as a percentage. Recovery is dependent upon several factors ***(5)*** which are summarized in **Table 1**; the most important among them are the perfusion flow rate (Q) and the properties of the external medium (tissue factors).

Early studies of MD processes assumed that the membrane used to construct the probe constituted the greatest resistance (and therefore the limiting factor) to drug movements. This led to the use of the results of simple *in vitro* calibration experiments in a quiescent aqueous medium to calculate the corresponding E value. The *in vitro* E value thus calculated, was then used to transform *in vivo* C_d into C_{ecf}, assuming that the membrane properties, probe geometry, and flow-rate were not different in the *in vitro* and *in vivo* situations ***(27,28)***.

It is now clear that this procedure is not valid, however. Several investigations have shown the existence of factors, in biological tissues, that serve to either impede or enhance mass transport of the drug from the ECF to the probe ***(19, 23, 24, 29)***.

In a simple medium (for example an aqueous solution), the flux of diffusion, *J*, measured in relation to the total area is given by Fick´s law as:

$$J = -D \cdot \frac{\partial C}{\partial r} \tag{1}$$

where *D* is the diffusion coefficient in the free solution, *C* is the drug concentration in a unit volume, and *r* is the distance measured perpendicularly to the area considered. However, in a solid tissue, flux must be calculated by the following equation:

$$J = -\alpha \cdot D^{*} \cdot \frac{\partial C}{\partial r} \tag{2}$$

where α is the effective volume fraction and D^{*} the diffusion coefficient in the tissue ECF (it is assumed that the drug does not penetrate into the cells). As can be seen, flux of diffusion in a solid tissue is directly dependent on the α value, which is usually lower than 1 (for example, in most brain regions $\alpha \approx 0.2$) ***(21)***. On the other hand, the diffusion path length *in vivo* is clearly increased compared to that existing in a free solution, because of the tortuosity (λ) of the route. In this context, λ, which describes the increased diffusion pathway *in vivo* due to the presence of impermeable cell membranes, is equal to $(D/D^{*})^{1/2}$ (in brain $\lambda \approx 1.6$). Therefore, the diffusion coefficients in a free liquid medium (D) are usually clearly higher than those found in a porous matrix such as a solid tissue ***(20,22)***. The above theoretical considerations clearly suggest that flux *in vivo* is always smaller than *in vitro*. Because MD is essentially a diffusion process, it would be expected recovery *in vitro* being greater than that obtained *in vivo* at steady state. In fact, this has been empirically demonstrated for many substances.

Thus, for example, the ratio for the recoveries of mannitol and sucrose *in vitro* versus *in vivo* (determined in brain) was found to be 2.93 ***(30)*** and 3.50 ***(31)***, respectively.

Nevertheless, the above assertions must not be taken as a rule of thumb; several authors have also reported *in vivo* recoveries exceeding those obtained *in vitro*. For example, the ratio for the recoveries was 0.82 for cocaine ***(32)*** and 0.72 for dopamine ***(29,33)***. In these latter examples, the discrepancies between *in vitro* and *in vivo* recoveries are largely due to the existence of potent drug clearance processes as it will be explained later.

It is clear that *in vitro* calibration has scant application in MD *in vivo*. However, *in vitro* experiments can be very useful for detecting drug-probe membrane interactions and for establishing the absence or existence of probe-to-probe differences - particularly in the case of in-house manufactured probes ***(6,7)***. *In vitro* experiments in combination with *in vivo* studies can also yield relevant information on the tissue parameters involved in mass transfer phenomena in MD ***(34,35)***.

At present it is well established that tissue is the most important factor in determining the mass transfer in the majority of MD-PK experiments, and that the calculation of C_{ecf} requires *in vivo* probe calibration. In order to perform *in vivo* calibration of the probes, it is customary to calculate, in the tissue of interest, the E value. Originally, E was defined as the ratio between dialysate concentration (C_d) and ECF concentration at far field distance from the probe (C_{ecf}) (Eq. 1):

$$E = C_d/C_{ecf} \quad (3)$$

A more generalized form of the above equation is that corresponding to the so-called extraction fraction, extraction ratio or extraction efficiency, which is designated with the same symbol (E) in the remaining discussion below:

$$E = (C_{in}-C_d)/(C_{in}-C_{ecf}) \quad (4)$$

where C_{in} is the inflowing drug concentration. When no drug is added to the perfusate (i.e., when we perform a classical recovery experiment, $C_{in}=0$), diffusion of the drug is from the external medium into the dialysate, and equation 4 is reduced to equation 3.

