Journal of Pharmaceutical and Biomedical Analysis 47 (2008) 876–881

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Microdialysis as a tool to determine free kidney levels of voriconazole in rodents: A model to study the technique feasibility for a moderately lipophilic drug

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ARTICLE INFO

Article history:
Received 4 September 2007
Received in revised form 21 February 2008
Accepted 22 February 2008
Available online 29 February 2008

Keywords:
Microdialysis
Lipophilic compounds
In vitro and *in vivo* recoveries
Voriconazole

ABSTRACT

Microdialysis has been employed for the *in vivo* measurement of endogenous compounds and a variety of drugs in different tissues. The applicability of this technique can be limited by drug lipophilicity which can impair the diffusion through dialysis membrane. The objective of this study was to evaluate the feasibility of using microdialysis to study kidney penetration of voriconazole, a moderately lipophilic antifungal triazolic agent (Log $D_{7.4}$ = 1.8). Microdialysis probe recoveries were investigated *in vitro* by dialysis and retrodialysis using four different drug concentrations (0.1–2 μg/mL) at five flow rates (1–5 μL/min). Recoveries were dependent on the method used for the determination as well as on the flow rate, but independent of drug concentration. The average apparent recoveries determined by dialysis and retrodialysis, at flow rate of 2 μL/min, were 21.1 ± 1.5% and 28.7 ± 2.0%, respectively. Recovery by retrodialysis was bigger than the recovery by dialysis. The average apparent dialysis/retrodialysis recovery ratio *in vitro* was 0.73 for all concentrations investigated. The differences between retrodialysis and dialysis recoveries were attributed to the drug's binding to the plastic tubing before and after the dialysis membrane which was experimentally evaluated and mathematically modeled. The *in vivo* apparent recovery determined by retrodialysis in healthy Wistar rats' kidney was 38.5 ± 3.5%, similar to that observed *in vitro* using the same method (28.7 ± 2.0%). The *in vivo* apparent recovery after correcting for plastic tubing binding (25.1 ± 2.8%) was successfully used for determining free kidney levels of voriconazole in rats following 40 and 60 mg/kg oral dosing. The results confirmed that microdialysis can be used as sampling technique to determine free tissue levels of moderately lipophilic drugs once the contribution of tubing binding and membrane diffusion on the apparent recovery are disentangled.

1. Introduction

Microdialysis (MD) is a sampling technique that allows to access substances in the interstitial fluid and to measure free concentrations of exogenous and endogenous compounds [1,2]. The main innovation introduced by this technique was the possibility to determine drugs free fraction at the biophase, which has been changing some misconceptions about drug tissue distribution [3,4].

One critical step to apply microdialysis is to determine the probe's relative recovery. As MD system works in sink condition, where diffusion equilibrium is never reached, the levels measured in the dialysate will always be lower than the actual levels in tissue, leading to the need of determining the probe's relative recovery, that express the ratio between measured and real concentrations in the investigated media [1]. The relative recovery, which must be determined *in vitro* and *in vivo* because of the differences in drug diffusion in solution and at the intercellular fluid, can be evaluated by retrodialysis and dialysis, among other methods, as reported previously [2].

Several factors affect drug's relative recovery including perfusion fluid flow rate, probe's characteristics such as type, membrane length and diameter, drug's physico-chemical properties, experimental conditions such as temperature and sampling time and matrix tortuosity of the tissues of interest, when the recovery is determined *in vivo* [5].

The physico-chemical properties of the drug under investigation, specially the partition coefficient which affects permeability, have a significant influence on the diffusion process through the

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doi:10.1016/j.jpba.2008.02.025

probe's membrane and, consequently, on drug's relative recovery [6]. For hydrophilic drugs, the diffusion across the microdialysis membrane leads to equal values of relative recovery by dialysis and retrodialysis [7,8]. For moderately or highly lipophilic drugs ($\text{Log}\,P \approx 1$–4), however, retrodialysis and dialysis recoveries are not similar, probably due to drug binding to microdialysis probe tubing, making it difficult to determine the real recovery *in vivo* [6,9].

The first study showing recovery by dialysis and by retrodialysis could differ depending on the drug investigated was conducted by Groth and Jorgensen who observed that the degree of recovery *in vitro* was reduced proportionally to the increase in drug lipophilicity [6]. Nowadays, other authors have shown that lipophilicity is an important determinant of drug's binding to the microdialysis device [9,10].

