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RAPID AND SIMPLE METHOD FOR THE SIMULTANEOUS DETERMINATION OF 3,4-DIHYDROXYPHENYLACETIC ACID, 5-HYDROXYINDOLE-3-ACETIC ACID AND 4-HYDROXY-3-METHOXYPHENYLACETIC ACID IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A simple procedure for the simultaneous determination of major metabolites of dopamine and serotonin in plasma, i.e. 3,4-dihydroxyphenylacetic acid, 5-hydroxyindole-3-acetic acid, and 4-hydroxy-3-methoxyphenylacetic acid, was developed. The method is based on rapid isolation of the compounds by one-step clean-up on a small C_{18} column, followed by high-performance liquid chromatography with dual electrochemical detection. The system is readily used for clinical applications.

INTRODUCTION

Abnormalities of biogenic amine metabolism are implicated in various pathological states including psychiatric [1] and neurological disorders [2]. A number of investigations have concentrated on measurement of the metabolites of biogenic amines in biological fluids, particularly cerebrospinal fluid (CSF) and urine. This seems to be helpful for the diagnosis and interpretation of the disorders. Unexpectedly, information on plasma monoamines and their metabolites, except for catecholamines [3, 4], is scanty. The plasma concentrations of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), major metabolites of dopamine [5], have been suggested to reflect changes in the brain dopaminergic system [6]. 5-Hydroxyindoleacetic acid (5-HIAA), another acidic compound, is a major metabolite of serotonin which is known as a neurotransmitter and vasoconstrictor. Interaction between the dopaminergic

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and serotonergic systems has been suggested [7]. Modifications of these plasma metabolites have been documented in hepatic encephalopathy [8] and psychoses [9]. However, to our knowledge, no method for the simultaneous determination of DOPAC, HVA and 5-HIAA in blood appears to have been available.

The development of high-performance liquid chromatography (HPLC) with electrochemical detection (ED) facilitated the establishment of highly sensitive and selective assay procedures for these metabolites. Under certain conditions, direct injection of the deproteinized brain tissue [10], CSF [11], or urine [12] into the HPLC system has been reported to permit a clear separation and detection of these compounds, which is not easily applicable to plasma metabolites owing to the much lower levels and various interfering substances relative to other matrices as described above. The difficulty in determining the plasma metabolites could be overcome by the use of Sephadex G-10 [13, 14] or organic solvent extraction [15, 16] prior to HPLC. However, our preliminary test with a small C_{18} column revealed that the purification or enrichment procedure became less time-consuming and simpler compared to the use of Sephadex G-10 or organic solvent extraction.

This paper describes a simple and rapid procedure for the simultaneous determination of DOPAC, 5-HIAA and HVA in plasma by one-step purification on a small C_{18} column followed by HPLC—ED.

EXPERIMENTAL

Reagents

3,4-Dihydroxyphenylacetic acid (DOPAC) and 5-hydroxy-2-indolecarboxylic acid (5-HICA) were obtained from Aldrich (Milwaukee, WI, U.S.A.); 5-hydroxyindole-3-acetic acid (5-HIAA), 4-hydroxy-3-methoxyphenylacetic acid (HVA), and other related compounds were from Sigma (St. Louis, MO, U.S.A.). Stock solutions of these compounds were prepared in 0.1 *M* hydrochloric acid in a concentration of 100 μg/ml. Standard solutions were diluted from their corresponding stock solutions on the day of experiment. All other reagents were of analytical reagent grade.

Chromatography

The LC—ED system was purchased from Yanagimoto Mfg. Co. (Kyoto, Japan), and consisted of a Yanako Model L-4000W pump, a 250 × 4.6 mm I.D. 7-μm Yanapak ODS-A reversed-phase column, and a Model VMD-501 dual electrochemical detector with series-adjacent twin glassy carbon electrodes. Applied electrode potentials were 0.6 V and 0.75 V vs. Ag/AgCl at the upstream (W_1) and downstream (W_2), respectively. The mobile phase consisted of 0.1 *M* potassium phosphate buffer (pH 3.2), containing EDTA · 2Na (10 μM) and methanol (18%). The flow-rate was 1.2 ml/min, and the column temperature was set at 40° C.

