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SIMULTANEOUS ASSAY OF 3,4-DIHYDROXYPHENYLALANINE, CATECHOLAMINES AND O-METHYLATED METABOLITES IN HUMAN PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

TADASHI ISHIMITSU* and SHINGO HIROSE

Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607 (Japan)

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SUMMARY

We devised a procedure for the simultaneous determination of 3,4-dihydroxyphenylalanine, catecholamines and O-methylated metabolites using a reversed-phase liquid chromatographic system. Detection is achieved by an electrochemical detector and a fluorescence detector connected in series. Sample preparation is kept to a minimum, and involves precipitation of proteins with trichloroacetic acid and perchloric acid, and subsequent neutralization, thus omitting the commonly adopted adsorption step. Chromatographic peaks were identified on the basis of retention behaviour and the ratio of responses at several oxidation potentials. The method was applied to the quantitative determination of 3,4-dihydroxyphenylalanine, catecholamines and O-methylated metabolites in human plasma.

INTRODUCTION

Analytical techniques for the catecholamines in tissues and body fluids are extremely useful in medicine and biochemistry. Analysis of compounds such as dopamine (DA) and 3,4-dihydroxyphenylalanine (DOPA), which are neurotransmitters in various tissues such as the brain, is important in the understanding of the central nervous system. Many analytical procedures have been devised for DOPA, catecholamines and their O-methylated metabolites such as 3-methoxy-4-hydroxyphenylalanine (3-O-methyl-DOPA), metanephrine (MN), normetanephrine (NMN) and 3-methoxy-4-hydroxyphenethylamine (3-O-methyl-DA). The quantitative methods for determining these compounds employ gas chromatography—mass spectrometry [1—4], radioenzymatic assay [5—7], and high-performance liquid chromatography (HPLC) with fluorescence detection [8—10] and electrochemical detection (ED) [11—17]. Since the metabolic pathways of DOPA and catecholamines have been

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established, their measurement has become clinically important for certain disease states; for example, patients with Parkinson's disease have lower levels of DA than normal [18]. The diagnosis of some tumours may be made via catecholamine analysis; a useful monitor for neuroblastoma is the increased secretion of DA or noradrenaline (NA) [19]. In addition, the O-methylated metabolites were recognized as major intermediates of DOPA and catecholamines, which play a part in their therapeutic action [20—22]. Therefore, it is necessary to measure these compounds simultaneously. However, no simple HPLC method exists for the simultaneous determination of DOPA, catecholamines and O-methylated metabolites in plasma. Further, with all these methods an extensive clean-up procedure, sometimes followed by a concentration step, is inevitable in order to obtain accurate and precise data.

The present report describes a method for the simultaneous determination of free and conjugated DOPA, catecholamines and O-methylated metabolites in plasma. The procedure requires minimal sample pretreatment utilizing a C_{18} reversed-phase column [23, 24]. Also, the sensitivities afforded by ED and fluorescence detection are compared.

EXPERIMENTAL

Reagents

Adrenaline (A), NA, MN, NMN, DOPA, DA and *o*-tyrosine were purchased from Nakarai, Kyoto, Japan. 3-O-Methyl-DA was obtained from Calbiochem, U.S.A. 3-O-Methyl-DOPA was purchased from Kyowa Hakko, Japan.

