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Simultaneous monitoring of levodopa, dopamine and their metabolites in skeletal muscle and subcutaneous tissue in different pharmacological conditions using microdialysis*

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Abstract: Microdialysis, in combination with ion-pair reversed-phase liquid chromatography and electrochemical detection is described for the simultaneous determination of levodopa, dopamine, 3-*O*-methyldopa and 3,4-dihydroxyphenylacetic acid in the extracellular space of skeletal muscle and subcutaneous tissue *in vivo* in beagle dog. The relative recoveries *in vitro* for levodopa, dopamine, 3-*O*-methyldopa and 3,4-dihydroxyphenylacetic acid with a 16 mm probe at a flow rate of 5 μl min^{-1} were 29.1, 25.1, 34.7 and 30.1%, respectively. This technique was then applied for three types of pharmacological experiments. In the first experiment L-dopa was administered without carbidopa pretreatment, in the second one, L-dopa was administered following carbidopa pretreatment, and in the last experiment, following pretreatment with both carbidopa and the catechol-*O*-methyltransferase inhibitor, OR-611. After the administration of levodopa without carbidopa pretreatment, all four compounds could be detected in dialysates from skeletal muscle, whereas dopamine and 3,4-dihydroxyphenylacetic acid were not found in dialysates from subcutaneous tissue. After the administration of levodopa following carbidopa pretreatment and following pretreatment with both carbidopa and OR-611 all compounds could be measured except for dopamine. This method enables the pharmacokinetics and metabolism of levodopa to be studied in subcutaneous tissue and skeletal muscle simultaneously.

Keywords: *Levodopa; dopamine; microdialysis; subcutaneous tissue; muscle.*

Introduction

Levodopa (L-dopa) in combination with the peripheral aromatic L-amino acid decarboxylase (ALAAD) inhibitor (carbidopa) is still the mainstay of treatment for Parkinson's disease. However, despite its unique clinical neuropharmacologic characteristics of completely reversing pronounced motor dysfunction, patients in a more advanced stage do respond clinically in an unpredictable way [1, 2]. This problem has created a renewed interest in the relationship between the peripheral pharmacokinetics and clinical response.

L-dopa is transformed in the peripheral tissues by ALAAD to dopamine (DA) and by catechol-*O*-methyltransferase to 3-*O*-methyldopa (3-OMD) (Fig. 1). Other metabolic pathways are unimportant [3]. The main metabolic route of dopamine is deamination by monoamine oxidase to 3,4-dihydroxyphenylacetic acid (DOPAC), which is finally converted by catechol-*O*-methyltransferase to the end metabolite, homovanillic acid (HVA). Conjugation reactions of L-dopa, DOPAC and DA may also occur but are of less importance in peripheral tissue [4].

3-OMD is the principal circulating metabolite found when L-dopa is administered to Parkinson's disease patients [5, 6]. Its long elimination half-life (15–18 h) in humans [7] results during chronic treatment in an accumulation reaching steady state levels which are proportional to the cumulative daily L-dopa dose. Deleterious effects of 3-OMD on the clinical status of the Parkinson's disease patients have been observed only when large oral doses (100 mg kg^{-1}) of this compound were administered [8]. Nonetheless, it is possible that the formation of 3-OMD constitutes an important metabolic sink for exogenously-administered L-dopa. Inhibition of this metabolic pathway might therefore increase the levels of L-dopa and eventually extend its

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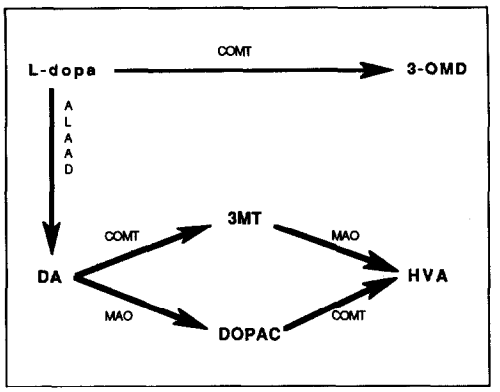


Figure 1
Main pathways of L-dopa and DA degradation. Abbreviations: L-dopa = levodopa; COMT = catechol-*O*-methyltransferase; 3-OMD = 3-*O*-methyldopa; ALAAD = aromatic L-amino decarboxylase; MAO = monoamine oxidase; DA = dopamine; 3MT = 3-methoxytyramine; DOPAC = 3,4-dihydroxyphenylacetic acid; HVA = homovanillic acid.

circulating half-life. One of these recent available potent, non-toxic and selective catechol-*O*-methyltransferase inhibitors is OR-611 which acts in peripheral tissue as well as in the brain [9–11].