As a consequence of drug's binding to the microdialysis probe phenomenon, the relative recoveries determined do not express the real drug's recovery from the medium, i.e., mass transfer in *sink* condition, but an apparent recovery composed by the analyte diffusion through the probe's membrane and its binding to the MD tubing. The apparent recovery determined restricts the use of microdialysis for moderately and highly lipophilic compounds.

Some alternatives have been proposed in the literature to overcome this limitation and allow the use of microdialysis for lipophilic compounds, such as the use of albumin in the perfusion fluid that appears to prevent drug binding to the probes tubing, after a time for saturation of the dialysis membrane is observed [10]. This approach, although has proven to be efficient in terms of eliminating the binding issue, seems to alter the composition of the perfusion fluid that no longer mimics the proteic composition of the extracellular fluid surrounding the probe, modifying the osmotic pressure and the physiology of the tissue under investigation [11].

Another approach to overcome the limitation of using microdialysis for lipophilic compounds is to mathematically model the drug's binding to the probe. Lindberger et al. [9] used a set of equations to separate the binding to the microdialysis tubing from the drug's diffusional process through the semi-permeable membrane and to obtain the real recovery from the apparent recovery determined experimentally.

Using an independent set of experiments the drug binding to the probe's tubing is determined experimentally and the results are expressed as binding coefficient, a constant that relates drug binding and perfusion fluid flow rate. By eliminating the binding component from the apparent recovery, determined by dialysis or retrodialysis, the diffusional part of the recovery, i.e., the real relative recovery is obtained.

For antifungal agents, lipophilicity is required to allow drug penetration into the fungal cell and promote the killing effect. The knowledge of tissue penetration of these compounds is crucial for determining their efficacy. The *Echinocandin caspofugin* was evaluated by microdialysis by Traunmüller et al. [10]. The authors observed significant differences between the recoveries determined *in vitro* by dialysis and retrodialysis, probably due to drug binding to the probe's tubing. The recoveries were equalized by adding albumin to the perfusion fluid.

Voriconazole is a triazolic compound introduced in the clinical practice recently as a therapeutic alternative to treat deep fungal infections such as invasive aspergilosis and hematogenous candidiasis, which usually disseminate to kidney, liver and brain. Deep fungal infections are the fourth most common cause of nosocomial bloodstream infections in the US [12], responding for 40–60% of the deaths by fungal sepsis in Brazil [13].

In this context, the present study aimed to investigate the feasibility of using microdialysis to evaluate the tissue penetration of voriconazole, a moderately lipophilic compound ($\text{Log}\,D_{7.4} = 1.8$).

2. Materials and methods

2.1. Chemicals

Voriconazole was donated by Mikatub (India) as a powder (102.3% purity). Sodium chloride, calcium chloride, potassium chloride and ammonium monobasic phosphate were purchased from Reagen® (Brazil). Acetonitrile and methanol, HPLC grade, were purchased from Merck® (Germany). HPLC water from Millipore's Milli-Q system was used throughout the analysis.

2.2. Standard solutions preparation

Standard stock solution of voriconazole was prepared on the day of the study by dissolving the drug in methanol. The stock solution (500 μg/mL) was diluted in Ringer's solutions viewing to obtain different final concentrations for the calibration curve and microdialysis studies.

Ringer's solution contained: 148 mM Na^+, 2.3 mM Ca^{++}, 4 mM K^+, and 157 mM Cl^-.

2.3. VRC quantification

The determination of VRC in microdialysate samples was conducted using a HPLC method adapted from previously published work [14]. The HPLC system consisted of a Waters® 600 pump, a Waters® UV 2487 dual λ absorbance detector, a Waters® Plus 717 autosampler and a Waters® 746 integrator. The mobile phase, acetonitrile:ammonium monobasic phosphate buffer (60:40, v/v, pH 6.0), was pumped at a flow rate of 1 μL/min. A Shimadzu® C18 reverse phase column (4 mm × 150 mm) preceeded by a pre-column filled with the same packing material was used as stationary phase. VRC was detected at 254 nm.

