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Sensitive high-performance liquid chromatographic method for the determination of the three main metabolites of selegiline (L-deprenyl) in human plasma

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Abstract

A high-performance liquid chromatographic (HPLC) method with fluorescence detection was developed for the determination in human plasma of the three main metabolites of selegiline (L-deprenyl): amphetamine, methamphetamine and norselegiline. The HPLC separation of the analytes was performed under isocratic conditions, after extraction from plasma and precolumn derivatization with 9-fluorenylmethyl chloroformate. The linearity, precision and accuracy of the method were evaluated; the limit of quantification for all three metabolites in plasma was 0.5 ng/ml.

1. Introduction

Selegiline, [(*R*)-(−)-N-methyl-N-(1-phenyl-2-propyl)-2-propinylamine] or L-deprenyl (DEP), is an irreversible and selective inhibitor of monoamine oxidase (MAO) type B [1–3]. The inhibition of MAO-B by DEP is thought to result in a decreased degradation of dopamine at the neuronal level [4,5]. Therefore, DEP is used as an adjuvant to L-dopa treatment in Parkinson's disease. An improvement in end-of-dose response fluctuations and a dose-sparing effect on L-dopa were observed following co-administration of DEP and L-dopa [6–8].

Several investigations have shown that DEP metabolism involves a biotransformation of the drug into amphetamine(AP), methamphetamine (MAP) and desmethyldeprenyl (DMD) (Fig. 1). After administration of DEP to experimental animals, these amine metabolites were found in dogs [9], mice [10] and rats [11]. During treatment of healthy volunteers or Parkinsonian patients with DEP, both AP and MAP were measured in urine and plasma by several methods [12–14]. Unchanged DEP was measured in plasma only by GC with nitrogen–phosphorus detec-

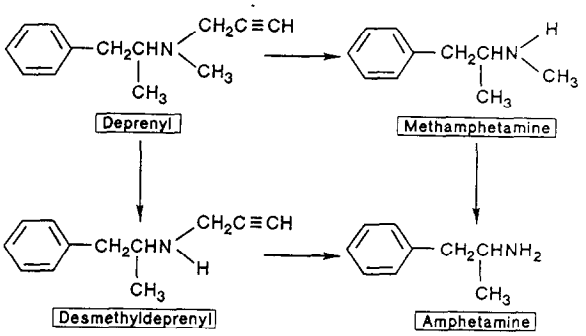

Fig. 1. A simplified metabolic pathway of DEP.

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tion [15]; however, this compound was shown to be rapidly cleared from plasma [16], and therefore in all subsequent pharmacokinetic studies with DEP only its main plasma metabolites were measured. In these studies, AP and MAP were the most abundant metabolites circulating in plasma and lower levels of DMD were found only using more sensitive methods such as gas chromatography–mass spectrometry (GC–MS) or with electron-capture detection (GC–ECD) [9,17,18]. In an attempt to evaluate the possible pharmacokinetic interaction between DEP and cabergoline (an ergoline derivative with dopamine D_2 receptor agonist activity) in *de novo* Parkinsonian patients, a method has been developed for the simultaneous determination of AP, MAP and DMD in human plasma by HPLC with fluorescence detection, after precolumn derivatization with 9-fluorenylmethyl chloroformate (FMOC).

Several reagents have been used for the precolumn derivatization of amino groups, including FMOC [19–22], phenyl isothiocyanate (PITC) [23–25], *o*-phthaldialdehyde (OPA) [26–28] and 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl-Cl) [29–31]. These derivatization agents have become increasingly popular, but some of them show several drawbacks: the PITC method requires a vacuum system to remove all traces of excess reagent, OPA reacts only with primary aminogroups and the reaction with dansyl-Cl is slow and difficult in complex matrices [32–34]. In contrast, FMOC reagent shows advantages over other derivatization reagents: it reacts with both primary and secondary amino groups (Fig. 2), it forms stable derivatives by a simple and rapid procedure and it allows a high detection sensitivity and reproducibility even in complex matrices. The only disadvantage of FMOC reagent is that it is fluorescent: because of this property, the excess of reagent has to be removed before injection into the HPLC column. This is usually achieved by solvent extraction or by reaction of the reagent in excess with a suitable amine.

The separation of FMOC derivatives was performed by reversed-phase HPLC and the method was shown to be free from interferences due to human plasma. Endogenous components present in blank human plasma did not interfere in the determination of the three analytes.