So far, measurements of tissue concentrations of a drug have been carried out in tissue slices and homogenates. Tissue slices clearly have limitations. The preparation procedure is time-consuming and traumatic, and therefore can hardly be used in human studies. The microdialysis technique can be applied for the monitoring and the study of *in vivo* pharmacokinetics and metabolism of endogenous and exogenous compounds, including drugs, in brain, blood and muscle tissue in animal [12] as well as in man [13]. In the present study the ability of microdialysis in monitoring L-dopa and DA, and their metabolites, DOPAC and 3-OMD, was evaluated *in vivo* in both the extracellular space of skeletal muscle and subcutaneous tissue. To evaluate the application of this technique for future pharmacokinetic studies, these experiments were performed under different pharmacological conditions.

Experimental

Chemicals and reagents

L-dopa (L-3,4-dihydroxyphenylalanine) and 3-OMD, for *in vitro* experiments were supplied by Roche (Basle, Switzerland). DA hydrochloride was purchased from UCB (Brussels, Belgium) and DOPAC from Sigma (St Louis, MO). L-dopa and carbidopa for *in vivo* experiments were supplied by Merck Sharp and Dohme Research Laboratories (Rahway, NJ). The catechol-*O*-methyltransferase inhibitor, OR-611 (*N*,*N*-diethyl-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl) propaneamide) was provided by Orion Pharmaceutica (Espoo, Finland). 1-Octane sulphonic acid sodium salt was obtained from Janssen Chimica (Beerse, Belgium). All other reagents were analytical reagent grade and purchased from Merck (Darmstadt, Germany). Stock solutions (0.01%, w/v, kept at 4°C) of the standards were prepared in 0.01 M HCl containing 0.1% (w/v) $Na_2S_2O_5$ and 0.01% (w/v) Na_2EDTA. Further dilutions were freshly prepared from the stock solutions by appropriate dilution in 0.5 M acetic acid.

Carbidopa and L-dopa for intravenous administration were prepared as described before [14]. OR-611 (a total dose of 15 mg kg^{-1}) was dissolved in phosphate buffer (pH 7.4; 0.067 M) and was given intravenously 1 h prior and 1 h after L-dopa administration, to provide an optimal inhibition of the O-methylation.

Instrumentation

Analyses were performed on an HPLC system consisting of a Gilson (Villiers-le-Bel, France) Model 302 pump with a Rheodyne 7010 (Cotati, CA) injection valve using a 100-μl injection loop. The Chromatofield Eldec 201 electrochemical detector (Chromatofield, Châteauneuf-les-Martigues, France) was equipped with a thin-layer electrochemical cell fitted with a dual glassy carbon working electrode positioned in parallel and a Ag/AgCl reference electrode. Separation was performed on a 250 mm × 4.6 mm i.d. reversed-phase analytical column (Ultrasphere ODS-column, 5 μm particle size) (Beckman, San Ramon, CA) protected by a μBondapak C_{18}/Corasil 37–50 μm (Waters Chromatography Division, Milford, MA) guard column (30 mm × 4 mm i.d.). Integration of the chromatograms was achieved with a dual channel integration computer program (Integration Pack®, Kontron, Milan, Italy).

Chromatographic conditions

The mobile phase consisted of acetate-citrate buffer containing 0.1 M sodium acetate, 20 mM citric acid monohydrate, 1 mM octane

sulphonic acid, 0.1 mM Na_2EDTA and 1 mM dibutylamine. The pH of the buffer was adjusted to 2.87 with concentrated phosphoric acid. All separations were performed isocratically at room temperature (18–22°C) at a flow-rate of 1 ml min^{-1}. Both electrochemical cells were operated in the oxidative mode with a detector potential of +0.75 V vs 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 one working electrode (W1) at 1 nA V^{-1} and the other (W2) at 10 nA V^{-1}. Standard curves of peak area vs standard concentrations for L-dopa, DA, DOPAC and 3-OMD covering the range 0–1500 ng ml^{-1} were constructed.

Microdialysis experiments

In vitro *experiments*. The microdialysis probes (Carnegie Medicin, Stockholm, Sweden) had a 0.52 mm outer diameter and a membrane length of 16 mm, with a molecular weight cut-off point of 20,000 D. The probes were connected to a microinjection pump (CMA 100; Carnegie Medicin) and continuously perfused at a flow rate of 5 μl min^{-1}. Dialysates were collected every 20 min. A mixture of 0.01 M HCl, 0.1% $Na_2S_2O_5$ and 0.01% Na_2EDTA, used as antioxidant, was added to the vials prior to the sampling. Volumes of 100 μl were injected into the chromatographic system. *In vitro* calibration of the probes was carried out by placing the probes in a standard mixture containing the compounds of interest. The relative recovery was calculated by comparing the concentration found in the dialysate with that in the standard solution. All dialysis experiments *in vitro* were carried out at 37°C.