HPLC conditions

The HPLC system consisted of a Yanagimoto L-2000 high-speed liquid chromatograph equipped with a Yanagimoto VMD 101 electrochemical detector [25] and a Shimadzu RF-530 fluorescence spectrophotometer. The ED cell was a thin-layer design fitted with a glassy carbon working electrode and an Ag/AgCl reference electrode. The two detectors were connected in series, with the electrochemical detector downstream. The column consisted of a Yanapak ODS (Yanagimoto Manufactory, 10 μm) prepacked column (250 mm × 4.0 mm I.D.). A short precolumn (10 mm × 4.0 mm I.D.) packed with Nucleosil C_{18} (Macherey-Nagel, 10 μm) was used as a guard column. The temperature of the column was set at 25°C, the flow-rate was 0.54 ml/min. A working electrode potential of +0.6 V relative to the Ag/AgCl reference electrode was used for the first 17 min, and then increased to +0.9 V. The change in the working potential caused a significant change in the background current. Accordingly, prior to the analysis, this procedure was repeated several times until the baseline became stable within 6—7 min following the change of potential. The chromatographic system was programmed as follows: 0—6 min: 0.05 *M* phosphate buffer (pH 3.1); 6—36 min: linear gradient of increasing methanol concentration (up to 15%) in phosphate buffer; after 36 min: 15% methanol in phosphate buffer. The fluorescence was monitored using an excitation wavelength of 282 nm and emission wavelength of 322 nm. The mobile phase was passed through a 0.22-μm Millipore filter before introduction into the system and degassed in a vacuum prior to use. The retention of chromato-

graphic peaks was expressed as the capacity ratio, k', which was calculated by the formula $k' = (t_R - t_0)/t_0$, where t_0 and t_R are the retention of an unretained solute and of the solute in question, respectively. Retention times were measured with a Shimadzu C-RIA Chromatopac. The column's dead time, t_0, was measured by noting the first baseline disturbance after hydrochloric acid was injected.

Peak identification

The peaks of DOPA, catecholamines and O-methylated metabolites were identified by a combination of methods. Initially, peak identification of the compounds was performed on the basis of liquid chromatographic retention behaviour and co-injection with the reference compounds. Secondly, ratios of responses at several oxidation potentials (DOPA and catecholamines +0.3V to +0.6 V; metabolites +0.5 V to +0.9 V) were calculated for the reference compounds and compared with those of the peaks in the plasma samples.

Quantitative analysis

The ratios for peak height of compound to that of internal standard were calculated and calibration curves were constructed for each compound; in each case a linear relationship between compound concentration and peak height ratio was observed over the concentration ranges studied. The equations for the calibration curves were constructed by linear regression analysis. Calibration curves for each compound were linear over the range 0.25—100 ng/ml with ED and 1.0—100 ng/ml with fluorescence detection.

Plasma

The five subjects (three men, two women) were members of our university staff and students. The blood samples (venous blood) were obtained from the subjects between 10.00 and 11.00 a.m. Samples of blood (12 ml) were

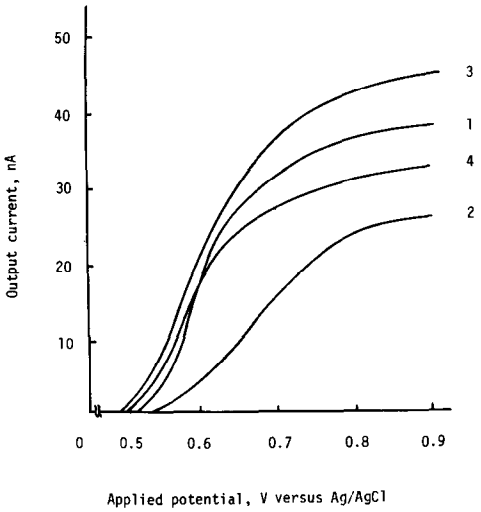

Fig. 1. Relationship between electrochemical detector response and applied potential curves. 1 = NMN; 2 = MN; 3 = 3-O-methyl-DOPA; 4 = 3-O-methyl-DA.

collected into a syringe containing 0.1 ml of sodium heparin (1000 U/ml) and centrifuged immediately at 1000 *g* for 5 min at 4°C.