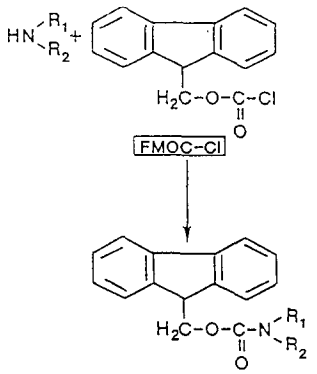

Fig. 2. Reaction of FMOC-Cl with primary or secondary amino groups.

2. Experimental

2.1. Apparatus

The HPLC system consisted of an Isochrom isocratic pump with a 200-μl loop, an FP 821 fluorescence detector, an SP 4270 integrator and a Winner 386 data acquisition system with Labnet software. All these instruments were supplied by Spectra-Physics (Santa Clara, CA, USA), except the detector, which was purchased from Jasco (Tokyo, Japan).

2.2. Reagents and materials

Amphetamine sulphate was supplied by Sigma (St. Louis, MO, USA), methamphetamine sulphate by Recordati (Milan, Italy), desmethyldeprenyl hydrochloride by Farmitalia Carlo Erba (Milan, Italy), L-proline and FMOC-Cl by Fluka (Buchs, Switzerland). All the other reagents and solvents were of analytical-reagent grade from Carlo Erba Reagents (Milan, Italy).

AP, MAP and DMD stock standard solutions were prepared by dissolving a weighed amount of each substance in water. All these stock solutions were stable for at least 1 month when stored at a nominal +4°C in the dark. Working

standard solutions were obtained daily by diluting the stock standard solutions in 0.1 *M* HCl.

Borate buffer (0.5 and 1 *M*) was adjusted to pH 11 and 8, respectively, with 2 *M* sodium hydroxide solution; 0.05 *M* phosphate buffer for the mobile phase was adjusted to pH 6.0 with 1 *M* sodium hydroxide solution.

2.3. HPLC conditions

The chromatographic separation was performed with a Nova-Pak Phenyl column (150 × 3.9 mm I.D., particle size 4 μm) (Millipore–Waters, Milford, MA, USA) equipped with a precolumn filter (2 μm) (Millipore–Waters). The mobile phase was acetonitrile–50 m*M* phosphate buffer (pH 6.0) (50:50, v/v); the mixture was prepared daily and degassed under vacuum prior to use. The flow-rate of the mobile phase was 1.0 ml/min and the column was kept at room temperature. The fluorescence detector (17-μl flow cell) was set at an excitation wavelength of 260 nm and an emission wavelength of 315 nm and sent a 1 V signal to the integrator.

2.4. Sample extraction procedure

Plasma (1.0 ml) was placed into a 10-ml conical glass tube and mixed with 1.0 ml of 0.5 *M* borate buffer (pH 11). After addition of 2.5 ml of diethyl ether the tubes were shaken on a vortex mixer for 5 min and centrifuged at 1200 *g* for 5 min, then the organic phase was collected. This extraction step was repeated and the combined organic phase was back-extracted with 200 μl of 0.1 *M* HCl by mixing on a vortex-mixer for 2 min. After centrifugation at 1200 *g* for 5 min, the organic phase was discharged and the aqueous phase was subjected to the derivatization reaction.

2.5. Derivatization procedure

Calibration, quality control and unknown samples (200 μl in 0.1 *M* HCl) were mixed with 150 μl of 1 *M* borate buffer (pH 8); 100 μl of FMOC-Cl solution (4 m*M* in acetonitrile) were added, shaken and allowed to react at 50°C. After 5 min, 20 μl of proline (20 m*M* in water) were added and allowed to stand for 2 min at 50°C. Aliquots (200 μl) of the reaction mixture were injected directly into the HPLC system.

2.6. Determination of calibration, quality control and unknown samples

Analyses of blank human plasma spiked with known amounts of AP, MAP and DMD were carried out applying the above-described procedure. Linearity was evaluated from five calibration graphs prepared and run on five different days in the concentration range 0.5–80.0 ng/ml for all three compounds. Precision and accuracy were evaluated by repeated analyses of the three compounds in plasma at three concentrations (1.0, 16.0, 64.0 ng/ml) in three replicate samples analysed on four different days.

All chromatograms obtained were evaluated by peak-height measurement. The quality controls and the unknown samples were calculated with the calibration graph generated on each day by least-squares linear regression (weighting factor $1/y$) of the peak height of the analytes against their concentration in plasma. To evaluate the extraction recovery, the peak height of extracted plasma samples was compared with that obtained with unextracted standard solution injected directly into the chromatograph.

