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Continuous intravenous monitoring of levodopa and 3-O-methyldopa by microdialysis and high-performance liquid chromatography with electrochemical detection

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Abstract: Microdialysis, in association with ion-pair reversed-phase high-performance liquid chromatography with electrochemical detection, was applied *in vitro* for the determination and quantification of levodopa and 3-O-methyldopa in blank spiked plasma and blood. The method presented gave accurate results; the calibration graphs for plasma were linear over the range of the expected values for both compounds. When using a dialysis probe with a membrane length of 1.6 cm at a 5 μl min^{-1} perfusion speed, the recovery rate in plasma for levodopa was 30.1% and 68.5% for 3-O-methyldopa. However, less reproducible results were obtained for plasma levodopa levels in the range of 0.5 μg ml^{-1} and lower. The microdialysis technique was subsequently successfully applied for the continuous intravenous monitoring of levodopa and 3-O-methyldopa in a levodopa-treated dog.

Keywords: *Levodopa; 3-O-methyldopa; microdialysis; plasma; reversed-phase high-performance liquid chromatography; electrochemical detection.*

Introduction

Levodopa in combination with carbidopa has shown its beneficial effects in the treatment of Parkinson's disease. However, long-term treatment with levodopa/carbidopa is associated with a variety of untoward effects, including "end-of-dose" hypokinesia or wearing-off phenomenon, "on–off" phenomenon, dyskinesia and dystonia. Investigations into the pharmacokinetic behaviour of levodopa have greatly contributed to our understanding of these response fluctuations [1–3]. Age-related delayed gastric emptying, dietary factors, variable transit time and erratic absorption of the drug at the proximal part of the small intestine are some of the contributing factors to the decreased bioavailability of this drug, leading to unpredictable patterns of motor fluctuation [4, 5]. Furthermore, elevated blood levels of 3-O-methyldopa, the 3-O-methylated metabolite of levodopa, have been associated with the occurrence of levodopa-induced dyskinesias and even in the induction of off-periods [6, 7]. The bioavailability of levodopa and 3-O-methyldopa is not routinely evaluated for individual patients as yet because of a lack of fast, reliable and sensitive assays required during long-term monitoring periods.

Microdialysis is a recently introduced sampling technique allowing the *in vivo* study of extracellular levels of neurotransmitters in brain [8, 9], although apparently it has not yet been applied for the intravenous monitoring of levodopa and 3-O-methyldopa. Although this method was initially developed for *in vivo* sampling, the present probes are not yet suitable and safe for intravenous drug monitoring in man.

This paper describes a rapid, sensitive and specific method for the simultaneous determination of levodopa and 3-O-methyldopa, in plasma and blood *in vitro*, using a microdialysis technique and high-performance liquid chromatography (HPLC) with electrochemical detection. The microdialysis technique was subsequently successfully applied to the continuous intravenous monitoring of levodopa and 3-O-methyldopa, in an experimentally levodopa-treated dog. From an analytical point

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of view this approach offers the advantage that neither deproteinization nor extraction of the clinical samples is required. From a clinical point of view the method is of interest since it does not require blood sampling, making it more acceptable for continuous intravenous drug monitoring with multiple sample analyses. The experiments reported will probably form the basis for intravenous drug monitoring in man once suitable probes are available.

Experimental

Chemicals

Levodopa (L-3,4-dihydroxyphenylalanine) and 3-O-methyldopa for *in vitro* experiments were supplied by Roche (Basel, Switzerland) and carbidopa was purchased from Merck Sharp & Dohme (Haarlem, The Netherlands). Levodopa and carbidopa for *in vivo* use were supplied by Merck Sharp & Dohme Research Laboratories (Rahway, NJ, USA). 1-Octane sulphonic acid sodium salt was obtained from Janssen Chimica/Aldrich Europe (Beerse, Belgium). All other reagents were analytical reagent grade and purchased from Merck (Darmstadt, FRG). Stock solutions (100 mg 100 ml^{-1}, kept at 4°C) of the standards were prepared in 0.01 M HCl containing 0.1% $Na_2S_2O_5$ and 0.01% Na_2 EDTA stored at 4°C. Subsequent working dilutions were freshly prepared from the stock solutions by appropriate dilution in Ringer solution (Travenol Laboratories, Lessines, Belgium). Standards were prepared by spiking 2 ml of blank plasma or citrated blood with each compound to give final concentrations covering the expected *in vivo* values of the two compounds of interest.