Extraction

Blood was collected in a tube containing 0.1% EDTA · 2Na and 0.1% sodium metabisulphite and put on ice. Plasma was separated by centrifugation at 600 *g* for 7 min at 4° C, and stored at −80° C until analysed.

Extractions were performed under vacuum using Bond-Elut columns prepacked with 100 mg of C_{18} bonded silica (40 μm) in a 1-ml capacity disposable syringe (Analytichem International, Harbor City, CA, U.S.A.). The columns, which were inserted into a vacuum chamber connected with water aspirator, were prepared by washing with 2 ml of methanol followed by 2 ml of water.

To 1 ml of plasma, 100 μl of a solution of the internal standard (equivalent to 10 ng), 5-hydroxy-2-indolecarboxylic acid (5-HICA), and 250 μl of 1 *M* hydrochloric acid were added. Samples were then applied to and passed through the columns, followed by a 1-ml water wash to rinse off residual samples and easily eluted hydrophilic compounds. The adsorbed materials were eluted with 200 μl of methanol and 5—20 μl of this solution were injected into the HPLC system.

Calibration curves were generated by processing authentic standard substances through the entire procedure of extraction and comparing the relative peak heights to the internal standard.

RESULTS AND DISCUSSION

The usefulness of electrochemical detection for endogenous monoamines including catecholamines and related compounds has been repeatedly emphasized in recent years [17]. Many aspects can be further improved with dual amperometric detection [18].

In the present chromatographic conditions, DOPAC and 5-HIAA are oxidized readily at potentials above 0.6 V (Fig. 1). Since higher selectivity and lower background noise can be obtained at lower potentials, we adopted 0.6 V for the determination of DOPAC and 5-HIAA. However, HVA showed a poor response at 0.6 V but gave a quantifiable response current at potentials above 0.7 V (Fig. 1). Thus, the upstream (W_1) and downstream (W_2) potentials were selected at 0.6 V for the detection of DOPAC, 5-HIAA and 5-HICA (internal standard) and 0.75 V for HVA, respectively.

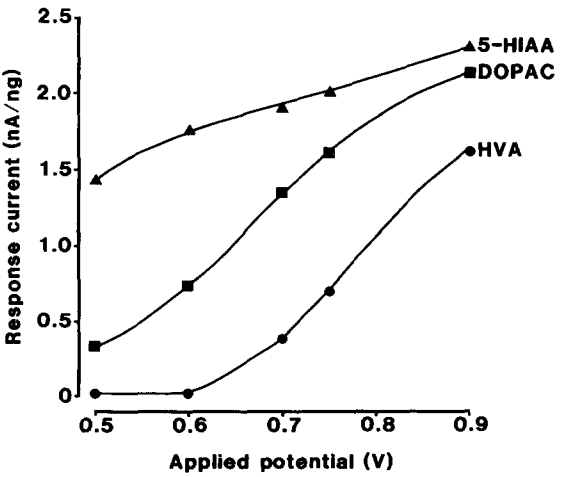

Fig. 1. Hydrodynamic voltammograms of DOPAC, 5-HIAA and HVA. The details of HPLC conditions are described in Experimental.

Chromatograms demonstrating the resolution of authentic DOPAC, 5-HIAA, 5-HICA and HVA are shown in Fig. 2. To achieve an optimal separation of the compounds from interfering substances, the pH of the mobile phase was critical. The retention times of monoamine-related compounds examined using the system described above are listed in Table I. All the compounds determined herein, which are acidic metabolites of biogenic amines, had much longer retention times than any other amines and neutral compounds examined, except for tryptophan which was eluted immediately after DOPAC using this system but did not interfere with the detection of DOPAC because of little or no response at 0.6 V. The retention times of these acidic compounds were shortened at higher pH values and substantial overlap of peaks occurred.

