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Simultaneous determination of levodopa, carbidopa, 3-*O*-methyldopa and dopamine in plasma using high-performance liquid chromatography with electrochemical detection

Y. MICHOTTE,[1]* M. MOORS,[1] D. DELEU,[2] P. HERREGODTS[2] and G. EBINGER[2]

[1] *Pharmaceutical Institute, Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium*
[2] *Department of Neurology, University Hospital, Vrije Universiteit Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium*

Abstract: An analytical method is described which allows a fast, reliable and precise determination of levodopa and its metabolites 3-*O*-methyldopa and dopamine, as well as the peripheral aromatic amino acid decarboxylase inhibitor carbidopa, in a single 1 ml plasma sample of Parkinsonian patients. The compounds are quantitatively isolated on small Sephadex G-10 columns and determined by HPLC with electrochemical detection. Dihydroxybenzylamine or α-methyldopa are used as the internal standard. An example of therapeutic drug monitoring in a patient with fluctuations in motor performances is given. It is confirmed that interference with absorption of levodopa from the stomach by food can be partly responsible for these observed fluctuations.

Keywords: *Levodopa; 3-O-methyldopa; dopamine; carbidopa; reversed-phase liquid chromatography; electrochemical detection; plasma.*

Introduction

Fluctuations in motor performances of patients with Parkinson's disease receiving levodopa in combination with the aromatic amino acid decarboxylase inhibitors carbidopa or benserazide, are a major problem in the treatment of this disorder. Monitoring of drug plasma levels is therefore of great importance in the management of this disease. Hypokinesia is usually associated with low plasma levels of levodopa. This situation may occur when the time interval between two doses is too long and is termed "end of dose hypokinesia", or when levodopa is not absorbed from the stomach, e.g. after a high-protein meal [1, 2]. Inadequate drug absorption does not however explain discrepancies observed between the actual clinical state and plasma levodopa concentration. Competition for transport across the blood–brain barrier between levodopa and large neutral amino acids may offer a clue to the understanding of these observed discrepancies. The levodopa metabolite 3-*O*-methyldopa, may be one of the amino acids

* Author to whom correspondence should be addressed.

involved in this phenomenon [3]. The absorption of the aromatic amino acid decarboxylase inhibitors such as carbidopa, and their efficacy as reflected in the circulating plasma dopamine levels, has been less well studied.

Therefore, there seems to be a need to develop a technique that would permit the simultaneous extraction and quantitative determination of levodopa, carbidopa, dopamine and 3-*O*-methyldopa in a single plasma sample from patients exhibiting a fluctuating clinical response. The determination of free circulating dopamine would allow the detection of individual variations in the efficiency of the decarboxylase inhibitor and the study of the correlation between circulating dopamine levels and side-effects such as nausea or cardiovascular symptoms.

Ion-pair reversed-phase HPLC with electrochemical detection is now the most frequently applied analytical technique for the determination of catechol compounds and their metabolites. A number of isolation procedures from biological fluids or tissues are based on their selectivity towards catechol groups. These include adsorption on alumina [4–6] or on boric acid gels [7] and, more recently, the solvent extraction of the diphenylborate–catecholamine complex as an ion-pair with tetraoctylammonium bromide [8]. Nissinen and Taskinen [9] have reported an HPLC method for the simultaneous determination of the catechol compounds levodopa, carbidopa, dopamine and 3,4-dihydroxyphenylacetic acid in plasma after isolation by alumina adsorption. Ogasahara *et al.* [10] have determined levodopa and 3-*O*-methyldopa in plasma of Parkinsonian patients after successive adsorption on alumina and on a Dowex column. This paper describes an HPLC assay for the simultaneous determination of levodopa, carbidopa, dopamine and 3-*O*-methyldopa in plasma of Parkinsonian patients, after a single isolation step using Sephadex G-10.