A linear calibration curve was obtained in the range of 25–2500 ng/mL using drug peak area. The method was validated by performing three calibration curves on each of two consecutive days and by analyzing quality control samples (75, 1000 and 2000 ng/mL). VRC retention time was approximately 2.9 min. The intra-assay and inter-assay precision were bigger than 94.9 and 95.8%, respectively. The method showed accuracy bigger that 89.1%. The results obtained for VRC was within the acceptable limits stated for bioanalytical methods validation [15].

The microdialysate samples obtained were directly injected by the autosampler (30 μL) without previous preparation. All samples were kept frozen at −20 °C until analysis by HPLC.

2.4. Microdialysis system

The microdialysis system consisted of a syringe infusion pump, MD-1001 Baby Bee Syringe Drive-connected to a MD-1020 Bee Hive Controller (Bioanalytical, USA). A microliter syringe (1 mL, gas-tight) was used to provide the perfusate solution. CMA/20 microdialysis probes (membrane length: 4 mm, cutoff: 20 kDa, CMA/Microdialysis AB, Sweden) were employed in this study.

2.5. Microdialysis experiments

The microdialysis experiments were carried out to investigate VRC binding effect to the microdialysis device (inlet and outlet tubing), as well to investigate flow rate and concentration influence

on the relative recovery determined by dialysis and retrodialysis methods.

2.5.1. Influence of perfusion flow rate and VRC concentration on in vitro recovery

The influence of flow rate on the relative recovery of voriconazole was evaluated using five distinct flow rates: 1, 2, 3, 4 and 5 μL/min.

For the determination of VRC *in vitro* recovery by retrodialysis the probes ($n = 3$), continuously perfused with Ringer's solutions containing voriconazole 1 μg/mL, were inserted into tubes containing Ringer's solution devoid of drug, kept at 37 ± 1 °C. The system was allowed to equilibrate for 1 h and, 30 min interval samples were collected up to 2 h. For determination of recovery by dialysis similar procedure was used, but the probes were perfused with Ringer's solution devoid of drug and the medium surrounding the probes consisted of voriconazole 1 μg/mL in Ringer.

For the determination of VRC concentration influence on recoveries the flow rate was fixed at 2 μL/min and four different VRC concentrations were used to determine the recovery by retrodialysis and dialysis: 0.1, 0.5, 1.0 and 2.0 μg/mL. The systems were allowed to equilibrate for 1 h before 30 min interval samples were collected up to 2 h.

All experiments were conducted in triplicate using different microdialysis probes ($n = 3$).

The relative recovery by dialysis (RR_D) was calculated as [1]:

$$RR_D\,(\%) = \left(\frac{C_{dial}}{C_{ext}} \times 100\right) \quad (1)$$

where C_{dial} is the drug concentration on the dialysate and C_{ext} is the drug concentration on the medium surrounding the microdialysis probe.

The relative recovery by retrodialysis (RR_{RD}) was calculated as [1]

$$RR_{RD}\,(\%) = \left(\frac{C_{perf} - C_{dial}}{C_{perf}} \times 100\right) \quad (2)$$

where C_{perf} is the drug concentration in the perfusate solution.

2.5.2. Binding experiments in the inlet/outlet tubing

To investigate the drug's binding to the microdialysis device, three polyurethane inlet/outlet tubing, with a length of 200 mm, were separated from the probes dialysis membranes and perfused with 1 μg/mL of voriconazole solution at flow rates of 1, 2, 3, 4 and 5.0 μL/min. This experiment was conducted twice.

Binding (B) to the tubing was expressed as the proportion of drug lost during perfusion thought the tube [9]:

$$B = 1 - \frac{C(l_b = L_b)}{C(l_b = 0)} \quad (3)$$

where C is the drug concentration, l_b is the position variable along the tubing, expressed in mm, and L_b is length of tubing, also expressed in mm.

After the establishment of the tubing's binding, the drug-binding coefficient (K_b) was calculated by the relationship [9]:

$$K_b = \frac{-\ln(1 - B)\phi}{L_b} \quad (4)$$

where ϕ is the flow rate.

When drug binds to the tubing the apparent recovery determined is the product of binding and dialysis process that are independent but additive phenomena. In this case, the determination of K_b is crucial for the establishment of the real value of drug's diffusional mass transfer coefficient (K_d), which is employed to correct the apparent recoveries determined *in vitro*.