E may also be calculated by including a known concentration of the drug in the perfusion fluid while C_{ecf} is zero (i.e., the so-called delivery experiment). In this case, drug diffusion occurs from the perfusate into the ECF, and equation 4 is reduced to:

$$E = (C_{in}-C_d)/C_{in} \quad (5)$$

Theoretically, under identical experimental conditions for a given MD probe and drug, E values calculated from a recovery experiment or from a delivery experiment are identical.

1.2. Properties of recovery

A basic and important property of E is its dependence on perfusion flow rate (Q) (Fig. (**2**)). As mentioned above, E is inversely dependent on Q. The greater Q, the lower the concentration in dialysate. As can be seen in Fig. 2 (*top*), E attains its maximum value (i.e., 100%) at zero flow. At this point C_d equals C_{ecf} (Fig. 2, *bottom*). As we will comment below, the dependence of E on Q was mathematically formulated by Jacobson et al. in 1985 ***(11)***, and constitutes the basis for the calculation of E used in one of the empirical methods more commonly used in quantitative MD.

Another important property of E is related to the time dependency of this parameter. MD is essentially a diffusion process. The insertion of a MD probe, perfused at a flow rate Q, in a tissue in which C_{ecf} is at steady state (the common situation in a classical *in vivo* calibration experiment), induces the appearance of a depletion zone around the probe. In other words, a concentration gradient is formed around the probe as the drug is removed by the perfusion fluid. For any given diffusion coefficient, the E value is

determined by the magnitude of this concentration gradient. The latter will be more or less steeper depending on the existence of additional tissue factors such as irreversible metabolism, microvasculature transport processes and so on. The faster the drug is metabolized in the tissue or is removed by microvascular transport, the steeper the gradient formed, and, consequently, the greater the value of E.

The formation of the concentration gradient is not an instantaneous phenomenon. In a classical recovery experiment, drug diffusion occurs first through the tissue ECF space, then through the membrane, and finally into the dialysate. Attending to the usual geometry of MD probes (cylindrical), the progression to steady state concentration profile can be very slow, principally if there are no other mechanisms for removing the drug other than extraction through the probe. As E depends on the concentration gradient around the probe, the time dependency of E is, therefore, an inherent characteristic and is related to the temporal development of spatial concentration gradients in the probe and in the milieu adjacent to the probe. This phenomenon is known as mass transfer transient ***(36)***. The time dependency of E has been confirmed by experimental data proceeding from both *in vitro* ***(31)*** and *in vivo* experiments ***(15)***.

Theoretical predictions suggest very significant changes in E value over time. So, predictions indicate that E will decrease progressively over time, starting with the maximum value at time = 0, and, finally, falling to its steady state value, E^{ss} as can be appreciated in Figure **3**.

The magnitude and rapidity of the time-dependent variation of E are mainly dependent on the drug clearance processes (for example, efflux transport, local tissue metabolism…,globally represented by k_{clr} in Figure **3**) taking place in the tissue interstitium. As observed in the top panel of Figure **3**, a decrease in k_{clr} value could imply: (i) a decrease in the magnitude of E^{ss}; and (ii) an increase in the time to reach the E^{ss} value, being, in some circumstances, this second trend the most prominent: for example a change in two orders of magnitude in the k_{clr} value is able to induce a 60-fold increase in the time to approach E^{ss}, whereas the same change in k_{clr} induces only

a 3-fold change in the E^{ss} value. Since this property of E is very relevant, it should be adequately considered by the users of MD in PK studies.

The scenario is clearly more complicated when we consider other normal situations in MD-PK studies. In many cases, drug administration can involve different modalities (intragastric administrations, intraperitoneal injections, intravenous administration (bolus or infusions), etc.), in which C_{ecf} is not at steady state in the course of the experiment. In these cases, the changes in E over time are due not only to the above mentioned mass transfer transients (inherent to MD) but also to the time variation of the C_{ecf} values: the so-called pharmacokinetic transients ***(36)***. The theoretical limitations derived from this E property in determining C_{ecf} in MD-PK studies had not been studied in depth until some simulations using two kinds of *in vivo* MD-PK experiments involving either a loading intravenous dose followed by an intravenous constant infusion or intravenous bolus drug administration were performed ***(36)***. The most relevant conclusion drawn from these simulations is that the time variation of E depends on the nature of the PK experiment. As can be appreciated in the bottom panel of figure 3, E^{ss} tended towards the same value obtained in the simulation of the pure mass transient in the case of constant intravenous infusion, though this situation was reached over longer times. The E^{ss} value in bolus administration was also achieved over longer times, though, as can be observed, its value is lower than that obtained in the pure mass transfer transient simulations. In other words, the time variation of E and its steady state values can be clearly different depending on the mode of administration; this suggests that some methods of probe calibration *in vivo* could not be correctly used in some types of MD-PK experiments - specifically those in which C_{ecf} is not under steady-state conditions. Thus, some of the usual *in vivo* calibration methods commented below (in particular, the variation in flow rate method, the delivery and retrodialysis methods, and the no net flux method) may be severely limited depending on the mode of administration assayed.