Experimental

Materials

The HPLC system consisted of a Varian 8500 (Varian Instruments, Walnut Creek, CA, USA) pump equipped with a 100-μl injection loop (Valco, Houston, TX, USA) and a 100 × 4.6 mm i.d. 5-μm Partisil-5 ODS-3 column (Whatman Ltd., UK). The amperometric detection system consisted of a LCC 231 thin-layer flow-through electrochemical cell with a glassy carbon working electrode and a calomel reference electrode, type E 230 (Bruker Spectrospin, FRG). Integration of the chromatographic peaks was achieved with a Vista CDS 401 integrator (Varian).

The mobile phase consisted of methanol–aqueous 0.1 M sodium acetate and 20 mM citric acid (1:99, v/v) containing 1 mM 1-octanesulphonic acid, 0.1 mM Na_2EDTA, 1 mM dibutylamine and adjusted to pH 3.0 with phosphoric acid. Flow-rate was 1 ml/min, the detector potential was +0.80 V and the sensitivity 50 or 100 nA.

Chemicals and reagents

Levodopa (L 3,4-dihydroxyphenylalanine), and 3-*O*-methyldopa were generously supplied by Roche (Basel, Switzerland), α-methyldopa and carbidopa by Merck Sharp & Dohme (Brussels, Belgium). Dopamine HCl was purchased from UCB (Brussels, Belgium), 3,4-dihydroxybenzylamine.HBr (DHBA) and 1-octanesulphonic acid sodium salt from Janssen Chimica/Aldrich Europe (Beerse, Belgium) and Sephadex G-10 from Pharmacia (Sweden). All other reagents were of analytical reagent grade and were purchased from Merck (Darmstadt, FRG).

Stock solutions of the standards were prepared in 0.01 M HCl containing 0.1% $Na_2S_2O_5$ and 0.01% Na_2EDTA and could be stored at 4°C for 3 months without degradation. Standard solutions were freshly prepared from stock solutions by appropriate dilution with 0.01 M HCl.

Extraction procedure

The extraction columns are prepared by filling long Pasteur pipettes as described previously by Westerink and Korf [11]. The pipettes are never allowed to become dry and were stored in 0.02 M ammonia when not in use. They may be reused for several months. To 1 ml plasma, 1 ml of 400 ng/ml DHBA or α-methyldopa (internal standard) was added and deproteinisation performed with 1 ml 1 M perchloric acid. Following centrifugation (20 min, 1900 **g**), 1.5 ml of the supernatant was applied on the top of a Sephadex G-10 column (10 cm × 0.5 cm i.d.) which had been washed with 3 ml 0.02 M ammonia and then with 3 ml 0.01 M formic acid [11]. The column was eluted with two × 2.5 ml 0.01 M formic acid fractions, the first of which was rejected and 100 μl of the second collected fraction injected into the HPLC.

Standard curves

Linear calibration graphs of peak area ratio of the standards to the internal standard, versus the concentration of the standards were constructed by analysing spiked blank plasma by the extraction and HPLC procedure described, over the concentration range of 25–2000 ng/ml. Regression equations gave good linearity ($r = 0.9999$).

Results and Discussion

As our previous experience with the ion-pair extraction of biogenic amines had been successful, the methods described by Smedes [8] and Herregodts [13] were tested. However even after modification of the experimental conditions, no satisfactory extraction recovery could be obtained for all the compounds using a single procedure. Therefore an extraction procedure based on the adsorption of aromatic compounds on Sephadex was examined because some authors have applied this method for the isolation of biogenic amines and their metabolites from brain tissue samples [10–12]. The isolation on Sephadex was found to be very rapid as all compounds and the internal standard were eluted with a single formic acid fraction. The percentage recovery from blank plasma spiked with 100 ng/ml of each standard was high and reproducible (Table 1), and yielded clean extracts. The peak areas of the extracted standards were compared with the areas obtained for the standards of the same concentration injected directly into the chromatograph.

The capacity of the Sephadex columns was tested because the total amount of the listed compounds in plasma, especially 3-*O*-methyldopa, may be very high. It was found that a total amount of at least 20 μg could be quantitatively eluted with 2.5 ml formic acid solution.