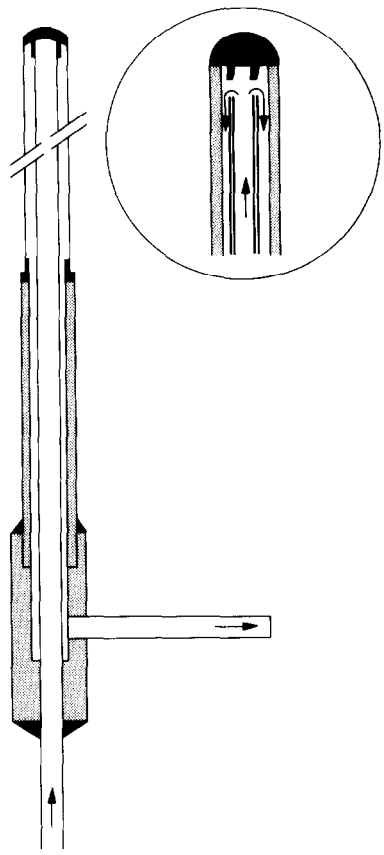

Figure 1
Diagram of the microdialysis probe. A tubular membrane is mounted via a double steel cannula. The probe inlet is connected to the perfusion pump (not shown on the figure). The perfusion fluid (Ringer solution) enters the inner cannula, reaches the bottom from where the fluid flows upwards between the inner cannula and the tubular membrane. The perfusate leaves the probe via the side arm from where it is collected in a suitable vial. The outer diameter of the dialysis membrane is 0.5 mm and its length is 1.6 cm. The insert shows in detail the tip of the tubular membrane. The perfusion fluid reaches the bottom of the inner cannula and flows, via two holes in its wall, upwards between the tubular membrane and the inner cannula.

Microdialysis probe

The microdialysis probe (Carnegie Medicin, Stockholm, Sweden) consisted of a tubular membrane mounted via a double steel cannula (Fig. 1). The perfusion fluid enters the probe through the inner cannula and leaves the probe through an outer cannula via a sidearm, from which the perfusate is collected. The outer diameter of the dialysis membrane is 0.52 mm, the molecular weight cut-off point is 20,000 and the length of the membranes used during the analyses was 0.5 and 1.6 cm. During dialysis, the probe was mounted on a holder and introduced into the blank spiked plasma (or blood) containing the compounds of interest. The probes were connected to a microinfusion pump (CMA 100, Carnegie Medicin, Stockholm, Sweden) and perfused with Ringer solution, which was subsequently collected from the probe outlet in polyethylene tubes. The influence of the perfusion rate was tested by varying the pump speed (2 and 5 $\mu l\ min^{-1}$) and samples were collected at 20 and 8 min intervals, respectively. Forty microlitres of the samples were obtained after using these conditions. Eighty microlitres of Ringer solution were then added to the perfusate, to yield a final quantity of 120 μl for HPLC analysis. All dialysis experiments *in vitro* were carried out either at 25 or 37°C.

Instrumentation

Analyses were performed on an isocratic HPLC system consisting of a Gilson 302 pump (Middleton, WI, USA) with a Rheodyne (Cotati, CA, USA) injection valve using a 100 μl injection loop. The Gilson 141 amperometric detector was equipped with a 4 μl thin-layer flow-through electrochemical cell with a glassy carbon working electrode. Integration of the chromatographic peaks was achieved with a Trio chromatographic computing integrator (Trivector, West Chester, PA, USA). Chromatography was carried out on a 250 × 4.6 mm i.d. Ultrasphere ODS-column (Altex, Beckman, USA) packed with a 5 μm particle size spherical silica gel and protected by a Bondapack C_{18}/Corasil 37–50 μm (Waters Association, Milford MA, USA) guard column (30 × 4 mm).

