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Simultaneous determination of levodopa methyl ester, levodopa, 3-O-methyldopa and dopamine in plasma by high-performance liquid chromatography with electrochemical detection

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Abstract

A new procedure is described for the simultaneous determination of levodopa methyl ester (LDME) and its biotransformation products levodopa (L-DOPA), 3-O-methyldopa (3-OMD) and dopamine (DA) in stabilized plasma samples, using reversed-phase high-performance liquid chromatography. A coulometric detector equipped with a dual-electrode system operating in the redox mode was used to simultaneously quantitate all compounds. This system generated a double signal monitored by a dual-channel acquisition data system and allowed quantitation of compounds at the nanogram level. The intra- and inter-assay precision varied in the 2.4–6.9% and 3.2–9.1% ranges respectively, whereas the recoveries were close to 85% for L-DOPA and 3-OMD and 70% for DA and LDME. Samples may be stored at −80°C for 15 days before analysis. The method was applied to plasma samples after oral administration of LDME to rats, but it may also be suitable for human pharmacokinetic studies.

1. Introduction

Levodopa (L-DOPA) alone or in combination with peripheral decarboxylase inhibitors is currently used as the treatment of choice for Parkinson's disease [1]. Nevertheless, there are often complications associated with chronic therapy, such as on-off effect, dyskinesia and psychiatric symptoms [2]. It has been demonstrated that with a continuous intravenous infusion of L-DOPA, a constant capability of movement was maintained in such patients [3], as stable plasma levels were attained. L-DOPA itself is not useful for long-term systemic use since it is relatively insoluble and it has to be diluted in large volumes of solvent before administration. For these reasons there has been interest in developing the L-DOPA methyl ester (LDME). This is a highly soluble pro-drug produced by esterification of the carboxylic function of L-DOPA . This pro-drug is suitable for chronic systemic infusion and as a fast acting oral liquid preparation [4,5].

In order to study the pharmacokinetic properties of different L-DOPA formulations, many methods have been developed for the determination of L-DOPA and its metabolites 3-O-methyldopa (3-OMD) and dopamine (DA).

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Both radioenzymatic assays [6,7] and high-performance liquid chromatography with electrochemical detection (HPLC–ED) [8–15] are currently used. Generally the latter is the method of choice for the separation and detection of these compounds.

Among the works on LDME published so far, only two describe the determination of the prodrug and L-DOPA in plasma [16] and in brain microdialysis samples [17], but L-DOPA metabolites are not determined in these studies. To date, no analytical techniques have been developed for the simultaneous determination of LDME and its major metabolites.

The new analytical method reported here, allows a rapid quantitation of LDME and its biotransformation products L-DOPA, 3-OMD and DA in plasma samples using HPLC–ED and 3,4-dihydroxybenzylamine as internal standard (I.S.).

2. Experimental

2.1. Chemicals and reagents

LDME (L-3,4-dihydroxyphenylalanine methyl ester) hydrochloride was synthesized in our laboratories. L-DOPA, 3-OMD, DA, I.S. and the external standard (E.S.) (homovanillic acid) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol were of HPLC grade and were obtained from Baker (Phillipsburg, NJ, USA). All other reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany).

2.2. Standard solutions

Stock solutions of LDME, L-DOPA, DA, 3-OMD and I.S. were prepared by dissolving the compounds in 0.4 *M* perchloric acid (PCA). Working standard solutions containing LDME, L-DOPA, DA (0.6, 1, 2, 4, 8, 14, 20 μg/ml), 3-OMD (1.2, 2, 4, 8, 16, 28, 40 μg/ml) and I.S. (2 μg/ml) were prepared by diluting the stock solutions in 0.4 *M* PCA. They were stored at +4°C and used within one month of preparation.

2.3. Instrumentation and chromatographic conditions

The HPLC system consisted of a Waters (Milford, MA, USA) Model 6000 A solvent delivery pump equipped with an SSI pulse damper, a Waters Wisp 712 automatic injector and a Spherisorb C_{18} reversed-phase column (250 × 4.6 mm I.D., particle size 5 μm). The system was connected to an ESA (Bedford, MA, USA) Model 5100 coulometric electrochemical detector equipped with an ESA Model 5020 guard cell placed in line before the injector and an ESA Model 5010 dual-electrode analytical cell operating in a redox mode.

The potential of the first electrode (DET 1) was set at +0.35 V and that of the second electrode (DET 2) at −0.35 V. The guard cell potential was set at +0.6 V.

The signals of the two electrodes (DET 1 and DET 2) were monitored by connecting them to a data acquisition and processing system (Waters Maxima 820) and for each analysis two chromatograms were simultaneously obtained.

