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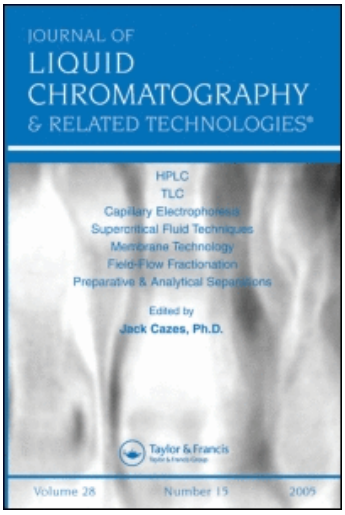

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Analysis of Biogenic Amines and Their Acidic Metabolites by Reversed-Phase Liquid Chromatography with Electrochemical Detection

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ANALYSIS OF BIOGENIC AMINES AND THEIR ACIDIC METABOLITES BY REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

An improved reversed-phase liquid chromatography analysis with an electrochemical detector was developed to determine concurrently concentrations of the basic neurotransmitters, serotonin, dopamine, norepinephrine and epinephrine and their acidic metabolites. A combination of extraction procedures and a novel method of peak identification using applied potentials from 0.35 to 0.95 volts are described.

INTRODUCTION

Reproducible, sensitive analytical methods were needed for mental illness investigations for the determination of the concentrations the neurotransmitters, serotonin (5HT), dopamine (DA), norepinephrine (NE), and epinephrine (E) [1, 2], along with their acidic metabolites 5-hydroxyindoleacetic acid (5HIAA), homovanillic acid (HVA), 3-methoxy-4-hydroxyphenylethylene glycol (MHPG), vanillylmandelic acid (VMA) and 3, 4-dihydroxyphenylacetic acid (DOPAC) (Fig. 1a and b). Abnormal amounts of 5HT and 5HIAA in blood and urine have been associated with many diseases, such as depression [1], migraine [3], carcinoid syndrome [4, 5], and essential hypertension [6]. In addition, scientists have found altered levels of the catecholamines

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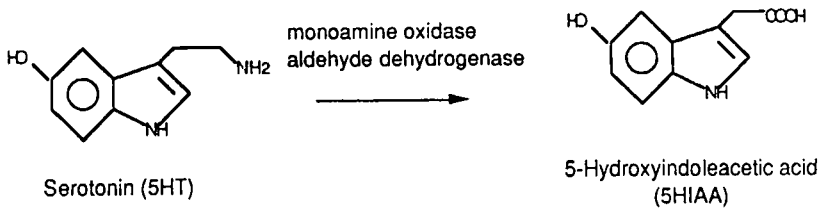
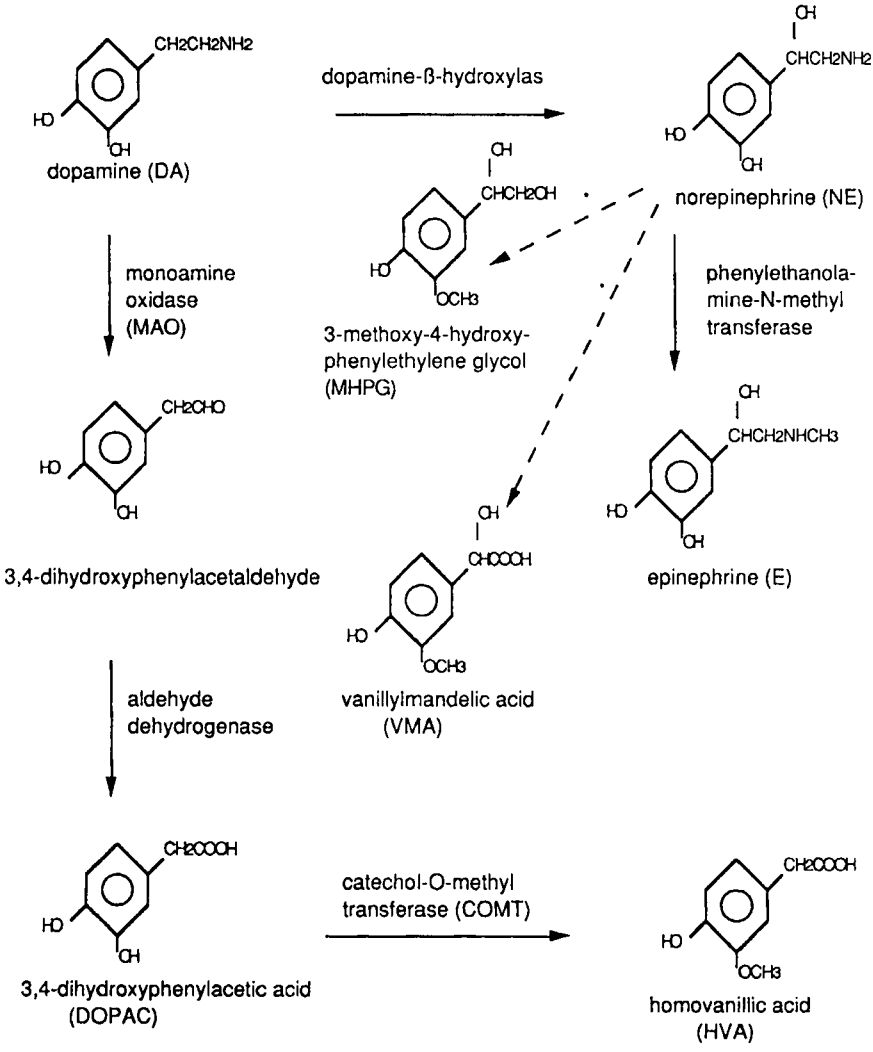


