Quantitative Microdialysis for Studying the *in Vivo* L-DOPA Kinetics in Blood and Skeletal Muscle of the Dog

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Received Sept 6, 1994; accepted Dec 6, 1994

In this study the microdialysis technique, using α-methyldopa as internal standard (IS), is introduced for the *in vivo* determination of L-DOPA, dopamine (DA), and their metabolites dihydroxyphenylacetic acid (DOPAC) and 3-O-methyldopa (3-OMD) in blood plasma and skeletal muscle extracellular fluid (ECF), in anaesthetised beagle dogs, after i.v. administration of L-DOPA. In a first calibration experiment, the *in vivo* relative losses (RL) of the compounds and the IS were determined. These were lower in skeletal muscle than in blood plasma. K was defined as the ratio of the RL of the IS to the RL of the compound of interest and was shown to be constant for a certain compound within one tissue. However, except for DA, a significant difference was seen in K values between blood plasma and skeletal muscle. In a second step, the method was validated in blood plasma. The $AUC_{0\rightarrow 3}$ values for the non-protein bound L-DOPA did not differ significantly between the dialysis (141.3 ± 16.0 nmol.h/ml) and traditional whole blood sampling (145.3 ± 18.7 nmol.h/ml), confirming that microdialysis combined with accurate calibration is a reliable technique for studying the kinetics of drugs *in vivo* in different tissues.

KEY WORDS: quantitative microdialysis; *in vivo* calibration; L-DOPA; plasma; skeletal muscle.

INTRODUCTION

The use of microdialysis sampling in pharmacokinetic studies requires the *in vivo* calibration of the microdialysis probes in order to obtain accurate estimates of kinetic variables such as AUC and C_{max}.

In this study, the internal reference technique (1-3) is used for the determination of the *in vivo* recoveries of L-DOPA, dopamine (DA), and their metabolites dihydroxyphenylacetic acid (DOPAC) and 3-O-methyldopa (3-OMD) from blood plasma and skeletal muscle extracellular fluid (ECF), in anaesthetised beagle dogs, after the i.v. administration of L-DOPA. This calibration technique is based on the principle that the relative loss (RL) of a carefully chosen internal standard (IS) (also called *in vivo* marker) added to the perfusate, is related to the recovery of the compounds of interest. The advantage of this technique is that real extracellular tissue concentrations can be calculated for each dialysate sample, throughout the entire experiment. This provides a tool to detect changes in recovery, due to changes in probe or tissue characteristics during the experiment. Of utmost importance, however, is the choice of the IS, which should have similar structural and diffusional characteristics (e.g. lipophilicity and molecular weight) to the compounds of interest (4). In a first set of calibration experiments, the RLs of the compounds and of α-methyldopa were determined. Then, validation of the technique was done by comparing kinetic data of L-DOPA and 3-OMD obtained from i.v. microdialysis (corrected for recovery) and traditional whole blood sampling.

EXPERIMENTAL

Chemicals and Drugs

L-DOPA and 3-OMD were supplied by Roche (Basel, Switzerland). DA hydrochloride was purchased from UCB (Brussels, Belgium) and DOPAC from Sigma (St. Louis, MI, USA). 1-Octane sulphonic acid sodium salt was obtained from Janssen Chimica/Aldrich Europe (Beerse, Belgium). Ringer's solution was purchased from Travenol (Lessines, Belgium). Human plasma was obtained from the Blood Transfusion Centre of the University Hospital A.Z.-V.U.B. L-DOPA, carbidopa, 3-OMD and α-methyldopa for the *in vivo* experiments were supplied by Merck Sharp and Dohme Research Laboratories (Rahway, NJ, USA). All other reagents were of analytical grade and were purchased from Merck (Darmstadt, FRG).

Stock solutions (0.01% (w/v), kept at 4°C) of the standards were prepared in an antioxidant mixture i.e. 0.01 M HCl, containing 0.1% (m/v) $Na_2S_2O_5$ and 0.01 % (m/v) Na_2EDTA. Subsequent working solutions were freshly prepared from the stock solutions by appropriate dilution in 0.5 M acetic acid. L-DOPA and carbidopa for i.v. and/or oral use were prepared as previously described (5).

