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Simultaneous measurement of L-DOPA, its metabolites and carbidopa in plasma of Parkinsonian patients by improved sample pretreatment and high-performance liquid chromatographic determination[a]

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ABSTRACT

A procedure is described for the determination of L-3,4-dihydroxyphenylalanine (L-DOPA), its metabolites and carbidopa (CD) in plasma of Parkinsonian patients by high-performance liquid chromatography with dual working-electrode coulometric electrochemical detection. An efficient sample preparation scheme is presented for the isolation of L-DOPA, its metabolites and the catecholamines from the same plasma aliquot. After a simple deproteinization with methanol containing 2% of 0.5 *M* perchloric acid and evaporation of the solvent, L-DOPA, its metabolites and CD were separated with a 5-μm Nucleosil C_{18} column. Catecholamines were extracted from the supernatant of the deproteinized plasma by ion exchange on small columns and adsorption on alumina. Recoveries were close to 100% for L-DOPA, its metabolites and CD and 70% for catecholamines. The use of the same mobile phase for the concurrent assay of L-DOPA, its metabolites and catecholamines considerably increased the throughput of samples in the chromatographic system. The dual-electrode coulometric detector afforded peak identification by comparing current ratios. Monitoring of data from patients under L-DOPA therapy is reported.

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INTRODUCTION

Since L-3,4-dihydroxyphenylalanine (L-DOPA) alone or in combination with carbidopa (CD), a peripheral decarboxylase inhibitor (PDD), was introduced for the treatment of Parkinson's disease, there have been many studies on its metabolism and pharmacokinetics that have greatly improved the dosage form of the drug.

Many methods have been developed for the determination of these compounds and their metabolites in biological materials, such as spectrophotometric[1], fluorimetric[2,3], gas chromatographic[4,5], radioenzymatic[6,7] and high-performance liquid chromatographic (HPLC) methods with ultraviolet[8,9], fluorescence[10–12] and electrochemical detection (ED)[10,12–29]. Most of these methods evaluated only a few of the interesting catechol compounds, although there is general agreement on the assumption that the complete profile is more informative in diseases involving abnormalities of catecholamine metabolism. In fact, only the simultaneous monitoring of L-DOPA, 3-O-methyl-DOPA (OMD), dopamine (DA), 3-4-dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE) and epinephrine (E) in plasma allows the study of the genesis of fluctuations in motor performance occurring in Parkinsonian patients undergoing long-term L-DOPA treatment[30]. In this regard, the role of L-DOPA metabolites (particularly OMD) that compete with the drug for the use of the blood–brain carriers is very important[31].

A new pro-drug, L-DOPA methyl ester (L-DOPA ME), is now in experimental use on patients with Parkinson's disease. The advantages of this pro-drug are its high solubility and low acidity[32], so that the drug can be diluted in small volumes of water and administered orally, intravenously or intraduodenally. The clinical effects of this drug are very similar to those obtained with standard L-DOPA but the dosage required are higher. This results in a higher production of OMD[32] and for this reason it is crucial to determine the OMD plasma level in treated patients. The measurement of CD is necessary to ensure that peripheral interfences remain minimal.

The two major problems involving the determination of these compounds are the very low levels of free catecholamines with respect to the amounts of L-DOPA and OMD and the difficult separation of OMD and CD in clinical samples. In previous work[12] these problems were overcome by using a weak cation-exchange column to extract the catecholamines and a double detection system for the determination of OMD and CD. In this paper we report an easy and inexpensive way to shorten the analysis time by an improved plasma prepurification procedure. A complete separation of all compounds is obtained by using an optimized mobile phase composition of appropriate pH. Moreover, the use of two detectors is not required, which is important in terms of cost and simplicity of the method.

The sample preparation simply includes deproteinization with cold methanol solution containing 2% of 0.5 *M* perchloric acid followed by centrifugation. An aliquot (200 μl) of the supernatant is used to measure L-DOPA, OMD, DOPAC and CD. The other part, employed for catecholamine determination, must be purified using a short column of CM-Sephadex and alumina adsorption. The two portions were analysed using the same chromatographic conditions. Detection was performed with a coulometric detector.