The mobile phase was 50 m*M* phosphate buffer pH 3.2 (containing 3.5 m*M* heptansulfonic acid and 0.05 m*M* EDTA)–methanol–acetonitrile (88:8:4, v/v/v). The flow-rate was 1.0 ml/min.

2.4. Sample preparation

Blood (rat or human) was directly transferred to an ice-cold collection tube containing sodium EDTA and 20 μl of 20% sodium metabisulfite and centrifuged at 1200 *g* for 5 min at +4°C. The samples were kept on ice throughout the preparation to avoid hydrolysis of LDME. Plasma aliquots (0.5 ml) were placed in screw-cap glass tubes containing 25 μl of 2 μg/ml I.S. solution. The plasma samples were immediately deproteinized by addition of 25 μl of 70% PCA. After vortex-mixing for 1 min, the samples were left on ice for 15 min and then stored at −80°C. On the day of analysis the samples were removed from the freezer and allowed to thaw at +4°C. The tubes were vortex-mixed for 1 min, then centrifuged at 1200 *g* for 15 min at +4°C.

The supernatant (300 μl) was added to 200 μl of 2 *M* citrate buffer (potassium salt) pH 4.5 in order to remove the excess PCA as precipitate. The samples were then centrifuged at 1200 *g* for 10 min at +4°C and 50 μl of the supernatant were injected onto the HPLC system.

2.5. Calibration and calculation

Calibration curves were obtained from a pool of drug-free rat plasma spiked with known amounts of LDME, L-DOPA, DA and 3-OMD. Seven concentrations were used for the calibration curve: 30, 50, 100, 200, 400, 700, 1000 ng/ml LDME, L-DOPA, DA and 60, 100, 200, 400, 800, 1400, 2000 ng/ml 3-OMD.

Peak-height ratios of each compound to the I.S., obtained from plasma standards, were plotted against the concentration of each substance to generate a linear least-squares regression line.

The responses related to each substance obtained on four different days were used to calculate a global linear least-squares regression line. The analysis of variance (*F* test, $\alpha = 0.05$) and lack of fit were used to confirm the significance of the regression and the adequacy of the linear model [18,19].

2.6. Precision and accuracy

The precision of the method was evaluated by calculating the intra-day and the inter-day coefficients of variation (C.V.), using plasma spiked with three different concentrations of LDME, L-DOPA, DA (100, 400, 700 ng/ml) and 3-OMD (200, 800, 1400 ng/ml).

To estimate the intra-day C.V., replicated spiked samples ($n = 5$) were analyzed; concentrations were calculated using the appropriate daily standard curve. The inter-day C.V. was calculated by analyzing, in duplicate, spiked plasma samples using the appropriate curve obtained daily on four consecutive days.

The accuracy was evaluated by calculating the relative error on the total number of samples assayed ($n = 14$) for each concentration.

2.7. Recovery

The recovery from plasma was determined at three different concentrations ($n = 5$ each). Two experiments were carried out. In the first, blank plasma samples (0.5 ml) were prepared as previously described. After precipitation of excess PCA, 200 μl of supernatant were added to 10 μl of 0.4 *M* PCA containing the compounds (corresponding to 100, 400, 700 ng/ml LDME, L-DOPA, DA and 200, 800, 1400 ng/ml 3-OMD), the I.S. (100 ng/ml) and the E.S. (500 ng/ml). Then 50 μl were injected onto the HPLC system.

In the second experiment, plasma samples were spiked with the same amounts of LDME, L-DOPA, DA, 3-OMD and I.S. employed in the first experiment, then prepared as described. After precipitation of excess PCA, 200 μl of supernatant were added to 10 μl of 0.4 *M* PCA containing E.S. (corresponding to 500 ng/ml) and, finally, 50 μl were injected onto the HPLC system.

2.8. Stability

The stability of the compounds in plasma (0.5 ml) was investigated. Spiked samples were prepared with drug-free rat plasma as previously described (see sample preparation) and, after deproteinization with PCA, they were stored at −80°C for 15 days. On the day of analysis the samples were analyzed together with the freshly spiked ones. For each concentration (the same as used in the recovery experiments) five freshly spiked and five stored samples were analyzed.

3. Results and discussion

The simultaneous quantitation of the compounds was obtained by means of the dual-electrode system of the analytical cell, operating in a redox mode. This mode of operation provides additional detector selectivity and, applied to plasma extracts, excludes interfering peaks from the signal of DET 2.