In Vivo Experiments

Four healthy male beagle dogs (body weights, 8–9.5 kg) were used for both the calibration and validation experiments that were carried out in one session. They were pretreated for one week with carbidopa (100 mg p.o. daily) and received an additional, i.v. dose of 100 mg shortly after the induction of the anaesthesia. After fasting overnight, the dog was anaesthetised with sodium pentobarbital (25 mg/kg i.v.). The temperature was monitored and kept at 37°C.

For i.v. microdialysis, the probe was inserted by means of an i.v. guide (1.4 mm i.d.; CMA Microdialysis, Stockholm, Sweden) into a peripheral vein of the foreleg, and then fixed in place. For intramuscular (i.m.) microdialysis, a small incision (2 cm) was made in the skin overlying the gluteal muscle of the hindleg. The guide cannula was pushed into the muscle and the microdialysis probe was inserted, sealed into the guide tube, and sutured in place.

The CMA 10 microdialysis probes (CMA Microdialysis, Stockholm, Sweden) were connected to the CMA/100 microinjection pump. The length of the dialysis membrane was 16 mm and the cut-off point was 20 000 D. The probes were

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0724-8741/95/0500-0746$07.50/0

perfused with the Ringer's solution at a flow rate of 5 μl/min for 0.5 h, after which three 8-min baseline samples were collected. Hundred μl of antioxidant mixture was added to the dialysates, yielding a final volume of 140 μl, and the samples were subsequently stored at −24°C until assayed.

For the calibration experiments, the *in vivo* RLs were determined by adding L-DOPA, DA, DOPAC, α-methyldopa (1 μg/ml each) and 3-OMD (3 μg/ml) to the perfusates of both the i.v. and i.m. probes. Five 8-min dialysate samples were simultaneously collected from blood plasma and skeletal muscle ECF. They were immediately diluted with 100 μl of antioxidant solution and frozen at −24°C until analysed. The RL was defined as the loss from the perfusate, and by this way represented a reversed mode of determining *in vivo* recovery. Therefore, the RLs of the different compounds in blood plasma and skeletal muscle ECF were calculated as the ratio of the difference between perfusate and dialysate concentration over the perfusate concentration. Subsequently, the K values = RL_{IS}/RL_a, with RL_a being the RL of the compound of interest (L-DOPA, DA, DOPAC or 3-OMD) were calculated. The actual concentration of the compound of interest in the extracellular space ($C_{e,a}$) was then given by

$$C_{e,a} = C_{d,a} \cdot K / RL_{IS}$$

The validation of the microdialysis technique was assessed by comparing the pharmacokinetic data from i.v. microdialysis with those from whole-blood sampling. The syringes containing the perfusion fluid for the *in vivo* calibration experiments were switched for syringes containing the modified Ringer's solution and α-methyldopa (1 μg/ml). Dialysates and whole blood samples were collected simultaneously.

New baseline dialysates were collected and subsequently, L-DOPA (20 mg/kg i.v.) was administered over 2 min. Dialysate samples were collected for another 3 hours. The samples were then diluted with antioxidant solution and stored at −24°C until analysed.

Whole-blood samples were simultaneously collected by means of a catheter placed into a peripheral vein of the hindleg. Blood-samples were collected before L-DOPA administration and half-way each i.v. microdialysis run. The blood samples were centrifuged 20 minutes at 2,500 g, the plasma was removed and frozen at −24°C until analysed.

Analysis

The separation and quantification of the compounds in the dialysates or plasma samples (after deproteinization) was carried out according to an assay previously reported (5), using LC with electrochemical detection. Dialysates were injected without any sample clean-up procedure. For the plasma samples 250 μl 1 M perchloric acid was added to each aliquot (500 μl) of plasma. The samples were then centrifuged 10 min at 2,000 g. Subsequently, 10 μl of the supernatant was diluted 100 times with 0.5 M acetic acid and immediately injected into the HPLC system.

