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Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 807–813
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

In vitro microdialysis sampling of docetaxel

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Received 13 April 2004; received in revised form 25 June 2004; accepted 4 July 2004
Available online 25 August 2004

Abstract

Microdialysis is a technique that allows sampling compounds from the extracellular fluid in different tissues, such as muscle, lung, and brain. However, the feasibility of using this technique with lipopohilic and high molecular weight compounds has been questioned, since these compounds are less likely to diffuse through the dialysis membrane. Therefore, it was the objective of this study to investigate the feasibility of doing microdialysis of docetaxel by determining its recovery by the microdialysis probe. Three different methods were investigated: extraction efficiency, retrodialysis, and no-net-flux. For the first two methods, three different concentrations were tested: 2.5, 5, and 9 mg/l. The recovery obtained for each concentration was 49.3 ± 6.7 (n = 4), 44.6 ± 5.4 (n = 3), and 34.7 ± 2.1 (n = 4) by extraction efficiency, and 53.4 ± 7.9 (n = 3), 61.4 ± 7.6 (n = 3), and 64.2 ± 1.9 (n = 3) by retrodialysis, respectively. The average recovery obtained by no-net-flux was 68.7 ± 9.6 (n = 5). Although it has been reported that microdialysis cannot be applied to lipophilic compounds, the results here show the opposite. The high recoveries obtained for docetaxel in all methods applied show that the compound can diffuse through the probe membrane. Overall, docetaxel seems to be very suitable for microdialysis despite its lipophilicity and high molecular weight.

Keywords: Microdialysis; Docetaxel; Lipophilic drugs; Recovery; Probe calibration

1. Introduction

Microdialysis is a technique that has been used for sampling compounds from the extracellular fluid in different tissues, such as muscle, brain, and lung, either in animals or humans [1]. It has been used to study different drugs, such as antibiotics, anti-inflammatory, and psychoactive compounds [2,3].

The feasibility of doing microdialysis of different analytes depends on the physical chemical characteristics of the compound. Lipophilic as well as high molecular weight compounds have been reported to be less likely to diffuse through the probe membrane and, therefore, may not be feasible for microdialysis [4]. Since the diffusion through the microdialysis membrane follows Fick's law, factors such as partition coefficient, particle size, and surface area of the compound will affect the drug permeability through the membrane [4,5]. Molecules with high molecular weight tend to have a lower diffusion coefficients through the dialysis membrane, which results in a decreased recovery [4]. Low recoveries observed for lipophilic compounds are also attributed to the solubility of the compound in the hydrophilic perfusate media, nonspecific binding to the probe, and high protein binding [4,6,7].

The fact that microdialysis could not be used for lipophilic compounds would limit its application for an important class of drugs. Currently, there are many drugs used in therapeutics that have high molecular weight and lipophilic characteristics, as steroids and anticancer drugs. Amongst those type of drugs is docetaxel, an anticancer drug used in the treatment of breast, ovarian, and non-small-cell lung tumors. Docetaxel (molecular weight of 807.9) is a semi synthetic analog of paclitaxel. Its chemical structure is composed of a bulky, extended fused ring with several hydrophobic substitutes that provide its lipophilicity (log P = 3) and poor water solubility (Fig. 1) [8,9]. The possibility of using microdialysis to study the distribution profile of this compound would allow one to investigate the drug's penetration into a tumor tissue,

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doi:10.1016/j.jpba.2004.07.007