In this method both signals were simultaneous-

ly monitored and two chromatograms were obtained from each injected sample. LDME and DA were measured at DET 2, L-DOPA and 3-OMD at DET 1. The choice of DET 1 for L-DOPA was due to the presence of an interfering peak at DET 2, while for 3-OMD it was due to the higher peak response.

Typical chromatograms obtained from plasma extracts are shown in Figs. 1 and 2. Similar chromatograms were obtained for human and rat plasma.

Deproteinization is a commonly used procedure to prepare plasma samples for the determination of L-DOPA and metabolites [8–15]. As catechols are easily oxidized at room temperature and at physiological pH, the addition of EDTA-sodium metabisulfite and centrifugation at +4°C were necessary [13]. Also the enzymatic hydrolysis of LDME, which is dependent on the temperature and the pH [20], had to be avoided.

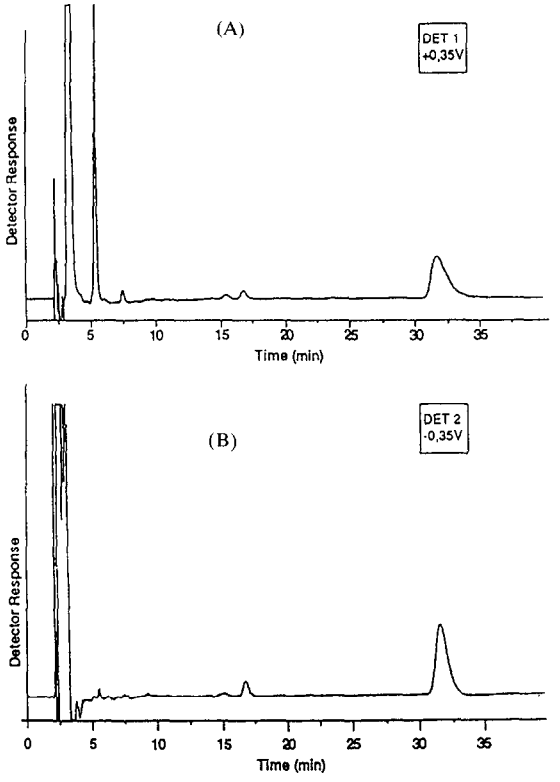

Fig. 1. Representative chromatograms of extracts from drug-free plasma. Applied potentials: DET 1 = +0.35 V (A); DET 2 = −0.35 V (B).

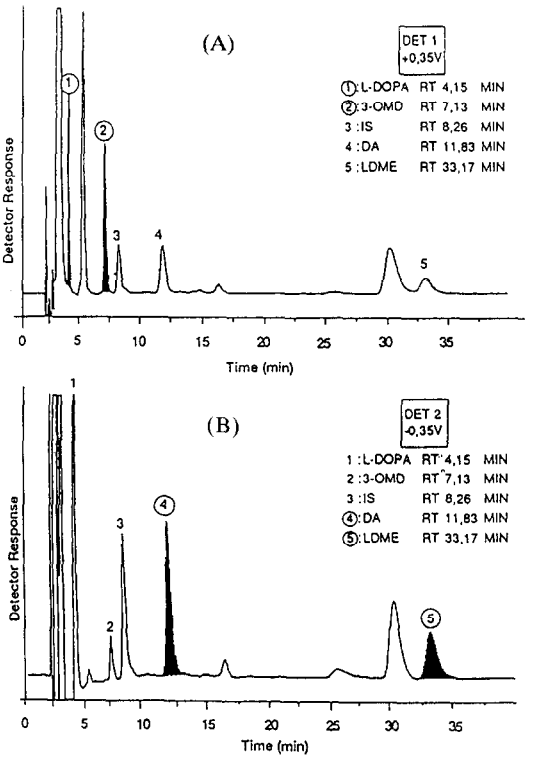

Fig. 2. Representative chromatograms of extracts from plasma spiked with 200 ng/ml of L-DOPA, 400 ng/ml of 3-OMD (A), and 200 ng/ml LDME, DA (B).

Centrifugation at +4°C for 5 min, adopted in the plasma separation step, was irrelevant for the hydrolytic process. The prompt and effective deproteinization obtained by adding PCA [21] to the sample after centrifugation, destroyed the *ex vivo* esterase activity, and maintained the acidic conditions, avoiding the chemical hydrolysis of the ester during analysis.

These assumptions were verified by evaluating the stability of (i) LDME in plasma (400 ng/ml, $n = 5$) at +4°C for 4 h before PCA addition, and (ii) in the autosampler at room temperature for 16 h after the complete sample preparation procedure, by rechromatographing 200 ng/ml standard samples ($n = 5$). In both conditions a small and not significant (Student's t-test) decrease in analyte concentration was observed (−4.8 and −2.6% respectively).