Figure 1(a): Metabolic pathway of serotonin



* The dashed lines indicated that there are multiple steps of metabolism.

Figure 1(b): Metabolic pathway of catecholamines

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(CAs), DA, NE, E and their metabolites in body fluids of patients with other diseases [2, 7]. Hypotheses on the pathophysiological roles of altered catecholaminergic functions have been proposed. Although these hypotheses have not yet been proven, they have led to intensive research. Because of inadequate analytical methods, large studies involving the concentrations of these particular biogenic amines and their metabolites have not been reported, although many studies have been done on one or several of these compounds.

Recently, reversed-phase high performance liquid chromatography (RP-HPLC) coupled with electrochemical (EC) detection was successfully employed to determine one or several of the nine compounds (5-HT, DA, NE, E, 5-HIAA, HVA, MHPG, VMA, DOPAC) in samples of body fluids. e.g., simultaneous determination of CAs and 5-HT [7], of three CAs [8-24], of HVA, VMA, DOPAC, and 5-HIAA [25], and of MHPG [26-29]. However, because the amines (5HT, DA, NE, E) are basic and the metabolites of interest are acidic, it is difficult to analyze the nine compounds simultaneously. Although the simultaneous analysis of the amines and their metabolites has been reported by Taylor et al. [30], the catecholamines were eluted together at the begining of the chromatogram. Therefore, they used two isocratic ion-pairing assays: one to separate the amines and one to separate the acidic metabolites. Since the mental illness investigations planned involve the use of ratios of the concentrations of neurotransmitters to their metabolites, we needed a method in which the levels of the acidic metabolites could be determined simultaneously with the levels of the basic parent biogenic amines. Therefore we investigated an ion-paring RPLC method with electrochemical detection for use in these studies.

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EXPERIMENTAL

Apparatus: A Perkin-Elmer Series 3B liquid chromatography (Perkin-Elmer Corp., Norwalk, CT, USA) was used. Detection was performed by means of a Perkin-Elmer LC-4B amperometric detector with Ag/AgCl reference electrodes and glassy carbon working electrodes. Injections were made via a Micromeritics Model 725 Autoinjector with a 20µl loop (Micromeritics, Norcross, GA, USA). Peak heights were recorded with a strip chart Omniscribe recorder (Houston Instruments, Austin, TX, USA). Peak areas were electronically integrated with a Perkin-Elmer 3600 Data Station using CHROM2 software. The samples were centrifuged with a DYNAC Centrifuge (Clay Adams, Division of Becton, Dickinson and Company, Parsippany, NJ, USA).

Column: All separations were performed on a 250 x 4.6 mm, 5 µm Nucleosil C18 column (Prepacked HPLC Column, I. Molnar, Berlin, FRG). A guard column packed with sepralyte C18 (25µm, pore size 60Å) was used to protect the analytical column.

