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Validation of a new intraarterial microdialysis shunt probe for the estimation of pharmacokinetic parameters

Christian Höcht *, JavierA.W. Opezzo, CarlosA. Taira

Cátedra de Farmacología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, (C1113AAD) Buenos Aires, Argentina

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Abstract

The aim of our study was to compare pharmacokinetic parameters of a highly bound protein drug, irbesartan, obtained from microdialysis data (MD) of arterial blood and conventional blood samples (BS). A new vascular shunt microdialysis probe was inserted into the carotid artery and one femoral vein was cannulated for i.v. administration of irbesartan. Microdialysis samples were collected every 15 min. Blood samples were taken every 15 min. Levels of drug were measured by HPLC. Pharmacokinetic parameters were estimated using TOPFIT program. Corrected MD were compared with BS taken at same time to determine protein binding. The irbesartan protein binding did not change during the experiment. The estimated Ke from MD and BS were similar (MD: 1.8 ± 0.3 h^{-1}, $n = 5$; BS: 1.7 ± 0.2 h^{-1}, $n = 5$). After protein binding correction for the MD, the estimated values of volume of distribution (Vd) (MD: 1.2 ± 0.4 l, $n = 5$; BS: 1.1 ± 0.4 l, $n = 5$), clearance (Cl) (MD: 32.3 ± 7.3 ml min^{-1}, $n = 5$; BS: 30.7 ± 8.2 ml min^{-1}, $n = 5$) and AUC (MD: 7.7 ± 3.2 μg ml^{-1} h, $n = 5$; BS: 8.8 ± 3.4 μg ml^{-1} h, $n = 5$) were similar between MD and BS. In conclusion, these results show that our new probe inserted in the carotid artery provides accurate MD to estimate pharmacokinetic parameters of a highly bound protein drug like irbesartan. On the other hand, MD were also useful to the in vivo study of drug protein binding and saturation in protein binding.

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1. Introduction

Microdialysis has been developed during the last 25 years by several authors (for review see Ungerstedt [1]), primarily to study brain function and changes in levels of endogenous compounds such as neurotransmitters or metabolites. The development of microdialysis for the purpose of measuring drugs was initiated during the late eighties [2–4]. This technique also provides a means of continuous plasma sampling without repeated blood sampling and the applicability to the study of drug metabolism and pharmacokinetic in rats has been demonstrated in several reports [5,6].

* Corresponding author. Tel.: +54-11-4964-8265; fax: +54-11-4508-3645.

E-mail address: chocht@ffyb.uba.ar (C. Höcht).

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When compared with traditional blood sampling methods, blood microdialysis provides a powerful tool to continuously monitor the extracelular free drug concentration in the blood of animals for metabolic and pharmacokinetic purpose [7,8]. Several characteristics of microdialysis make it suitable to pharmacokinetics studies: continuos sampling is possible since the dialysis process does not change the blood volume or composition; and the experiments may be performed during long periods of time. On the other hand, the microdialysis technique allowed the in vivo determination of protein binding of drugs [9–11]. However, the placement of microdialysis probes was usually the jugular vein or the inferior cava.

In this work, we report a shunt microdialysis probe, which is placed in the carotid artery and allows frequent blood sampling in experimental animals. This probe has a long dialysis section and allows the pharmacokinetic study of drugs with high protein binding. The performance of this microdialysis probe was examined in vivo and compared with traditional blood sampling for determining irbesartan concentration in blood and the estimation of its pharmacokinetic parameters. On the other hand, we also studied the utility of this microdialysis shunt probe for the determination of protein binding of irbesartan. Irbesartan was chosen as the model drug for this study because its high protein binding and because we are currently studying the cardiovascular effects of irbesartan using the designed microdialysis probe.