Chromatographic conditions

All separations were performed isocratically at room temperature (18–22°C) at a flow-rate of 1 ml min^{-1}. The electrochemical cell was operated in the oxidative mode with detector potential set at +750 mV vs a Ag/AgCl reference electrode. The gain of the detector was 5 nA for the measurement of levodopa and 3-O-methyldopa. The composition of the mobile phase used in this study was similar to that described for the HPLC determination and measurement of levodopa and its metabolites in plasma samples, using Sephadex G-10 as the extraction procedure [10]. The mobile phase consisted of methanol–aqueous 0.1 M sodium acetate and 20 mM citric acid buffer (3:97, v/v) containing 0.1 mM Na_2EDTA, 1 mM 1-octane sulphonic acid and 1 mM dibutylamine. The pH of the buffer was adjusted to 3.0 with concentrated phosphoric acid.

Calibration and recoveries

Four-point standard curves of peak area or peak height vs plasma concentrations for levodopa and 3-O-methyldopa, covering the range of expected values (usually between 0.1–20 and 1–50 μg ml^{-1} for levodopa and 3-O-methyldopa, respectively) were constructed using the method of least-squares regression. The standards used consisted of spiked blank plasma (and blood) analysed as described above. Recoveries were determined by comparing the peak areas or peak heights from the dialysates with those of the standards from which the dialysates were yielded, obtained by direct injection into the HPLC system, after deproteinization. Deproteinization was performed by adding 1 ml of 1 M perchloric acid to 2 ml of blood or plasma. Following centrifugation (20 min, 2400*g*) a dilution of the supernatant was made and injected directly into the HPLC system. Data are expressed as means with standard deviation (SD).

In vivo *experiment*

A male beagle dog (18 kg) was anaesthetized with a 25 mg kg^{-1} intravenous dose of sodium pentobarbital and supplementary doses if required, after being fasted overnight. Temperature was monitored and kept at 37°C. One single dose of 0.3 ml Fraxiparine (glycosaminoglycan heparin fractions, 4500 Dalton) was administered subcutaneously 3 h prior to the monitoring, to prevent coagulation around the dialysis membrane.

Subsequently, the dog was positioned on his left side and the microdialysis probe (membrane length, 1.6 cm) was inserted by an intravenous guide (Carnegie Medicin, Stockholm, Sweden) into the cutaneous brachial vein of the right foreleg. Pretreatment with carbidopa (100 mg orally day^{-1}) was initiated 1 week before the monitoring and an additional dose of 100 mg was given intravenously 30 min prior to the monitoring. Levodopa and carbidopa were administered intravenously via an indwelling catheter, placed in the femoral vein of the right hindleg.

Carbidopa as well as levodopa, for intravenous administration, were prepared in the same way. Levodopa (a total dose of 25 mg kg^{-1}) was dissolved in 1 N HCl (100 mg of drug ml^{-1}) and diluted with 10 ml, 0.9% NaCl just before infusion. This solution (pH 1) was given at a rate of 1 ml min^{-1} via the indwelling catheter. Monitoring was started 30 min before administration of levodopa and was continued during a 4 h period. Dialysis was performed at a perfusion speed of 5 μl min^{-1} during 8 min (40 μl dialysate collected). Ringer solution (120 μl) was added to the dialysate, which was then stored until assayed.

Results

Three types of *in vitro* analyses were performed: the evaluation of the probe using aqueous standards, blank spiked plasma and blank spiked citrated blood.

Aqueous standards. According to the length of the dialysis membrane, different recoveries were obtained. For a 0.5 cm dialysis membrane length, at a perfusion rate of 2 μl min^{-1}, the recovery of levodopa was 9.8% (SD 1.8%, $n = 10$) and of 3-O-methyldopa 10.6% (SD 1.6%, $n = 10$). The relative standard deviations (RSD) were 17.9 and 14.8% respectively). Better recoveries [57.2% (SD 6.2%, $n = 10$) for levodopa and 62.2% (SD 3.9%, $n = 10$), for 3-O-methyldopa] were obtained when using a probe with a membrane length of 1.6 cm. Relative standard deviations for repeated determinations from the same sample were 10.9% for levodopa and 6.4% for 3-O-methyldopa.