Free DOPA, catecholamines and O-methylated metabolites. A 2-ml sample of plasma, 100 µl of 1 µg/ml *o*-tyrosine as internal standard and 0.45 ml of

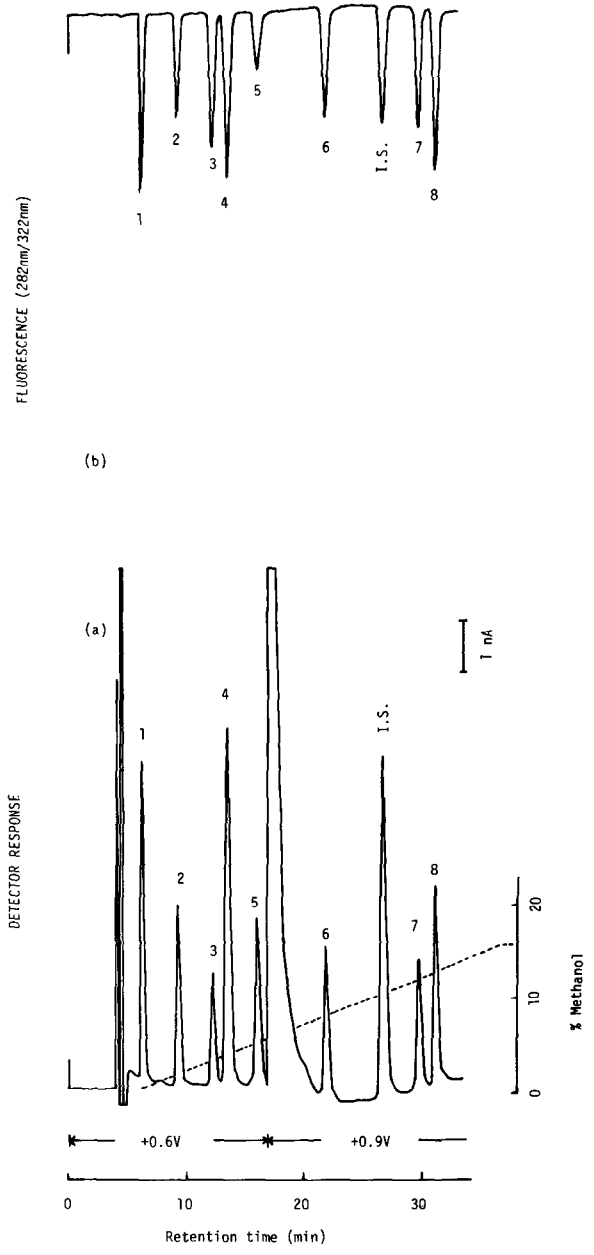

Fig. 2. Chromatograms of a standard solution obtained by HPLC with electrochemical detection (a) and native fluorescence (b). Injection sample: 100 µl of standard solution, containing 20 ng/ml of each compound, and 0.1 µg/ml of *o*-tyrosine. Chromatographic conditions as described in Experimental. Peaks: 1 = NA; 2 = A; 3 = NMN; 4 = DOPA; 5 = DA; 6 = MN; 7 = 3-O-methyl-DOPA; 8 = 3-O-methyl-DA; I.S. = *o*-tyrosine, internal standard.

1.0 *M* trichloroacetic acid were added to a centrifuge tube. The plasma was centrifuged at 10,000 *g* for 10 min. Next, the supernatant was treated with 0.05 ml of 5% potassium hydroxide and centrifuged again.

Total DOPA, catecholamines and O-methylated metabolites. A mixture of 2 ml of plasma containing internal standard and 0.3 ml of 9 *M* $HClO_4$ was heated in a boiling water bath for 20 min. Further, the supernatant was treated with 0.2 ml of 20% potassium hydroxide and centrifuged again.

RESULTS AND DISCUSSION

The response characteristics of the detector system with respect to the applied potentials over the range +0.3 V to +0.9 V versus nA of output current generated for the detection of O-methylated standards are shown in Fig. 1.