The only modification to the previously described method is the use of dual electrochemical detection. Both electrochemical cells were operated in the oxidative mode with a detector potential of + 750 mV versus a Ag/AgCl reference electrode. For each injection, a dual chromatogram was recorded; one at high sensitivity for DA and DOPAC and one at low sensitivity for L-DOPA and 3-OMD. This was achieved by setting the amplifier for one working electrode (W1) at 1 nA/V and that of the other (W2) at 10 nA/V. The chromatograms were evaluated with a dual channel integration computer program (Integration Pack^R, Kontron, Milan, Italy).

Data analysis

Calibration Experiments

The data are expressed as arithmetic means ± SD (n = 20, 20 dialysates collected, 5 from each dog). The precision of the RLs and the K values was estimated by the residual variance from a one-way analysis of variance (16 d.f.). The statistical significance of differences between the K_{IV} and K_{IM} were determined by using the two-tailed paired *t*-test at the 5 % significance level.

Validation Experiments

The data obtained from the 2 different sampling methods are expressed as arithmetic means ± standard error on the mean (SEM)(n = 4; 4 dogs). The midpoints of the dialysis collection periods were taken as the time points for graphical representation. The area under the concentration vs. time curve between 0 and 3 h ($AUC_{0\rightarrow3}$) for L-DOPA and 3-OMD were used as pharmacokinetic parameter to compare the two sampling methods. The $AUCs_{0\rightarrow3}$ were calculated by using the trapezoidal rule.

The significance of the differences between the $AUCs_{0\rightarrow3}$ obtained by the two different sampling methods, was estimated by using the two-tailed paired *t*-test at the 5% significance level.

RESULTS

Intravenous and i.m. Microdialysis Samples

The LC method for L-DOPA and its metabolites (5) was also shown to be selective for the IS, α-methyldopa. The limit of detection for α-methyldopa was 142 fmol on-column and its capacity factor was 15.2. The *in vitro* RL for α-methyldopa (1 μg/ml) determined as described by Sarre et al. (1992) (6), in the same microdialysis conditions, was 39.4 ± 4.4% (concentration range 1–10 μg/ml, n = 10) in plasma and 40.5 ± 1.8% in Ringer's solution (concentration range 1–10 μg/ml, n = 10). The RL was not concentration-dependent over the range examined.

Estimation of the RL *in Vivo*

The RLs of the compounds (RL_a) from blood plasma and skeletal muscle ECF dialysates are given in Table I. As for all compounds a one-way analysis of variance (α = 0.05) showed a significant interindividual variance, the residual variance was used as an estimate of the overall precision of the microdialysis technique. The change in RL_{IS} with time for α-methyldopa in blood plasma and in skeletal muscle ECF in one of the dogs (dog 2) is shown in Fig 1. The K values (= RL_{IS}/RL_a) from i.v. (K_{IV}) and i.m. (K_{IM}) dialysates are given in Table II. In some cases, it was also ob-

Table I. *In Vivo* Calibration Experiments

	RL_{IM} (%)	RSD RL_{IM} (%)	RL_{IV} (%)	RSD RL_{IV} (%)
L-DOPA	22.50 ± 5.05	7.2	37.78 ± 5.87	12.4
DOPAC	19.81 ± 4.61	8.2	37.68 ± 6.09	12.8
DA	26.53 ± 3.96	11.7	56.05 ± 5.74	6.4
3-OMD	16.40 ± 3.87	11.1	27.54 ± 5.98	17.3
α-methyldopa	16.66 ± 4.00	13.6	33.2 ± 6.25	15.1

The RL of L-DOPA, DOPAC, DA, 3-OMD and α-methyldopa in dialysates obtained from blood plasma (RL_{IV}) and skeletal muscle ECF (RL_{IM}). Values are means ± SD (n = 20; 20 dialysates collected, 5 from each dog). The precision of the relative loss (RSD) for respectively i.m. and i.v. dialysis is estimated from the residual variance calculated in the one-way ANOVA.

served that the mean K-values were significantly different between the dogs. Therefore, the data were pooled in order to obtain a better estimate of the K values within the group of dogs studied. The difference between K_{IV} and K_{IM} was statistically significant for L-DOPA ($p = 0.0001$), DOPAC ($p = 0.02$) and 3-OMD ($p = 0.0001$).