In vivo *experiments*. Two male beagle dogs were used for these experiments. One beagle dog (18 kg) received in a first experiment L-dopa without carbidopa pretreatment and in a subsequent experiment (more than 1 month later) L-dopa following pretreatment with carbidopa, while the second beagle dog (12 kg) was used for the experiment with L-dopa administration following pretreatment with both carbidopa and OR-611. The dog was anaesthetized with a 25 mg kg^{-1} intravenous dose of sodium pentobarbital and supplementary doses during the experiment, if required, after kept fasting overnight. Temperature was monitored and kept at ±37°C by means of a warming plate covering the anaesthetized animal.

Intramuscular microdialysis. A small incision (2 cm) was made in the skin overlying the gluteal muscle of the hindleg. The guide cannula (1.4 mm) (Carnegie Medicine, Stockholm, Sweden) was introduced into the muscle and the microdialysis probe was entered, sealed into the guide, and fixed.

Subcutaneous microdialysis. A small incision was made in the skin above the groin and the microdialysis probe was implanted subcutaneously and fixed. After insertion of the microdialysis probes, a stabilization period was introduced, during which the probes were continuously perfused with Ringer's solution at a flow rate of 5 μl min^{-1}. After collecting the baseline dialysates, L-dopa (25 mg kg^{-1}) was administered intravenously and the dialysates were collected at 20-min intervals for a further 3 h. The perfusates were stored at −24°C until assayed. They were analysed for L-dopa, 3-OMD, DA and DOPAC.

Pharmacological experiments. Three types of experiments were performed all drugs were administered intravenously. Firstly, L-dopa metabolism was studied simultaneously in skeletal muscle and subcutaneous tissue after the intravenous administration of the L-dopa (25 mg kg^{-1}) without carbidopa pretreatment. Secondly, a similar experiment was performed but after the administration of L-dopa (25 mg kg^{-1}) plus carbidopa. Finally, in the last experiment L-dopa (25 mg kg^{-1}) was administered after both carbidopa and OR-611 (15 mg kg^{-1}) pretreatment. The latter was given 1 h prior to the L-dopa administration and was repeated after 2 h, in order to provide an optimal inhibition of the formation of 3-OMD.

Pretreatment with carbidopa (100 mg orally per day) was initiated, in both experiments, 1 week before the monitoring, with an additional dose of 100 mg intravenously 30 min prior to the start of the monitoring, providing an optimal inhibition of the conversion of L-dopa to DA [15, 16].

Results and Discussion

The utility of the microdialysis technique in

the continuous monitoring of acute or chronic changes, can provide information on the compartmental and metabolic fate of endogeneous as well as exogenous compounds. A major advantage of using microdialysis as a sampling technique is that no sample preparation is required, eliminating the most time-consuming steps of an analytical procedure. Furthermore, since this concerns a single-step procedure the use of an internal standard is not necessary and therefore would not improve the accuracy of the analytical method.

Although our *in vitro* experiments revealed good relative recoveries with small standard deviations we did not express the concentrations of the compounds of interest as interstitial concentrations, but as concentration per dialysate. Nevertheless the relative recoveries *in vitro* of the probes, determined after the experiments were comparable with the *in vitro* values preceding these experiments. Furthermore, the parameters that govern both stability and diffusional behaviour of the analytes across the membrane, among them temperature and pH were similar *in vivo* and during the *in vitro* experiments. The estimation of the *in vivo* recovery forms an alternative to the present method. However, the former depends too much on the diffusional behaviour of the substance in the tissue as well as on its tissue clearance. Furthermore, in some pathophysiological conditions the *in vivo* recovery might change [17].

The dose of L-dopa administered was equivalent to the common daily dose of 1750 mg in a patient with advanced Parkinson's disease. A smaller dose would probably have interfered with the detection limit of the system, which already formed a problem for DA in most of the dialysates. The sampling interval, sample volume, recovery and flow rate are all interrelated. A low flow rate provides a higher recovery but results in a smaller sample volume. All depends eventually on the detection limit (signal-to-noise ratio = 3) of the analytical system which was for L-dopa, DOPAC, DA and 3-OMD was 6, 100, 80 and 500 fmol on-column, respectively [18]. A representative dual chromatogram showing the separation of a standard mixture containing the compounds of interest is shown in Fig. 2. Figure 3 is the dual chromatogram of a dialysate collected from skeletal muscle, following the administration of L-dopa without pretreatment with carbidopa. For comparison