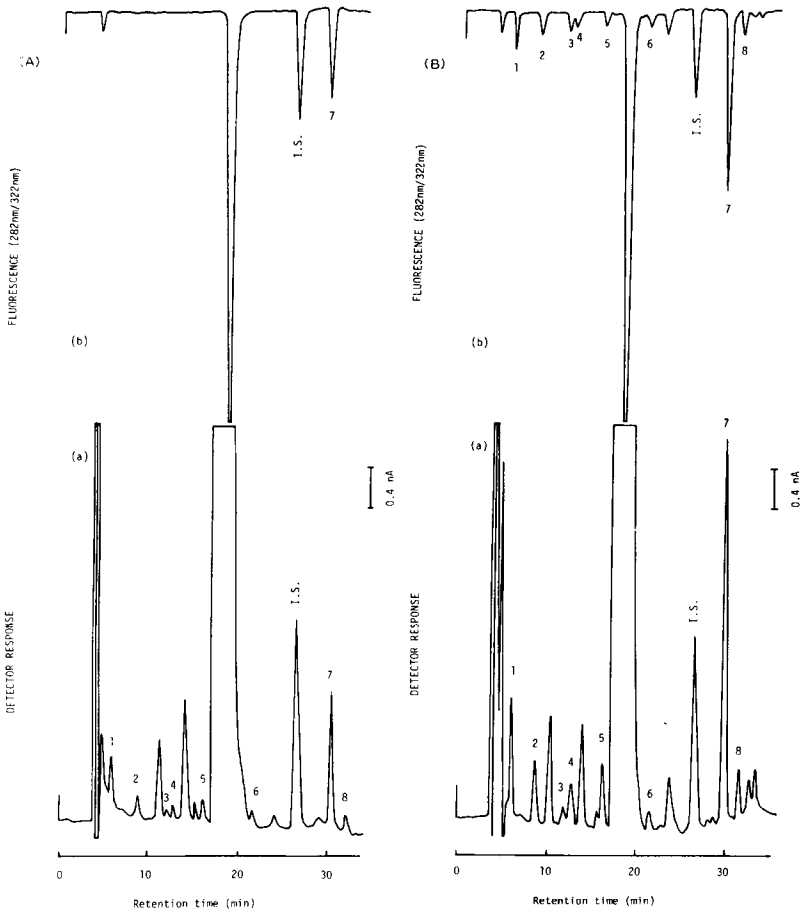

Fig. 3. Chromatograms of a free plasma sample (A) and a total plasma sample (B) obtained by HPLC with electrochemical detection (a) and native fluorescence (b). Injection sample 100 µl of plasma containing (ng/ml); (A) 1, 0.65; 2, 0.37; 3, 0.57; 4, 0.91; 5, 0.39; 6, 0.42; 7, 19.22; 8, 0.42; (B) 1, 2.08; 2, 1.44; 3, 2.55; 4, 2.33; 5, 2.14; 6, 1.88; 7, 45.42; 8, 2.76. Chromatographic conditions as described in Experimental. Peaks: 1 = NA; 2 = A; 3 = NMN; 4 = DOPA; 5 = DA; 6 = MN; 7 = 3-O-methyl-DOPA; 8 = 3-O-methyl-DA; I.S. = *o*-tyrosine, internal standard.

DOPA and catecholamines are easily oxidized at a potential of +0.5 V [13], while O-methylated standards are not readily oxidized at a low potential. Hence, a potential of +0.9 V provided sufficient sensitivity for the determination of O-methylated standards. However, the peak of NMN was rapidly eluted third and had an almost identical retention time to that of DOPA. In the present method, the working electrode operating at a potential of +0.6 V instead of +0.5 V proved to be optimal for the oxidation of DOPA and catecholamines; it was then increased to +0.9 V, the optimum potential for O-methylated metabolites. These settings provide peak/current ratios ranging from 0.5 for NMN to near 1.0 for DOPA and catecholamines. The reversed-phase HPLC separation of the synthetic mixture of DOPA, catecholamines and O-methylated standards, detected amperometrically and by measuring their native fluorescence, is shown in Fig. 2a and b, respectively. When the working potential changed from +0.6 V to +0.9 V, the potential caused a significant change in the background current. Complete separation of individual components of the mixture was obtained. Then, the separation and detection methods were tested in the analysis of plasma samples. Chromatograms of a normal plasma sample prior to, and after, hydrolysis at 100°C are shown in Fig. 3. *o*-Tyrosine served as an internal standard. The chromatogram of the plasma sample was recorded at the most sensitive setting of the electrochemical detector (4 nA full scale). The background seems to be almost free of interfering substances, thus allowing the determination of DOPA, catecholamines and O-methylated metabolites. As evident from Fig. 3A, only 3-O-methyl-DOPA could be detected using the fluorescence detector; its free concentrations in plasma of a normal subject are too low to be detected by the present system. The absolute detection limits for the compounds are listed in Table I. The electrochemical detector is more sensitive than the fluorescence detector. However, the total levels of DOPA, catecholamines and O-methylated metabolites can be determined by both detectors. However, when the working electrode was operated at a potential of +0.9 V from the start, NA in plasma samples could not be determined due to interference by the adjacent peak.