Representative chromatograms of an extract from normal human plasma are shown in Fig. 3. DOPAC, 5-HIAA and 5-HICA are clearly separated from other biological materials (which could not be identified) and selectively detected at 0.6 V (W_1). Only HVA needed to be detected at 0.75 V (W_2), while the peak of DOPAC was overlapped by tryptophan (whose recovery was not

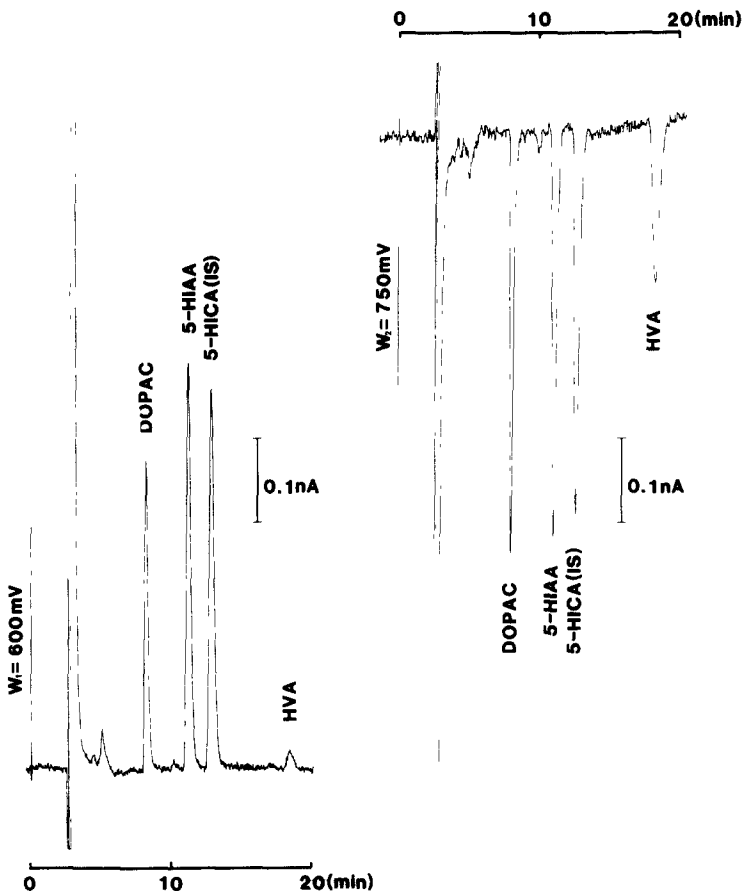

Fig. 2. Chromatogram demonstrating the separation of DOPAC, 5-HIAA, HVA and 5-HICA (internal standard) in the standard solution (0.3 ng each) using a dual electrochemical detector.

TABLE I

RETENTION TIMES OF SOME MONOAMINE-RELATED COMPOUNDS

Compound	Retention time (min)
Norepinephrine Epinephrine	2.79
l-DOPA	3.02
Dopamine	3.08
Tyrosine	3.37
3,4-Dihydroxyphenylglycol	3.38
Serotonin	3.87
Vanillylmandelic acid	4.12
5-Hydroxytryptophan	4.22
4-Hydroxy-3-methoxyphenylglycol	4.80
DOPAC	8.06
l-Tryptophan	8.18
5-HIAA	11.14
5-HICA	12.70
HVA	18.20