Chromatograms with fewer interfering peaks were obtained by the elimination of the excess

PCA as potassium salt. The small peak detected in the blank chromatogram of DET 1 at the retention time of 3-OMD is due to the endogenous plasma levels of this compound. Therefore, the peak height of the endogenous 3-OMD was subtracted from that of the calibration samples to avoid effect at lower concentrations.

Linear calibration curves were obtained in the 30–1000 ng/ml concentration range for LDME, L-DOPA, and DA and in the 60–2000 ng/ml range for 3-OMD. Table 1 shows the mean (±S.D.) slope, intercept and correlation coefficient for each compound obtained from four different curves. The analysis of variance with the F-test ($\alpha = 0.05$) and lack of fit were used to confirm the significance of the regression and the adequacy of the linear model.

The intra- and inter-assay precision (C.V.%) and the accuracy of the method at different concentrations of each compound are summarized in Table 2. The results showed a good reproducibility between experiments.

Plasma recovery is reported in Table 3. The average recovery of each compound and the I.S. ranged from 70 to 85% and the recovery ratio of the compounds to the I.S. was reproducible at different concentrations. Stability results are reported in Table 4. No significant variations in concentration (<10% nominal value) were detected when the substances were stored in plasma for 15 days at −80°C.

Table 2
Precision and accuracy of the method

Amount added (ng/ml)		Precision (C.V., %)		Accuracy (R.E., %) ($n = 14$)
		Intra-day ($n = 5$)	Inter-day ($n = 2$, 4 days)	
L-DOPA	100	5.40	8.55	+1.85
	400	3.83	5.28	−3.13
	700	2.44	7.10	−1.79
3-OMD	200	4.53	8.32	+3.67
	800	5.00	3.20	−3.16
	1400	5.19	7.23	−5.19
DA	100	6.85	4.89	+3.15
	400	2.69	9.14	+1.77
	700	3.29	7.10	+1.45
LDME	100	5.37	7.35	+3.63
	400	2.39	8.18	+2.39
	700	3.37	8.90	+2.47

The limits of quantitation, defined as the amount of each compound per ml of plasma giving a signal-to-noise ratio of 10 [22] were 9.7 ng/ml for LDME, 19.6 ng/ml for L-DOPA, 2.9 ng/ml for DA and 47.2 ng/ml for 3-OMD.

An example of the application of this method to the determination of the compounds in plasma after oral administration of 50 mg/kg of LDME to rats ($n = 2$) is shown in Fig. 3. They received an aqueous solution of LDME by gavage (1 ml/kg body weight). At each sampling time, rats were anesthetized with diethyl ether and blood

Table 1
Linear regression parameters (mean ± S.D.) of the standard curves ($n = 4$) and analysis of variance of the global calibration lines

	L-DOPA	3-OMD	DA	LDME
Range (ng/ml)	30–1000	60–2000	30–1000	30–1000
Slope ($\cdot 10^{-3}$)	20.49 ± 1.14	8.40 ± 0.46	4.97 ± 0.16	1.71 ± 0.06
Intercept ($\cdot 10^{-2}$)	7.62 ± 5.09	10.33 ± 2.22	2.06 ± 1.20	−2.10 ± 0.58
r^2	0.9992 ± 0.0009	0.9982 ± 0.0011	0.9989 ± 0.0016	0.9957 ± 0.0040
F_1	6885.62[a]	3042.53[a]	11627.34[a]	3877.77[a]
F_{tab}	4.23	4.23	4.23	4.23
F_2	0.68[b]	0.39[b]	1.15[b]	2.49[b]
F_{tab}	2.68	2.68	2.68	2.68

F_1 = MS regression (df = 1)/MS residual (df = 26); F_2 = MS lack of fit (df = 5)/MS pure error (df = 21); MS = mean squares; df = degrees of freedom.

[a] $F_1 > F_{tab}$ (1, 26, 0.95) = 4.23: regression significant.

[b] $F_2 < F_{tab}$ (5, 21, 0.95) = 2.68: lack of fit not significant.