EXPERIMENTAL

Materials

Norepinephrine (NE), epinephrine (E), dopamine (DA), DOPAC and N-methyldopamine (NMDA, internal standard) were purchased from Sigma (St. Louis, MO, U.S.A.). L-DOPA and CD were gifts from Merck Sharp & Dohme (Darmstadt, F.R.G.). 1-Octanesulphonic acid sodium salt (OSA) and ethylene glycol-O,O′-bis-(2-aminoethyl-N,N,N′,N′-tetraacetic acid (EGTA) were purchased from Fluka (Buch s, Switzerland). Methanol (HPLC grade) and all other chemicals, of analytical-reagent grade, were obtained from Carlo Erba (Milan, Italy). CM-Sephadex C-25 was purchased from Pharmacia (Uppsala, Sweden) and acid alumina AG-4 from Bio-Rad Labs. (Richmond, CA, U.S.A.). All solvents used in the HPLC system were solubilized with distilled water treated with a Milli-Q system (Millipore, Milford, MA, U.S.A.). The two anticoagulant–antioxidant solutions tested for sample treatment contained EGTA and reduced glutathione[12,33] or EDTA and sodium metabisulphite[26].

High-performance liquid chromatography

The HPLC system consisted of a Model M-45 solvent delivery system (Waters–Millipore, Bedford, MA, U.S.A.) and a Model 7125 injector (equipped with a 100-μl loop). The column was reversed-phase Nucleosil C_{18} (12.5 cm × 4.6 mm I.D.), particle size 5 μm (Macherey, Nagel & Co., Duren, F.R.G.). The Coulochem 5100 A electrochemical detection system (ESA, Bedford, MA, U.S.A.) had a 5011 A analytical cell. The potentials were +0.25 V for the first electrode and −0.35 V for the second. The detector gain was set to 6000 for the first electrode and 20 000 for the second with a full-scale sensitiviy of 1.7 and 0.5 nA, respectively. Chromatograms were analysed with a Chemresearch chromatographic data management computer (ISCO; Lincoln, NE, U.S.A.) monitoring both detector signals. The mobile phase consisted of 0.013 *M* sodium acetate containing 100 mg/l of OSA (ion-pairing reagent), 200 mg/l of disodium EDTA and 15% of methanol (organic modifier) (pH 2.82). The elution of the compounds was carried out isocratically at room temperature with a flow-rate of 0.8 ml/min.

Sample separation

Blood samples from patients receiving L-DOPA were drawn by venipuncture and collected in tubes containing 50 μl of EGTA–reduced glutathione solution and immediately centrifuged (2000 *g*, 5 min, 4°C). The supernatants were stored at −80°C prior to the analysis.

Samples were allowed to thaw at room temperature and a plasma aliquot (1 ml) was spiked with 50 μl of internal standard solution (NMDA, 80 ng/ml). Deproteinization was performed by adding three volumes of ice-cold methanol containing 2% of 0.5 *M* perchloric acid and centrifugation (4000 g, 3 min, 4°C). A volume of 0.2 ml of the supernatant (first aliquot) was aspired and was evaporated to dryness under vacuum and the residue was dissolved in 0.2 ml of the mobile phase. A 5–50-μl volume of the solution was injected into the chromatographic system for the determination of L-DOPA, DOPAC, OMD and CD. Isolation of catecholamines (NE, E, DA) was carried out in the remaining supernatant (second aliquot) according to the previously published method[12]. A 3-ml volume of 0.1 *M* phosphate buffer (pH 7) was added to

the solution and the mixture was poured onto a CM-Sephadex C-25 column (2 cm × 0.5 cm I.D.). Before use the column was washed with 5 ml of 0.1 *M* hydrochloric acid and 10 ml of distilled water and buffered with 10 ml of 0.1 *M* phosphate buffer (pH 7). After the sample had passed through, the column was washed with 5 ml of distilled water. The catecholamines were eluted with 3 ml of 1.5 *M* perchloric acid, collecting the effluent in conical tubes with caps. A 2-ml volume of 1.5 *M* Tris buffer (pH 9.3) containing 0.06 *M* EDTA and 20 mg of acid-washed alumina[34] was added to the solution. The tube was vortex mixed for 2 min on a whirlmixer, the supernatant removed by vacuum aspiration and the alumina washed three times with 1 ml of water. The catecholamines were eluted with 100 μl of 0.1 *M* acetic acid, vortex mixed for 2 min, allowed to settle and centrifuged at 3000 *g* for 2 min. The supernatant was removed and 25 μl were injected.