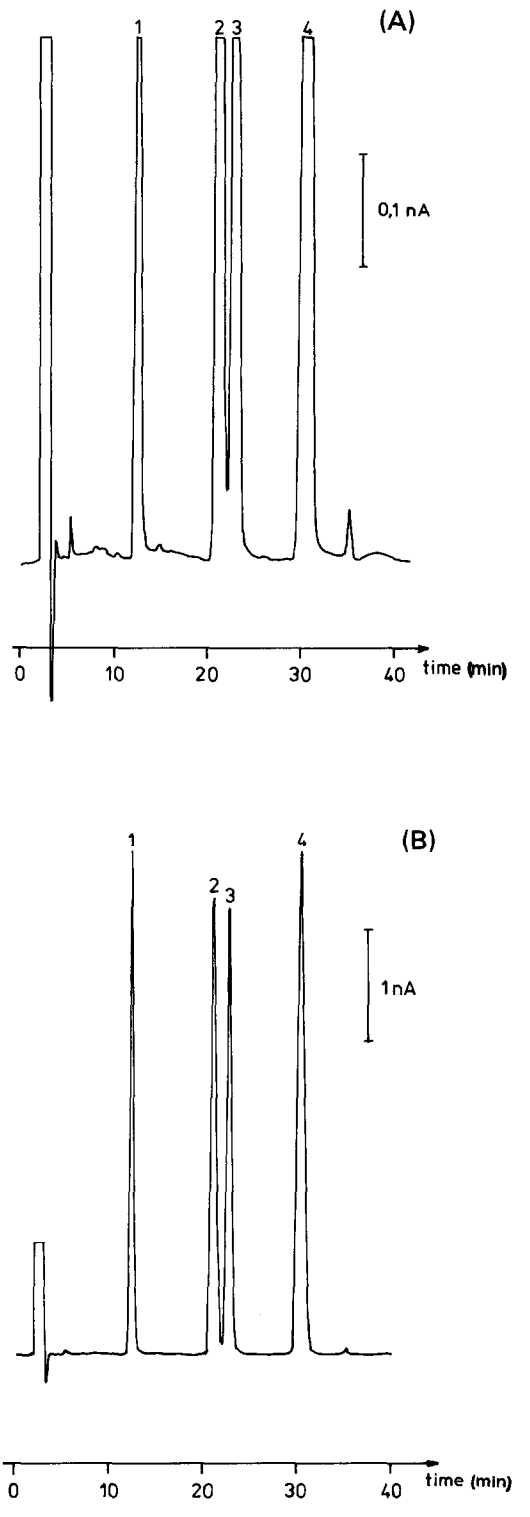
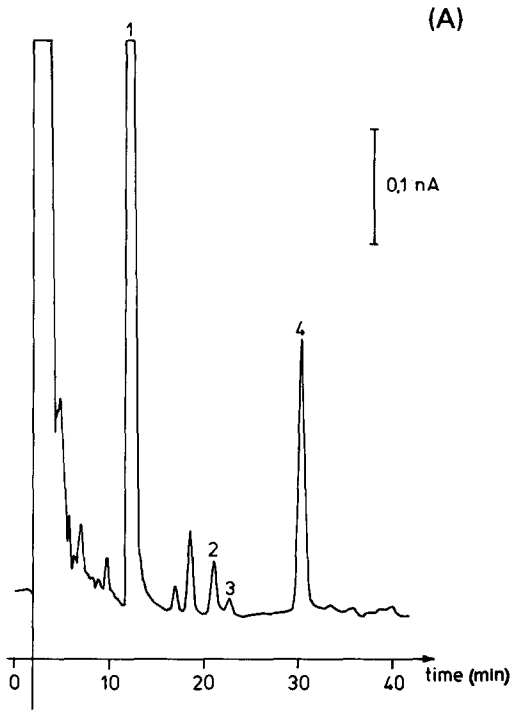


Figure 2
Dual chromatogram of a standard solution (50 ng on-column for L-dopa, DA and DOPAC) (150 ng on-column for 3-OMD). (A) Corresponds with working electrode (W1) at 1 nA V^{-1} and (B) with working electrode (W2) at 10 nA V^{-1}. Peaks: 1 = L-dopa; 2 = DOPAC; 3 = DA; 4 = 3-OMD.



