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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS WITH DOUBLE DETECTION SYSTEM FOR L-DOPA, ITS METABOLITES AND CARBIDOPA IN PLASMA OF PARKINSONIAN PATIENTS UNDER L-DOPA THERAPY

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SUMMARY

An analytical method is described for measuring L-3,4-dihydroxyphenylalanine (L-DOPA), 3-O-methyl-DOPA, dihydroxyphenylacetic acid, free catecholamines and the peripheral DOPA decarboxylase inhibitor, carbidopa, in plasma samples of Parkinsonian patients by using high-performance liquid chromatography. A sample preparation method is presented for the isolation of the catecholamines and L-DOPA with its metabolites. Catecholamines are extracted by weak cation exchange on small columns and subsequent adsorption on alumina. L-DOPA, 3-O-methyl-DOPA, dihydroxyphenylacetic acid and carbidopa contained in the column effluents are directly injected in the chromatographic system. The eluates are separated on a reversed-phase column, monitored by both a coulometric electrochemical detector and a fluorescence detector, connected in series. Chromatographic peaks were identified on the basis of their retentions and response ratios of the two detectors. Two examples are presented of therapeutic drug monitoring in Parkinsonian patients treated with oral doses and continuous intravenous infusion of L-DOPA.

INTRODUCTION

After 5–7 years of L-3,4-dihydroxyphenylalanine (L-DOPA) therapy, 50–70% of Parkinsonian patients show signs and symptoms which have never been observed during the natural course of this disease. One of the earliest signs is a gradual decrease in efficacy of single-drug administration, clinically expressed by a shortening of the periods of good motor performance after which Parkinsonian symptoms re-emerge (wearing-off). These fluctuations frequently precede the onset of dyskynetic/dystonic involuntary movements, which appear at the beginning and at the end of the periods of efficacy of each single dose. Both the wearing-off and these dyphasic involuntary movements are strictly related to L-DOPA plasma levels. The pathogenesis of fluctuations in motor performance in Parkinson's disease is not fully understood[1,2]. Oscil-

lations in plasma L-DOPA levels and factors interfering with its transport into the brain have been pointed as major problems in the genesis of fluctuations. Maintenance of the L-DOPA plasma concentration is crucial. In fact, by maintaining the plasma level of the drug constant, it is possible to control most of the on/off phenomenon.[3,4].

The transport of L-DOPA through the blood–brain barrier is another important factor. Large neutral amino acids and some L-DOPA metabolites, such as 3-O-methyl-DOPA (OMD), can compete with L-DOPA for the utilization of the barrier carrier[5,6]. The detection of carbidopa (CD), a peripheral decarboxylase inhibitor, is also very important in ensuring that peripheral interferences remain minimal. Simultaneous monitoring of plasma levels of L-DOPA, its metabolites, catecholamines and CD is of great importance in the management of Parkinson's disease and thus there seem to be a definitive need for a simple method of quantitation of these compounds.

Investigation of the metabolism of L-DOPA has been facilitated by the introduction of high-performance liquid chromatography (HPLC), coupled with electrochemical detection (ED). The development of this analytical technique facilitated the establishment of highly sensitive and selective assay procedures for L-DOPA and its metabolites in plasma of Parkinsonian patients[7–21]. Under certain conditions, direct injection of deproteinized plasma into the HPLC system has been reported[11,13,14]. However, this is not applicable to the catecholamines, owing to their much lower levels. The use of C_{18} cartridges for plasma prepurification has also been described[15], but dopamine (DA) cannot be detected. Plasma extraction on alumina allows the determination of CD, L-DOPA, its metabolites and catecholamines, but the recovery is very low especially for dihydroxyphenylacetic acid (DOPAC) and CD[12,18], or OMD determination is not included[21].