Chemicals and Supplies: Chromatographic standards were serotonin, dopamine, norepinephrine, 5-hydroxyindoleacetic acid, homovanillic acid, 3-methoxy-4-hydroxyphenylethylene glycol, vanillylmandelic acid, and 3, 4-dihydroxyphenylacetic acid. They were obtained from Sigma Chemical Company (St. Louis, MO, USA). Ethylenediamine tetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), Tris[hydroxymethyl]aminomethane, and sodium octanesulfonate were also obtained from Sigma Chemical Company. Sodium chloride, ethyl acetate, and acetonitrile were obtained from Fischer Scientific (Fair Lawn, NJ, USA). Acid washed alumina was purchased from Bioanalytical Systems Inc. (West Lafayette, IN, USA). Mobile phases were prepared with doubly distilled deionized water and filtered through a 0.45 µm Millipore membrane filter (Millipore Corp., Bedford, MA, USA). The reducing reagent, sodium metabisulfite was obtained from Mallinckrodt Chemical Works (St. Louis, MO, USA).

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Chromatographic Conditions: A step gradient with mobile phase A and mobile phase B were used to separate the nine compounds with a flow rate of 1.0 ml/min. Mobile phase A was 500 ml of an aqueous solution containing 0.0500 g SDS (sodium dodecyl sulfate), 0.0500 g NaCl, and 0.0500 g EDTA and 88 ml acetonitrile. Mobile phase B contained 0.0500 g SDS, 0.0500 g NaCl, 0.0500 g EDTA, 0.1000 g sodium octanesulfonate, and 214 ml acetonitrile. The mobile phases were adjusted to pH 3.3 with 0.15 M H_3PO_4. The HPLC system and EC detector were first stabilized by mobile phase A for about 30-40 minutes. The mobile phase was then changed to mobile phase B and a sample was injected.

Sample Preparation: Morning urine samples from a normal person were collected in plastic bottles on different dates. Sodium metabisulfite was added as a reducing reagent. The samples were refrigerated and then filtered with 0.45 µm Millipore filter. Extraction was carried out in three steps.

Step 1. Extraction of VMA, MHPG, DOPAC, 5HIAA and HVA with ethyl acetate: 1.5 g NaCl was added to 10 ml filtered urine in a glass vial. The pH was adjusted to ~4 with 0.15 M H_3PO_4 since the optimal pH value for 5HIAA was found to be pH 4.0 [31]. 5 ml ethyl acetate was added and the vial was shaken for ~5 min. The organic layer (organic A) was transferred into another vial. The pH of the aqueous layer was adjusted to 1-2 with 0.15 M H_3PO_4 and 5 ml ethyl acetate was added to the solution. The vial was shaken for ~5 min and the organic layer (organic B) was transferred into the vial of organic A. The organic solution was evaporated to dryness under reduced pressure.

Step 2. Extraction of DA, NE and E with acid washed alumina: 5 ml Tris buffer (pH 8.6) and 0.4 g acid washed alumina were added to 10 ml urine sample in a glass vial. The vial was shaken for ~5 min. The sample was centrifuged for 3 minutes at high speed and the supernatant was transferred into another vial for the extraction step 3. Alumina was washed by 10 ml doubly distilled water. 1.5 ml 5% HAc was added into the vial to dissolve the absorbates on alumina. Then, the HAc solution was transferred to the ethyl acetate extract of step 1.

Step 3. Extraction of 5HT with 1-heptanol: 5 ml 1-heptanol was added to the vial of the supernatant from step 2 and the vial was shaken for ~5 min. The organic layer (organic C) was transferred into another vial. 1.5 ml 5% HAc was added to the solution of organic C and the vial was shaken for ~5 min. The aqueous layer was transferred to the mixture of extracts from step 1 and step 2.

Sample recovery: The recovery of the nine compounds was determined by addition of known amounts of standards to a urine matrix. The compounds were extracted by the sample preparation procedures described above. The concentration of standards in the matrix was 10^{-5} M for VMA, MHPG, DOPAS, 5HIAA, and HVA, and 10^{-6} M for DA, NE, E, and 5HT.

Peak Identification: Identification of the peaks of interest was based on retention time, co-injection of standards with the samples, and determination of concentrations at applied poterntials of 0.35, 0.45, 0.55, 0.65, 0.75, 0.85, and 0.95 V.