2. Materials and methods

2.1. Microdialysis probe manufacture

Fig. 1 shows a diagram of the designed microdialysis shunt probe. The intravascular probe was constructed as follows: a 25 mm long (1.90 mm I.D., 3.10 mm O.D.) plastic tube was used to make the body of the probe. A 23G needle was inserted into the body (or the interior of the tube), 2.5 mm from one extreme. The tip of the needle was drawn out until 2.5 mm from the other extreme of the probe, at the same wall, and another hole was made. This hole was made in such a way that a 35-mm-long length of an hemodialyzer fiber (cuproammonium rayon, cutoff 10 000, 206 μm o.d.) was inserted into the needle. Then, the needle was extracted carefully of the body of the probe, so the dialysis fiber remained crossing the body of the probe. Two fused silica tubing (10-mm-long, 143 μm I.D., 144 μm O.D.) were partially inserted in each end of the hollow fiber and then they were sealed with glue. To secure the fiber to the body it was sealed using glue. Two 80-mm-long (500 μm I.D., 800 μm O.D.) plastic tube were inserted into both end of the hollow fiber and sealed with glue. These junctions were allowed 20 min to cure at room temperature before to proceed with the next step. Finally, two 50-mm-long (500 μm I.D., 800 μm O.D.) plastic tube were partially (5 mm) inserted in one end of the body of the probe and one 50-mm-long (500 μm I.D., 800 μm O.D.) plastic tube was partially (5 mm) inserted in the other end of the body. Both end of the body and the tubes were sealed with care taken to avoid glue spreading to the tip of the tubes. The probe was allowed to cure at room temperature for at least 1 day before the surgical procedure. (Pat. pending. no. AR010102592, INPI, Argentina).

2.2. Animal studies

Male Wistar rats (250–350 g) were anesthetized with a mix of chloralose (50 mg kg^{-1} i.p.) and urethane (500 mg kg^{-1} i.p.). Supplements of anesthesia were administered when it was necessary. The shunt microdialysis probe was filled with heparinized saline solution (50 U ml^{-1}) before the surgical procedure. The inlet was inserted into the left carotid artery with the tip directed to the heart and one outlet was inserted into the artery with the tip oriented to the brain. The remaining outlet was connected to a Statham Gould P23ID pressure transducer coupled to a Grass79D polygraph. The remaining outlet was also used to inject supplements of the heparinized saline solution when it was necessary. The shunt vascular probe was perfused with a Ringer solution consisted of 147 mM NaCl, 2.4 mM $CaCl_2$, 4.0 mM KCl, pH 7.3, pumped at a rate of 2 μl min^{-1} and 15 min