2.7. Chromatographic system suitability test

On each day of analysis the performance of the chromatographic system was checked in order to ensure that controlled conditions were used in the assay. Two parameters, determined according to the US Pharmacopeia [35], were used to define the suitability of the chromatographic system. The parameters monitored were column efficiency and peak symmetry. Column efficiency was evaluated as the number of theoretical plates of the column calculated from the equation $N = 5.54(t_R/W)^2$, where t_R is the retention time (mm) of the compound tested and W is the peak width (mm) at half-height. The value of N must be at least 5000 for each compound. Peak symmetry was evaluated as the

symmetry factor (SF), calculated from the equation $SF = W_{0.05}/2A$, where $W_{0.05}$ is the peak width (mm) measured at 1/20th of the peak height and A is the distance (mm) between the perpendicular dropped from the peak maximum and the leading edge of the peak at 1/20th of the peak height. SF must be ≤ 1.3.

3. Results and discussion

3.1. Derivatization procedure

Optimization of the reaction conditions for derivatization was pursued. In preliminary studies, borate and phosphate buffers adjusted to different pH values were tested for use in the derivatization reaction; borate buffer at pH 8 gave the highest reaction yield.

Acetonitrile was preferred to acetone as the FMOC-Cl solvent, in order to avoid the precipitation of hydrophobic amino acid derivatives [33,36]. The time and temperature of the reaction were selected to minimize the interferences with derivatized plasma compounds.

Proline addition rather than solvent extraction was preferred for removal of the excess FMOC-Cl in order to eliminate the risk of partial extraction of the derivatized analytes by the solvent [21,22,31]; the FMOC–proline derivative is soluble in the reaction mixture and does not interfere in the determination of DEP metabolites as it is poorly retained under the chromatographic conditions adopted.

3.2. Linearity

The retention times of AP, MAP and DMD derivatives were *ca.* 12, 15 and 19 min, respectively; at these times no significant interfering peaks from blank human plasma were detected (Fig. 3).

The mean calibration graphs obtained for the three compounds on five different days were described by the equations $y = 33\,320x + 1925$ (slope C.V. = 5.19%) for AP, $y = 31\,706x + 4613$

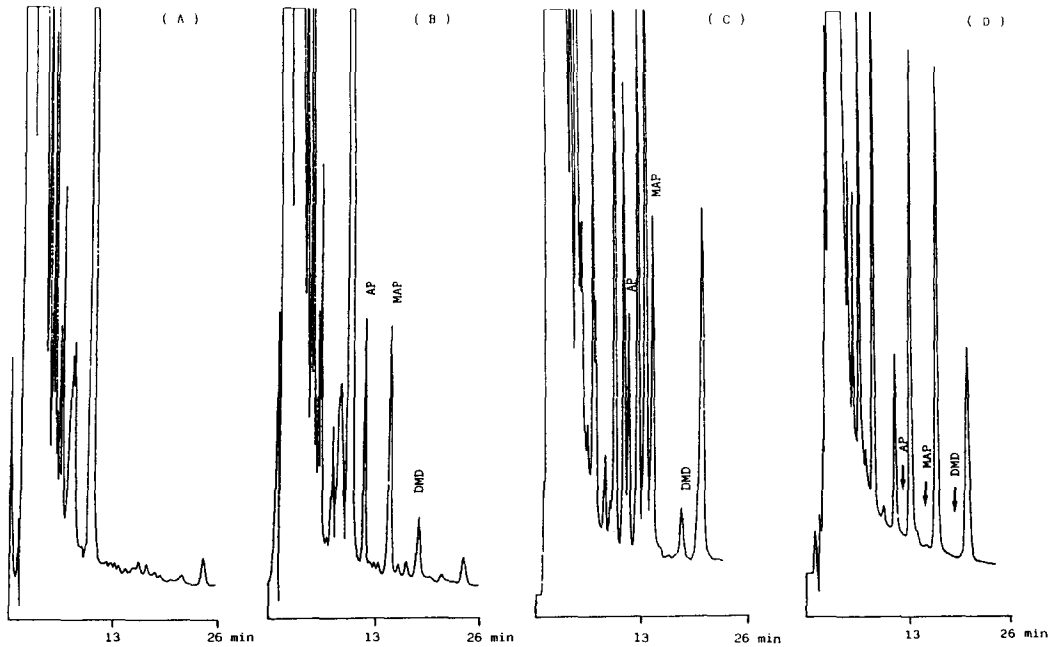

Fig. 3. Chromatograms of (A) extract from 1 ml of blank human plasma, (B) extract from 1 ml of blank human plasma with 2.0 ng of AP, MAP and DMD, added (C) extract from 1 ml of plasma from one patient (subject 4) obtained on day 8, 24 h after a daily dose of 10 mg of DEP (see text), and (D) extract from 1 ml of plasma from one patient (subject 4) obtained on day 52, 24 h after the last dose of 1 mg of cabergoline (see text).