2. MATERIALS

The use of MD in PK studies requires no specific materials other than those commonly used in other applications of MD.

3. METHODS

Methods for probe calibration under *in vivo* conditions

As we have commented above, since the vast majority of tissues in which MD probes are implanted do not constitute a simple and passive environment, and different kinetic processes can take place, it is usual for quantitative work in PK studies to calibrate MD probes directly in the tissue/s of interest. Several *in vivo* calibration methods have been developed and studied in the last years. In general, the *in vivo* MD calibration procedures that we will present below require C_{ecf} to be zero or a constant value (i.e., they require C_{ecf} to be at steady state). This requirement is very important because it will preclude the use of the E value calculated to correctly convert C_d into C_{ecf} when the tissue drug concentrations are not at steady state.

3.1. Variation of perfusion flow rate (extrapolation to zero flow rate) method

The exponential dependence of recovery on flow rate (Q) was mathematically formulated by Jacobson et al in 1985 ***(11)***:

$$E = \frac{C_d}{C_{ecf}} = 1 - \exp^{\left(-\frac{Km \cdot S}{Q}\right)} \quad (6)$$

where *Km* is the average mass transfer coefficient, unique to the drug analyzed and the membrane properties, and *S* is the membrane surface. This model assumes that

Km is constant and does not vary with *Q*. By extrapolating the curve to zero flow, E will attain a value of 100%, i.e., at zero flow C_d is equal to C_{ecf} (Fig. (**2**), *bottom*).

From a practical point of view, calculations using this method imply measuring C_d in the tissue of interest at several *Q* values. Using a nonlinear regression analysis program, the parameters of equation (6), $Km \cdot S$ and C_{ecf}, can be obtained.

The variation of perfusion flow rate method has been extensively used in the MD-PK literature. Numerous reports have utilized this method for their calculations. In general, it can be considered a good method for estimating C_{ecf} concentrations, although some authors ***(37,38)*** have shown that when used in *in vitro* experiments, it yields smaller estimates of C_{ecf} and higher estimates of E.

An alternative method that overcomes the above problems is based on the fact that at very slow perfusion rates, the residence time of the perfusion fluid through and in the membrane lumen is greatly increased. In such cases, C_d is near to the *in vivo* drug concentration in ECF. While theoretically it would require zero flow for concentrations to equilibrate, in practice E values higher than 0.9 (90%) are obtained at slow perfusion rates (of around 50 nl/min or lower). In this situation, quantification of C_d in dialysate samples directly yields the C_{ecf} value. The validity of this slow perfusion rate method has been ascertained by several authors both *in vitro* and *in vivo* under steady-state C_{ecf} conditions ***(32,39)***. To our knowledge, this method has not been applied to MD-PK studies using non-steady state conditions, despite its great advantage. Obviously, the method is only applicable when the analytical technique employed to quantify the drug in dialysates is highly sensitive and capable of accurately manipulating sample volumes of less than 1 µl. Other problems also derived from the very slow flow rate used are: (i) experiments are time consuming as for example on removing dead volumes from the MD system; (ii) sample evaporation can occur during the long collection times and (iii) the lack of stability and accuracy of the perfusion flow rates used. At present, technology exists to manipulate and analyze low volume samples and to overcome some of the problems mentioned above.

In fact, a modified version of the ultraslow microdialysis has been recently described ***(40)***. In this new method, the conventional microdialysis probe setup has been modified by introducing an additional flow line (exclusively acting as a carrier flow) that enters into the probe through a different channel in order to merge with the ultraslow dialysate immediately downstream from the microdialysis membrane. The carrier fluid is delivered at a higher flow rate (typical 0.9 µl/min). This shortens the lag time (making possible the *in vivo* application of the technique) and increases the final sample volume for easier handling. This new type of probes, named MetaQuant by the authors, have been able to generate drug concentration-time curves useful for PK analysis in medial prefrontal cortex of rats, with the use of a small numbers of rats. It seems that this *in vivo* MD methodology will become more common in application to MD-PK studies in the near future.