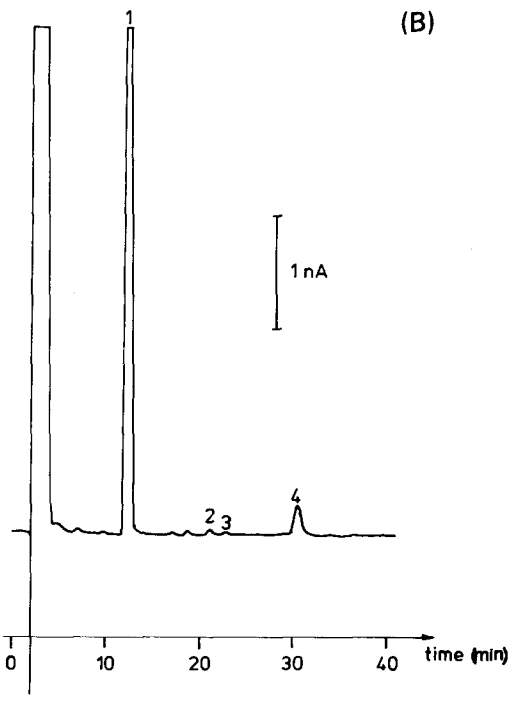
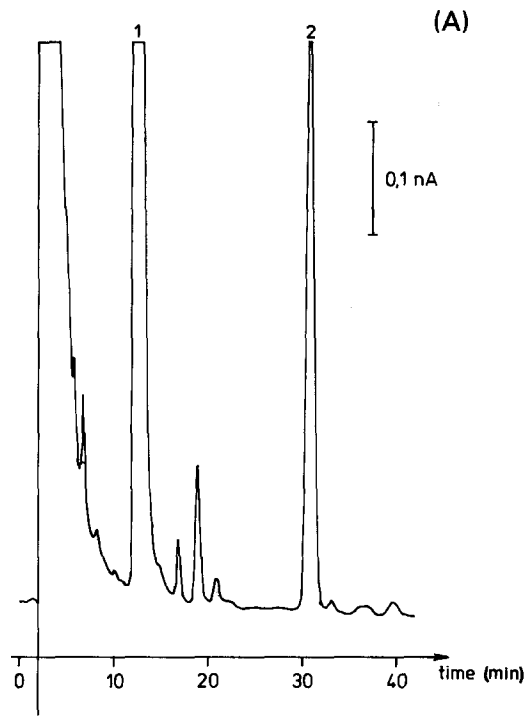


Figure 3
Dual chromatogram of a skeletal muscle dialysate after the administration of L-dopa without carbidopa pretreatment. (A) Corresponds with working electrode (W1) at 1 nA V^{-1} and (B) with working electrode (W2) at 10 nA V^{-1}. Peaks: 1 = L-dopa; 2 = DOPAC; 3 = DA; 4 = 3-OMD.



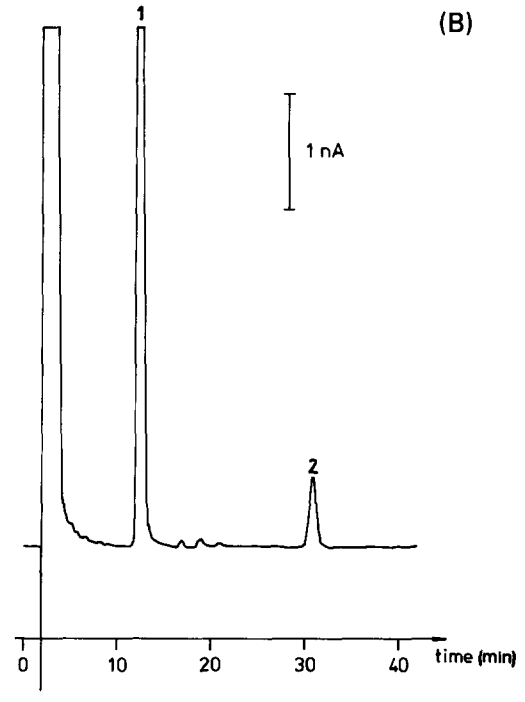


Figure 4
Dual chromatogram of a subcutaneous dialysate during the experiment described in Fig. 3. (A) Corresponds with working electrode (W1) at 1 nA V^{-1} and (B) with working electrode (W2) at 10 nA V^{-1}. Peaks: 1 = L-dopa; 2 = 3-OMD.

the dual chromatogram of the dialysate collected from subcutaneous tissue during the same experiment is shown (Fig. 4). All these recordings showed an optimal separation of the compounds of interest.

The suitability of the system was tested by repeat injection of a standard solution (250 ng ml^{-1}) of the compounds into the chromatograph. The relative standard deviation (RSD) of the peak areas was 2.3, 3.4, 3.0 and 4.9% for L-dopa, DOPAC, DA and 3-OMD, respectively. The regression equations for the compounds of interest were highly linear ($r = 0.99$ for all compounds) over the range of concentrations (0–1.5 μg ml^{-1}) examined.