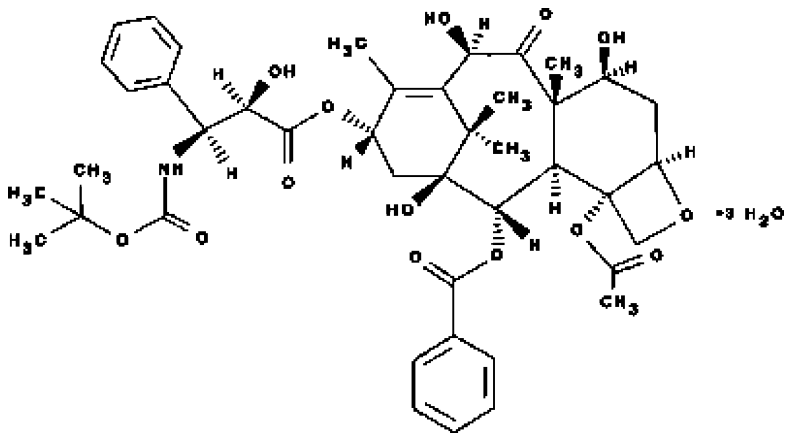

Fig. 1. Docetaxel chemical structure.

which has different physiological characteristics compared to normal tissue.

Therefore, it was the objective of this study to investigate the feasibility of doing microdialysis of docetaxel, a very lipophilic compound, by determining the in vitro recovery of this compound by the microdialysis probe.

2. Material and methods

2.1. Chemicals

Docetaxel reference standard was obtained from Aventis. Paclitaxel was purchased from Sigma-Aldrich. Both compounds were stored at 4 °C in amber containers.

Acetonitrile and methanol (HPLC grade) and phosphoric acid 85% used in the HPLC analysis were purchased from Fisher (Springfield). Lactated Ringer's solution USP was purchased from Abbott and used in the microdialysis experiments. Ethanol 70% was obtained from LabChem Inc. Human plasma was obtained from Civitan Lab.

2.2. Standards in lactated Ringer's solution

A stock solution of 100.0 mg/l of docetaxel in methanol (stock solution A) was used to prepare the standard curve and quality controls (QC) in lactated Ringer's solution for analysis of the microdialysis samples. The stock solution A was stored at −20 °C for up to 6 months [10]. The standards for the calibration curve were prepared every analysis day by diluting the stock solution A with lactated Ringer's solution to obtain a concentration of 10.0 mg/l. This solution was further diluted with lactated Ringer's solution in order to achieve the following final concentrations: 0.5, 1.0, 3.0, 5.0, and 7.0 mg/l. The quality control standards were prepared by diluting stock solution A with lactated Ringer's solution in order to obtain the final concentrations of 0.8 (QC1), 4.0 (QC2), and 9.0 mg/l (QC3).

The standards in lactated Ringer's solutions were directly injected into the HPLC/UV system described below.

2.3. Standards in plasma

A stock solution of 1 mg/ml of docetaxel in methanol (stock solution B) was used to spike plasma and prepare the standard curve and QCs for the analysis of the plasma samples from the no-net-flux experiment. Stock solution B was stored at −20 °C when not in use. The standards for the calibration curve were prepared every analysis day by diluting the stock solution B with plasma to get a concentration of 100.0 mg/l (stock solution B1). This plasma solution was further diluted with plasma to achieve the following final concentrations: 10, 25, 50, and 75 mg/l. The QC standards were prepared by making another dilution of stock solution B with plasma to get the concentration of 100.0 mg/l (stock solution B2). Stock solution B2 was further diluted with plasma in order to obtain the final concentrations of 15.0, 35.0, and 60.0 mg/l (QC1, QC2, and QC3, respectively).

The plasma solution used in the microdialysis experiment (no-net-flux) was prepared by spiking 7 ml of plasma with docetaxel stock solution B to obtain the final total concentration of 62.5 mg/l.

The plasma standards as well as the plasma samples obtained in the no-net-flux experiment were extracted by solid phase extraction before injecting into the HPLC/UV system described below.

2.4. Solid phase extraction (SPE)

The plasma standards were extracted by a solid phase extraction (SPE) method [11]. A SPE column LC18 6 ml 0.5G (Supelclean®) was used for the plasma extractions. A 50-μl aliquot of internal standard (IS) paclitaxel (50 mg/l in methanol) was added to 200 μl of plasma spiked with docetaxel standard as described above. The plasma was diluted with 1 ml of an acetonitrile:water (30:70) solution. The solution was vortex and centrifuged at 3500 rpm for 15 min. One milliliter of the supernatant was used in the extraction.