3.2. Delivery method

Lindefors et al ***(18)*** presented this method in 1989. In this approach to calibrating an MD probe *in vivo*, the amount of drug that diffuses out of the membrane relative to the amount in the perfusate is determined, and it is assumed that the same proportion exists for the amount of drug that will diffuse into the probe relative to the concentration in the ECF. In other words, in an *in vivo* delivery experiment, the drug is perfused through the MD probe while C_{ecf} is zero, and it is assumed that E is independent of drug mass transport direction.

As indicated above, the validity of the *in vivo* delivery method as a calibration protocol depends on the assumption that the E value calculated for diffusion out of the probe is the same as E for diffusion into the probe. Several papers support the above assertion for a wide range of compounds ***(41-43)***, although some exceptions have been reported ***(18,44)***.

3.3. Retrodialysis method

The retrodialysis method constitutes an alternative to the above commented *in vivo* delivery experiment. In this method, a calibrant or internal standard is included in the perfusion fluid.

Another valid approach is the utilization of close structural analogs of the drug with a similar molecular size, degree of ionization, lipophilicity and interaction with the probe membrane and dialysis components. In this case, it is crucial to check not only the diffusivity of the calibrant *in vivo*, but also to consider the potential differences in microvasculature transport, metabolism in tissue, and intra/extracellular space exchange. In addition, the calibrant must not kinetically interact with the drug. For example, it has been demonstrated that that zidovudine is able to decrease the E value of aluvodine from 10.6% to 8.7% in brain, but not in adipose tissue ***(45)***. This phenomenon was attributed to competition for an active transport site in brain, and demonstrates the importance of appropriate selection of the calibrant.

A retrodialysis experiment can be performed simultaneously with the MD-PK experiment. This is in fact its main feature, and also the most common practice. Retrodialysis can offer several advantages over other *in vivo* calibration methods. The first advantage is a shortening of the study duration. Methods such as no net flux method (NNF), for example, can only be used when C_{ecf} is at steady state – a fact that may lengthen the experiment. A second advantage is that retrodialysis can be used to continuously monitor and, consequently, to correct for changes in E among and within MD probes during *in vivo* microdialysis ***(46-49)***.

Despite the advantages of retrodialysis, it may be severely limited for *in vivo* probe calibration when the drug of interest is not under steady state conditions ***(36)***. It must be remembered that transient C_{ecf} conditions will prevail unless the drug is administered according to zero order kinetics. Thus, if the calibrant is neither initially present in the tissue ECF nor systemically administered, the calibrant will exhibit a pure mass transfer transient (see above), and the E^{ss} value obtained for the calibrant by using equation 5 might be clearly different to E^{ss} exhibited for the study drug. In this

case, E^{ss} obtained for the pure mass transfer transient (i.e., E^{ss} obtained for a calibrant in a retrodialysis experiment) does not correctly convert the individual C_d values to the corresponding C_{ecf} values.

As we commented above, although the MD parameters of the calibrant agree with those of the drug and, theoretically, it is possible to achieve equality in E^{ss} for the two solutes, the time courses of drug and calibrant recoveries will agree only if, additionally, both solutes exhibit the same plasma kinetics and are administered systemically in identical fashion. However, if so administered, then C_{ecf} for the calibrant would be non-zero during the course of the experiment and, therefore, E could not be calculated by using equation 5. This suggests that the retrodialysis method for in vivo calibration in PK transient experiments is severely limited.

3.4. No-Net-Flux (NNF) and Dynamic No-Net-Flux (DNNF) methods

Another commonly used calibration method employed under *in vivo* conditions is the so called no net flux or zero net flux method (NNF) ***(50)***. In this method the drug is added to the perfusion fluid at different concentrations (C_{in}) (higher and lower than the expected C_{ecf}), and the different C_d are measured in the dialysates. C_{in} and C_d are presumed to be equal if there is no net exchange of drug between ECF and the perfusate in the probe. However, when C_{in} is higher than C_{ecf}, the drug will diffuse out into the ECF, resulting in a decrease in C_d in relation to C_{in}. On the other hand, when C_{in} is lower than C_{ecf}, drug will diffuse into the probe from the ECF and, therefore, C_d will increase.

For assessment of this C_{ecf}, the difference between C_{in} and each particular C_d must be calculated. Linear regression of ($C_{in} - C_d$) versus C_{in} is used to determine the intercept with the abscissas axis (the point of NNF) i.e., C_{ecf}. The slope of the regression line provides the *in vivo* E value (Fig. (**4**)).