RESULTS AND DISCUSSION

As catechols are liable to oxidize[35], antioxidants such as sodium metabisulphite or reduced glutathione are commonly added to blood together with the anticoagulants, before the preparation of plasma. We compared two different anticoagulant–antioxidant pairs, EGTA–reduced glutathione and EDTA–metabisulphite, and no significant differences were observed. We preferred to use EGTA–reduced glutathione as these constituents have been demonstrated to produce negligible chromatographic interferences on the front peaks.

The sample pretreatment for measuring the compounds of main clinical interest such as L-DOPA, OMD, DOPAC and CD is faster than that used previously, as proteins are simply separated by centrifugation and the drying solvent (methanol) is highly volatile. Moreover, the extract is more concentrated, which is particularly useful in detecting CD, the plasma level of which is often lower than the detection limit. Deproteinization by addition of trichloroacetic acid, perchloric acid, acetonitrile, methanol and methanol containing 2% of 0.5 or 1.0 *M* perchloric acid was examined. The use of methanol containing 2% of 0.5 *M* perchloric acid to prepare protein-free samples from the plasma gave highly reproducibile recoveries of L-DOPA, its metabolites and CD. It was necessary to evaporate the supernatant to dryness in order to obtain unaltered chromatographic characteristics. The recovery was determined by comparing the peak heights of known amounts of standards added to a pool of plasma from healthy subjects carried through the assay procedures with those resulting from the analysis of the same amount of standard stock solution. As reported in Table I, satisfactory recoveries were obtained with good relative standard deviations (R.S.D.). Table I also gives the regression equations of amount of catechol added *versus* amount found. The regression coefficient is indicative of the recovery and the intercept corresponds to a good approximation to the endogenous concentration.

Several approaches can be used to improve the chromatographic separation of catechols by the reversed-phase ion-pairing technique as a fine balance must be obtained between ion-pairing reagent, organic solvent and pH. The pH of the mobile phase can perhaps provide a means of separating the various substances as it can modify the charges of functional groups. On increasing the pH the retention times of the carboxylic acids and amino acid catechols decreased[35]. The retention time of OMD and CD is the most sensitive to modest pH manipulations in the range 2.75–3.0,

TABLE I

RECOVERY AND REGRESSION LINES OF CATHECHOLS OBTAINED BY STANDARD ADDITION TO A NORMAL PLASMA POOL

The values represent the means of five experiments at three different concentrations. The recovery of L-DOPA, DOPAC, OMD, CD and NMDA[a] was obtained after deproteinization and evaporation steps; the recovery of NE, DA, E and NMDA[b] was obtained after extraction by weak cation exchange and subsequent adsorption on alumina. The internal standard (NMDA) was used in both methods.

Compound	*Recovery (mean ± S.D.)*	*Relative standard deviation (%)*	*Concentration range*[c]	*Regression equation*[d]	*r*
L-DOPA	100.0 ± 7.3	6.7	10–40	$y = 0.853x + 2.028$	0.9999
DOPAC	93.3 ± 4.6	4.6	10–40	$y = 0.889x + 0.954$	0.9968
NMDA[a]	94.4 ± 4.5	4.8	20–80	$y = 0.915x + 0.099$	0.9978
OMD	150.0 ± 5.1	5.1	400–1600	$y = 1.000x + 2.520$	0.9992
CD	93.2 ± 1.6	1.7	200–800	$y = 0.892x + 0.056$	0.9997
NE	74.5 ± 4.5	6.0	152–1249	$y = 0.525x + 160.7$	0.9719
E	68.9 ± 3.3	4.8	15–855	$y = 0.679x + 7.38$	0.9997
DA	69.1 ± 4.2	6.1	13–889	$y = 0.704x + 12.28$	0.9968
NMDA[b]	66.0 ± 5.2	5.2	400–1600	$y = 0.668x - 1.27$	0.9990

[a] NMDA internal standard used for the determination of L-DOPA, DOPAC, OMD, CD.