Table 3
Plasma recovery (mean ± S.D., $n = 5$)

Compound	Added (ng/ml)	Recovery (%)		Compound/I.S. recovery ratio
		Compound	I.S.	
L-DOPA	100	79.2 ± 2.5	81.0 ± 1.9	0.98 ± 0.02
	400	85.5 ± 1.4	80.9 ± 3.3	1.06 ± 0.05
	700	87.8 ± 3.4	90.5 ± 2.5	0.97 ± 0.03
3-OMD	200	85.5 ± 2.8	–	1.05 ± 0.02
	800	86.9 ± 0.5	–	1.07 ± 0.05
	1400	88.2 ± 2.4	–	0.97 ± 0.03
DA	100	65.7 ± 2.3	–	0.88 ± 0.02
	400	71.9 ± 1.5	–	0.95 ± 0.05
	700	73.4 ± 3.0	–	0.89 ± 0.02
LDME	100	62.7 ± 2.7	–	0.84 ± 0.02
	400	73.5 ± 0.9	–	0.98 ± 0.05
	700	74.7 ± 3.1	–	0.91 ± 0.01

In all instances 100 ng/ml internal standard was added.

was collected from the abdominal aorta. Plasma was then obtained as described in the sample preparation section.

Table 4
Stability of compounds stored in plasma for 15 days at −80°C

	Control concentration (ng/ml)	After storage (ng/ml)	Deviation (%)
L-DOPA	97.1 ± 5.1	96.1 ± 7.3	−1.0
	406.4 ± 10.2	389.0 ± 9.2	−4.3
	727.8 ± 33.3	682.1 ± 11.5	−6.3
3-OMD	193.5 ± 8.3	192.3 ± 12.9	−0.6
	805.5 ± 30.7	798.5 ± 28.5	−0.9
	1399.8 ± 86.3	1385.9 ± 102.4	−1.0
DA	96.4 ± 3.8	94.4 ± 2.4	−2.0
	397.8 ± 24.1	392.9 ± 14.5	−1.2
	713.3 ± 38.6	716.7 ± 18.4	+0.5
LDME	99.2 ± 8.2	100.1 ± 6.8	+0.9
	390.9 ± 12.3	405.8 ± 18.5	+3.8
	712.9 ± 28.1	703.4 ± 11.6	−1.3

Mean values (±S.D.) calculated from five replications at each concentration level.

Plasma levels of LDME always remained under the limit of quantitation, confirming the rapid enzymatic hydrolysis of the pro-drug.

4. Conclusions

The method developed allows a reliable, fast and precise determination of the compounds studied. The sample preparation and HPLC technique allow the simultaneous determination of LDME and the main metabolites L-DOPA, 3-OMD and DA. The method is suitable for the verification of the rapid hydrolysis of the pro-drug after oral administration and for investigating the pharmacokinetics of the drug after continuous intravenous infusion.

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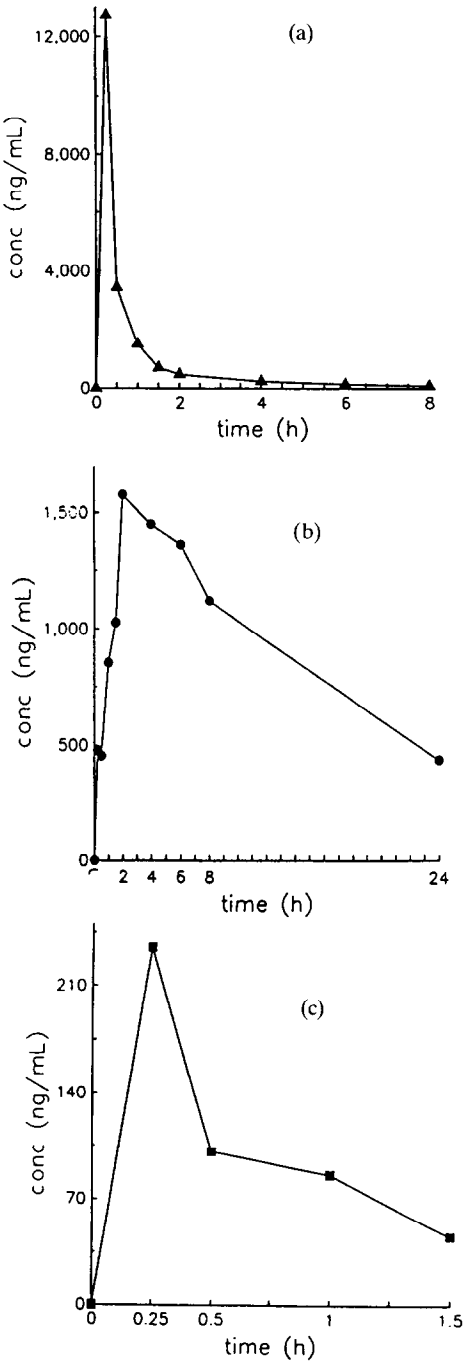

Fig. 3. Plasma levels of L-DOPA (a), 3-OMD (b), DA (c), after oral administration of 50 mg/kg of LDME to rats.

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