Either DHBA or α-methyldopa may be used as the internal standard, as their extraction recovery is high and reproducible (Table 1). In our HPLC conditions however, α-methyldopa was not completely separated from 3-*O*-methyldopa.

The mobile phase used in this study has been described by us previously for the HPLC analysis of biogenic amines and their metabolites [13]. However the concentration of methanol, the pH value and the concentration of the counter-ion have been optimised

Table 1
Percentage recovery from spiked plasma using the Sephadex extraction procedure

Compound	% recovery (mean ± S.D.; $n = 6$)
Levodopa	93.8 ± 2.3
3-*O*-methyldopa	98.6 ± 6.2
Carbidopa	103.4 ± 4.7
Dopamine	105.6 ± 5.9
DHBA	94.9 ± 5.1
α-methyldopa	95.9 ± 2.1

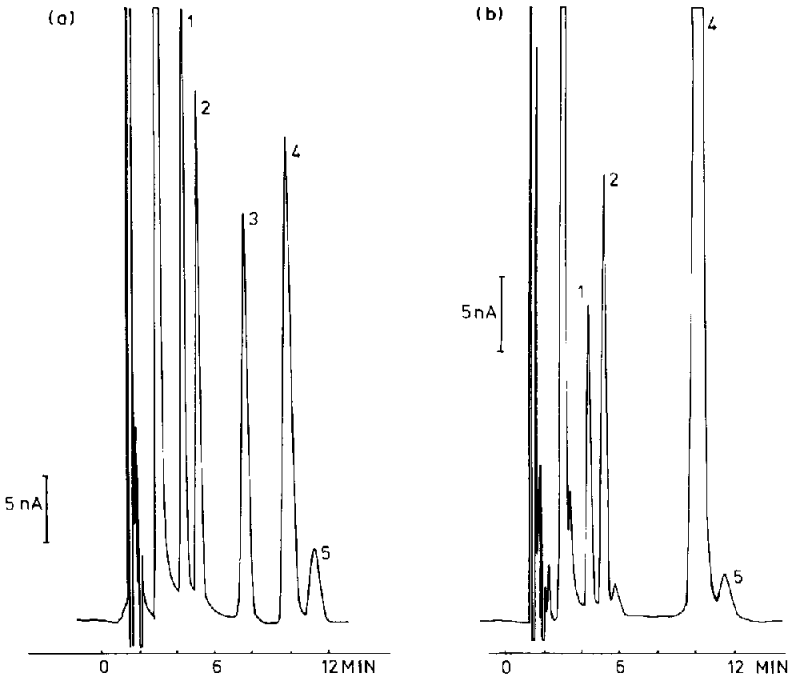

Figure 1
(a) Chromatogram of an extract of blank plasma spiked with standards 1 = levodopa (200 ng/ml); 2 = dihydroxybenzylamine (200 ng/ml); 3 = dopamine (200 ng/ml); 4 = 3-*O*-methyldopa (400 ng/ml); 5 = carbidopa (100 ng/ml); (b) Chromatogram of a plasma extract collected from a patient 1 h after intake of levodopa and carbidopa.

for this particular application. As seen in Fig. 1, all the compounds studied were completely separated in 12 min. Figure 1a shows a chromatogram of an extract of blank plasma spiked with the standards. No interfering peaks were detected. Figure 1b shows a chromatogram of a plasma extract from a patient after 1 h of oral administration of levodopa (100 mg) and carbidopa (12.5 mg).