For transient conditions, an alternative protocol exists ***(51)***. In this method, named Dynamic-No-Net-Flux (DNNF) method, the drug of interest is systemically administered

to different groups of animals using both the same dose and route of administration. After dosing, each subject belonging to a concrete group is continuously perfused with one of the perfusion concentrations selected around the expected ECF concentrations and the difference C_{in}-C_d is calculated over time. Different groups are perfused with different C_{in} concentrations and, therefore, different C_{in}-C_d values are obtained at each time-point. To calculate the C_{ecf} and the E values as a function of time, the results must be combined at each time point and subjected to regression analysis as described in the NNF method.

4. NOTES

4.1. Uses and limitations of the variation of perfusion flow rate method.

To accurately apply this method when used under *in vivo* conditions, the existence of a constant C_{ecf} is needed in order to measure the parameters of equation (6). This is not a problem when the drug is administered following a zero order kinetics for an enough period of time (for example, via constant intravenous infusions, Alzet® osmotic minipumps…). However, it is not a workable method when C_{ecf} is not at steady state. Moreover, the use of E values obtained under steady state conditions with this method to correct other C_d values obtained when the same drug is administered by another mode of administration (for example a bolus injection) could lead to errors, according to the above commented properties of E.

4.2. Uses and limitations of the delivery method.

The delivery method is stable over time, allowing flexibility for incorporation to an experimental design. Obviously, the delivery experiment cannot be carried out simultaneously during the MD-PK experiment. The measurement must be conducted before (or after) the start (end) of the pharmacokinetic experiment. It must then be assumed that the E value does not change as a result of the MD-PK experiment.

Finally, it must be remembered that the E value calculated in a delivery experiment could be inadequate for correctly converting C_d obtained under transient pharmacokinetic conditions.

4.3. Selection of a calibrant: the key step in the application of the retrodialysis method.

Retrodialysis requires the use of a calibrant with physical and biological properties identical to those of the study drug. Specifically, the ideal calibrant should have diffusion and PK properties similar to those of the assayed drug. This requirement can be achieved by using a radiolabelled version of the drug molecule; theoretically, a radiolabelled form of the drug will exhibit the same diffusion coefficient and PK properties as the actual drug substance.

4.4. Uses and limitations of the NNF method.

The NNF method requires a stable steady state drug ECF concentration and unfortunately, a long time to be performed. Another important limitation (in a way similar to the two above commented methods) is related to use of the E value derived by this method to correctly convert C_d into C_{ecf}. Again, this inconvenience only manifests when the drug administered in the PK experiment is not at steady state.

4.5. DNNF method for non steady-state conditions in PK.

The DNNF method overcomes the important problem of the time dependency of E. It can be used independently of the route and modality for drug administration. However, this is a very complex and time-consuming protocol, with another important disadvantage from an ethical point of view: the requirement of a significantly larger number of experimental animals than other methods. Moreover, authors with a long standing experience with the use of the DNNF indicate that often this method generates highly variable outcomes ***(40)***.

4.6. Do the different calibration methods offer comparable results?

In recent years several authors have examined the suitability and agreement of the different calibration methods both *in vitro* and *in vivo.*

The *in vitro* performance of the different methods has been repeatedly compared. In general, good agreement among the results obtained with the different methods has been reported. For example, it has been found the estimates of E, obtained by the variation of flow rate method and the NNF method, to be practically identical ***(38)***. In the same manner, the *in vitro* comparisons of the NNF, variation in flow rate and slow perfusion rate methods seem to demonstrate a good correlation among the three methods ***(32)***.

When comparisons between calibration methods are conducted in the *in vivo* setting, the results are, in general, also coincident. Some illustrative exceptions have been, however, reported. As examples of good agreement we should mention two classical studies. In the first, Menacherry et al ***(32)*** compared the E values for cocaine in brain tissue estimated by the NNF, variation in flow rate and very slow perfusion rate methods. The authors did not detect statistically significant differences in E values with the three methods. Another important comparative study was published by Wang et al in 1993 ***(48)***. These authors compared the retrodialysis and NNF methods for the *in vivo* calibration of MD probes. Two antiviral nucleosides (zidovudine (AZT) as the drug of interest and 3´-azido- 2´,3´-dideoxyuridine (AZdU) as calibrant) which differ structurally by only a methylene group, were used in the experiments. The comparison of the results obtained with the two methods in CSF and in the thalamus ECF of rabbits showed no significant differences. Moreover, concentrations of AZT, measured directly in ventricular CSF, agreed well with those determined by NNF or retrodialysis methods.