[b] NMDA internal standard used for the determination of NE, E, DA.

[c] L-DOPA, DOPAC,NMDA[a], OMD and CD are expressed in ng/ml and NE, E, DA and NMDA[b] in pg/ml.

[d] x = added amount of L-DOPA, DOPAC, OMD, CD, NMDA[a] (expressed as ng/ml) and NE, E, DA, NMDA[b] (expressed as pg/ml); y = found amount.

whereas the retention times of amines were not influenced. Fig. 1 shows the effect of pH on the retention times of the compounds in the acidic range. For OMD and CD, optimum separation was obtained at a mobile phase pH of 2.82, and these conditions allowed the simultaneous separation of all the analytes tested without prolonging their retention times too much.

DA was normally detected in the second aliquot, purified by CM-Sephadex C-25 and alumina (see *Sample separation*). When the DA plasma concentration was particularly high it could also be found in the first aliquot, that used to measure L-DOPA, OMD, DOPAC and CD. For this reason, the chromatogram (Fig. 2A) illustrating resolution of standard mixture of L-DOPA and its metabolites also includes DA. As an effect of pharmacological treatment, the plasma levels of L-DOPA, OMD and (sometimes) DOPAC can be 1000–10 000 times higher than that of the other compounds. Fig. 2B shows a chromatogram of an extract from a plasma sample from a Parkinsonian patient after administration of a dose of L-DOPA and CD. As some peaks of interest were out of the range the extract was diluted 20-fold and re-injected (Fig. 2C).

Fig. 3 shows the separation of the catecholamines (A) in a standard mixture and (B) in the plasma extract analysed in Fig. 2. The results demonstrate that all compounds are conveniently separated and can be determined avoiding the previously necessary double detection system. With the chromatographic conditions used in this method, OMD can also be easily detected with the coulometric detector using a higher sensitivity than that employed in the previous work[12] (a gain of 20 000 *vs.* a gain of

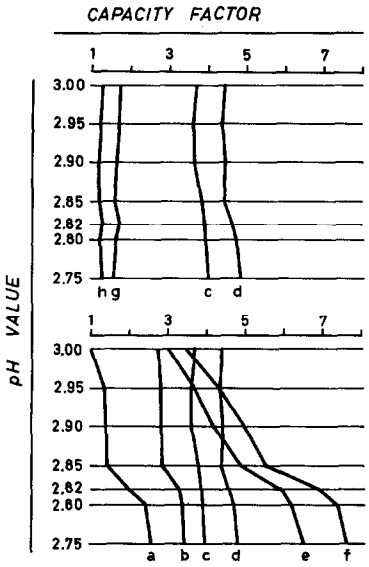

Fig. 1. Influence of pH on the capacity factor of (top) (h) NE, (g) E, (d) NMDA and (c) DA (bottom) (a) L-DOPA, (b) DOPAC, (c) DA, (e) OMD and (f) CD. Column, Nucleosil C_{18}, 5 μm; mobile phase, 0.013 *M* acetate containing 100 mg/l of OSA, 200 mg/l of disodium EDTA and 15% of methanol; flow-rate, 0.8 ml/min. DA was included in both groups as it is often concentrated enough to be detectable in the aliquot used for L-DOPA and the other main compounds (OMD, DOPAC and CD).