2.5.3. Determination of diffusional mass transfer coefficient (K_d) in different flow rates

After the establishment of the drug binding coefficient (K_b) and the apparent recovery (R_{app}) by dialysis and retrodialysis for the different flow rates investigated, the diffusional mass transfer coefficient (K_d), expressed in mm^2/min, was calculated using Eq. (5) [9]:

$$R_{app} = \left[1 - \exp\left(\frac{-K_d L_d}{\phi}\right)\right] \exp(1 - B) \quad (5)$$

where R_{app} is the apparent recovery (determined experimentally) and L_d is the length of dialysis membrane, expressed in mm.

By the determination of R_{app}, K_d and K_b constants it was possible to calculate the real relative recoveries determined *in vitro* and *in vivo*. VRC binding (B) to the outlet tubing was considered negligible and no correction was used for the recovery determined by dialysis. The binding to the inlet tubing (B) was used to determine the real perfusion concentration (C_{perf}) in order to calculate the real recovery by retrodialysis.

2.5.4. In vivo recovery

The experiments involving animals were approved by UFRGS Ethics in Research Committee (protocol #2004300). Specific-pathogen-free male Wistar rats, weighting 200–250 were used for all studies. Animals were maintained in accordance with the criteria of the Canadian Council of Animal Care [16].

VRC *in vivo* recovery was determined by retrodialysis. Three male Wistar rats were anesthetized with uretane (1.25 g/kg i.p.). After anesthesia, the animals were put in the lateral decubitus position. Skin was surgically removed, the kidneys were exposed and the probes were inserted into the kidney cortex. The microdialysis probes were perfused with Ringer's solution at a flow rate of 2.0 μL/min and were allowed to equilibrate inside the kidneys for 1 h. After equilibration, plain Ringer's solution was replaced by Ringer's solutions containing VRC 1 μg/mL, which was perfused at the same flow rate. Microdialysate samples were collected from each probe in 30 min intervals, up to 2 h, after 1 h equilibration. Drug concentrations in the dialysate sample (C_{dial}) and in the perfusate solution (C_{perf}) were determined by HPLC.

The *in vivo* apparent recovery by retrodialysis (R_{app}) was calculated using Eq. (2). For the flow rate used in the *in vivo* experiments (2 μL/min) VRC binding (B) to the outlet tubing was considered negligible and no correction was used. The binding to the inlet tubing (B) determined *in vitro* was used to calculate the real perfusion concentration (C_{perf}) in order to estimate the real *in vivo* recovery by retrodialysis.

2.5.5. Determination of free renal concentrations of VRC in rats

Two groups of six animals were used. After anesthesia with urethane (1.25 g/kg i.p.) the animals had the carotid artery catheterized for blood sampling. MD probes were inserted into the kidney cortex as described previously and allowed to equilibrate for 1 h. VRC 40 or 60 mg/kg was administered orally by gavage to each group. Blood (200 μL) and microdialysate samples (60 μL, 30 min intervals) were collected at pre-determined time-points up to 18 h. Plasma was separated, frozen and stored at −20 °C until assayed by an LC–MS/MS validate method [15]. MD samples were frozen and assayed by HPLC.

The total plasma and free kidney concentrations *versus* time profiles were analyzed individually. The $AUC_{0-18\ plasma}$ and $AUC_{0-18\ kidney}$ were calculated by trapezoidal rule, employing Excel® v. 2000 software (Microsoft®). The ratio $AUC_{kidney}/AUC_{plasma}$ was used as a measure of drug penetration into the organ.

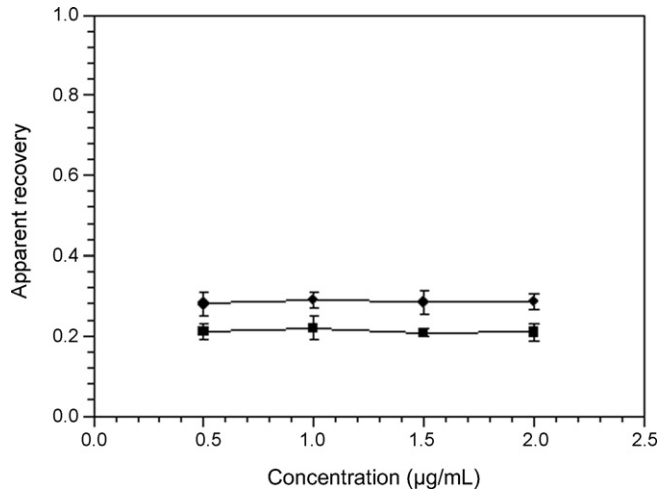

Fig. 1. Concentration effect on VRC relative recovery by dialysis (■) and retrodialysis (●) (average ± S.D.) (n = 3 probes).