The relative recoveries *in vitro* for the type of probe used (16 mm) at a flow rate of 5 μl min^{-1} were 29.1% ± 3.9% for L-dopa, 30.1% ± 3.5% for DOPAC, 25.1% ± 4.3% for DA and 34.7% ± 4.5% for 3-OMD. With respect to the recovery of the probe and the detection limit of the system, it could be concluded that at least a 16 mm membrane length was required for the monitoring of the extracellular space in muscle and subcutaneous tissue.

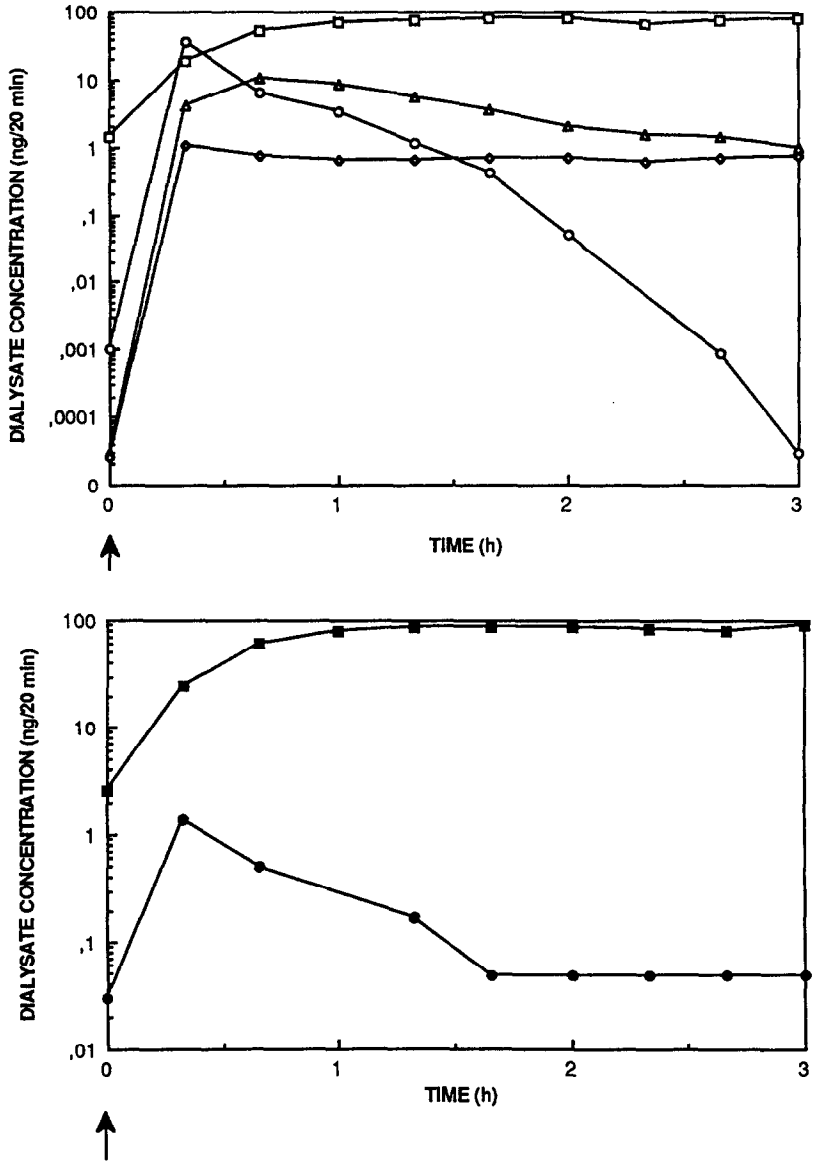


Figure 5
Dialysate concentrations (ng 20 min^{-1}) of L-dopa (circles), DOPAC (triangles), DA (quadrangles) and 3-OMD (squares) in muscle (upper panel) and subcutaneous tissue (lower panel), plotted on a semilogarithmic scale, following intravenous administration of L-dopa (25 mg kg^{-1}) (arrow) without carbidopa-pretreatment.

The time course of the amounts of L-dopa, 3-OMD, DA and DOPAC in dialysates collected from the skeletal muscle and subcutaneous tissue after the administration of L-dopa without carbidopa pretreatment are shown in Fig. 5 and those after L-dopa with carbidopa pretreatment in Fig. 6. Very low baseline dialysate concentrations of L-dopa were measured in both tissues after L-dopa administration without carbidopa pretreatment. Furthermore, L-dopa, DA and their metabolites could be detected simultaneously in dialysates from skeletal muscle, in contrast to subcutaneous tissue, where DA and DOPAC could not be detected. L-dopa levels were much lower in subcutaneous tissue compared to skeletal muscle, in contrast to 3-OMD concentrations which were comparable.