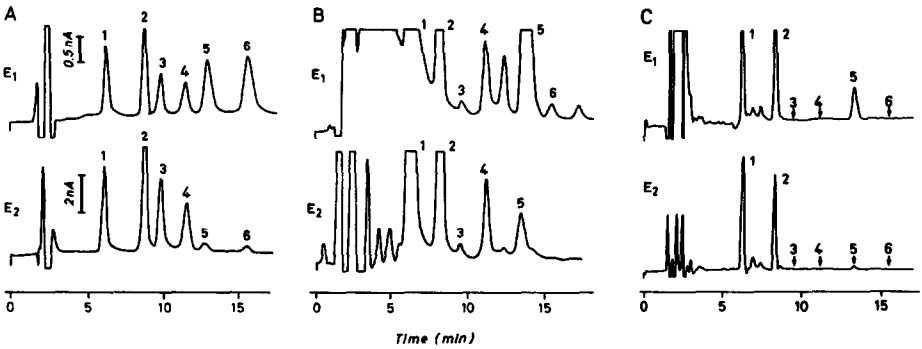

Fig. 2. Chromatograms of (1) L-DOPA, (2) DOPAC, (3) DA, (4) NMDA, (5) OMD and (6) CD after injection of (A) 10 μl of standard solution containing 0.2 ng each of L-DOPA, DOPAC, DA and NMDA, 8.0 ng of OMD and 0.8 ng of CD; (B) 20 μl of the solution obtained after deproteinization of plasma from a patient under L-DOPA therapy containing 2.5 ng of L-DOPA, 2.6 ng of DOPAC and 91.8 ng of OMD; and (C) the same extract as analysed in (B) diluted 20-fold. Chromatogram B does not allow the quantificaton of DA as its peak is not completely resolved and sited on the trailing side of the large tailing peaks of L-DOPA and DOPAC. Column, Nucleosil C_{18}, 5 μm; mobile phase, 0.013 *M* sodium acetate containing 100 mg/l of OSA, 200 mg/l of disodium EDTA and 15% of methanol (pH 2.82). The potential applied was +0.20 V at the first electrode (upper part of the chromatogram) and −0.35 V at the second (lower part). The detector gain was set to 6000 for the first electrode and to 20 000 for the second, with a full-scale sensitivity of 1.7 and 0.5 nA, respectively.

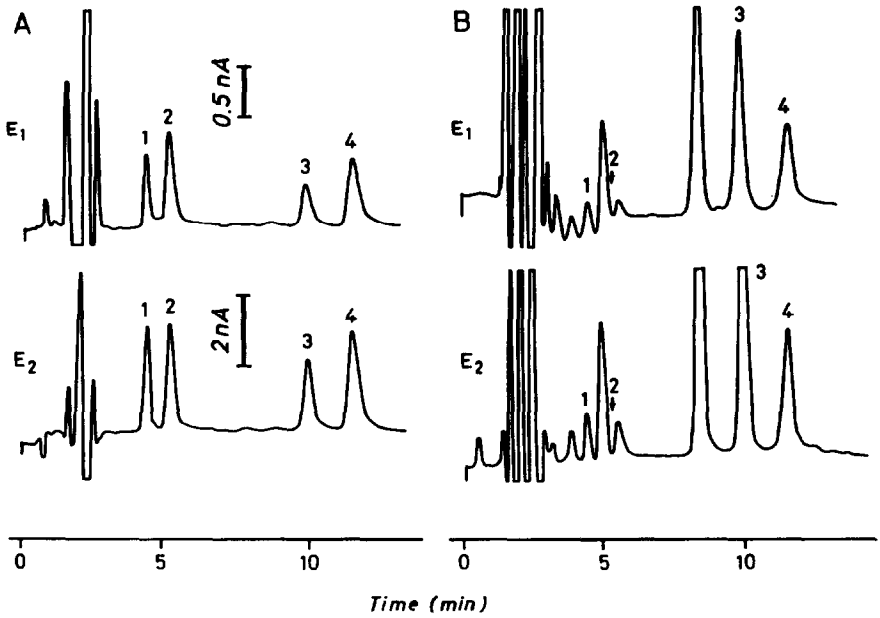

Fig. 3. Chromatograms of (1) NE, (2) E, (3) DA and (4) NMDA after injection of (A) 10 μl of standard solution containing 0.1 ng each of NE and E, 0.2 ng each of DA and NMDA and (B) 25 μl of alumina extract of plasma obtained from a patient under L-DOPA therapy containing 0.05 ng of NE and 0.9 ng of DA. Other details as in Fig. 2.