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RESULTS AND DISCUSSION

The two groups of compounds are difficult to separate in one analyses because of their different acidities; the parent compounds are basic and their metabolites are acidic. However, good resolution of all the compounds of interest was achieved using a step gradient (Fig. 2). Fig. 2 shows a chromatogram of 5HT, DA, NE, E and their five metabolites, VMA, MHPG, DOPAC, 5HIAA and HVA. Most of the peaks are baseline resolved. The peaks appeared as two groups, the acidic metabolite group (VMA, MHPG, DOPAC, 5HIAA, HVA) and the amine group (NE, E, DA, 5HT). The negative peak between the two groups is caused by changing the mobile phase from mobile phase A to mobile phase B. The retention times, peak areas, and peak shapes were highly reproducible on ten samples of 10^{-5} M standards (Table 1).

Table 2 shows detection limits of all the nine compounds at a potential of 0.75 V. The detection limits are comparable to those previously reported and are adequate for studying the nine compounds of interest in human urine. There was good linearity of

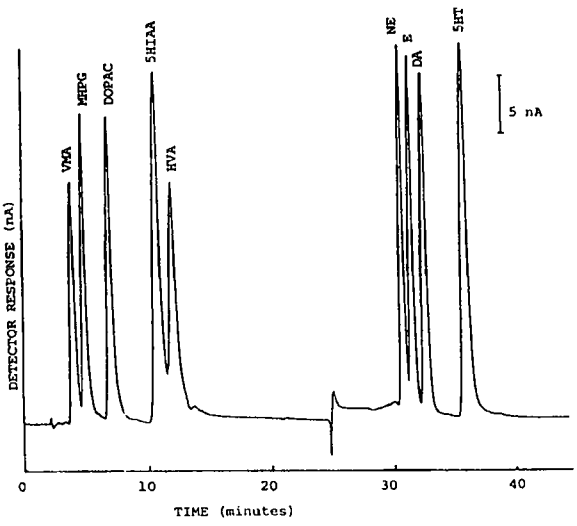

Figure 2: HPLC separation of a standard 10^{-5} M mixture of vanillylmandelic acid (VMA), 3-methoxy-4-hydroxyphenylethylene glycol (MHPG), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5HIAA), homovanillic acid (HVA), norepinephrine(NE), epinephrine(E), dopamine(DA), and serotonin (5HT). Chromatographic conditions as described in Experimental. Applied potential of EC detector is 0.75 V. Sensitivity is 50 nA full scale.

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response in a concentration range of 5 x 10^{-5} M to 10^{-7} M. The regression coefficients for these plots were 0.999 for DOPAC, 5HIAA, E, and DA, 0.998 for NE and 5HT, 0.988 for HVA, 0.987 for VMA, and 0.974 for MHPG (n = 27).

The stability of 5HT, DA, NE, and E was investigated. There was almost no degradation of 5HT after one day at 0°C or at ambient temperature upon exposure to indirect sunlight. There was little degradation of DA, NE and E after two days at 0°C; however, after three days DA and E showed significant degradation and NE showed degradation after one day at ambient temperature upon exposure to indirect sunlight. Therefore, degradation of the amines is not a problem if samples are kept at 0°C and analyzed within one day.

TABLE 1
Abbreviation, Retention Times and Peak Areas

Compound	Abbreviation	Retention time* in min (SD)	Peak Area (SD)
Metabolites			
vanillylmandelic acid (4-hydroxy-3-methoxymandelic acid)	VMA	4.3 (±0.0)	9428 (±171)
3-methoxy-4-hydroxyphenylethylene glycol (MOPEC)	MHPG	5.5 (±0.1)	10026 (±493)
3,4-dihydroxyphenylacetic acid	DOPAC	7.6 (±0.1)	11299 (±453)
5-hydroxyindoleacetic acid	5-HIAA	11.6 (±0.1)	16131 (±1268)
homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid)	HVA	13.0 (±0.1)	13702 (±504)
Amines			
norepinephrine (noradrenaline)	NE	31.9 (±0.1)	9824 (±235)
epinephrine (adrenaline)	E	32.8 (±0.1)	9988 (±178)
dopamine	DA	33.8 (±0.1)	10915 (±215)
Serotonin	5-HT	36.7 (±0.1)	11026 (±1047)

* Retention times and peak areas were determined for 10 separate samples (10^{-5} M)

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TABLE 2
Detection Limits of the Compounds Under Study

Compound	Molecular Weight	Mass Limits (ng)
VMA	198.2	2.00
MHPG	227.3	0.45
DOPAC	168.1	0.34
HVA	191.2	0.38
5HIAA	182.2	0.36
NE	205.6	4.10
E	183.2	1.80
DA	189.6	1.90
5HT	212.7	2.10