However, there have also been studies reporting important disagreements among several calibration methods when applied to particular drugs. As an illustrative example, mention will be made of the work of Song and Lunte ***(51)***. These authors compared the delivery and NNF methods in determining the E values for caffeine and

acetaminophen in muscle and in brain tissue. Both caffeine and acetaminophen cross the blood-brain barrier. However, while caffeine is actively transported by a saturable carrier-mediated process, passive diffusion is the only process used by acetaminophen to cross the blood-brain barrier. No differences were found between the two methods when determinations were made in muscle for either caffeine or acetaminophen. However, the E value determined in brain by the delivery method was higher for caffeine and lower for acetaminophen than E determined by the NNF method. For caffeine this discrepancy was attributed to the saturable active transport across the blood-brain barrier, which resulted in E being dependent upon the concentration of caffeine in brain. These results are illustrative of the difficulties of selecting a calibration method. In our opinion, this study clearly shows that any MD calibration procedure must be validated for each compound in each studied tissue.

Another illustrative example is offered by Sjöberg et al ***(52)***. These authors compared the estimated unbound steady state concentration of theophylline in blood and brain tissue in rats. They used the tritium method (not commented in this review), the low perfusion rate method and the NNF method. Curiously, all three methods accurately predicted the steady state theophylline concentrations. However, the E value obtained with the NNF method was clearly lower than 100% (the expected *in vivo* recovery for theophylline at a flow rate of 0.1 µl/min). As the authors pointed out, this observation did not agree with the consistent results in predicting the steady state concentration of the drug obtained with the three methods. The question of whether active processes, such as uptake and release, influenced the slope of the regression line of the NNF method without affecting the estimation of steady state drug concentration (as other authors have also suggested ***(53)***), remain to be elucidated.

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Figures and tables

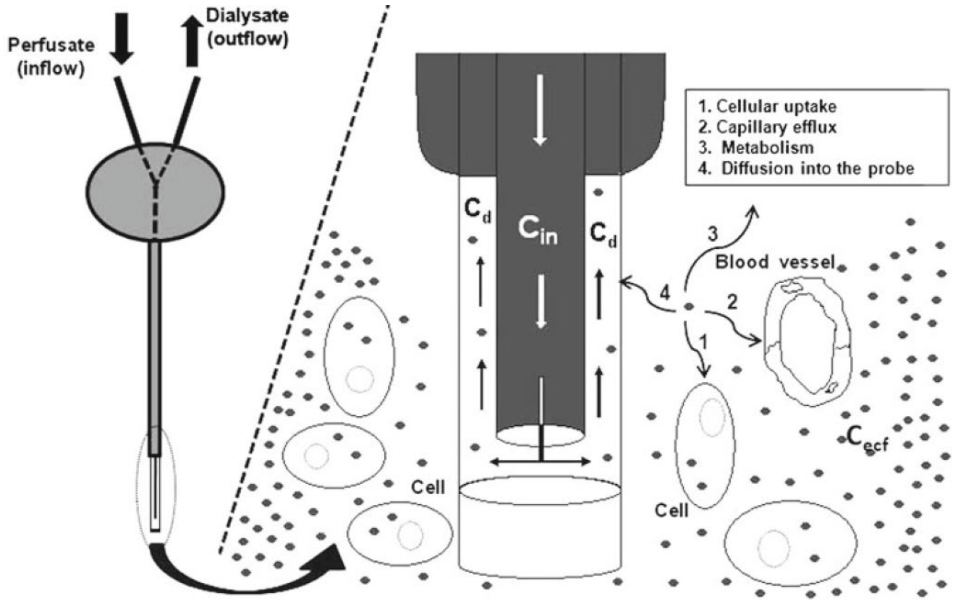

Fig. 1. Schematic representation of a microdialysis concentric probe implanted in a solid tissue, sampling a hypothetic drug systemically administered. The diffusion paths and routes of elimination for a hypothetical drug molecule are also shown. Abbreviations: *ECF* extracellular fluid of the tissue, C_d drug concentration in dialysate, C_{ecf} drug concentration in the ECF at far field distance from the probe, C_{in} drug concentration in the perfusate, Q flow rate.