The SPE columns were mounted in a Visiprep Solid Phase Extraction Vacuum Manifolds and conditioned with two 1.5-

ml aliquot of acetonitrile followed by two 1.5-ml aliquot of water. The plasma standards and samples were then added to the SPE columns. Afterwards, the columns were washed with two 1.5-ml aliquots of water. Docetaxel and the IS were eluted twice from the column with 2 ml of acetonitrile and collected in a glass tube. The acetonitrile solutions eluted from the column were evaporated to dryness in a vacuum centrifuge (Jouan Inc.). The dried residues were reconstituted with 200 μl of mobile phase and a volume of 25 μl was injected into the HPLC system.

2.5. HPLC system

The standard solutions prepared in lactated Ringer's solution and the microdialysis samples were analyzed by an HPLC system consisted of a ConstaMetric IIIG LDC pump, a spectromonitor LDC analytical 3200 set to 225 nm, an HP 3396 integrator, and a Perkin Elmer Serie 200 autosampler. A 25 μl sample was injected onto an Inertisil ODS-2 column (150 mm × 4.6 mm, 5 μm), connected to a guard column filled with Pellicular C18 material (30–40 μm), at a flow rate of 1 ml/min. The mobile phase consisted of 0.3% phosphoric acid:methanol (32.5:67.5).

The same HPLC system was used for the analysis of the standards prepared in plasma and for the plasma samples. However, the column used for the analysis was a Discovery C18 reversed phase column (250 mm × 4.6 mm, 5 μm) from Supelco (Bellefonte, PA). A different column was used in the plasma analysis in order to improve the separation of the IS paclitaxel and the standard docetaxel, which was not good with a 150 mm × 4.6 mm column.

2.6. Microdialysis system

A CMA/20 microdialysis probe (CMA Microdialysis, Stockholm), with a membrane length of 10 mm and molecular cutoff of 20 kDa, was used in this study. The probe was connected to a 1000 μl gastight syringe by a catheter connector (BBraun). A microinfusion pump (Harvard apparatus, model 22, South Natick, MA) was used to keep the flow constant through the probe.

2.7. Microdialysis experiments

The in vitro recovery of docetaxel was determined by three different methods: extraction efficiency (EE), retrodialysis (RD), and no-net-flux (NNF). All methods were carried out at 37 °C. Each procedure is described in the following sections.

2.7.1. Extraction efficiency method (EE)

In this experiment, blank lactated Ringer's solution was pumped through the microdialysis probe, which was placed into a glass tube filled with approximately 4 ml of drug solution prepared in lactated Ringer's solution. Three different docetaxel concentrations were tested in this experiment, 2.5, 5, and 9 mg/l. The flow through the membrane was initially set to 5 μl/min for 10 min, and afterwards changed to 1.5 μl/min for 1.5 h (equilibration period). Subsequently, dialysate samples were collected every 25 min. A total of five samples were collected for each experiment, and a total of three to four replicates were performed for each concentration. The concentration of docetaxel in the dialysates and in the tube before and after the experiments was determined by the HPLC/UV method described above. The probe recovery determined by the extraction efficiency method was calculated by the equation:

$$R\,(\%) = \frac{C_{\text{out}}}{C_{\text{sol}}} \times 100 \quad (1)$$

where R (%) is the recovery in percentage; C_{out} the concentration in the dialysate; and C_{sol} the average drug concentration in the tube before and after the experiment.