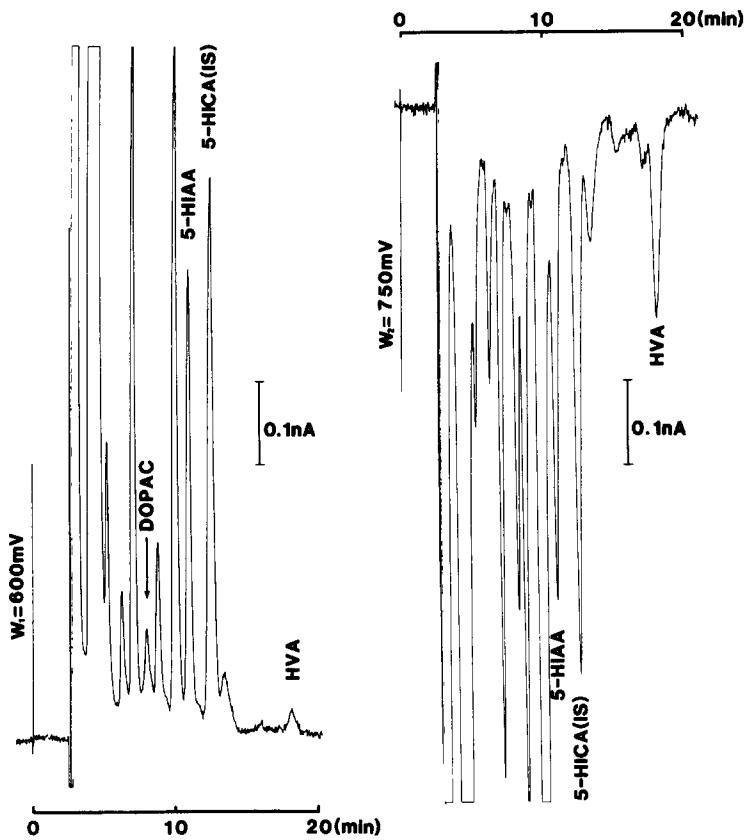

Fig. 3. Typical chromatograms demonstrating the separation of DOPAC, 5-HIAA and HVA in an extract from human plasma using a dual electrochemical detector. 5-HICA was used as an internal standard.

determined) at this potential. Identities of the peaks were confirmed by chromatographic behaviour using several solvent systems differing in pH and percentage of organic solvent and electrochemical characteristics. The dual ED system revealed another advantage that the peak identities were confirmed by comparing the polarities and relative magnitudes of current responses of standards and unknowns [18].

To establish the most effective extraction procedure from plasma various adsorbents for the extraction columns were tested in preliminary experiments. When the authentic standard solution was used, C_{18}, phenyl and cyclohexyl columns showed a high recovery to a similar extent for all three compounds, i.e. 5-HIAA, DOPAC and HVA, compared with cyanopropyl, aminopropyl, quarternary amine, and C_8 columns. When using blood samples, the C_{18} column proved to be better than or not different from phenyl or cyclohexyl columns in terms of selectivity against possible interfering endogenous substance(s) and recoveries. We chose a C_{18} column on the basis of this finding and also because of its general distribution and ready availability. The recovery rates of substances when using the extraction columns were further influenced by the differences in extraction conditions (Table II). The recoveries of the substances were all around 90% when the plasma samples were treated according to the standard method. Deproteinization of plasma samples with perchloric acid prior to application on to the extraction columns (Method 1) markedly reduced those recoveries, although the acidic condition was indispensable. Differences in the composition of the eluent also seriously influenced recovery. Extraction with a much more polar eluent containing more than 50% of aqueous solution in place of methanol (Method 2) reduced the recoveries of less hydrophilic compounds. Although fairly good recoveries were obained with another eluent containing less than 50% of aqueous solution (Method 3), this eluent extracted interfering substances simultaneously, which interfered with the determination of DOPAC. We can prepare a sample for HPLC injection through the entire extraction procedure within a few minutes.