TABLE II

REPRODUCIBILITY OF THE METHOD

Between- and within-assay reproducibility. A plasma sample was spiked with known amounts of L-DOPA, its metabolites, CD and catecholamines.

Compound	*Concentration*[c]	*Within-assay R.S.D. (%)*[d]	*Between-assay R.S.D. (%)*[d]
L-DOPA	18.2	5.5	6.7
DOPAC	38.3	4.1	5.8
NMDA[a]	747.2	3.3	4.2
OMD	855.1	5.6	5.9
CD	370.4	5.7	6.1
NE	292.1	4.9	5.3
E	156.1	4.8	5.5
DA	151.7	5.9	6.8
NMDA[b]	278.1	5.2	6.3

[a] NMDA internal standard used for the determination of L-DOPA, DOPAC, OMD, CD (deprotcinization and evaporation steps).

[b] NMDA internal standard used for the determination of NE, E, DA (cation exchange and alumina extraction).

[c] L-DOPA, DOPAC, NMDA[a], OMD and CD are expressed in ng/ml and NE, E, DA and NMDA[b] in pg/ml.

[d] Within-assay $n = 6$; between-assay $n = 20$; 5 runs.

TABLE III

REVERSIBILITY RATIOS OF CATECHOLS

The values represent the ratios of the detector responses (oxidation current/reduction current) of L-DOPA, its metabolites, catecholamines and CD. Results are means ± S.D. of ten experiments. E: plasma level was not detectable under our experimental conditions.

Compound	*Standards*	*Plasma*
L-DOPA	0.98 ± 0.05	1.02 ± 0.05
DOPAC	0.77 ± 0.06	0.74 ± 0.03
NMDA	0.93 ± 0.05	0.90 ± 0.03
OMD	6.07 ± 0.17	5.89 ± 0.21
CD	11.18 ± 0.38	11.45 ± 0.43
NE	0.68 ± 0.04	0.71 ± 0.05
E	1.01 ± 0.1	–
DA	0.76 ± 0.06	0.78 ± 0.05

5000) where the compound can only be detected spectrofluorimetrically. The use of a dual electrode system constitutes a powerful tool for optimizing selectivity because the potential of each electrode can be controlled independently. This permits oxidizable and reducible sustances to be detected simultaneously. For our determinations, the oxidation potential was kept as low as possible in order to minimize the background noise and to reduce the interference of the large amounts of OMD normally present in plasma from patients on L-DOPA therapy with the determination of CD, taking advantage of the fact that OMD is not readily oxidized at low potential[10].

The reproducibility of the method, evaluated from multiple analysis of a pooled plasma samples, is satisfactory. Table II gives the between- and within-assay reproducibilities.

TABLE IV

L-DOPA, ITS METABOLITES, CD AND CATECHOLAMINE PLASMA LEVELS IN A PARKINSONIAN PATIENT RECEIVING A CONTINUOUS INTRAJEJUNAL INFUSION OF L-DOPA ME

Each value represents the mean of three determinations. Infusion rate = 180 mg/h plus oral CD 25 mg every hour. CD levels are constistently higher than the minimum amound detectable after the administration of a further 25 mg of the drug (240 min). E: plasma level was not detectable under our experimental conditions.