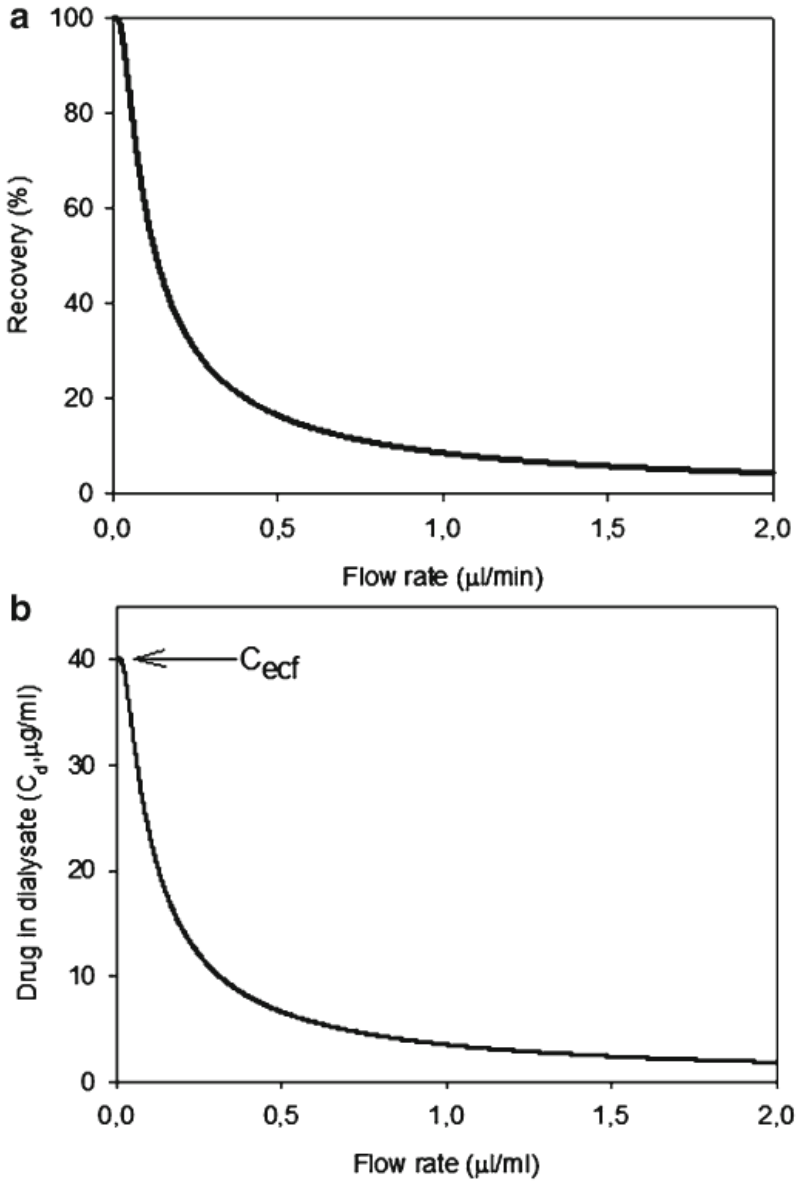

Fig. 2. (**a**) Effect of flow rate (Q) on relative recovery (E) and (**b**) concentration in dialysate samples (C_d) for a hypothetic drug.