As expected, when applying a higher perfusion rate (5 μl min^{-1}), recoveries decreased to 29.1% (SD 3.9%, $n = 6$) for levodopa and to 34.7% (SD 4.5%, $n = 12$) for 3-O-methyldopa. However, these values were still appropriate for detection, since the detection limit was 750 pg on the column for levodopa and 1 ng on the column for 3-O-methyldopa. Levodopa concentrations of 0.5 μg ml^{-1} gave less reproducible recoveries, explaining the smaller number of relevant measurements for levodopa (Table 1). The RSD was 13% for both compounds.

There was no major change when using room temperature compared to 37°C. However, it should be emphasized that the probe had to be conditioned before use, since the device was stored at 4°C.

The regression equations for the compounds of interest were linear ($r = 0.98$ for levodopa and $r = 0.99$ for 3-O-methyldopa) over the range examined. The use of the dialysis membrane was limited to 10–15 clinical samples; after this there was a loss of recovery: up to 5% for a 0.5 and 11% for a 1.6 cm length of dialysis membrane.

Plasma. Recoveries from plasma for levodopa and 3-O-methyldopa are given in Table 1. The RSD was 23% for levodopa and 12.3% for 3-O-methyldopa. These high values were due to the less reproducible recoveries obtained in the range of 0.5 μg ml^{-1}, especially for levodopa. The regression equations were linear over the concentration ranges examined ($r = 0.98$ for both compounds).

Blood. The same *in vitro* analyses were performed on citrated blood. Recovery rates for the two compounds of interest were inconsistent. On withdrawing the probe after dialysis was performed, a blood-clot was observed on the surface of the dialysis membrane.

Figure 2 shows two typical chromatograms; (a) a dialysate from plasma *in vitro* (b) the dialysate obtained during continuous intravenous microdialysis. Chromatograms showed complete separation of the compounds and no interfering plasma peaks were detected.

Figure 3 shows the pharmacokinetic profile of levodopa and 3-O-methyldopa after administration of a single intravenous dose of levodopa and during continuous intravenous microdialysis in an experimentally treated dog. No levodopa and 3-O-methyldopa were detected in the dialysates prior to the administration of levodopa (not represented in the figure).

Discussion

The use of microdialysis, as the sampling method for intravenous drug monitoring, renders sample preparation unnecessary, eliminating some time-consuming steps such as: centrifugation of blood samples, deproteinization and centrifugation of plasma and, if required, extraction of the compounds of interest. Furthermore, its properties permit the

Table 1
Recovery and reproducibility for the determination of levodopa and 3-O-methyldopa in aqueous solution and plasma (membrane length of probe 1.6 cm, perfusion rate 5 μl min^{-1}, dialysis time 20 min, at 37°C)

	Compound	Recovery (%)	Relative standard deviation (%)
Aqueous solution	Levodopa	29.1 (SD 3.9) ($n = 6$)	13
	3-O-Methyldopa	34.7 (SD 4.5) ($n = 12$)	13
Plasma	Levodopa	30.1 (SD 7.2) ($n = 18$)	23.0
	3-O-Methyldopa	68.5 (SD 8.4) ($n = 18$)	12.3