Here, we report a purification procedure permitting a selective isolation of free L-DOPA, OMD, CD, DOPAC, norepinephrine (NE), epinephrine (E) and DA. The sample preparation involves extraction of catecholamines by ion exchange on small columns and subsequent adsorption on alumina. The ion-exchange column was washed with water to collect L-DOPA, OMD, DOPAC and CD. The eluate after deproteinization is directly injected in the chromatographic system. The use of the same mobile phase and column for the concurrent assays of catecholamines, L-DOPA and its metabolites increases the throughput of samples in the chromatographic system. Detection is achieved by a coulometric detector and a fluorescence detector, connected in series. Fluorimetric detection of catecholamines is a well known procedure that has been applied to HPLC assays[13,22,23]. The catecholamines, L-DOPA and its metabolites have been monitored by their natural fluorescence but at rather low sensitivity[13]. However during L-DOPA therapy, the levels of these compounds are high enough[19,20] so that detection by both detectors is possible. In regard to those compounds responding to both detectors, the specificity of our detection system, already rendered high thanks to the double potential barrier of the coulometric detector, is further enhanced by the presence of the second detector. The identity of L-DOPA, NE and DA was confirmed by the response ratios of the two detectors. The detection of the other compounds was performed only by ED (CD and DOPAC) or by fluorescence spectrometry (OMD).

EXPERIMENTAL

Materials

NE, E, DA, DOPAC and N-methyldopamine (NMDA, internal standard) were obtained 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 obtained from Fluka (Buchs, Switzerland). Acetonitrile and methanol (HPLC grade) were obtained from Erba (Milan, Italy). Water was purified with the Milli-Q purification system (Millipore, Bedford, MA, U.S.A.). The column materials used for plasma purification were acid alumina AG-4, activated as described by Anton and Sayre[24], from Bio-Rad Labs. (Richmond, CA, U.S.A.) and CM-Sephadex from Pharmacia (Uppsala, Sweden). The anticoagulant/antioxidant solution used for sample treatment contained EGTA (60 mg/ml) and reduced glutathione (90 mg/ml), adjusted to pH 7 with 0.1 *M* sodium hydroxide.

The HPLC system consisted of a M-45 pump (Waters-Millipore, Bedford, MA, U.S.A.), an electrochemical detector (Coulochem 5100 A; ESA, Bedford, MA, U.S.A.) with a 5010 A analytical cell and a fluorescence spectrometer (LS-4; Perkin-Elmer, Norwalk, CT, U.S.A.). The two detectors were connected to a Chemresearch Chromatographic Data Management computer (ISCO, Lincoln, NE, U.S.A.). The samples were introduced with an injection valve (Rheodyne 7125, Berkely, CA, U.S.A.) having a 100-μl loop. The column was a 25 cm × 4.6 mm I.D. Nucleosil C_{18} (particle size 5 μm; Macherey-Nagel, Düren, F.R.G.).

Methods

The eluent was a mixture of 92% 0.025 *M* sodium acetate, 4% methanol and 4% acetonitrile, containing $2 \cdot 10^{-4}$ *M* OSA and $3 \cdot 10^{-4}$ *M* disodium EDTA. The eluent was adjusted to pH 3 with acetic acid. Isocratic elution was carried out at room temperature at a flow-rate of 0.9 ml/min. Potentials of +0.25 and –0.30 V were applied at the first and the second electrode of the analytical cell, respectively. The fluorescence was monitored using an excitation wavelength of 282 nm and an emission wavelength of 322 nm[13]. The two detectors were connected in series, with the fluorescence detector downstream, as its flow cell has a recommended maximum working pressure (450 p.s.i.)[25] lower than that of the ED flow cell (600 p.s.i.)[26].