2.7.2. Retrodialysis method (RD)

In the retrodialysis experiment the probe was placed into a blank lactated Ringer's solution and drug solution was pumped through the probe. The same equilibration period as in the EE method was followed and, after it, a total of 10 dialysate samples were collected every 25 min. A total of three replicates were performed for each concentration tested (2.5, 5, and 9 μg/ml). The drug concentration in the microdialysis samples as well as in the syringe (perfusate) before and after the experiment were determined by HPLC/UV.

The recovery in this experiment was calculated by the equation:

$$R\,(\%) = \frac{C_{\text{in}} - C_{\text{out}}}{C_{\text{in}}} \times 100 \quad (2)$$

where R (%) is the recovery in percentage; C_{in} the average concentration in the perfusate before and after the experiment; and C_{out} the concentration in the dilaysate.

2.7.3. No-net-flux method (NNF)

In the NNF experiment the microdialysis probe was placed into a plasma solution containing 62.5 mg/l of docetaxel. The microdialysis probe was perfused, in a sequence, by three docetaxel solutions (2.5, 5, and 10 mg/l), all prepared in lactated Ringer's solution. An equilibration time of 10 min at 5 μl/min followed by 3 h at 1.5 μl/min was allowed before the first dialysate sample was collected for the initial concentration of 2.5 μg/ml. For every change in the perfusate concentration the probe was allowed to re-equilibrate with the new concentration for 10 min at 5 μl/min followed by 1.5 h at 1.5 μl/min. A total of three dialysate samples were collected for each concentration. The drug concentration in the syringe (perfusate) before and after the experiment was also determined. A total of five replicates were performed.

Plasma samples from the tube were also collected before starting the NNF experiment, at the end, and in every perfusate concentration change. The plasma samples were extracted by SPE and analyzed by the HPLC/UV method described before.

The recovery was determined by plotting the net change in the docetaxel perfusate and dialysate concentrations versus the perfusate concentration. The slope of the curve represents the recovery and the intercept of the curve represents the point of no-net-flux, which is equal to the free docetaxel concentration in the plasma solution.

3. Results

3.1. Analytical methods

The analytical method developed for the plasma samples showed to be linear at the range 10–75 mg/l (r^2 = 0.999). The mean regression curve was $y = 0.0202x - 0.0243$ (x: docetaxel concentration, y: standard to internal standard peak height ratio). The lowest concentration of the standard curve showed a coefficient of variation (CV (%)) within day below 1.5% and was accepted as the lowest limit of quantification of the method. The recovery of the extraction method was determined by comparing the peak heights of four standard concentrations prepared in mobile phase with those obtained for the standards prepared in plasma and extracted by SPE. The SPE method showed an average recovery of 91.7 ± 9% (n = 6 for each concentration tested). The intra and inter day precision was determined by the CV (%) obtained after injecting three times four concentrations representing the entire range of the standard curve. The intra day precision ranged from 0.2 to 1.8% and the inter day precision ranged from 8.3 to 12%. The assay accuracy was determined by comparing the nominal QC concentration to the concentration measured using the standard curve. The accuracy of the method ranged from 80.9 to 106.5% for all three concentrations tested.

The analytical method developed for the microdialysis samples was validated as described for the plasma samples. The method showed to be linear in the concentration range of 0.5–10 mg/l (r^2 = 0.998). The mean regression curve was $y = 6802x - 1529$ (x: docetaxel concentration, y: peak height). The lower concentration of the curve was accepted as the lower quantification limit since it showed a CV (%) within 15%. The intra day precision ranged from 0.6 to 12.1% and the inter day precision ranged from 3.6 to 12.3%, with higher variability observed for the lower concentration of 0.5 mg/l. The accuracy of the method ranged between 83.6 and 112.7%, with lower accuracy observed for the lower QC (0.8 mg/l).

The two methods applied for the analysis of docetaxel showed to be within an acceptable range for precision and accuracy and, therefore, were accepted for analysis [12].