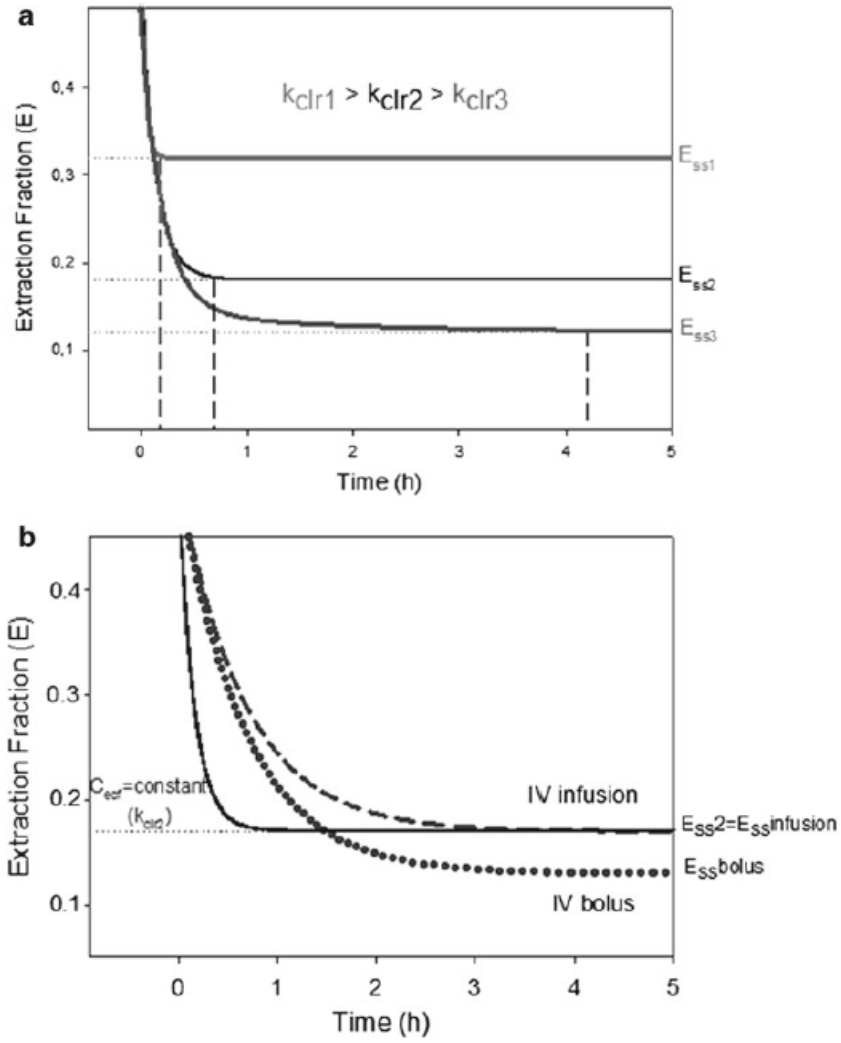

Fig. 3. (**a**) Time course of E for a hypothetical microdialysis experiment in which C_{ecf} = constant. The right E^{ss} values indicate the respective steady-state recoveries obtained for three hypothetical drugs that only differed in their overall rate constant for clearance from the tissue (k_{clr}). (**b**) Time course of E for a hypothetical drug administered by constant iv infusion (*dashed line*) or iv bolus (*dotted line*). At *right* are indicated the corresponding E^{ss} values. For comparison, the time variation of E for the same drug is also shown, with a rate constant equal to k_{clr2}, in the case of C_{ecf} = constant shown in the upper panel (*solid line*). Adapted from (36).

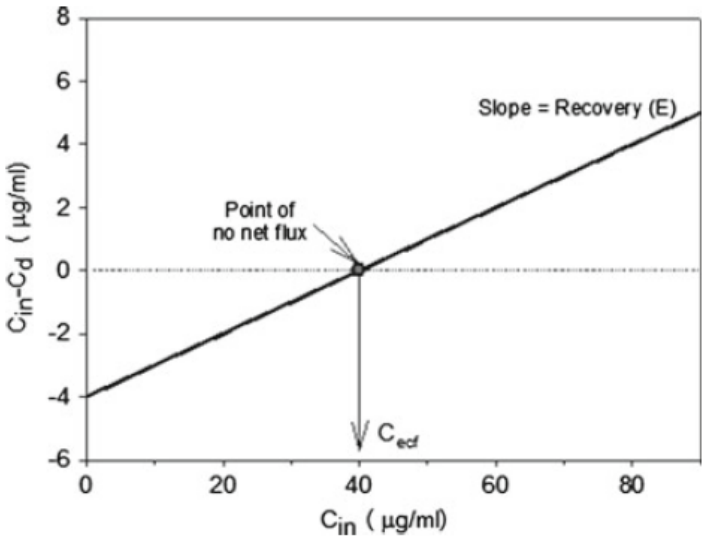

Fig. 4. Typical plot from an NNF experiment for determining E and C_{ecf}.

Table 1
Factors exerting an influence upon recovery (E)

Membrane properties: • Composition and surface charge (6, 7) • Molecular weight cutoff (MWC) (8) • Membrane surface area (S): Outer diameter (OD), length, thickness (9) For example, Polyethersulfone (OD: 0.50 mm; MWC 100 kDa) Cuprophan®(OD: 0.24 mm; MWC 6 kDa) Polyarylethersulfone (OD: 0.50 mm; MWC 20 kDa) HOSPAL AN69® Polyacrylonitrile/sodium methallyl sulfonate copolymer (OD: 0.31 mm; MWC 40 kDa)	Perfusate: • Perfusion flow rate: ↑flow rate implies ↓recovery (9, 11) • Ionic composition (12–16) • Osmolality (17) For example, Saline solution, ringers solution, buffered ringers solution, artificial-cerebrospinal fluids, Krebs Ringer solution
Drug properties: • Molecular weight (8) • Charge (10) • Shape	Tissue properties: • Tissue diffusivity: Diffusion coefficients in tissue are directly dependent on effective volume fraction of the distribution space (α) (18–22) and inversely dependent on tortuosity (λ^2) • Tissue clearance processes: Capillary exchange, cellular uptake processes, local metabolism (23–26)