For comparison, Table III shows the *in vitro* RRs of the compounds of interest in Ringer's solution and plasma determined as described by Sarre et al (6).

Comparison of i.v. Microdialysis with Whole-Blood Sampling

The pharmacokinetic curves for L-DOPA, obtained by whole-blood sampling and i.v. microdialysis, matched perfectly (Fig. 2). There was no significant difference between the $AUC_{0\rightarrow3}$ for L-DOPA obtained by whole-blood sampling (145.27 ± 18.72 nmol.h/ml) and that yielded by i.v. microdialysis (141.28 ± 16.04 nmol.h/ml). The $AUC_{0\rightarrow3}$ values for 3-OMD were 159.14 ± 12.11 nmol.h/ml and 65.15 ± 11.12 nmol.h/ml ($p = 0.001$), respectively. The concentrations of DOPAC and DA in blood plasma were beyond the limits of detection in the dialysates during the validation experiments.

Plasma Protein Binding (PB)

The pharmacokinetic curves of L-DOPA obtained from whole blood collection and *corrected* dialysate concentrations were practically identical and therefore demonstrate that L-DOPA is in an unbound state in the plasma. However, the plasma concentrations for 3-OMD were higher with whole-blood collection at any given time point (Fig. 3). This difference reflected the extent of binding of 3-OMD to plasma proteins. The protein binding of 3-OMD varied between 47–78%.

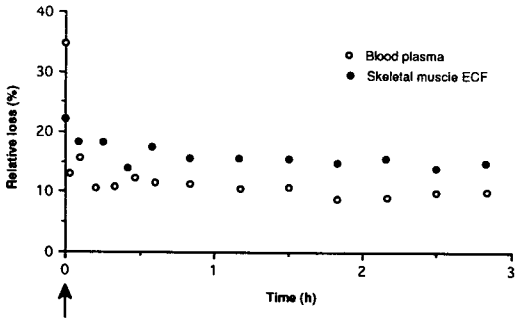

Fig. 1. The RL (%) for α-methlydopa in dialysates from blood plasma and skeletal muscle ECF, during a validation experiment in dog 2. (arrow, the i.v. injection of L-DOPA (20 mg/kg)).

DISCUSSION

The advantages of the use of microdialysis in pharmacokinetic studies instead of traditional procedures have been described in detail before (7). Some researchers still use *in vitro* recovery for estimation of the real ECF concentration of the drug under investigation. Recently, Stenken et al. (8) reported that in hydrodynamic environments *in vivo* such as blood, bile and liver, *in vitro* calibration of microdialysis probes is appropriate. However, our data revealed a substantial difference for most of the compounds between the RLs in the *in vivo* experiments and the RRs obtained in *in vitro* experiments, using a self-constructed artificial blood vessel to create dynamic conditions as described earlier (6)(see Table III). This can be explained by the fact that the tissue is the rate limiting-step in the diffusion process (4,9). Furthermore, variations in tissue *in vivo* recovery have been described by others (10,11). Indeed, in this study the *in vivo* RLs in skeletal muscle were lower than in blood plasma. Therefore the *in vivo* recovery must be experimentally estimated. Diffusion on the outside of the membrane might be influenced by several factors; whilst the concentration gradient can be affected by convection in *in vitro* conditions, *in vivo* diffusion must be restricted due to the small extracellular volume fraction and the tortuosity caused by the cell