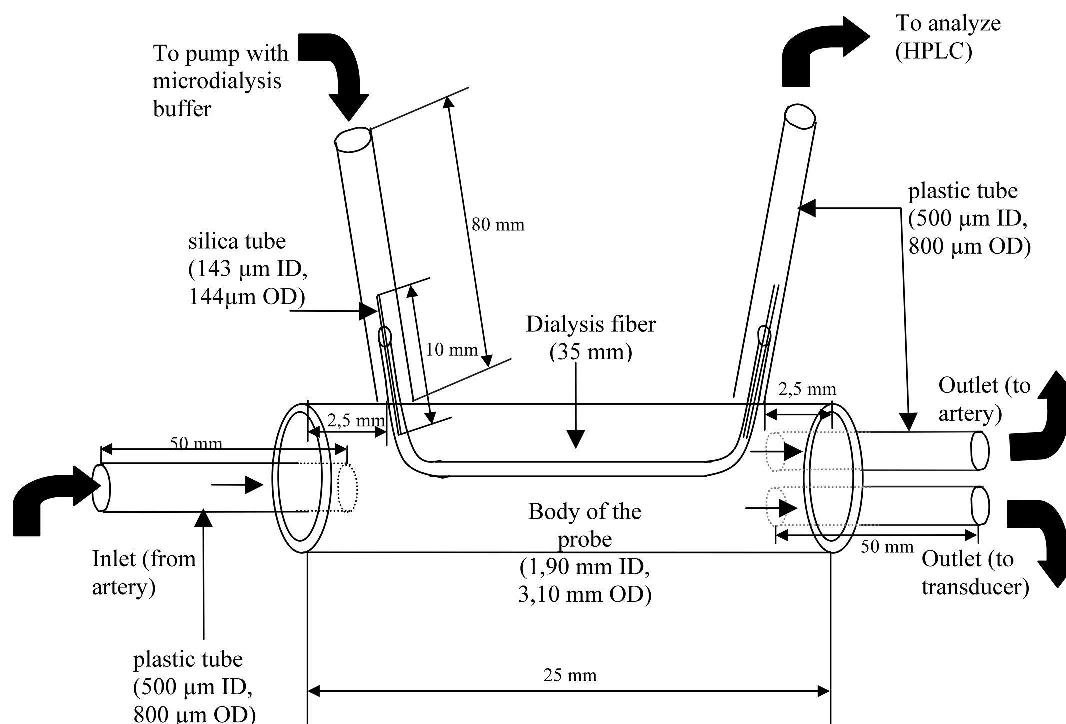

Fig. 1. Scheme of the designed microdialysis shunt probe.

samples were collected. The in vivo recovery of irbesartan from the tissue to the perfusion medium in the dialysis probe was determined before the intravenous injection by perfusing the microdialysis probe with a solution of irbesartan (200 ng ml^{-1}) and by taking the proportion of lost across the dialysis membrane as an estimate of the recovery.

After intravenous administration of irbesartan (10 mg kg^{-1}), microdialysis samples were taken every 15 min during 3 h. Blood samples (50 µl) were taken from the remaining outlet of the microdialysis probe at the following times: 15, 30, 60, 90, 120, 150 and 180 min.

2.3. Sample analysis

Arterial blood samples (BS) (50 µl) were collected in 0.5 ml PCR tubes containing 5 µl of heparinized solution and gently mixed. Blood samples were centrifuged at 10 000 rpm for 10 min under controlled temperature (4 °C) to avoid either decomposition or biological activity. The plasma supernatant (30 µl) was carefully separated and deproteinized with acetonitrile (30 µl) and 10 µl of zinc sulfate solution (10%). Microdialysis samples were injected without any pre-treatment.

Levels of irbesartan in microdialysis and BS were measured by liquid chromatography with fluorescence detection using a Phenomenex ODS column 5 µm, C18, 250 × 4.6 mm (LUNA) and a Model LC304 Fluorescence detector (Linear Instruments). The excitation and emission wavelengths used were 236 and 462 nm, respectively. The optimal composition of the mobile phase was achieved by mixture of pure acetonitrile and 5 mM sodium acetate buffer (60:40), pH 4. The mobile phase was filtered through a 0.2 µm type HVLP Durapore membrane filters (Millipore) and the residual air was removed from them by bubbling helium through. The volume injected into the chromatography system was 20 µl. The flow rate used was 1.4 ml min^{-1} and the retention time of irbesartan was approximately 6 min. The chromatographic method was adapted from the method developed by González [12].

The chromatographic method was tested for linearity, precision and reproducibility. The limit of quantification and detection were also determined. Linearity was determined at concentration in the range of 5–50 000 ng ml^{-1}. Three concentration within the linearity range (low, medium and high) were selected. Five standard solution of each concentration were prepared and analyzed in triplicate (repeatability assay). This assay was repeated for 5 days (reproducibility assay). Recovery of plasma samples was calculated at three concentration within the linearity range.

2.4. Determination of irbesartan concentration in plasma by microdialysis

To determine the unbound irbesartan concentration in the blood from the microdialysis data (MD), the concentration of irbesartan in the microdialysis samples were adjusted with the in vivo recovery of the probe.

The in vivo recovery of irbesartan from the tissue to the perfusion medium in the dialysis probe was determined before the intravenous injection by perfusing the microdialysis probe with a solution of irbesartan (200 ng ml^{-1}). The in vivo recovery of irbesartan was calculated with the following equation:

$$R = \frac{C_{in} - C_{dial}}{C_{in}}$$

where R is the irbesartan in vivo recovery, C_{in} is the concentration of irbesartan in the perfusate and C_{dial} is the concentration of irbesartan in the dialysate. The recovery was calculated four times in each experiment.

So, the unbound irbesartan concentrations in blood (C) were calculated using the following equation:

$$C = \frac{C_{out}}{R}$$

where C is the calculated unbound irbesartan concentration, C_{out} is irbesartan concentration in the dialysate and R is the in vivo recovery of the microdialysis probe.