Blood samples were drawn by venipuncture and transferred immediately to tubes containing 20 μl of EGTA–reduced glutathione solution per ml of blood. Samples were centrifuged at 4°C and the plasma was removed and stored at –80°C until analyzed. Plasma samples (1 ml) were spiked with 30 μl of internal standard solution (NMDA, 80 ng/ml) and applied to a small polypropylene column (2 cm × 0.5 cm) packed with CM-Sephadex pre-swollen in distilled water. Before use, the packed column was washed with 5 ml of 2 *M* hydrochloric acid and 10 ml of distilled water and buffered with 10 ml 0.1 *M* phosphate (pH 7). After sample application the column was rinsed with 5.5 ml of distilled water, discarding the first ml and collecting the next 4.5 ml of effluent in a conical tube, containing 0.5 ml of 0.5 *M* perchloric acid. After centrifugation of the deproteinized solution, the supernatant was removed and 10 μl of the solution were injected into the chromatograph for the determination of L-DOPA and its metabolites.

The catecholamines were eluted from the cation-exchange column with 3 ml of 0.5 *M* perchloric acid and the eluate was collected in 15-ml conical tubes. A 2-ml volume of Tris buffer (1.5 *M*, pH 9.3) containing 0.06 *M* EDTA and 20 mg of alumina were added. The tube was vortexed for 2 min, the supernatant was removed and the alumina was washed twice with 2 ml of water, centrifuging (3000 *g*) for 3 min between each washing. After the second washing, as much water as possible was removed. The catecholamines were eluted from the alumina with 100 µl of 0.1 *M* acetic acid after vortex mixing for 2 min. After centrifugation, the supernatant was removed and 25 µl were injected[27].

RESULTS AND DISCUSSION

The specific and sensitive method reported here for the determination of free L-DOPA, its metabolites and some catecholamines in human plasma employs HPLC with the use of two different detection systems (ED and fluorimetric detection) connected in series. The chromatographic conditions for all compounds of interest, expecially CD and OMD, were greatly improved compared with the case of only one detector. The retention times of these compounds, as described by other authors[20], were similar in a variety of previously used mobile phases, and their simultaneous determination was previously impossible in clinical samples without the use of the two detectors. This is evident from Fig. 1 A and B, where the separation of a standard