Table II. *In Vivo* Calibration Experiments

	K_{IM}	RSD K_{IM} (%)	K_{IV}	RSD K_{IV} (%)
L-DOPA	0.74 ± 0.06	6.5	0.87 ± 0.04§§	4.5
DOPAC	0.84 ± 0.05	6.1	0.88 ± 0.05§	3.7
DA	0.62 ± 0.11	9.2	0.60 ± 0.05	7.3
3-OMD	1.02 ± 0.06	4.9	1.22 ± 0.07§§	3.8

The ratio (K) of the RL of α-methyldopa (RL_{IS}) to the RL of L-DOPA, DA, DOPAC and 3-OMD (RL_a) from blood plasma (K_{IV}) and skeletal muscle ECF (K_{IM}) dialysates (n = 20; 20 dialysates collected, 5 from each dog). Values are means ± SD. The precision of the K values for respectively i.m. and i.v. dialysis are estimated from the residual variance calculated in the one-way ANOVA. Levels of significance vs. corresponding K_{IM} value: t-test: §$p \leq 0.05$; §§$p \leq 0.0001$.

Table III. The RRs of the Compounds of Interest in Standard Mixtures of Plasma and Ringer's Solution over the Concentration Range of 5 ng/ml to 50 μg/ml for L-DOPA, DOPAC and DA and 15 ng/ml to 150 μg/ml for 3-OMD

	Compound	RR (%)
Plasma	L-DOPA	39.5 (SD 2.8)
	DOPAC	48.5 (SD 3.6)
	DA	35.5 (SD 3.1)
	3-OMD	36.0 (SD 3.2)
Ringer's solution	L-DOPA	41.5 (SD 2.2)
	DOPAC	59.3 (SD 2.8)
	DA	39.7 (SD 1.7)
	3-OMD	38.9 (SD 2.5)

Microdialysis conditions; perfusion rate 5 μl/min, dialysis time 8-min, at 37°C. Mean ± SD (n = 10; 10 dialysate samples taken over the concentration range examined).

membranes, resulting in the prolongation of the diffusion pathway. The tissue clearance of the compound under investigation may also influence *in vivo* recovery.

Scheller and Kolb (2) demonstrated that RL is a phenomenon independent of concentration, therefore the RL *in vivo* was determined for only one concentration of both the compounds and IS (1 μg/ml for L-DOPA, DA, DOPAC and α-methyldopa, and 3 μg/ml for 3-OMD). In addition, our *in vitro* experiments (Table III) also disclosed that the RR was not a concentration-dependent phenomenon.

Surprisingly, in the *in vivo* calibration experiments, the RL of α-methyldopa was markedly different from the RL of L-DOPA. α-Methyldopa differs structurally from L-DOPA by one methyl group only. As their structural differences probably do not influence their diffusion properties (12), these differences in RL explicitly demonstrate that the diffusional behaviour may be a more tissue-dependent phenomenon and that a substance may interact with a tissue in a highly specific way.

There was a clear disparity between the RLs for L-DOPA, DOPAC, DA and 3-OMD and that for the IS, in blood plasma and in skeletal muscle ECF, resulting in different K values between the 2 tissues. In view of these considerable differences, it seems appropriate to estimate *in vivo* K values separately for each tissue. Although, the chemical and physicochemical properties of the compounds of interest

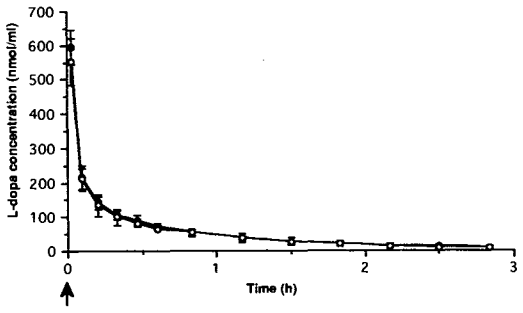

Fig. 2. Validation experiment. Concentrations of L-DOPA (nmol/ml) in blood plasma determined by microdialysis (open circles) and whole-blood sampling (closed circles) following the i.v. injection (arrow) of L-DOPA (20 mg/kg) after pretreatment with carbidopa. Data are arithmetic means ± SEM (n = 4; experiments in 4 dogs).