Microdialysis generated data that are the integral of the concentration surrounding the probe during the sampling interval, while BS provided a point measurement during the same interval. To estimate pharmacokinetic parameters, the MD must to be transformed from a series of integrals to a series of points corresponding at the same time of BS collection.

It is necessary to calculate first the time point during the sample interval at which the mean microdialysis samples concentration is attained (T) using the following equation developed by Ståhle [13]:

$$T = \frac{[\ln(k\Delta t) - \ln(1 - e^{-k\Delta t})]}{k}$$

where Δt is the sample interval and k the elimination rate constant.

Next, to calculated the concentration of irbesartan in the microdialysis sample at the end time of the sample interval, the following equation was used:

$$C_{(t)} = C_{(0)} e^{-k(\Delta t - T)}$$

were $C_{(t)}$ is the concentration of irbesartan at the end of the sample interval, $C_{(0)}$ is the mean concentration of irbesartan in the microdialysis sample, Δt is the sample interval and T is the time point during the sample interval at which the mean microdialysis samples concentration is attained.

2.5. Pharmacokinetic analysis

The protein binding of irbesartan at different time points was calculated by dividing the corrected concentration of irbesartan in the microdialysis sample through the concentration of irbesartan in the BS at the same time.

The irbesartan concentration–time profiles obtained from corrected MD (unbound concentration) and BS (total concentration) following bolus dosing were described by a one compartment, first-order elimination model. Nonlinear least-squares regression analysis was performed using the TOPFIT program. Areas under the curves (AUC) of irbesartan levels in plasma and dialysate versus time were calculated by using the trapezoi-

dal rule. The parameters clearance (Cl) and volume of distribution (Vd) were calculated by standard methods [13], where Cl = dose/AUC and Vd = Cl/k. To compare the estimation of pharmacokinetic parameters of the total irbesartan plasma concentration obtained from MD and from blood sampling, the obtained pharmacokinetic parameters from MD (unbound irbesartan concentration) of each rat were corrected with the corresponding mean unbound fraction of irbesartan.

2.6. Statistics

Normal distribution of the variables of the study was verified using the Kolmogorov–Smirnov (K–S)-test. Protein binding of irbesartan at different time was compared by one way ANOVA with post hoc tests performed using a Bonferroni test. A paired t-test was performed for each of the pharmacokinetic parameters to compare the parameters determined using MD with those parameters determined using traditional blood sampling. The statistical tests were performed using GRAPHPAD PRISM version 3.02 for Windows (GraphPad Software, San Diego, CA, USA).

Data are expressed as mean ± S.E.M. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Validation of chromatographic methods

Fig. 2 show a representative chromatogram at 20 ng ml^{-1} of irbesartan. The results obtained in the validation assay procedure in plasma and Ringer solution are summarized in Table 1. The regression equations obtained by unweighted least-squares linear regression are represent by PA = $a + b$C, where PA is the peak area and C is concentration. Good linearity was observed for irbesartan in Ringer solution ($r = 0.999$) and plasma ($r = 0.995$). The mean recovery of irbesartan from plasma samples was 102, 108 and 98% for high, medium and low concentration. The limit of quantification and detection were 1 and 2 ng ml^{-1} in Ringer solution and 2 and 5 ng ml^{-1} in plasma.