3.2. Microdialysis

3.2.1. Extraction efficiency method (EE)

The average perfusate concentration measured before and after each experiment as well as the recovery obtained for each experiment is depicted in Table 1. The average recovery calculated by applying Eq. (1) was 49.3 ± 6.7%, 45.1 ± 4.7%, and 38.5 ± 2.1% for the concentrations of 2.5, 5, and 9 mg/l, respectively.

3.2.2. Retrodialysis method (RD)

The average perfusate concentrations measured before and after the experiment are depicted in Table 1 for all three concentrations tested. The recovery, calculated for each concentration by applying Eq. (2), is also described in Table 1. The recovery ranged from 53.4 to 64.2% for all three concentrations tested.

3.2.3. No-net-flux (NNF)

The net change in the dialysate and perfusate concentrations was plotted against the perfusate concentration. The

Table 1
Average docetaxel concentration, measured before and after each extraction efficiency (EE) and retrodialysis (RD) experiments, and the respective average recovery calculated for each concentration

EE			RD		
Averaged measured concentration[a] (mg/l) ± S.D.[b]	Intra day R (%) ± S.D.	Average R (%)	Averaged measured concentration[a] (mg/l) ± S.D.	Intra day R (%) ± S.D.	Average R (%) ± S.D.
2.0 ± 0.2	43.5 ± 4.9	49.3 ± 6.7	1.9 ± 0.1	46.5 ± 1.7	55.4 ± 8.0
2.3 ± 0.2	57.8 ± 2.5		2.2 ± 0.1	57.7 ± 6.4	
2.0 ± 0.3	44.3 ± 5.9		2.1 ± 0.3	61.9 ± 4.6	
2.1 ± 0.3	51.5 ± 5.3				
4.4 ± 0.6	47.3 ± 2.7	44.6 ± 5.4	4.8 ± 0.1	52.6 ± 1.8	61.4 ± 7.6
4.2 ± 0.2	38.4 ± 4.8		4.4 ± 0.1	66.2 ± 3.8	
4.0 ± 0.1	48.2 ± 5.9		4.5 ± 0.03	65.4 ± 3.7	
7.8 ± 1.1	34.6 ± 4	38.5 ± 6.3	8.5 ± 0.1	62.1[c] ± 3.6	64.2 ± 1.9
8.0 ± 0.5	34.3 ± 7		8.3 ± 0.2	65.2 ± 2	
7.8 ± 0.2	37.4 ± 7.2		9.5 ± 0.1	65.4 ± 1.2	
8.8 ± 0.7	47.7 ± 5.9				

[a] Average of the concentration measured before and after the experiment.
[b] Standard deviation.
[c] n = 4.

Table 2
Average perfusate concentration and plasma concentration obtained in the NNF experiments

Experiment	Perfusate concentration (mg/l)			Plasma samples (mg/l)			
	2.5	5	10	Initially	2.5–5	5–10	End
NNF-A	2.3 ± 0.2	4.4 ± 0.4	9.3 ± 0.9	54.7	54.4	55.0	39.0
NNF-B	2.4 ± 0.03	4.3 ± 0.0	9.2 ± 0.2	54.5	57.3	49.5	55.8
NNF-C	2.4 ± 0.01	4.8 ± 0.1	9.1 ± 0.7	62.1	46.2	41.3	45.5
NNF-D	2.4 ± 0.1	4.6 ± 0.03	10.0 ± 0.1	52.2	48.2	45.8	43.3
NNF-E	1.9 ± 0.1	3.8 ± 0.0	7.7 ± 0.01	52.1	52.8	44.3	41.2

The plasma samples were obtained every time the perfusate concentration was changed to a higher concentration.