After the administration of L-dopa following carbidopa pretreatment, all compounds, except DA, were measured. Furthermore DOPAC concentrations were almost comparable in both tissues. Similar findings were observed after the administration of L-dopa following pretreatment with both carbidopa and OR-611 (Fig. 7). This coadministration

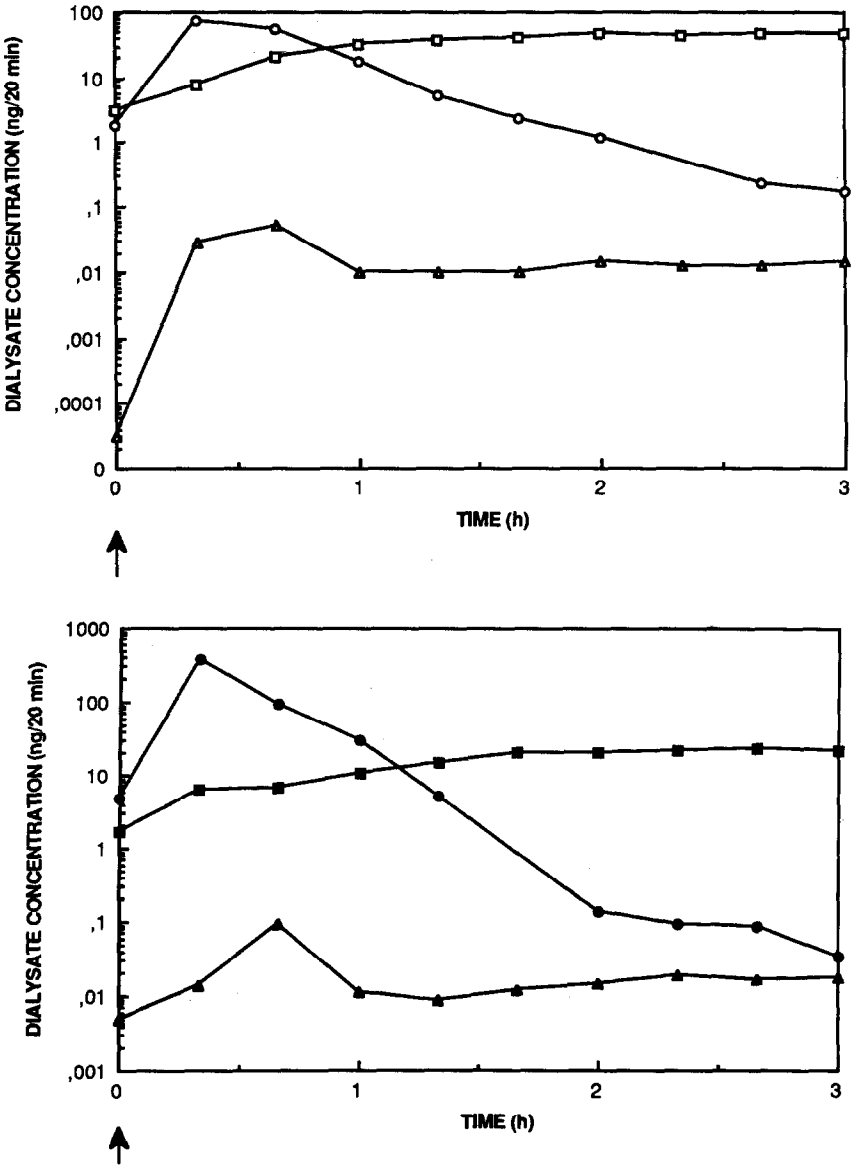


Figure 6
Dialysate concentrations (ng 20 min^{-1}) of L-dopa (circles), DOPAC (triangles) and 3-OMD (squares) in muscle (upper panel) and subcutaneous tissue (lower panel), plotted on a semilogarithmic scale, following intravenous administration of L-dopa (25 mg kg^{-1}) (arrow) after pretreatment with carbidopa.

