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Note

Determination of L-DOPA and 3-O-methyl DOPA in human plasma by extraction using C_{18} cartridges followed by high-performance liquid chromatographic analysis with electrochemical detection

M. GERLACH, N. KLAUNZER and H. PRZUNTEK*

Department of Neurology, University of Würzburg, Josef-Schneider-Strasse 11, D-8700 Würzburg (F.R.G.)

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The treatment of Parkinson's disease is still based on substitution therapy with L-3,4-dihydroxyphenylalanine (L-DOPA) in spite of the side-effects (dyskinesia, on—off phenomena and psychoses) that arise, particularly during long-term therapy [1—3]. The causes of these long-term side-effects are still not fully understood. Reduced vesicular storage capacity for dopamine has been discussed for supersensitization of dopamine receptors and post-receptor processes [2, 4]. For peak dose dyskinesia in particular, a role is attributed to the increased blood level of 3-O-methyl DOPA (3-OMD), a principal L-DOPA metabolite. Various authors have put forward a hypothesis that the level of 3-OMD in plasma and/or the plasma 3-OMD/L-DOPA ratio may be taken as a predictive indicator of the long-term effects of L-DOPA therapy [5, 6]. This hypothesis has not yet been confirmed experimentally.

An optimization of parkinsonian therapy (avoidance of frequent medication, of the occurrence of dyskinesia as well as of the on—off effect) could possibly be achieved by the development of a delayed-action preparation of L-DOPA. In order to establish the bioavailability of L-DOPA during medication of a large proband collective with such a delayed-action preparation and in order to test the above-mentioned hypothesis, it was necessary to develop a rapid, simple and reliable method for determining L-DOPA and 3-OMD in human plasma. The methods for determination of plasma catecholamines that had been published at that time were, for various reasons, unsuitable for routine analysis in extensive test series [7—14]. They involved, for example, elaborate sample preparation and poor recoveries after catecholamine adsorption on alumina, or

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expensive and time-consuming radioenzymatic methods. A method for the determination of L-DOPA and 3-OMD in human blood that was published recently [15] was not available at the outset of our project.

Here, we report a simple, rapid and specific method for the simultaneous determination of free L-DOPA and 3-OMD in human plasma. The sample preparation is simple, essentially representing an extraction of the deproteinized plasma from C_{18} cartridges with 15% aqueous ethanol. The method is characterized by high recovery and good reproducibility.

EXPERIMENTAL

Materials

L-DOPA and 3-OMD standards were purchased from Sigma (Taufkirchen, F.R.G.). The 50-mg benserazide capsules [1-DL-seryl-2-(2,3,4-trihydroxybenzyl)-hydrazine · HCl; Ro 04-4602/007, Ch.-B.: G HK 080 CO 1 LO 124] for clinical testing were from Hoffmann-La Roche (Basle, Switzerland). All other chemicals were of analytical grade and obtained from Merck (Darmstadt, F.R.G.).

Apparatus

The high-performance liquid chromatographic (HPLC) system was from Milton-Roy (Hasselroth, F.R.G.) and comprised a ConstaMetric III pump, a Rheodyne injection valve with a 100-μl loop, a CI 10 integrator and a Sekonic S 200 GP plotter. The chromatographic column (250 mm × 4.6 mm I.D.) was packed with Sep-Pak 120-5 C_{18}, particle size 5 μm, from Macherey-Nagel (Düren, F.R.G.). A cartridge head with interchangeable cartridges (20 mm × 4.6 mm I.D.) packed with Shandon Hypersil, particle size 5 μm, from Bischoff Analysentechnik (Stuttgart, F.R.G.) was screwed on to the column head. The amperometric detector consisted of a Model 656 detector cell and a Model 641 current/voltage meter, both from Metrohm (Filderstadt, F.R.G.). The working and auxiliary electrodes were glassy carbon. The potential was adjusted to 0.75 V vs. Ag—AgCl at a sensitivity of 10—50 nA/V.

Chromatography

The Sep-Pak C_{18} cartridges were from Millipore (Eschborn, F.R.G.).