(slope C.V. = 5.37%) for MAP and $y = 6952x + 1183$ (slope C.V. = 10.81%) for DMD, where y represents the peak height (counts) and x the amount of analyte (ng) added to 1.0 ml of plasma. When submitted to Student's t-test, the y-intercept values were not significantly different from zero ($p > 0.05$) for all three analytes. Correlation coefficients (r) for the regressions were higher than 0.99 for all the three substances. The back-calculated standard values exhibited a C.V. lower than 13.5% for all the compounds.

3.3. Precision and accuracy

To determine the assay precision and accuracy, quality control samples at three concentrations (1.0, 16.0 and 64.0 ng/ml plasma) were analysed on four different days. The data obtained for AP, MAP and DMD are reported in Tables 1, 2 and 3, respectively.

The inter-day precision (expressed as C.V.) ranged from 4.04 to 9.80% for AP, from 3.46 to 9.15% for MAP and from 6.14 to 10.78% for DMD. The intra-day precision was better than 10.43%, 9.55% and 11.78% for AP, MAP and DMD, respectively.

Accuracy, expressed as found/added amount (%), ranged from 92.0 to 113.0% for AP, from 95.3 to 114.0% for MAP and from 88.1 to 111.2% for DMD.

3.4. Sensitivity

To evaluate the limit of quantification (LOQ) for each analyte in plasma, the signal-to-noise ratio and the precision for the lowest concentration of standard samples were considered. The LOQ for AP, MAP and DMD was 0.5 ng/ml plasma; at this concentration the signal-to-noise ratio was better than 5:1 and the C.V. of replicate determinations was 13.5% ($n = 5$).

3.5. Extraction recovery

The mean extraction efficiency, evaluated in the concentration range 0.5–80.0 ng/ml plasma ($n = 5$), ranged from 74.2 to 89.4% (C.V. <9%) for AP, from 72.0 to 90.2% (C.V. <7%) for MAP and from 56.6 to 72.2% (C.V. <13%) for DMD.

3.6. Application

The method was successfully applied to the analysis of samples obtained in a clinical study carried out to evaluate a possible pharmacokinetic interaction between DEP and cabergoline

Table 1
Accuracy and precision of the method for the determination of amphetamine in plasma

Control Sample (ng/ml)	Day	n	Accuracy				Precision	
			Mean found (ng/ml)	S.D. (ng/ml)	Found/added (%) (intra-day)	Found/added (%) (inter-day, $n = 12$)	C.V. (%) (intra-day)	Pooled C.V. (%) (inter-day, $n = 12$)
1.00	1	3	1.02	0.017	102.0		1.70	
	2	3	1.13	0.031	113.0		2.70	
	3	3	1.08	0.113	108.0		10.43	
	4	3	0.92	0.078	92.0	103.75	8.44	9.80
16.00	1	3	15.90	0.916	99.4		5.76	
	2	3	15.93	0.850	99.6		5.34	
	3	3	15.60	0.693	97.5		4.44	
	4	3	16.00	0.264	100.0	99.12	1.65	4.04
64.00	1	3	65.73	4.481	102.7		6.82	
	2	3	60.80	1.539	95.0		2.53	
	3	3	64.07	2.417	100.1		3.77	
	4	3	61.90	1.836	96.7	98.62	2.96	4.94

Table 2
Accuracy and precision of the method for the determination of methamphetamine in plasma

Control Sample (ng/ml)	Day	n	Accuracy				Precision	
			Mean found (ng/ml)	S.D. (ng/ml)	Found/added (%) (intra-day)	Found/added (%) (inter-day, $n = 12$)	C.V. (%) (intra-day)	Pooled C.V. (%) (inter-day, $n = 12$)
1.00	1	3	1.04	0.015	104.0		1.44	
	2	3	1.14	0.051	114.0		4.50	
	3	3	1.12	0.107	112.0		9.55	
	4	3	0.97	0.072	97.0	106.75	7.46	9.15
16.00	1	3	15.63	0.503	97.7		3.22	
	2	3	15.90	0.721	99.4		4.53	
	3	3	15.50	0.781	96.9		5.04	
	4	3	15.77	0.321	98.6	98.15	2.04	3.46
64.00	1	3	66.27	4.252	103.5		6.42	
	2	3	61.00	2.100	95.3		3.44	
	3	3	63.67	2.281	99.5		3.58	
	4	3	63.03	2.065	98.5	98.67	3.27	4.90