The capacity ratio and chromatographic peak height ratio of the eight

TABLE I

TYPICAL DETECTION LIMITS

Compound	Detection limit* (ng/ml)	
	Amperometric	Fluorescence
NA	0.10	0.50
A	0.10	0.50
NMN	0.20	0.95
DOPA	0.10	0.75
DA	0.15	0.75
MN	0.15	0.95
3-O-Methyl-DOPA	0.20	1.00
3-O-Methyl-DA	0.20	1.00

*Injected quantity giving a signal-to-noise ratio of 2.0.

TABLE II

HPLC CAPACITY RATIOS AND PEAK CURRENT RATIOS AT VARIOUS OPERATING POTENTIALS OF DOPA, CATECHOLAMINES AND O-METHYLATED METABOLITES

	NA	A	NMN	DOPA	DA	MN	3-O-Methyl-DOPA	3-O-Methyl-DA
Capacity ratio	0.6	1.3	2.1	2.4	3.1	4.5	6.5	6.8
300/600 mV	0.02	0.10	N.D.*	0.03	0.10	N.D.	N.D.	N.D.
350/600 mV	0.29	0.26	N.D.	0.38	0.33	N.D.	N.D.	N.D.
400/600 mV	0.90	0.92	N.D.	0.91	0.92	N.D.	N.D.	N.D.
500/600 mV	0.99	0.98	N.D.	0.98	0.99	N.D.	N.D.	N.D.
550/900 mV	1.01	1.01	0.19	1.01	1.01	0.08	0.22	0.20
600/900 mV	1.03	1.02	0.51	1.02	1.03	0.16	0.55	0.58
700/900 mV	1.02	1.01	0.89	1.01	1.02	0.64	0.88	0.89
800/900 mV	1.02	1.01	0.94	1.01	1.02	0.90	0.93	0.95

*N.D., Ratio is approximately 0.0.

reference compounds are shown in Table II. For electrodes operating at 800/900 mV, the ratios for all peaks are nearly unity. However, at 500/600 mV, DOPA and catecholamines gave ratios of 0.98, whereas O-methylated metabolites gave ratios of 0.0.

The amounts of free and total DOPA, catecholamines and O-methylated metabolites found in the plasma of five healthy subjects are listed in Table III. These levels agree with those reported in the literature, except for A and DA, which are slightly higher. This might be attributed to the manner in which the sample was taken, which may have influenced the actual concentration of A and DA in the plasma. The levels of O-methylated metabolites, particularly that of 3-O-methyl-DOPA, in the plasma of normal individuals are higher than those of the other compounds. Since the free levels were determined by ED, the absolute quantity of conjugated compounds present in a given specimen is the difference between the total (determined after hydrolysis) and free (determined prior to hydrolysis) compounds.

The recovery was measured using the free and total plasma procedure. The compounds were added to plasma at concentrations of 0.5 and 5.0 ng/ml and the recovery results are shown in Table IV. The present method gives reproducible and high recoveries (greater than 95%) when compared to those usually obtained (about 70%) after alumina or ion-exchange resin separation. Therefore, this method permits the elimination of the extraction step by direct injection of the deproteinized and neutralized plasma supernatant into the reversed-phase HPLC system. Hence, a possible source of error and a loss in sensitivity is eliminated. A drawback of the direct injection of biological materials is a shortened life of the column, especially the column top [26]: a remedy may be the insertion of a precolumn [27, 28]. In general, the columns used were adequate for about 500 injections, after which the resolution between NMN and DOPA and between 3-O-methyl-DOPA and 3-O-methyl-DA deteriorates.