Time (min)	*L-DOPA (μg/ml)*	*DOPAC (μg/ml)*	*OMD (μg/ml)*	*CD (ng/ml)*	*DA (ng/ml)*	*NE (ng/ml)*
0	0.1 ± 0.01	n.d.[a]	5.2 ± 0.2	n.d.[a]	73.0 ± 6.1	0.5 ± 0.03
20	19.7 ± 0.8	4.5 ± 0.2	5.5 ± 0.3	26 ± 1.6	28.3 ± 1.7	1.6 ± 0.10
40	9.9 ± 0.3	4.7 ± 0.1	6.7 ± 0.2	28 ± 1.5	17.2 ± 1.2	0.5 ± 0.02
60	5.9 ± 0.2	2.8 ± 0.2	8.5 ± 0.3	34 ± 2.4	12.0 ± 0.9	n.d.[a]
180	6.3 ± 0.4	0.5 ± 0.3	8.7 ± 0.3	39 ± 2.6	1.2 ± 0.1	0.2 ± 0.01
300	5.2 ± 0.2	0.4 ± 0.3	8.7 ± 0.6	60 ± 3.4	4.7 ± 0.3	0.5 ± 0.03
420	5.6 ± 0.3	0.4 ± 0.2	12.4 ± 0.4	58 ± 4.5	2.6 ± 0.2	0.6 ± 0.04
540	3.4 ± 0.2	0.3 ± 0.1	10.2 ± 0.4	41 ± 3.2	4.5 ± 0.9	0.9 ± 0.04
660	4.5 ± 0.4	0.3 ± 0.2	16.4 ± 0.7	57 ± 2.1	5.2 ± 0.2	0.4 ± 0.03

[a] n.d. = not detectable (< 5 ng/ml for CD and 0.5 ng/ml for catecholamines).

TABLE V

L-DOPA, ITS METABOLITES AND CATECHOLAMINE PLASMA LEVELS IN A PARKINSONIAN PATIENT RECEIVING A CONTINUOUS INTRAVENOUS INFUSION OF L-DOPA

Each value represents the mean of three determinations. CD levels below the minimum amount detectable could be due to the incomplete inhibition of PDD. Infusion rate = 60 mg/h plus oral CD (25 mg 4-hourly). E: plasma level was not detectable under our experimental conditions.

Time (min)	*Plasma level (ng/ml)*					
	L-DOPA	*DOPAC*	*OMD*	*CD*	*DA*	*NE*
0	128 ± 8	7.8 ± 0.6	6845 ± 444	n.d.[a]	0.133 ± 0.011	0.075 ± 0.005
30	2529 ± 164	9.8 ± 0.8	7219 ± 288	n.d.	0.231 ± 0.011	0.152 ± 0.007
60	2233 ± 179	20.6 ± 1.1	7330 ± 513	n.d.	0.436 ± 0.030	0.164 ± 0.013
90	2470 ± 185	25.3 ± 2.3	5843 ± 351	n.d.	0.608 ± 0.048	0.194 ± 0.009
120	2771 ± 150	32.2 ± 1.4	7498 ± 440	n.d.	0.562 ± 0.035	0.177 ± 0.012

[a] n.d. = not detectable (<5 ng/ml for CD and 0.5 ng/ml for the catecholamines).

Two approaches were used to establish the peak identification. Initially, the peaks were identified on the basis of liquid chromatographic retention behaviour and co-injection with standard compounds. Later, the identity of the separated compounds was tested by recording both the first detector response (E_1, oxidation current) and the second (E_2, reduction current). Comparison of the detector response ratios obtained with standard compounds and plasma samples can confirm peak identity and purity[36,37]. The detector response ratios of standard compounds and of plasma samples are given in Table III. In the case reported in Fig. 3, the peak with a position close to the retention time of standard E (arrow 2) could be easily mistaken for endogenous E, as a small shift of the peak might occur. The comparison of the detector response ratios allowed us to avoid an incorrect identification.

Assuming a signal-to-noise ratio of at least 3, the detection limits of the assay described in this paper were 10, 10, 10, 10, 100 and 40 pg for L-DOPA, DOPAC, DA, NMDA, OMD and CD, respectively.

The continuous intrajejunal administration of L-DOPA ME provided constant blood levels of L-DOPA (Table IV), very similar to the continuous intravenously administered standard L-DOPA (Table V).

From the data in Table IV, it can be seen that the plasma OMD levels in patients receiving L-DOPA ME, even if high, remained constant as soon as they reached the steady state (360 min). The plasma CD levels could be a useful means of monitoring the inhibition of PDD. The higher CD levels detectable after 240 min could indicate complete inhibition of PDD after the administration of another 25 mg of the drug. Table V reports CD levels below the minimum amount detectable, perhaps owing to the incomplete inhibition of PDD.

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