(an ergoline derivative with dopamine D_2 receptor agonist activity). In this study six *de novo* Parkinsonian patients, five men and one woman aged between 48 and 66 years, received once a day DEP (10 mg) for 8 days, then DEP (10 mg) and cabergoline (1 mg) for 22 days, and then cabergoline alone (1 mg) for 22 days. Blood samples were collected on days −1, 8, 30 and 52, immediately before and 0.5, 1, 2, 4, 8 and 24 h after dosing. Blood samples were drawn into heparinized tubes and immediately centrifuged at 4°C at 1200 *g* for 10 min, then the separated plasma was stored at −20°C until assayed.

The method proved to be sensitive and specific and allowed the evaluation of AP, MAP and DMD pharmacokinetic parameters. The results of this study will be published elsewhere. A chromatogram obtained from an *in vivo* sample collected on day 8 of the study from one patient (subject 4), 24 h after the last daily DEP dose, is

Table 3
Accuracy and precision of the method for the determination of desmethyldeprenyl in plasma

Control Sample (ng/ml)	Day	n	Accuracy				Precision	
			Mean found (ng/ml)	S.D. (ng/ml)	Found/added (%) (intra-day)	Found/added (%) (inter-day, $n = 12$)	C.V. (%) (intra-day)	Pooled C.V. (%) (inter-day, $n = 12$)
1.00	1	3	0.95	0.074	95.0		7.76	
	2	3	1.09	0.010	109.0		0.92	
	3	3	0.96	0.064	96.0		6.70	
	4	3	0.98	0.085	98.0	99.50	8.68	8.11
16.00	1	3	14.10	0.380	88.1		2.89	
	2	3	16.19	1.014	101.2		6.26	
	3	3	14.50	1.178	90.6		8.12	
	4	3	17.80	0.149	111.2	97.77	0.84	10.78
64.00	1	3	58.63	6.907	91.6		11.78	
	2	3	58.59	3.132	91.5		5.35	
	3	3	57.80	3.363	90.3		5.82	
	4	3	58.67	1.113	91.7	91.27	1.90	6.14

shown in Fig. 3C. It is interesting that although this sample shows some chromatographic peaks close to those of AP and MAP (these peaks were absent in blank human plasma and in most samples obtained from the other subjects), the two metabolites could be reliably determined even 24 h after dosing (the last sampling time in the study).

4. Conclusions

An HPLC method has been developed for the simultaneous determination of the three main metabolites of DEP in plasma. It proved to be linear, precise and accurate in the concentration range 0.5–80.0 ng/ml. The procedure was successfully applied to the determination of AP, MAP and DMD plasma levels in *de novo* Parkinsonian patients during treatment with DEP and allowed the evaluation of the pharmacokinetic parameters of the metabolites. As shown by the chromatogram obtained on the last day of the study (day 52) 24 h after the last daily cabergoline dose, no chromatographic interferences from the co-administered drug cabergoline and/or cabergoline metabolites were found (Fig. 3D). This shows that the selectivity of the whole procedure (extraction plus derivatization reaction plus chromatographic analysis) was appropriate for the determination of DEP metabolites in plasma after co-administration of cabergoline and DEP. The method might also be successfully employed to determine AP, MAP and DMD in other studies involving co-administration of DEP and levodopa in Parkinsonian patients as no chromatographic interference from levodopa and its main metabolites [37,38] in the assay is expected. Three main considerations support our hypothesis: (a) poor extraction recovery of levodopa and all its metabolites possessing a free carboxylic group and/or a free catechol group is expected at the pH used here; (b) the FMOC derivatives of levodopa and the 3-O-methyldopa formed should be eluted in the first part of the chromatogram (similarly to the FMOC derivative of proline); and (c) 3,4-dihydroxyphenylacetic acid and homovanillic acid (two levodopa metabolites that do not possess amino groups) would not react with FMOC and therefore should not be detected by fluorimetric monitoring. However, further studies will be needed to assess the possible interference in the assay of more lipophilic metabolites of levodopa (such as 3-O-methoxytyramine and dopamine) and of the peripheral dopa decarboxylase inhibitors (benserazide and carbidopa) usually given in combination with levodopa.

5. References

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