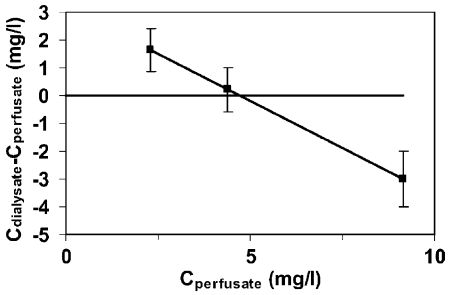

Fig. 2. Plot of the net change in the concentration between perfusate and dialysate versus the perfusate concentration. The slope of the curve represents the recovery and the intercept with the *x*-axis represents the free plasma concentration in the vial ($y = -0.679x + 3.187$; $r^2 = 0.9999$).

slope of the regression line represents the drug recovery by the probe. The recovery obtained by this method ranged from 54.5 to 79.6%. The average curve obtained in all five NNF experiments performed is shown in Fig. 2 ($y = 0.679x + 3.187$, $r^2 = 0.9999$). The average recovery obtained by this method was 67.9 ± 9.6% ($n = 5$). The point where the line crosses the *x*-axis represents the free plasma concentration inside the vial. The average free plasma concentration, calculated by the regression line obtained in each NNF experiment, was 4.7 ± 1.1 mg/l. The measured total plasma concentrations of the samples from the no-net-flux experiment as well as the concentration in the perfusate are shown in Table 2.

4. Discussion

The principle of microdialysis is based on the diffusion of compounds through the probe membrane, which is permeable to small compounds. Because the probe is constantly being perfused by a physiological solution, equilibrium between the drug concentration in the probe lumen and in the probe surrounding is never reached. Consequently, the concentration in the dialysate will always represent a fraction of the real concentration in the tissue [2]. Therefore, calibration of the probe is very important to determine how much drug can be recovered by the microdialysis probe. There are different ways one can determine the probe recovery [13,14], but the most frequently used methods are extraction efficiency, retrodialysis, and no-net-flux.

The recovery obtained by EE method (or recovery by gain) represents the fraction of the total amount of drug into solution that can be extracted by the probe. This method mimics in vitro the situation in vivo, when microdialysis is used to sample drugs from a specific site. In this case, the drug is present in the tissue and diffuses through the membrane into the probe. Because the drug concentration in the tissue is expected to change overtime, it is important to verify if the probe recovery remains constant over different drug concentrations. In this paper, three different concentrations were testes: 2.5, 5, and 9 mg/l. The concentrations were selected based on the assay sensitivity and on the drug's solubility in lactated Ringer's solution. Concentrations higher than 9 μg/ml were initially tested, however the solubility was a major problem for concentrations above 10 mg/l. The recovery by gain showed to be similar for all three concentrations studied with an average recovery of 44.2 ± 7.3%. However, there is a tendency of the recovery to drop at increasing concentrations, as showed by the lower recovery obtained for the concentration of 9 mg/l (Table 1). However, an ANOVA analysis of the recoveries showed that the difference observed is not significantly different ($P > 0.1$).

The recovery obtained by retrodialysis showed a higher range when compared to the recovery obtained by EE (Table 1). The average recovery obtained for all three concentrations by this method was 61.8 ± 4.2%. An ANOVA analysis of the results again did not reveal any difference in the recoveries obtained for all three concentrations tested for this method ($P > 0.1$). On the other hand, a comparison between methods for each concentration should also be performed, since a major assumption for using the retrodialysis method when calibrating the probe in vivo is that the drug diffusion between both sides of the membrane should be the same. When the recoveries for each concentration where compared, a significant difference was observed for the concentrations of 5 and 9 mg/l.

In the retrodialysis experiment, samples were collected over a longer period of time in order to determine how long it would take to reach equilibrium, when the diffusion of the drug through the membrane is constant. When performing microdialysis, the concentration in the first samples tends to be lower than the last samples. This difference is observed due to the dilution of the drug with the blank solution present in the tubing and due to steep concentration gradient through the membrane observed at the beginning of the MD procedure, when the system is not in equilibrium [4]. Before starting collecting samples when performing the probe calibra-