The elution buffer contained 84% 0.1 *M* sodium dihydrogen phosphate and 16% methanol with $2.6 \cdot 10^{-3}$ *M* sodium octylsulphonate, $1.0 \cdot 10^{-4}$ *M* disodium EDTA and $2.5 \cdot 10^{-4}$ *M* triethylamine [16]. The pH was adjusted to 3.35 with orthophosphoric acid. This solution was sterilized by ultrafiltration through a 0.22-μm Sterivex GS filter from Millipore (Eschborn, F.R.G.) using a peristaltic pump, and was degassed with helium. Isocratic elution was carried out at room temperature at a flow-rate of 1.5 ml/min.

Sample preparation

At fixed times of day, blood samples were obtained from probands in a horizontal position via an indwelling venous catheter. The blood was collected in EDTA-coated tubes containing 10 μl of a 0.5% sodium sulphite solution.

The blood was centrifuged (2500 *g* for 10 min at 4°C) and the plasma was

divided into three aliquots and frozen immediately at −20° C until analysis (all samples were analysed within two weeks of collection). A 1000-µl volume of thawed sample was deproteinized by treatment with 150 µl of 0.01 *M* hydrochloric acid, 300 µl of 0.7 *M* perchloric acid and 10 µl of 0.1% EDTA solution. After centrifugation of this mixture at 100 000 *g* and 4° C, the supernatant was decanted. A 750-µl sample of the supernatant was loaded onto a C_{18} cartridge, which had previously been washed and equilibrated according to the manufacturer's instructions with 2 ml of methanol followed by 5 ml of clean water. Loading was carried out by means of a 2-ml disposable syringe and was completed within 2 min. The column was then washed with 1 ml of water before eluting in less than 2 min with 1000 µl of 15% ethanol in water. A 100-µl aliquot of the ethanolic eluate was injected into the HPLC system.

RESULTS AND DISCUSSION

A representative chromatogram showing the separation of a standard mixture of L-DOPA and 3-OMD is shown in Fig. 1A. Fig. 1B and C are chromatograms of two plasma samples from a healthy male proband, prepared as described above. The separation and elution of the two compounds of interest was completed in 7 min. Other principal metabolites of L-DOPA, such as 3,4-