2.6. Statistical analysis

The relative recoveries determined by dialysis and retrodialysis were compared by two-way analysis of variance (ANOVA), where flow and recovery method were considered as factors. The differences were determined by Bonferroni test (α = 0.05). The AUCs were compared by one-way ANOVA (α = 0.05).

3. Results

The investigation of VRC concentration influence on MD probes recovery showed that the recoveries determined by retrodialysis and dialysis *in vitro* are concentration independent on the range investigated (Fig. 1). The recoveries determined by each method, however, were statistically different. The average relative recovery determined by dialysis was 21.1 ± 1.5%, while the average recovery by retrodialysis was 28.7 ± 2.0%, under the same experimental conditions.

The *in vitro* recoveries determined by dialysis and retrodialysis were different from each other and flow rate dependent, as shown in Fig. 2, decreasing with the increase of flow rate in both methods. For the slower flow rates (1 and 2 μL/min) the difference between

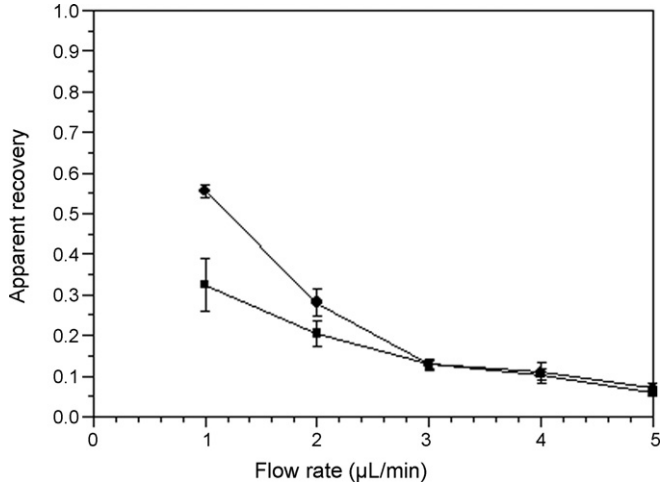

Fig. 2. Recoveries determined by dialysis (■) and retrodialysis (●) for different flow rates (n = 3).

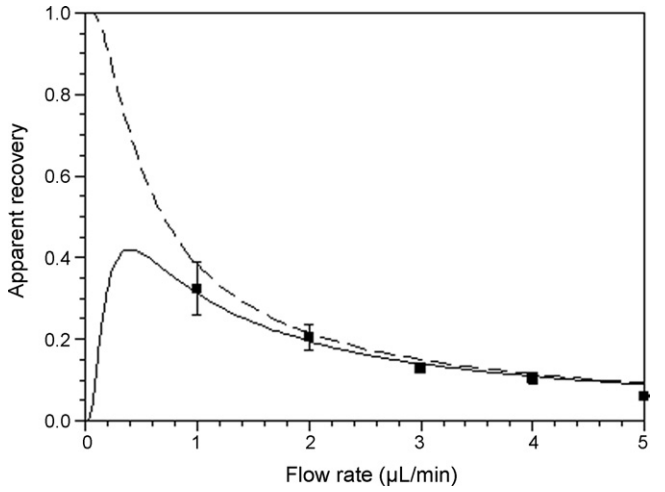

Fig. 3. *In vitro* apparent recoveries (R_{app}) determined experimentally (■) by dialysis method (average ± S.D.), and the values modeled by Eq. (5) using mean value of K_d (0.122 mm²/min) and K_b (0.0011 mm²/min) are described by solid line (—). Real recoveries, assuming no binding to the microdialysis device are shown by hatched line (- - -).

dialysis and retrodialysis recoveries was statistically significant (p < 0.001). For flow rates higher than 3 μL/min, no differences in recoveries for the two methods investigated were observed.