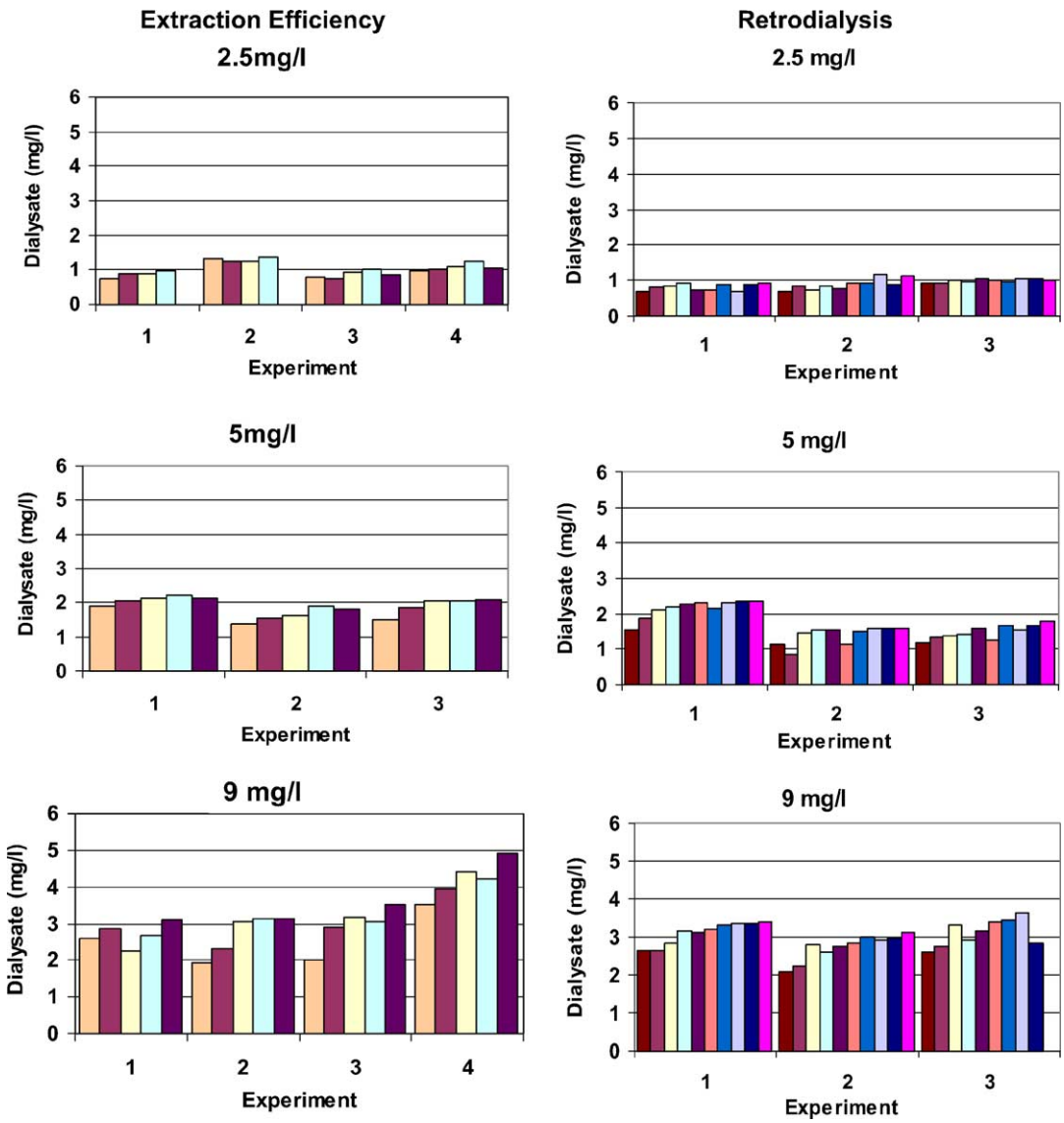

Fig. 3. Dialysate concentration obtained in the extraction efficiency (EE) and retrodialysis (RD) experiments performed for each concentration.