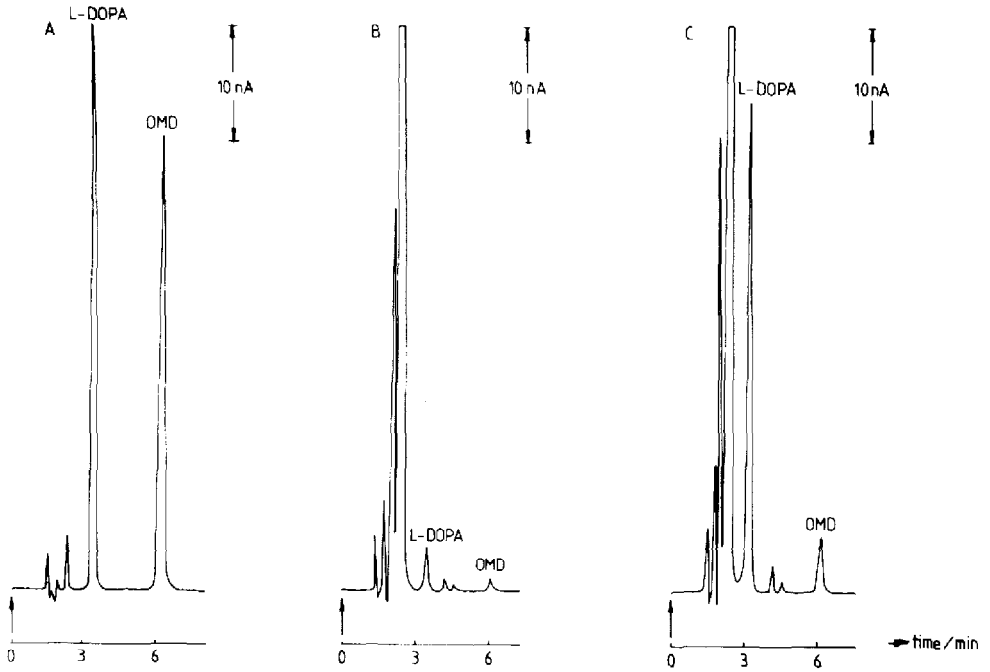

Fig. 1. HPLC chromatograms of: (A) 100-µl standard mixture of L-DOPA and 3-OMD (750 ng/ml L-DOPA and 2000 ng/ml 3-OMD in HPLC buffer); (B) 100-µl ethanolic eluate obtained from the plasma of a healthy male proband according to the methods described here (blood sample taken immediately before L-DOPA ingestion; the proband was treated with 50 mg of benserazide every 8 h on the previous day, i.e. three times, and then again 1 h before sample collection); (C) 100-µl ethanolic eluate obtained from a proband 1 h after ingestion of 100 mg of L-DOPA.

dihydroxyphenylacetic acid (DOPAC), vanillylmandelic acid (VMA) and homovanillic acid (HVA), did not interfere in the separation of L-DOPA and 3-OMD. Dopamine, which arises from decarboxylation of L-DOPA, cannot be detected under the conditions of this assay. There is also no interference from benserazide, a peripheral decarboxylase inhibitor, or from the EDTA and sodium sulphite, which are added to the blood as anticoagulant and antioxidant, respectively (these substances are eluted from the C_{18} cartridge in the aqueous eluent, as are the polar L-DOPA metabolites HVA, VMA and DOPAC). The peak at ca. 3.5 min, which is present in chromatograms B and C, was identified as norepinephrine from its retention time and by co-injection with a reference substance. In some cases, up to three other minor peaks were recorded in the HPLC elution profile after 7 min. These substances were not identified; they are probably amino acids such as 5-hydroxytryptophan and metabolites, which are eluted under these conditions with L-DOPA and 3-OMD. Therefore, the column was washed for 10 min after every separation to ensure a flat baseline and good chromatographic resolution.

So far, we have analysed ca. 2000 samples using the method described. With an average analysis time of ca. 20 min, up to twenty samples may be analysed during an average working day. The use of an automatic sampler with a thermostated sample compartment would make a daily work-load of up to 50 samples conceivable.

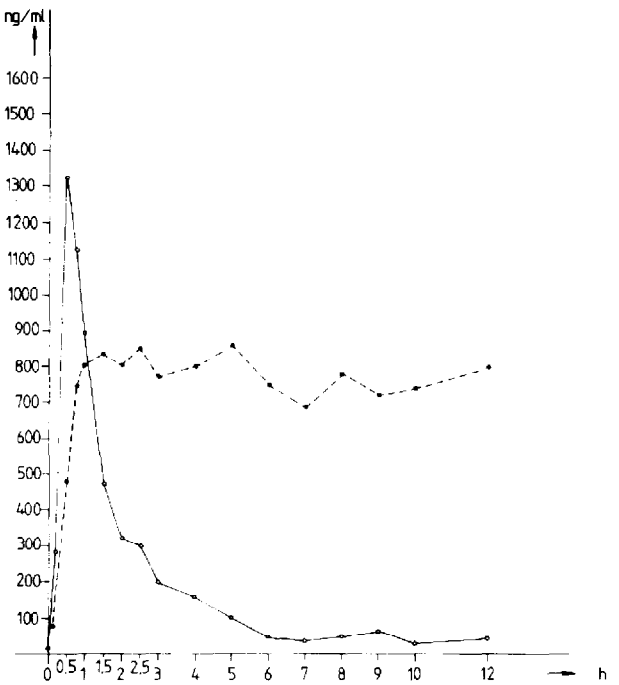

Fig. 2. Time course of the plasma concentrations of free L-DOPA (○——○) and 3-OMD (●- - -●) in a healthy male proband after ingestion of 100 mg of L-DOPA (the proband was treated with 50 mg of benserazide every 8 h on the previous day, i.e. three times, and then again 1 h before sample collection and once more after 6 h). Values immediately before ingestion of L-DOPA (ng/ml plasma): L-DOPA 19.6; 3-OMD 29.8.