TABLE II

COMPARISON OF RECOVERIES OF SUBSTANCES (10 ng/ml) ADDED TO NORMAL PLASMA UNDER VARYING EXTRACTION CONDITIONS

Procedures are similar to the standard method except for the following: in Method 1, plasma was deproteinized with perchloric acid (40 μg/ml) before applying to the Bond-Elut column; in Method 2, adsorbed materials were eluted from the Bond-Elut column with 0.1 *M* phosphate buffer (pH 3.2)—methanol mixture (60:40); and in Method 3, adsorbed materials were eluted from the Bond-Elut column with 0.05 *M* hydrochloric acid—methanol mixture (40:60)

	Recovery (%)			
	Standard method (mean ± S.D., n = 5)	Method 1	Method 2	Method 3
DOPAC	87.7 ± 4.1	57.0	87.7	95.6
5-HIAA	92.2 ± 4.1	52.4	80.7	93.1
5-HICA	87 1 ± 1.8	33.1	65.3	82.0
HVA	91.1 ± 3 8	75.6	55.6	85.6

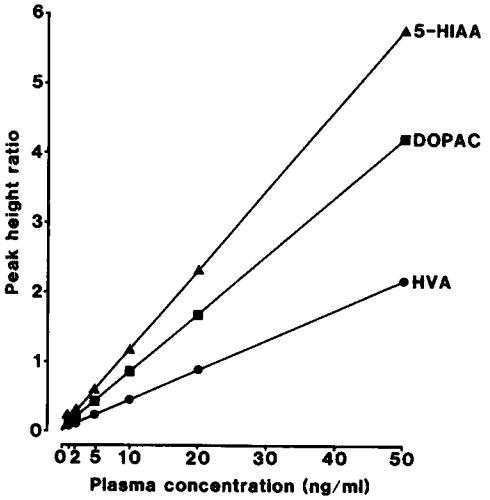

Fig. 4. Calibration curves of the peak height ratio of DOPAC, 5-HIAA and HVA vs. 5-HICA (10 ng/ml) in plasma. A three- or four-point standard curve was prepared by plotting the ratio of each compound's peak height to that of the internal standard for each concentration. Linear regression analysis of calibration data indicated no significant deviation from linearity (r = 0.9985—0.9990). In addition, the intercept did not differ significantly from zero.

The standard curves were run on each occasion with plasma standards carried through the entire procedure. Linearity was demonstrated by plotting the peak height ratio of compounds vs. 5-HICA over the range 1—50 ng/ml (Fig. 4). Standard curves had r values of 0.9985—0.9990 and passed through the origin. The sensitivity of the present method permitted the assay of at least 200 pg/ml for DOPAC and 5-HIAA and 800 pg/ml for HVA in plasma. The precision of the assay is given in Table III. High reproducibility was verified by the coefficients of variation which ranged from 2.1% to 4.3%.

The analytical results of human plasma from five healthy subjects are given in Table IV. The concentrations of DOPAC, 5-HIAA and HVA are compatible with the values recently reported with HPLC [3, 19, 20] and gas chromatography—mass spectrometry [9, 21—23].

The present method offers a means for the rapid and simultaneous determination of three monoamine metabolites, i.e. DOPAC, 5-HIAA and HVA, in human plasma. The readiness of the procedure may augment the utilization of these values as indices for evaluating the activities of dopaminergic and

TABLE III

PRECISION OF THE ASSAY FOR DOPAC, 5-HIAA AND HVA IN POOLED PLASMA

	Concentration ± S.D. (ng/ml)	n	C.V. (%)
DOPAC	1.87 ± 0.04	5	2.1
5-HIAA	11.56 ± 0 33	5	2.9
HVA	15.88 ± 0.68	5	4.3

TABLE IV

DETERMINATION OF MONOAMINE METABOLITES IN PLASMA FROM HEALTHY HUMAN SUBJECTS

Subject No	DOPAC (ng/ml)	5-HIAA (ng/ml)	HVA (ng/ml)
1	1.70	6.8	15.6
2	2.44	6.8	16.9
3	2.10	8.2	11 7
4	1.90	11.4	10.0
5	1.95	12 9	10.3
Mean	2.02	9.22	12.9
± S.D.	±0 28	±2.79	±3.2

serotonergic systems in diverse clinical conditions. The assay method presented here is also of sufficient sensitivity, precision, and accuracy for clinical application. In addition, the procedure may be applicable to other biological fluids or tissues with minor modifications.

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