tion, it is important to wait until the system is in equilibrium, which means that the drug diffusion through the probe membrane is constant [15]. The dialysate concentration in the first two samples obtained by RD is still increasing (Fig. 3), even though an equilibration period of 1.5 h at 1.5 μl/min was allowed before collecting the first sample. Similar situation was observed in the EE method, where the first dialysate sample showed lower concentration than the other samples (Fig. 3). The RD results also show that equilibrium is reached only after the third sample, which indicates that the best equilibration period could be as long as 3 h at a flow of 1.5 μl/min.

The recoveries measured by RD and NNF methods were a little higher than the recovery obtained by EE, which may indicate that there are some other factors interfering with the docetaxel recovery. It is known that docetaxel is unstable when stored in PVC bags at room temperature [16], however it is stable at room temperature in glass containers for 4 weeks and in plasma for 15–24 h [10,16]. The drug concentration before and after the RD and EE experiments were similar, as it can be observed by the low standard deviation obtained when these two concentrations were compared (Table 1). The similar concentrations obtained indicate that the compound was fairly stable in lactated Ringer's solution throughout the experiment period. On the other hand, the plasma samples obtained in the NNF experiment showed a decrease in the concentration towards the end of the experiment, which may be an indication of drug instability in plasma at 37 °C.

The drug also may bind to plastic materials, as it is observed for taxol, and the binding affinity depends on the partition of the compound to plastic and to the solvent used as perfusate [17]. This factor can affect the recovery of the compound, since it could bind to the probe tubing and membrane. The binding effect could explain why the recovery observed by RD is higher than the EE method. Since for RD the drug

needs to pass by the inlet, membrane, and outlet of the probe, getting in contact with longer plastic tubing, while for EE the drug gets in contact only with the probe membrane and outlet.

In the NNF method, the drug diffuses to both sides of the membrane, depending on the difference between concentration in the perfusate and in the tube. When the concentration of the analyte in the perfusate is higher than the concentration in the tube, some analyte will diffuse from the probe into the tube, resulting in a decreased dialysate concentration. On the other hand, when perfusate concentration is smaller than the solution concentration, drug will diffuse from the tube into the probe, resulting in increased dialysate concentration. At the point where the perfusate and solution concentrations are identical, there will be no-net-flux across the membrane. At this point the curve crosses the *x*-axis and the free concentration in the plasma solution can then be determined.

Because of this characteristic of having drug diffusion through both sides of the membrane, the NNF is considered a more precise method to determine the recovery. The recovery obtained for docetaxel by NNF was similar to the recovery obtained by retrodialysis (67.9 and 61.8%, respectively).

The initial drug concentration in plasma was chosen to be around 62.5 mg/l, which would give a free concentration of 5 mg/l, according to the reported protein binding of docetaxel [8]. This free concentration was considered adequate for the NNF experiment since it is seated right at in mid point among all concentrations tested and it is within the linear range of the assay. The free concentration in the plasma solution obtained by this method was 4.7 ± 1.1 mg/l ($n = 5$). If the free concentration is compared with the initial plasma concentration, the fraction bound to proteins can be estimated. The free fraction estimated in this case was $8.6 \pm 2.3\%$, or 91.4% bound, which is in excellent agreement to the protein binding reported in the literature for this compound of 92% [8].

In conclusion, the good recoveries obtained by all methods applied in this study show that the compound docetaxel has a good diffusion through the microdialysis membrane used in this study. However, due to the difference in the recoveries obtained for the extraction efficiency and retrodialysis methods, the probe calibration in vivo might be a challenge. Some other alternative to calibrate the microdialysis probe for in vivo studies should be considered, such as the use of the internal standard method. Overall, docetaxel seems to be very suitable for microdialysis despite its lipophilicity and high molecular weight.

Acknowledgements

Virna Schuck thanks the financial support granted by CAPES—Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil.

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