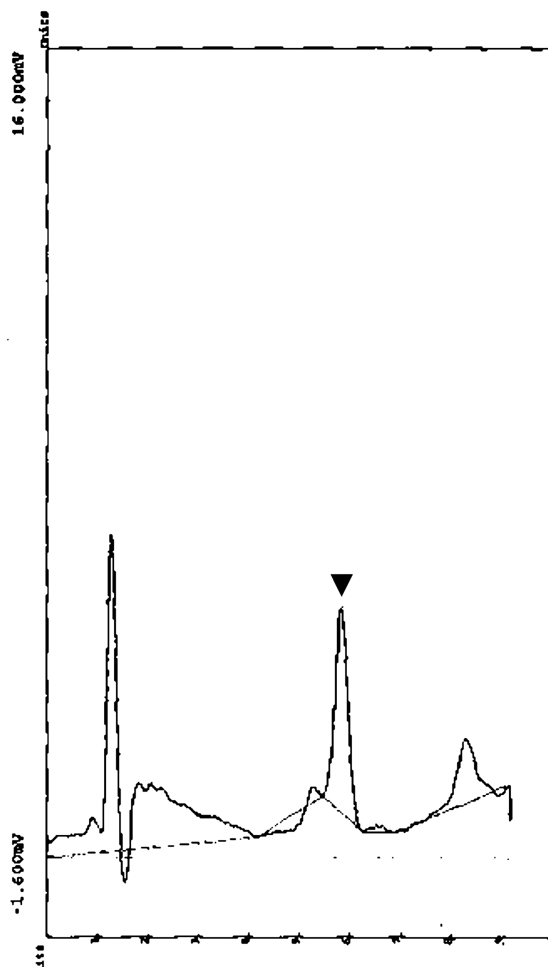

Fig. 2. Representative chromatogram of irbesartan at 20 ng ml^{-1}. Full-scale represents 0.1 fluorescense units. The triangle indicates irbesartan peak.

3.2. Microdialysis recovery in vivo

The mean ± R.S.D. of the recoveries by loss of irbesartan in blood are listed in Table 2. The relative standard deviation (R.S.D.) of the in vivo recovery data in all rats did not exceed the R.S.D. of the chromatographic methods. We observed a good repeatability of the in vivo recovery in each experiment since the R.S.D. of the in vivo recovery not exceed the R.S.D. of the analytical technique. So we can conclude that the in vivo recovery did not change during the experiment.

Table 1
Results obtained from validation assays

	Theoretical concentration (ng ml^{-1})	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Liner regression model
Ringer	1000	1.40	2.05	PA = 7.7C−0.92
	50	0.95	3.14	
	5	5.44	4.52	
Plasma	1250	4.45	2.35	PA = 7.82C−1.07
	250	3.21	4.17	
	5	2.65	5.32	

Table 2
Intraarterial microdialysis probe recovery in vivo

Rat	Recovery by loss	
	Mean ($n = 4$) [a]	R.S.D. (%)
1	0.46	4.63
2	0.56	1.48
3	0.51	4.85
4	0.44	0.88
5	0.61	1.48

[a] Number of determination in each rat.

3.3. Protein binding of irbesartan

The irbesartan protein binding–time profile determined using intraarterial microdialysis is showed in Fig. 3. No difference in the irbesartan protein binding were seen between the determined time points ($P > 0.05$). Table 3 showed the mean protein binding of irbesartan in each rat and the repeatability of the determination expressed as R.S.D. The R.S.D. obtained in the determination of the unbound fraction indicates a good precision of this technique. This results validates the use of the microdialysis technique to determine in vivo protein binding of drugs. On the other hand, after intravenous administration of 10 mg kg^{-1} of irbesartan, we did not observe saturation of protein binding.

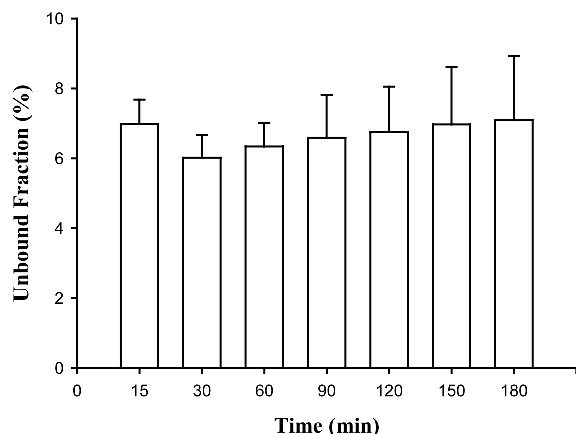

Fig. 3. Irbesartan protein binding–time profile after intravenous administration of 10 mg kg^{-1}. Each bar shows the mean ± S.E.M. of five rats.

3.4. In vivo comparison of arterial microdialysis sampling versus traditional blood sampling

The unbound (obtained from MD) and total (obtained from traditional blood sampling) concentration–time profiles of irbesartan are shown in Fig. 4. Pharmacokinetic parameters determined from the concentration–time data derived from each sampling method are listed in Table 3. There was no statistical difference in any pharmacokinetic parameter between these two sampling meth-

Table 3
Mean protein binding of irbesartan in each rat

Rat	Unbound fraction	
	Mean ($n = 6$) [a]	R.S.D. (%)
1	4.3	13.4
2	5.84	11.53
3	6.27	7.40
4	10.63	13.69
5	6.07	13.16

The unbound fraction of irbesartan in each rat corresponds to the mean of different time determination in the same animal.
[a] Number of determination in each rat.