Comparison of the two detectors indicates that the ED method is sensitive and specific for determining plasma levels of DOPA, catecholamines and their

TABLE III

CONTENTS OF DOPA, CATECHOLAMINES AND O-METHYLATED METABOLITES IN PLASMA DETERMINED USING ELECTROCHEMICAL AND FLUOROMETRIC DETECTORS

Results are expressed in ng/ml of plasma. Measurements made with the fluorometric detector are given in parentheses.

Subject		NA		A		NMN		DOPA	
Age (years)	Sex	Free	Total	Free	Total	Free	Total	Free	Total
37	M	0.65	2.08 (1.89)	0.37	1.44 (1.32)	0.57	2.55 (2.36)	0.91	2.33 (1.94)
32	M	0.41	1.86 (1.69)	N.D.*	0.84 (N.D.)	N.D.	1.81 (1.72)	0.82	2.19 (2.03)
21	M	0.39	1.48 (1.34)	0.29	1.03 (1.11)	0.51	2.24 (2.08)	0.48	2.01 (1.88)
23	F	N.D.	0.83 (N.D.)	N.D.	N.D. (N.D.)	0.42	1.07 (N.D.)	N.D.	1.16 (1.05)
20	F	0.27	1.18 (1.24)	N.D.	0.54 (N.D.)	0.33	1.54 (1.42)	0.44	1.62 (1.58)

*N.D., Not detectable.

TABLE IV

RECOVERY OF INVESTIGATED COMPOUNDS MEASURED BY ELECTROCHEMICAL DETECTION

Values represent mean ± S.D. of five experiments.

Compound	Recovery (%)	
	Free*	Total**
NA	95.1 ± 1.9	96.0 ± 2.1
A	95.8 ± 2.3	97.4 ± 2.7
NMN	95.3 ± 1.7	95.6 ± 1.8
DOPA	100.2 ± 2.1	101.2 ± 2.4
DA	102.4 ± 2.0	103.3 ± 1.9
MN	97.9 ± 2.4	96.8 ± 3.1
3-O-Methyl-DOPA	98.3 ± 1.3	98.4 ± 1.2
3-O-Methyl-DA	96.1 ± 1.8	95.3 ± 2.4
o-Tyrosine***	98.4 ± 2.0	97.8 ± 1.5

*Compounds were added at a concentration of 0.5 ng/ml.
**Compounds were added at a concentration of 5.0 ng/ml.
***Amount added was 0.1 μg/ml.

O-methylated metabolites. The fluorescence method was not as sensitive as ED, but it was less problematic in setting up and was found to be quite satisfactory for the measurement of high plasma concentrations of these compounds.

DA		MN		3-O-Methyl-DOPA		3-O-Methyl-DA	
Free	Total	Free	Total	Free	Total	Free	Total
0.39	2.14 (1.97)	0.42	1.88 (1.69)	19.22 (20.02)	45.42 (44.35)	0.42	2.76 (2.21)
0.41	2.96 (2.78)	0.37	1.43 (1.52)	20.36 (21.03)	52.18 (50.02)	0.56	3.01 (2.89)
N.D.	1.84 (1.91)	0.48	1.72 (1.63)	16.44 (15.98)	66.14 (63.82)	N.D.	1.33 (N.D.)
0.37	1.68 (1.56)	N.D.	0.98 (N.D.)	30.12 (28.97)	78.42 (77.56)	0.38	1.70 (1.56)
0.29	1.34 (1.27)	0.46	1.12 (1.21)	21.08 (20.48)	42.38 (40.14)	0.29	0.82 (N.D.)

CONCLUSIONS

The method reported gives a good separation of free and conjugated DOPA, catecholamines and O-methylated metabolites found in human plasma without a complex clean-up procedure. Also, it permits the simultaneous determination of these compounds by reversed-phase HPLC. The assay is suitable for research application and routine clinical analysis.

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