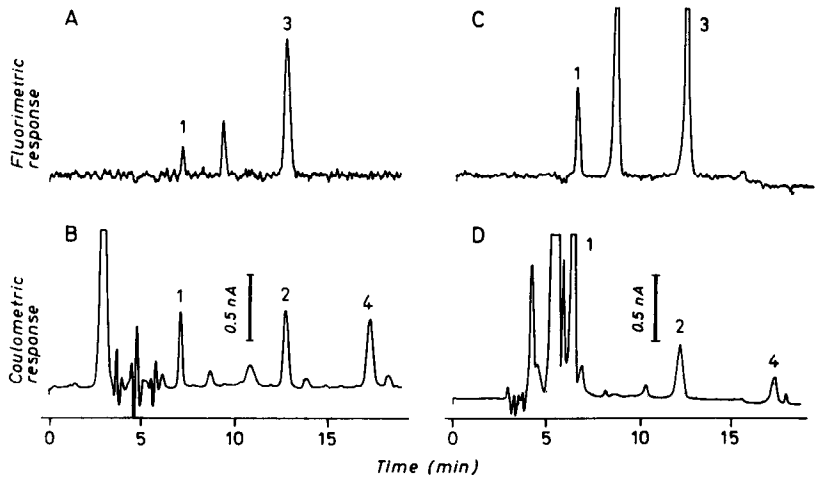

Fig. 1. Chromatograms of: (A),(B) 10 µl standard mixture of L-DOPA (20 ng/ml), CD (80 mg/ml), OMD (80 ng/ml) and DOPAC (20 ng/ml); (C),(D) 10 µl aqueous eluate obtained from the plasma of a patient undergoing L-DOPA therapy, according to the method described in the text. Peaks: 1 = L-DOPA; 2 = CD; 3 = OMD; 4 = DOPAC. The use of two detectors allowed the quantitative analysis of all compounds in the L-DOPA group. Since the retention times of OMD and CD were similar, we took advantage of their different detectabilities by the two detectors. Conditions: column, Nucleosil RP-C_{18}, 5 µm, 250 mm × 4.6 mm I.D.; room temperature; flow-rate, 0.9 ml/min; mobile phase, 0.0025 *M* sodium acetate–methanol–acetonitrile (92:4:4) (pH 3), detection, coulometric (E_1 +0.25 V, E_2 = –0.3 V), fluorimetric (excitation 282 nm, emission 322 nm). The signal-to-noise ratio of (C) seems to be different from that of (A) but really they are equal: it is simply the result of a stronger photoreduction of the original size, rendered necessary by the higher chart speed used in that experiment.

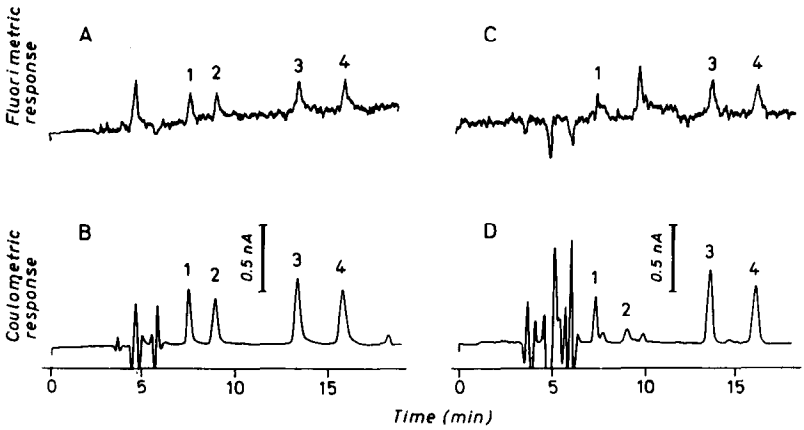

Fig. 2. Chromatograms of: (A),(B) 5 μl standard mixture of NE (34 ng/ml), E (13 ng/ml), DA (25 ng/ml) and NMDA (50 ng/ml, internal standard); (C),(D) 25 μl acetic eluate obtained from the plasma of a patient undergoing L-DOPA therapy according to the method described. For conditions see Fig. 1. Peaks: 1 = NE; 2 = E; 3 = DA; 4 = NMA.

mixture of L-DOPA, CD, OMD and DOPAC is shown. OMD and CD can be determined because the first is detected only by the fluorescence detector while the second is detected only by ED. The chromatograms were recorded at an expansion factor of 100 for the fluorescence detector and at a gain of × 5000 for the coulometric detector.

Fig. 2A and B show typical chromatograms of a standard solution of NE, E, DA and NMDA obtained with the two detectors. The chromatographic conditions were the same as in Fig. 1. Parts C and D of Figs. 1 and 2 show chromatograms of a plasma extract containing L-DOPA and its metabolites and the catecholamines, respectively.

The wide L-DOPA concentration range of the analyzed plasma samples (394–2351 ng/ml) forced us to utilize the coulometric detector with a gain not higher than × 5000. The low sensitivity allowed us to measure E, usually detectable in plasma by HPLC–ED[27], only in a few of the samples examined in this study. The case whose chromatogram is reported in Fig. 2C is one of them.

Recoveries of prepurification steps for L-DOPA, CD, DOPAC and NMDA were checked by analyzing plasma spiked with known amounts of substances ($n = 5$). As shown in Table I, the recoveries were nearly 100%. Consequently, the internal standard was not used routinely.

The reproducibility of the proposed method was established by the replicate analyses of a plasma pool, spiked with L-DOPA and its metabolites. Table II lists means, standard deviations and coefficients of variation ($n = 5$).