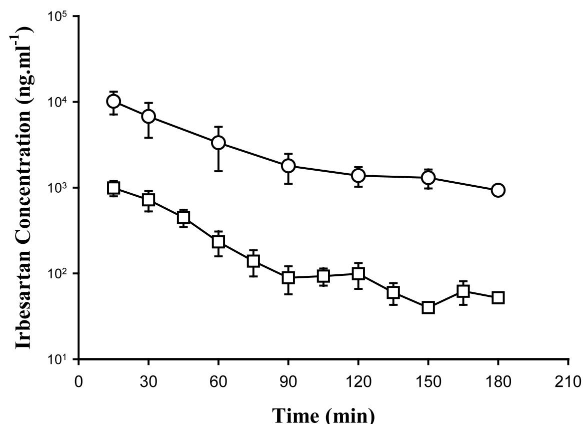

Fig. 4. Irbesartan concentration–time profile obtained from MD (squares) and blood sampling data (circles) after intravenous administration of 10 mg kg^{-1}. Each point shows the mean ± S.E.M. of five rats.

ods, as determined using a paired *t*-test. This result validates the use of the microdialysis technique to determine standard pharmacokinetic parameters of highly bound drugs Table 4.

4. Discussion

We have developed an intraarterial shunt microdialysis probe to sample unbound drug concentration in blood of experimental animals. This probe design have many advantages compared with prior intravenous microdialysis probe: (1) there is not possibility of damage in microdialysis membrane during the implantation, (2) the microdialysis membrane length allows the obtention of high recovery, (3) the developed probe allows the sample of arterial blood and the obtention of pharmacodynamic data through the connection of one outlet of the microdialysis probe to a pressure transducer [14–16]. The recording of arterial pressure permits to determine the effective circulation of the blood within the microdialysis probe. However, an important disadvantage of this probe design is the blood clotting within the microdialysis probe. To solve this problem the remaining outlet was also used to inject supplements of the heparinized saline solution when it was necessary.

This probe was originally designed in our laboratory for arterial microdialysis and simultaneous blood pressure recording in the same animal and with a minimal damage. Moreover, estimation of heart rate is possible by counting the blood pressure waves. Thus, by using this probe in this laboratory, it was seen a correlation between the cardiovascular effect of the antihypertensive drug methyldopa and their arterial blood concentration in sinoaortic denervated and control rats [15]. In other work, we reported a correlation between changes of heart rate and the temporary course of methyldopa concentration of arterial dialysates in aortic coarctated and control rats [16]. These previous works suggest that this intraarterial shunt microdialysis probe is suitable tool for pharmacokinetic–pharmacodynamics studies of drug because this probe made possible both studies in the same animal. However, the aim of this work is the validation of the probe as tool for pharmacokinetic studies.

The microdialysis technique is a powerful tool for the determination of extracellular free drug concentration in the blood of experimental animals for pharmacokinetic purpose. Advantages of this technique include: (1) frequent determinations

Table 4
Irbesartan pharmacokinetic parameters determined using intraarterial microdialysis versus traditional blood sampling

Pharmacokinetic parameter	Blood sampling ($n = 5$)	Microdialysis sampling ($n = 5$)	*P*
Ke (h^{-1})	1.72 ± 0.3	1.77 ± 0.23	0.821
Vd (l)	1.14 ± 0.4	1.20 ± 0.36	0.843
Cl (ml min^{-1})	30.7 ± 8.2	32.3 ± 7.3	0.851
AUC (μg ml^{-1} h)	8800 ± 3439	7735 ± 3154	0.421
U (%) [a]		7.09 ± 1.24	

Data are shown as mean ± S.E.M.
[a] Unbound fraction of irbesartan.

may be made, which can provide more information about the shape of the drug concentration–time profile and allow the use of the same animals for multiple experiments, without concern for blood loss from small animals, (2) continuous sampling without altering the pharmacokinetics due to physiological changes that result from blood sampling and (3) in vivo determination of unbound drug concentration in the blood can be performed [17,18].