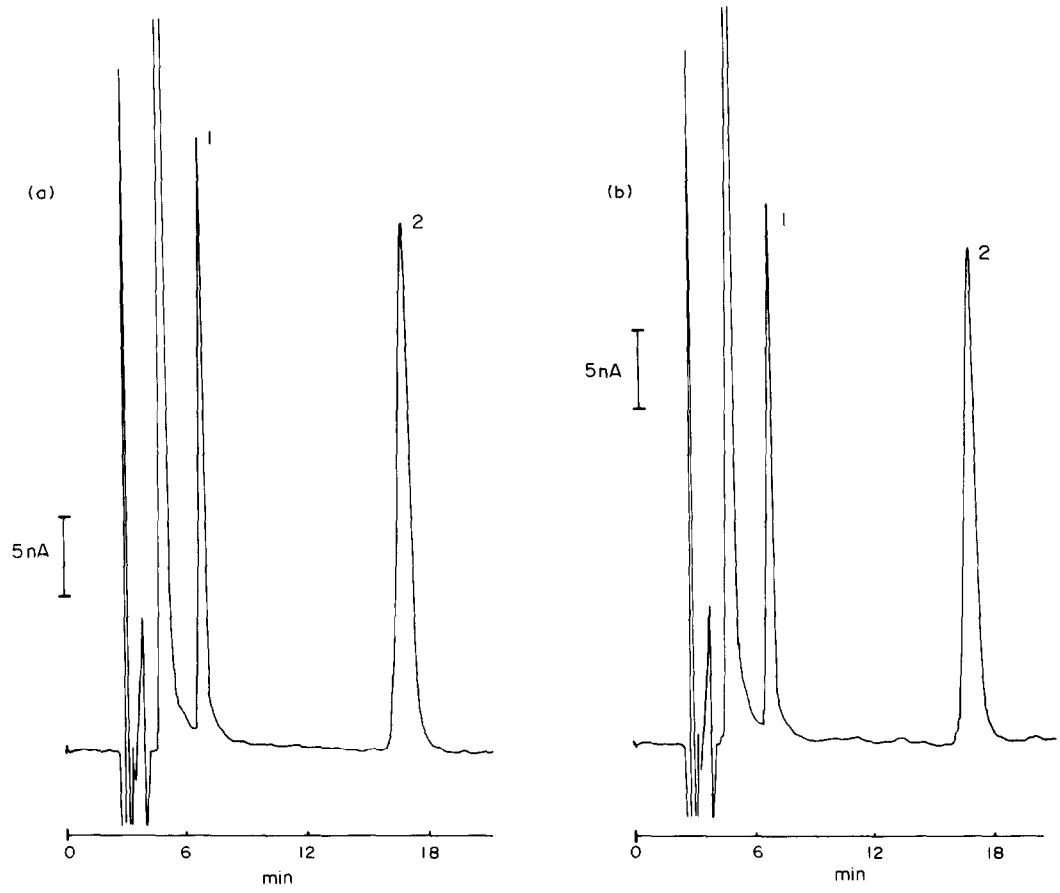

Figure 2
Chromatograms of: (a) a dialysate of spiked plasma, 5 μg ml^{-1} levodopa (Peak 1) and 30 μg ml^{-1} 3-O-methyldopa (Peak 2); (b) a dialysate obtained by intravenous microdialysis. Mobile phase, acetate–citrate buffer containing 1 mM 1-octanesulphonic acid at pH 3.0, methanol 3%, flow-rate, 1 ml min^{-1}, electrochemical detection at +750 mV, range 5 nA.

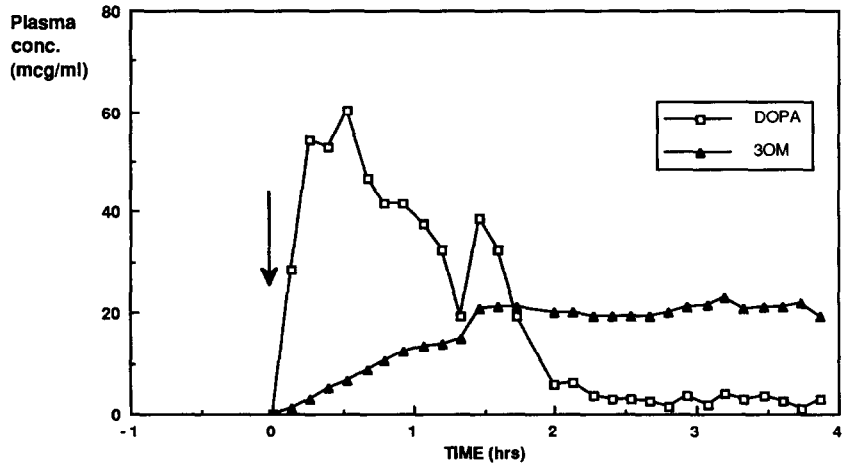

Figure 3
Levodopa and 3-O-methyldopa concentrations vs time curves obtained by continuous intravenous microdialysis in a levodopa-treated dog. (Arrow indicates the time of intravenous administration of 25 mg kg^{-1} levodopa.)

collection of other substances, including small peptides with molecular weight smaller than the cut-off value of the dialysis membrane. Finally, since the procedure involves only a single step, no internal standard is required. From the clinical point of view, it is of interest that microdialysis avoids blood sampling, which is important since drug monitoring in-

volves multiple sample analyses, especially in Parkinsonian patients with motor response fluctuations.