Voriconazole binds to the inlet and the outlet tubing devices of the microdialysis probes. The values of the binding coefficient (K_b) determined for each flow rate evaluated are shown in Table 1. The most important binding phenomenon was observed in the inlet tubing, which showed an average K_b of 0.0023 ± 0.0009 mm²/min, twice the average K_b observed for the outlet tubing (0.0011 ± 0.0004 mm²/min).

The influence of flow rate on the relative recovery was modeled by Eq. (5), taking the binding effect to the outlet tubing under consideration. The mean K_b in the outlet tubing (0.0011 ± 0.0004 mm²/min) was used in Eq. (5) to determine the diffusion mass transfer coefficient (K_d). The mean value of K_d was determined to be 0.122 ± 0.0133 mm²/min. The apparent recoveries (R_{app}) determined experimentally for the different flow rates investigated by dialysis and the real recoveries calculated using Eq. (5), considering no VRC binding to the probe's tubing are shown in Fig. 3.

VRC *in vivo* apparent recovery (R_{app}) determined by retrodialysis was 38.5 ± 3.5%. This recovery, however, cannot be used to determine the free fraction of VRC in tissue because the binding to the tube device observed *in vitro* has to be considered. To take binding into account, the percentage of inlet binding determined for the 2 μL/min flow rate (17.9%) was used to calculate the real perfused concentration (C_{perf}) in the *in vivo* experiment. In this way, the real relative recovery was determined to be 25.1 ± 2.8%, similar to the real recovery determined by retrodialysis *in vitro* using the same flow rate and approach, 23.1 ± 5.4% (α = 0.05). The real *in vivo* average recovery was used to back calculate VRC tissue levels in the animal experiments.

Fig. 4 shows the total plasma and free kidney profiles observed after VRC 40 and 60 mg/kg oral dosing. The pharmacokinetic parameters determined in plasma and tissues are presented in Table 2. The drug penetration calculated by the ratio $AUC_{0-18\ kidney}/AUC_{0-18\ plasma}$ was around 0.33–0.34, similar to the free fraction of voriconazole in rat plasma, reported to be 0.34 [17].

4. Discussion

Microdialysis is a very useful technique to access real tissue concentrations of drugs or endogenous compounds *in vivo*. Given that

Table 1
Results of *in vitro* voriconazole (1 μg/mL) binding experiments after perfusion of tubing (200 mm) with a at different perfusion rates

Flow (μL/min)	1	2	3	4	5
Inlet tubing					
Binding (*B*)	0.357 ± 0.166	0.179 ± 0.036	0.135 ± 0.049	0.132 ± 0.047	0.125 ± 0.010
K_b (mm²/min)	0.0023 ± 0.0009	0.0022 ± 0.0015	0.0022 ± 0.0009	0.0029 ± 0.0011	0.0027 ± 0.0002
Outlet tubing					
Binding (*B*)	0.193 ± 0.037	0.068 ± 0.016	0.074 ± 0.012	0.075 ± 0.015	0.075 ± 0.007
K_b (mm²/min)	0.0011 ± 0.0002	0.0007 ± 0.0002	0.0011 ± 0.0002	0.0015 ± 0.0003	0.0019 ± 0.0002

Three samples were collected from each tubing at each flow rate ($n = 6$). *B*, proportion of voriconazole bound to the tubing; K_b, calculated binding coefficient for voriconazole.