Linearity tests of L-DOPA, CD, OMD and DOPAC were performed by adding known amounts of the compounds to a pooled sample of plasma subjected to the complete preparation procedure. The detector response was linear for all compounds over the range 5–80 ng/ml for L-DOPA and DOPAC and 10–160 ng/ml for CD and OMD. Table III shows the regression lines for the four compounds. The values for catecholamines, absolute recovery, reproducibility and linearity, are in accord with data previously reported[27].

TABLE I

ABSOLUTE RECOVERY (%) AT DIFFERENT CONCENTRATIONS OF L-DOPA, OMD, DOPAC AND CD AFTER PURIFICATION BY USING A CATION-EXCHANGE COLUMN AND A DEPROTEINIZATION PROCEDURE

Each value is the average of five analyses ± standard deviation.

Concentration (ng/ml)	*Recovery (%)*			
	L-DOPA	*DOPAC*	*CD*	*OMD*
5	99.1 ± 3.2	97.2 ± 4.0		
20	106.1 ± 2.7	93.2 ± 4.7	99.3 ± 4.7	97.6 ± 5.1
40	102.1 ± 4.5	99.8 ± 3.5		
80			108.0 ± 2.1	98.4 ± 5.4
160			102.2 ± 4.1	100.0 ± 5.1

The peaks of L-DOPA, its metabolites and catecholamines were identified by a combination of methods. The peak identification for all compounds was performed on the basis of the chromatographic retention time and by simultaneous injection of a standard. Secondly, for compounds detectable by both detectors (L-DOPA, NE, E, DA and NMDA), the ratios of ED/fluorimetric response were calculated and compared with those obtained with plasma samples. The peak-height ratios of reference compounds and those obtained with plasma samples are reported in Table IV. If a signal-to-noise ratio of 2 is assumed, the detection limits of L-DOPA, CD, OMD, DOPAC, NE, E, DA and NMDA were (expressed as pg) 20, 40, 40, 20, 10, 10, 10 and 10 respectively. As explained before (see Methods) we had to position the fluorescence detector downstream. We did not investigate whether peak components detected by fluorescence are electrolyzed products according to a coulometric detector or unelectrolyzed. Nevertheless, from the values of the potentials applied (+ 0.25 V, –0.30 V at the first and the second electrode, respectively), we considered an oxidation (first) counter balanced by a reduction (secondly) with a negligible final effect. Whatever the case, it is reasonable that if the influence of any reaction would not matter since the standards and plasma extracts are analyzed under perfectly identical conditions.

TABLE II

REPRODUCIBILITY OF THE METHOD

Mean values, standard deviations and coefficients of variation (C.V.) of plasma samples spiked with known quantities of L-DOPA, its metabolites and CD, $n = 5$.

	Fluorescence detector		*ED*	
	Plasma (ng/ml)	*C.V. (%)*	*Plasma (ng/ml)*	*C.V. (%)*
L-DOPA	19.2 ± 0.2	4.9	18.7 ± 0.7	3.5
CD	–	–	83.1 ± 3.1	3.6
OMD	78.4 ± 3.0	3.8	–	–
DOPAC	–	–	79.6 ± 3.8	5.6

TABLE III

LINEARITY OF RESPONSE AS A FUNCTION OF CONCENTRATION

y = peak heights of L-DOPA, CD, OMD and DOPAC; x = amount (ng/ml). Values for L-DOPA, CD and DOPAC obtained by ED, values for OMD obtained by fluorimetric detector.

	Range tested	*Regression equation*	*r*
L-DOPA	5–80	$y = 8.42x + 0.91$	0.9998
CD	10–160	$y = 1.85x + 1.75$	0.9992
OMD	10–160	$y = 3.89x + 0.99$	0.9983
DOPAC	5–80	$y = 7.43x + 0.25$	0.997

TABLE IV

PEAK-HEIGHT RATIOS

The values represent the ratio of peak height obtained with ED to that obtained with the fluorimetric detector for standard solutions and for plasma. The data are used to confirm peak identity. Results are the mean ± S.D. of ten experiments. The E level in the majority of plasma samples examined was too low to be measured under our operating conditions.