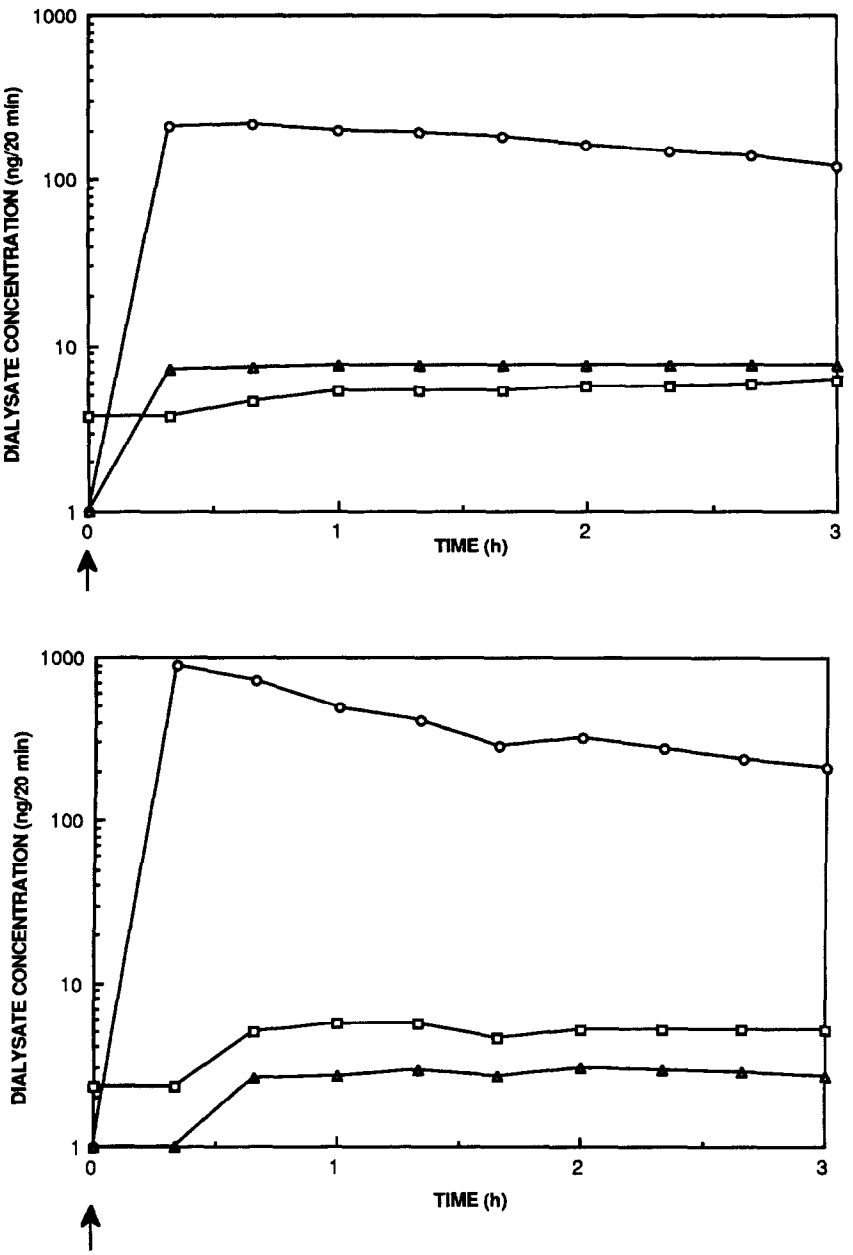


Figure 7
Dialysate concentrations (ng 20 min^{-1}) of L-dopa (circles), DOPAC (triangles) and 3-OMD (squares) in muscle (upper panel) and subcutaneous tissue (lower panel) plotted on a semilogarithmic scale, following intravenous administration of L-dopa (25 mg kg^{-1}) (arrow) after pretreatment with both carbidopa and OR-611 (15 mg kg^{-1}).

resulted in an increase in both the dialysate concentrations of L-dopa as well as its half-life, in both the tissues. This was indicated by the slow decline in L-dopa curve, compared to the profile of the curve after the administration of L-dopa following pretreatment with carbidopa alone. In addition, the 3-OMD formation was partially suppressed, in both the tissues, after pretreatment with carbidopa and OR-611, demonstrating the effectiveness of the latter. These results are encouraging, but it will be interesting to see whether this is true also in Parkinson's disease.

Thus far fewer compounds have been studied by subcutaneous microdialysis [19]. Pharmacologically it will be of interest to know if subcutaneous tissue forms a compartment different than that of the plasma. The extracellular space in skeletal muscle has shown to be a compartment other than plasma [12].

Skeletal muscle forms around 45% of our body mass in humans, therefore it is of importance to determine the metabolic fate *in vivo* of compounds which are extensively metabolized in this tissue such as glucose and amino acids, including L-dopa.

In conclusion, this study demonstrated that microdialysis in combination with HPLC and electrochemical detection can be used for the *in vivo* monitoring of L-dopa, 3-OMD, DA and DOPAC in skeletal muscle and subcutaneous tissue in dog. Furthermore, the method enables pharmacokinetic parameters and metabolism to be studied concomitantly in these two tissues simultaneously. The results indicated that the technique might not be optimal for the study of DA in both tissues.

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