This method has not yet been applied to the collection and subsequent determination and separation of levodopa and 3-O-methyldopa. *In vitro* recovery rates from aqueous solutions for the aromatic amino acids, tyrosine and phenylalanine, using microdialysis have been reported to be 20 and 26%, respectively, when using a probe with a 4 mm membrane length at a 2 $\mu l\ min^{-1}$ perfusion rate (data provided by Carnegie Medicin Laboratories). Taking into account the proportionally longer dialysis membrane a higher recovery rate for levodopa might be expected. However, the same non-linearity applied to these compounds when using probes with smaller membrane length. No major influence of temperature on dialysis was found. Recoveries were highest when using a 1.6 cm length of membrane. High recoveries permit a higher perfusion speed (e.g. 5 $\mu l\ min^{-1}$), which was eventually applied for *in vivo* intravenous levodopa drug monitoring. Even at this perfusion speed the recovery rate in plasma for levodopa was 30.1%, and 68.51% for 3-O-methyldopa. These higher perfusion rates might be useful in plasma levodopa monitoring of Parkinson's disease since motor response fluctuations in these patients are frequently associated with short-lasting fluctuating plasma levodopa levels. At present, no explanation is available for the nearly twofold increase in 3-O-methyldopa recovery in plasma compared to that obtained in aqueous solution. There is no evidence for the metabolization of levodopa since the recoveries were the same in plasma as in aqueous solution. Furthermore, the enzyme responsible for O-methylation, catechol-O-methyltransferase, is predominantly located in the liver and not in the plasma. No plasma peaks were observed which could interfere with 3-O-methyldopa.

From experience with *in vitro* work, it can be concluded that at least a 1.6 cm length of probe is necessary for intravenous microdialysis and therefore currently available probes might be useless for levodopa monitoring, particularly for low (below 0.5 $\mu g\ ml^{-1}$) plasma or blood levels. Plasma levodopa concentration in most patients ranges between 0.1 and 3 $\mu g\ ml^{-1}$. Little information was obtained from the experiments on citrated blood. The formation of a blood cell aggregate at the surface of the dialysis membrane resulted in an unacceptable reduction in the recovery of the probe. The probe was mainly immersed in the volume of blood cells and could not be evaluated in an appropriate dynamic model; therefore these analyses are probably irrelevant.

The technique was successfully applied for the intravenous monitoring of levodopa in a levodopa-treated dog. On this occasion, the anticoagulant was used in a prophylactic way, the same experiments will be repeated later without this drug, and it might be that little or no coagulation occurs at the surface of the dialysis membrane. The pharmacokinetic curves of the two compounds, revealed the profiles expected at least for 3-O-methyldopa, from a conventional type of monitoring [11]. More fluctuations were observed in the levodopa curve than expected. They occurred predominantly during the distribution phase of the drug and therefore, in contrast to the conventional type of monitoring, where blood samples are taken every hour or at most every 30 min [12], our technique might provide more detailed pharmacokinetic information. However, from this single experiment firm conclusions concerning the pharmacokinetic properties of the drug cannot be drawn.

Conclusions

A rapid, reliable and sensitive sampling method was presented for the determination of levodopa and 3-O-methyldopa in plasma using microdialysis and reversed-phase HPLC with electrochemical detection. The technique has been successfully applied for the intravenous monitoring of levodopa and 3-O-methyldopa. It would appear suitable for levodopa drug monitoring of Parkinsonian patients treated with levodopa/carbidopa, requiring multiple and serial sample analyses. Once dialysis probes suitable for human use are available, drug monitoring will be simplified.

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