Fig. 2 shows an example of the plasma levels of L-DOPA and 3-OMD from a healthy male proband after ingestion of 100 mg of L-DOPA [17]. Both the forms of the time-course curves and the normal levels (values obtained directly before ingestion of L-DOPA) and maximal levels reported in this paper agree with the previously published values [2, 8].

The absolute recovery of L-DOPA and 3-OMD from human serum was determined by comparison of peak heights from known amounts of standard mixture and of ethanol eluates prepared from control serum to which known amounts of standard have been added. As shown in Table I, the recoveries in the concentration range of 100—3000 ng/ml were approximately 100%. An internal standard was not used because it was established in experiments with α-methyl DOPA as standard that no improvement in precision was obtained [18].

TABLE I

ABSOLUTE RECOVERY OF L-DOPA AND 3-OMD AFTER PRE-PURIFICATION ON C_{18} CARTRIDGES

Substance	Concentration (ng/ml)	Recovery (%) (mean ± S.D., n = 5)
L-DOPA	100	101.4 ± 3.7
	300	100.5 ± 1.8
	750	100.2 ± 1.7
	1500	99.2 ± 1.8
	2000	98.7 ± 1.5
3-OMD	200	101.2 ± 3.9
	800	100.9 ± 2.8
	1200	100.5 ± 2.6
	2000	99.0 ± 2.5
	3000	97.9 ± 2.4

TABLE II

DETERMINATION OF THE REPRODUCIBILITY OF SINGLE VALUES FOR STANDARD MIXTURES IN HPLC BUFFER AND POOLED PLASMA SAMPLE AT TWO DIFFERENT CONCENTRATIONS

Substance	Concentration (ng/ml) (mean ± S.D., n = 9)	Coefficient of variation (%)
Standard mixtures in HPLC buffer		
L-DOPA	58.9 ± 2.833	4.80
	282.7 ± 9.891	3.49
	748.2 ± 11.821	1.58
3-OMD	78.1 ± 4.134	5.29
	389.4 ± 15.576	4.00
	1985.7 ± 36.995	1.86
Pooled plasma sample (analysed over a 10-day period)		
L-DOPA	410.2 ± 8.380	2.00
	360.4 ± 14.360	3.98
3-OMD	1710.4 ± 29.607	1.73
	3708.6 ± 130.673	3.52

To determine the reproducibility of the assay, known amounts of standard mixtures were analysed nine times. As shown in Table II, the coefficients of variation were 1.58—4.80% for L-DOPA and 1.86—5.29% for 3-OMD.

Analyses of plasma pools at two different concentrations in the therapeutic range over a 10-day period (the pools were stored in aliquots at —20°C between assays) yielded a coefficient of variation of 2.00—3.98% for L-DOPA and 1.73—3.52% for 3-OMD (see Table II). All plasma samples were prepared under identical conditions. The determinations with the ethanol eluate demonstrated that the values remained essentially constant for a day if the eluate was stored at 4°C.

Linearity was assessed from injections of L-DOPA/3-OMD standard mixtures in the concentration range of 10—3000 ng/ml. For both substances, a linear relationship between the amount injected and the detector signal was demonstrated. Under the assay conditions (sensitivity 10—50 nA/V and electrode potential +750 mV, with a signal-to-noise ratio of 5), the detection limits were 5 and 10 ng/ml for L-DOPA and 3-OMD, respectively.

CONCLUSIONS

The specific and sensitive method described here for simultaneous determination of free L-DOPA and free 3-OMD in human plasma employs HPLC with electrochemical detection and yields reliable results rapidly. The sample preparation is simple, essentially representing an extraction of the deproteinized plasma from C_{18} cartridges with 15% aqueous ethanol. The method is characterized by high recovery and good reproducibility; it is well suited for routine operation with extensive test series.

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