Compound	*Standards*	*Plasma*
L-DOPA	2.68 ± 0.21	2.62 ± 0.15
NE	1.95 ± 0.18	1.85 ± 0.27
E	2.05 ± 0.14	-
DA	2.58 ± 0.19	2.47 ± 0.15
NMDA	2.20 ± 0.18	2.31 ± 0.20

TABLE V

DETERMINATION OF L-DOPA METABOLITES AND CATECHOLAMINES IN PLASMA FROM PARKINSONIAN PATIENTS TREATED BY CONTINUOUS L-DOPA INFUSION

Each value represents the mean of three determinations. The values are expressed as ng/ml of plasma; n.d. = not detectable (< 0.5 ng/ml for the catecholamines and < 5 ng/ml for the other compounds). CD and DOPAC levels were not detectable (L-DOPA infusion rate = 80 mg/h).

Time (min)	*L-DOPA*	*OMD*	*NE*	*DA*
0	n.d.	2630 ± 210	n.d.	0.51 ± 0.03
30	394 ± 41	2036 ± 190	0.60 ± 0.05	0.74 ± 0.08
60	635 ± 38	2346 ± 244	0.51 ± 0.07	0.58 ± 0.04
90	563 ± 59	2012 ± 153	0.62 ± 0.04	0.92 ± 0.09
120	818 ± 57	2217 ± 124	0.52 ± 0.07	1.19 ± 0.07
180	979 ± 49	2700 ± 249	1.39 ± 0.06	2.94 ± 0.16
240	1114 ± 105	2752 ± 263	1.46 ± 0.0	2.97 ± 0.23

TABLE VI

DETERMINATION OF L-DOPA METABOLITES AND CATECHOLAMINES IN PLASMA FROM A PARKINSONIAN PATIENT FOLLOWING ORAL ADMINISTRATION (125 mg of L-DOPA + 12.5 mg of CD)

Each value represents the mean of three determinations. The values are expressed as ng/ml of plasma; n.d. = not detectable (< 0.5 ng/ml for catecholamines and 5 ng/ml for the other compounds).

Time	*L-DOPA*	*CD*	*OMD*	*DOPAC*	*NE*	*DA*
0	n.d.	n.d.	3000 ± 248	n.d.	0.97 ± 0.05	0.27 ± 0.02
30	500 ± 40	1212 ± 145	2453 ± 245	25 ± 1.5	0.82 ± 0.06	0.77 ± 0.06
60	2351 ± 180	295 ± 27	1325 ± 427	126 ± 11.1	1.21 ± 0.05	3.27 ± 0.26
90	2001 ± 160	n.d.	3388 ± 277	n.d.	0.71 ± 0.02	2.67 ± 0.16
120	1065 ± 115	800 ± 96	2548 ± 129	n.d.	0.71 ± 0.04	1.99 ± 0.18
180	866 ± 53	n.d.	3740 ± 411	75 ± 3.2	0.45 ± 0.04	0.88 ± 0.04
240	587 ± 37	n.d.	3969 ± 357	82 ± 6.6	n.d.	n.d.

Plasma samples from nine Parkinsonian patients, obtained during L-DOPA therapy, were analyzed. The results demonstrated large individual differences. Here, we report two cases as an example of the applicability of this method in the study of Parkinson patients. The case shown in Table V was treated by continuous L-DOPA infusion. From the L-DOPA plasma level it appears that this technique is able to maintain a constant level of the drug and thus control motor fluctuations. The level of OMD never reached the critical point (600 ng/ml), at which the action of L-DOPA is inhibited[28].

The second case (Table VI) shows the pharmacokinetics of oral L-DOPA, which produces a peak blood level after 60 min which then rapidly declines.

Our technique also allowed us to detected DA. The values obtained indicate that, despite the peripheral decarboxylase inhibitor, some DA is still present in the peripheral circulation.

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