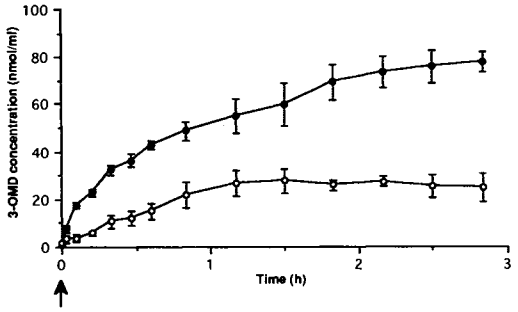

Fig. 3. Validation experiment. Concentrations of 3-OMD (nmol/ml) in blood plasma determined by microdialysis (open circles) and whole-blood sampling (closed circles) following the i.v. injection (arrow) of L-DOPA (20 mg/kg) after pretreatment with carbidopa. Data are arithmetic means ± SEM (n = 4; experiments in 4 dogs).

may differ from those of the IS, the most important guideline is that K should be constant for a compound within a certain tissue, because changes in RL of the IS are proportional to those of the compounds of interest.

The data of the K values were clearly more precise than those of the RLs confirming that the use of the internal standard is necessary for the determination of the *in vivo* recovery.

In the validation experiments, a dramatic decrease in RL for α-methyldopa in blood plasma, was observed between baseline (t_0) and the subsequent values. The RL at baseline (35%) confirmed the values found in the *in vivo* calibration experiments (Table I). However, the RL following the i.v. administration of L-DOPA (Fig. 1) was in the range of 10%. This dramatic reduction in RL of α-methyldopa could be explained by the administration of L-DOPA, which elicited a sympathomimetic response, including peripheral vasoconstriction, evidenced by the tachycardia observed in the experimental animals. There could also be an increase in endogenous adrenergic compounds. Another mechanism that might have contributed to this vasoconstrictor response of the peripheral vein is the presence of the probe itself. However, this phenomenon was not observed in skeletal muscle where the RL of the IS was similar to the RL determined during the *in vivo* calibration experiments. In this respect, it is worth mentioning that in brain dialysis, it has been observed that DA release following cocaine can decrease *in vivo* probe recovery by ±50% (13).

In our study, the pharmacokinetic curves of L-DOPA yielded by microdialysis (corrected for recovery) and whole-blood sampling matched perfectly. This confirmed that our technique was valid and provided accurate and precise pharmacokinetic data. Furthermore, it demonstrated that microdialysis can be used in combination with whole blood sampling for the determination of the PB *in vivo* because it determines only free plasma concentrations of drugs. Validation of the microdialysis sampling technique for the determination of PB has been established *in vitro* by comparing this technique with ultrafiltration or equilibrium dialysis, respectively (14-15). L-DOPA, being a large neutral amino acid, was not bound to proteins or other carrier substances in the plasma *in vivo*. Therefore, it is a very convenient drug to study by means of microdialysis. Unlike L-DOPA, 3-OMD was predominantly found in a bound state in blood plasma.

CONCLUSIONS

Microdialysis, as *in vivo* sampling technique, was successfully applied for the determination of L-DOPA, DA, and their metabolites in blood plasma and skeletal muscle ECF, in beagle dogs. The concentrations of the compounds of interest in each dialysate were corrected for the recovery by adding α-methyldopa, as *in vivo* marker, to the perfusates. The technique proved valid for the study of the L-DOPA disposition in blood plasma and revealed similar pharmacokinetic data compared with those from whole-blood collection. Unlike 3-OMD, L-DOPA was in a non-protein bound state in blood plasma.

ACKNOWLEDGMENTS

The authors wish to acknowledge the excellent technical assistance of Mr. G. De Smet, Mrs. R. Berckmans and Mrs. RM Geens. They are also grateful to the staff of the Animalarium VUB for their technical support during the *in vivo* experiments.

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