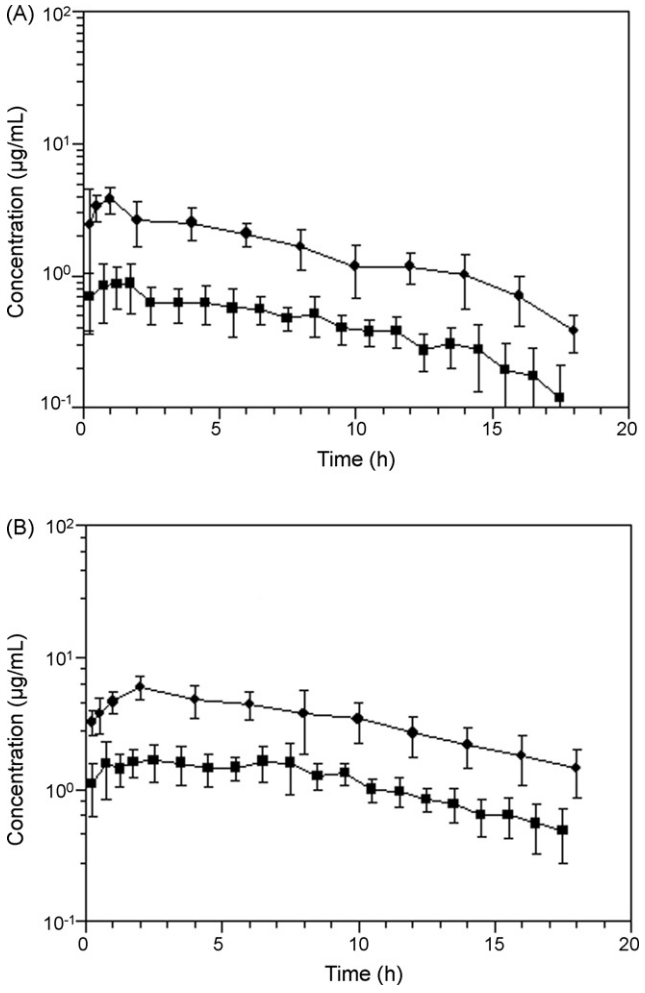

Fig. 4. Total plasma (●) and free kidney (■) levels of voriconazole after oral administration of 40 (A) and 60 (B) mg/kg to healthy Wistar male rats ($n = 6$/group) (average ± S.D.).

the probe is continuously flushed by the perfusate solution, the equilibrium between drug concentration in the tissue surrounding the probe and drug concentration in the internal medium is never reached, resulting in lower levels of the drug in the dialysate in comparison to the real levels in tissue, leading to the need of establishing probes recovery. The relative recoveries *in vitro* and *in vivo* can be determined by different methods, including dialysis and retrodialysis [3,4].

Drug's bindings to the microdialysis tubing have been reported for lipophilic drugs such as bethametasone dipropionate [6], calcipotriol [6], caspofungin [10] and antiepileptic drugs as phenytoin, carbamazepine and phenobarbital [9]. In the cases where the drug binds to the microdialysis device, the binding should be predictable and acceptably low in order to allow the use of this technique to determine the drug free tissue levels *in vivo*.

In the present work the feasibility of using microdialysis to determine free renal levels of the antifungal agent voriconazole was investigated. Relative recoveries determined by dialysis and retrodialysis were compared and the reason for the differences observed were investigated and mathematically analyzed.

Initially, the influence of VRC concentration on the relative recovery by both methods was investigated because *in vivo* tissue concentrations change over time and it is essential to confirm that the recovery is not affected by these fluctuating concentrations. The four concentrations investigated were selected based on the free plasma levels determined in rats after 30 mg/kg oral dosing [17]. VRC recoveries were not concentration-dependent regardless of the recovery method used. For a 2 μL/min flow rate, the average apparent recovery by dialysis was 21.1 ± 1.5% and by retrodialysis it was higher, 28.7 ± 2.0%. The ratio between retrodialysis and dialysis recoveries was constant for all concentrations evaluated (Fig. 1), showing that the recovery will remain constant during VRC pharmacokinetic profiling in tissue.

Dialysis and retrodialysis recoveries were influenced by the perfusion fluid flow rate used. The inverse effect of flow rate on the relative recoveries of drugs is extensively demonstrated in the literature [1–4] and is expected for hydrophilic and lipophilic drugs. Furthermore, the recoveries differ depending on the method used for its determination, being the gap less pronounced with increasing the flow rate (Fig. 2). VRC binding to the microdialysis tubing could explain the bigger recoveries observed by retrodialysis. Lindberger et al. reported that, for lipophilic compounds which bind to the microdialysis probes tubing, the influence of unspecific binding will be less pronounced when high flow rates are used because the contact time of the substance with the system tubing will be decreased [9]. The results obtained for voriconazole in the present study support this statement. For the lower flow rates investigated (1 and 2 μL/min), the differences between recoveries by dialysis and retrodialysis was more pronounced than those observed for higher flow rates (3, 4 and 5 μL/min), where similar recoveries were determined.