A fundamental difference between blood sampling and microdialysis sampling is that the former is concerned with concentration measured at discrete time-points while the microdialysis samples contain the mean concentration during the sampling interval [13]. In this way, to calculates slopes of the concentration–time curve from MD by standard methods, it is necessary to transform the data from a series of integrals to a series of points using the equation developed by Ståhle [13].

On the other hand, it is important to know the relative recovery in vivo to accurately determine drug concentration in the plasma. Several previous studies have indicates that the in vitro recovery usually is an overestimate of in vivo recovery [19–21]. The in vivo recovery of our microdialysis probe was determined by perfusing the dialysis probe with a solution of irbesartan and by taking the proportion lost across the dialysis membrane. This method for determination of in vivo recovery allows the determination of in vivo recovery and the concentration–time profile of irbesartan in the same animal. In this work, we have determined a good repeatability of the in vivo recovery of irbesartan, so that the recovery did not change during the experiment.

The microdialysis technique allows the determination of in vivo protein binding using microdialysis sampling in blood and simultaneous blood sampling [9–11]. The in vivo determination of protein binding using the microdialysis method permits a more accurate determination of protein binding, because it was found that the in vitro determination systematically underestimated the unbound fraction [11].

In this work we have studied the protein binding–time profile of irbesartan. We have determined the temporal course of protein binding in the same animal in order to evaluate saturation of the plasma protein binding. According with the results shown in Fig. 3 we did not observe a significant change of the unbound fraction of irbesartan during the experiment. We can conclude, that after an intravenous administration of 10 mg kg^{-1} of irbesartan, it is not observed a saturation of protein binding.

The results obtained with our probe shows a high mean protein binding (90–95%) of irbesartan. The protein binding of irbesartan have been extensively studied in human protein but not in rat and it is well known that this drug has a high protein binding. The unbound fraction of irbesartan determined in this work is similar to the protein binding observed by other authors in previous reports [22]. Therefore, this characteristic appears to be similar with rat plasmatic proteins.

The irbesartan protein binding–time profile obtained was remarcable constant. The R.S.D. obtained in the determination of the unbound fraction indicates a good precision of this technique as well as the good performance of our microdialysis probe. Our results could validate the use of the microdialysis technique to determine in vivo protein binding of drugs. Previous works have been applied this technique but using drugs with low protein binding. Moreover our results confirm the utility of microdialysis to determine protein binding of drugs with high protein binding.

In this work, we also have validated the use of the developed microdialysis shunt probe in the estimation of pharmacokinetic parameters of drugs with high protein binding. The pharmacokinetic parameters Cl, Vd and area under the curve (AUC) were estimated from the concentration–time curve obtained from blood sampling and microdialysis sampling. To compare the estimated Cl, Vd and AUC from each sampling, the obtained Vd and Cl from MD were multiply by the unbound fraction of irbesartan and the obtained AUC was divided by the unbound fraction of irbesartan. It is necessary to make this correction, because the pharmacokinetic parameters obtained from MD represent the parameters of the unbound concentrations of irbesartan. So the estimated pharmacokinetic parameters from MD

need to be corrected by the unbound fraction to represent the parameters of the total concentrations of irbesartan. After comparing the estimated parameters using a paired t-test, there was not statistical difference in any pharmacokinetic parameters between these two sampling methods. This result validates the use of the microdialysis technique to determine standard pharmacokinetic parameters for highly bound drugs.

5. Conclusion

In this work we have reported and validated a new intraarterial microdialysis shunt probe. This study shows that the developed microdialysis shunt probe is a powerful tool in the determination of the protein binding of highly bound drugs like irbesartan. However, this technique allows the determination of the temporal course of protein binding in the same animal in order to determine saturation of the plasma protein binding. On the other hand, the results show that the microdialysis sampling in carotid artery using this probe design provides an accurate method for determining blood pharmacokinetics of drugs with irbesartan characteristics that include, not only high protein binding, but also a long half-life.

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