The within-day precision of the entire analytical procedure was evaluated by analysing six replicate blank plasma samples, spiked with 100 ng/ml of each standard and the internal standard. A precision of 2.5% was found for levodopa, 6.3% for 3-*O*-methyldopa, 5.6% for carbidopa and 5.3% for dopamine. The proposed procedure was

successfully applied for monitoring the plasma of Parkinsonian patients showing response fluctuations. Figure 2 shows a typical example of plasma levodopa and 3-*O*-methyldopa concentrations after repeated oral administration of levodopa 100 mg and decarboxylase inhibitor 12.5 mg. The doses taken after fasting and 2 h after breakfast did produce a corresponding plasma levodopa peak within 1 h after administration. This rapid increase of the levodopa level was short-lasting, having an estimated half-life of about 1 h. The plasma peak was accompanied by an obvious clinical improvement, preceded and followed by dyskinesia symptoms, i.e. the dyskinesia–improvement–dyskinesia phenomenon. After ingestion of high-protein meal drug absorption was totally absent, and 3 h later drug absorption was still delayed. The clinical improvement observed at this time was also less marked than in the morning even though equivalent levels of circulating levodopa were measured.

After fasting for 12 h, the serum concentrations of 3-*O*-methyldopa remained very elevated. Oral administration of levodopa resulted in a slow increase of 3-*O*-methyldopa and this remained for several hours. This is caused by the long half-life (about 15 h) of 3-*O*-methyldopa [14]. The significance of permanently high 3-*O*-methyldopa levels occurring in Parkinsonian patients under levodopa treatment remains unclear. It may be that 3-*O*-methyldopa interferes with the penetration of levodopa into the brain [3], reducing its utilization at the synaptic level.

As far as plasma dopamine is concerned, it should be noticed that after administration of levodopa, *ca* 98% of the total plasma dopamine is sulphoconjugated and biologically inactive [15]. In this assay the detection limit of free dopamine is 15 ng/ml and for most of our patients the free dopamine concentration was below this level. Peripheral side-effects such as nausea and vomiting due to dopamine were also absent. The concentration of the decarboxylase inhibitor oscillated between 50 and 100 ng/ml.

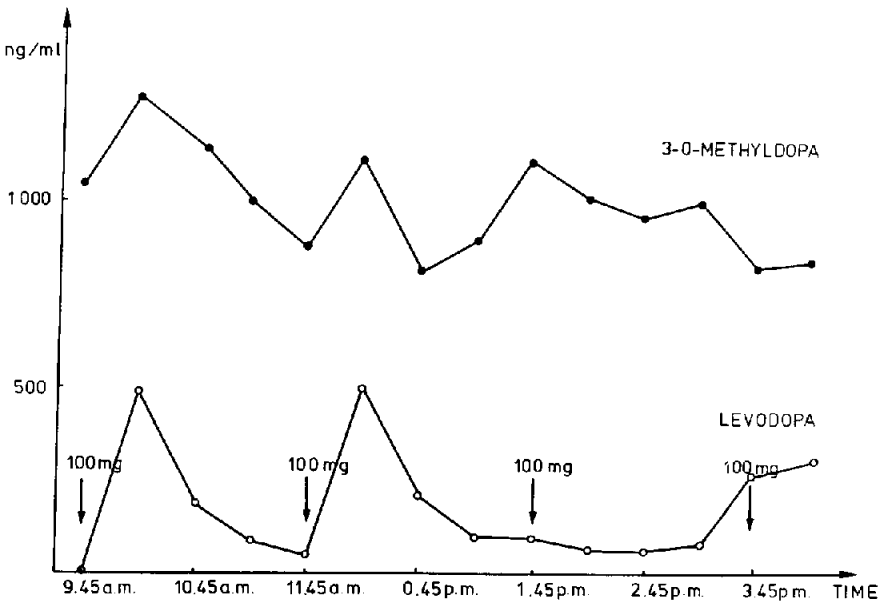

Figure 2
Time course of levodopa and 3-*O*-methyldopa levels in the plasma of a patient treated with levodopa 100 mg and decarboxylase inhibitor 12.5 mg.

Conclusions

Alterations in the absorption and the utilization of levodopa appear to be implicated in the fluctuations observed in the motor performances of some patients with this drug, so that therapeutic drug monitoring is required. The technique described allows a reliable, fast and precise determination of levodopa, carbidopa (the most commonly used inhibitor of the peripheral dopa decarboxylase), and the major levodopa metabolites dopamine and 3-*O*-methyldopa.

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