The hypothesis of VRC binding to the microdialysis device was confirmed by the tubing binding experiments. From Table 1 it is possible to verify that for lower flow rates, 1 and 2 μL/min, VRC binding to the inlet tubing was 35.7% and 17.9% and to the outlet tubing it was 19.3% and 6.8%, respectively. For flow rates higher than 3 μL/min binding was constant and two times more pronounced

Table 2
VRC pharmacokinetic parameters determined after oral administration of 40 or 60 mg/kg to Wistar rats ($n = 6$/group) (average ± S.D.)

Parameters	40 mg/kg	60 mg/kg
$AUC_{0-18\ plasma}$ (μg h/mL)	45.6 ± 10.8	77.0 ± 9.0
$AUC_{0-18\ kidney}$ (μg h/mL)	15.1 ± 3.1	26.2 ± 3.9
Kidney penetration	0.33 ± 0.03	0.34 ± 0.09

in the inlet tubing (13.3%), compared to the outlet tubing (7.5%). This finding showed that the drug binds to the plastic tubing of the microdialysis system, before and after the semi-permeable membrane, leading to an apparent recovery by retrodialysis bigger than that determined by dialysis. The increased recovery by retrodialysis does not represent higher drug diffusion from inside to outside the membrane but the drug's adherence to the tubing walls. For the flow rates higher than 2 μL/min the outlet binding was statistically similar probably because the contact time between the drug and the binding sites is decreased. The same principle holds true for the binding to the inlet tubing.

It can also be observed from Table 1 that the binding to the inlet and outlet tubing was different for the same flow rate considered although the polymer (polyurethane) and length of the tubing are the same. The only plausible hypothesis to explain these differences is that the adjutants used to prepare the blue inlet tubing are different from those used to prepare the transparent outlet tubing, resulting in the differences observed for VRC binding.

Although at 3 μL/min there is no difference between recovery by dialysis and by retrodialysis, the recovery is too small, around 13%, making it impossible to determine the free renal levels of voriconazole after 40 mg/kg oral dosing to rats due to the sensitivity of the HPLC method used. For this reason the flow rate selected for the *in vivo* experiments was 2 μL/min.

The minimal influence of VRC binding to the outlet tubing can be observed in Fig. 3, where the experimental data seems to be described by both curves the one that shows the apparent recovery and the one where the recovery was corrected for binding. Because the binding to the outlet tubing (∼7%) had a little impact on the recovery, it was neglected when the real recovery was calculated. The influence of VRC binding to the inlet tubing on relative recoveries by retrodialysis was overcome by using the perfusion concentration taking into account the tubes binding.

Assuming VRC's binding to the microdialysis probe is the same whether the recovery is determined *in vitro* or *in vivo*, retrodialysis was used to determine the probes recovery *in vivo*. The *in vivo* apparent recovery was found to be 38.5 ± 3.5%, similar to those determined *in vitro* using the same method (28.1 ± 5.0%) ($\alpha = 0.05$). The similarity between the recoveries observed for VRC in kidney and *in vitro* could be due to the high perfusion of this organ and also its histological characteristics, considering that kidney cortex is a soft and very loose tissue. For the determination of free VRC levels in renal tissue the *in vivo* apparent recovery was corrected by the binding constant (K_b). VRC recovery *in vivo* after correction was 25.1 ± 2.8%.

Using the real *in vivo* recovery of the antifungal, VRC penetration into the renal cortex was calculated by the ratio between $AUC_{0-18\ kidney}$ and $AUC_{0-18\ plasma}$. VRC penetration, independently of the dose investigated, was similar to the plasma free fraction reported in the literature for rats (0.34) [17]. This finding indicates that unbound VRC concentrations in plasma and renal tissue are comparable, and the free plasma levels provide a good estimate of the interstitial drug concentrations.

5. Conclusions

The experimental results indicated that voriconazole is a suitable drug to be evaluated by microdialysis, despite its moderate lipophilicity. Employing the mathematical modeling it was possible to account for the drug's binding to the probes tubing and to correct the apparent relative recoveries determined *in vitro* and *in vivo* allowing the investigation of free renal levels of voriconazole in healthy rats after administration of 40 and 60 mg/kg doses. VRC free renal and plasma concentrations were similar indicating that free plasma levels can be used to estimate the drug concentrations at the biophase.

Acknowledgements

The authors acknowledge CNPq-Brazil for financial support (# 473317/2004-4) as well as for the individual grants received. The authors wish to thank Roberto Debom from Cristália® (Brazil) for helping with the donation of voriconazole.

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