TABLE 3
Concentrations of All the Compounds of Interest in Urinary Study

Compounds	Concentration (M) (Range for N* = 6)
VMA	$1.0 - 1.3 \times 10^{-5}$
MHPG	$3.3 - 5.5 \times 10^{-6}$
DOPAC	$9.4 - 9.5 \times 10^{-6}$
5HIAA	$2.5 - 3.2 \times 10^{-5}$
HVA	$1.0 - 1.3 \times 10^{-5}$
NE	$1.7 - 2.1 \times 10^{-7}$
E	$1.3 - 1.4 \times 10^{-7}$
DA	$8.2 - 9.4 \times 10^{-7}$
5HT	$1.4 - 1.7 \times 10^{-7}$

* Number of samples processed and chromatographed

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To obtain precise analyses, contaminants must be removed from the sample. In addition, sample preparation procedures must be carefully carried out to maintain the lifetime of the column and to resolve the VMA peak from an unknown peak X, which has a retention time of 3.3 minutes in the urine samples. If the sample preparation procedures were not carefully carried out, the unknown peak was very large and merged with the peak of VMA. Table 3 shows the concentrations of all the compounds of interest in the urine samples.

To determine the recovery of the nine compounds from the matrix, concentrations of 10^{-5} M for the five metabolites and 10^{-6} M for the biogenic amines were used. The recoveries were calculated on the basis of the percent difference in peak height before and after sample preparation procedures were carried out. It was previously reported that with ethyl acetate, recoveries of MHPG, 5HIAA and HVA in NaH_2PO_4-H_3PO_4 buffer solution (pH 4) were 46%, 47%, and 78%, respectively [32]. By using 0.15 M H_3PO_4 to adjust the pH to 4 instead of using NaH_2PO_4-H_3PO_4 buffer solution, recoveries of MHPG, 5HIAA, HVA, VMA and DOPAC (10^{-5} M) were 101%, 56%, 91%, 102% and 68%, and of DA, NE, E and 5HT (10^{-6} M) were 70%, 73%, 87%, and 85% respectively (Table 4).

Fig. 3 shows a chromatogram of a urine sample under the same conditions as the standards. Since concentrations of the metabolites in urine are much higher than those of the amines, different sensitivity was used for the metabolite group (section A, 200 nA full scale) than for the amine group (section B, 20 nA full scale).

TABLE 4
Extraction Recoveries for the Compounds Under Study

Compound	Peak height (Standard Mixture) (nA)	Peak height (Recovered) (nA)	Recovery (%)
	10^{-5} M Standard Mixture		
VMA	25.6	26.0	102
MHPG	24.0	24.2	101
DOPAC	24.7	16.9	68
HVA	19.2	17.5	91
5HIAA	31.5	17.6	56
	10^{-6} M Standard Mixture		
NE	2.7	1.9	70
E	2.6	1.9	73
DA	2.3	2.0	87
5HT	2.8	2.4	85

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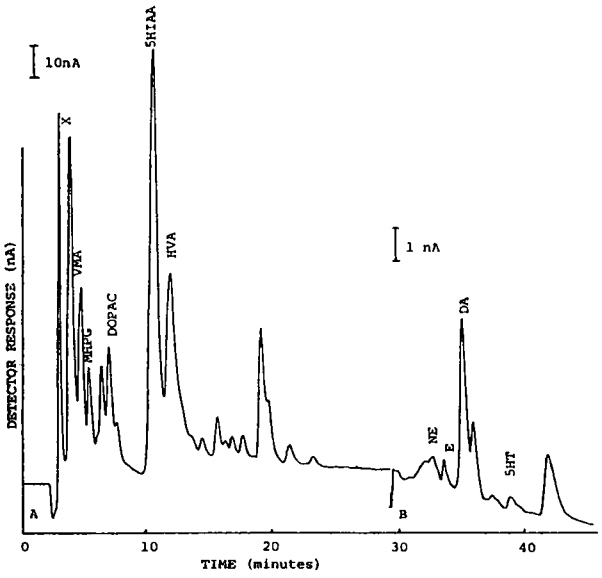

Figure 3: Chromatogram of 20 µl of a urine sample. Chromatographic conditions as described in Experimental. Applied potential of EC detector is 0.75 V. Sensitivity for section A is 200 nA f.s. and for section B 20 nA f.s..

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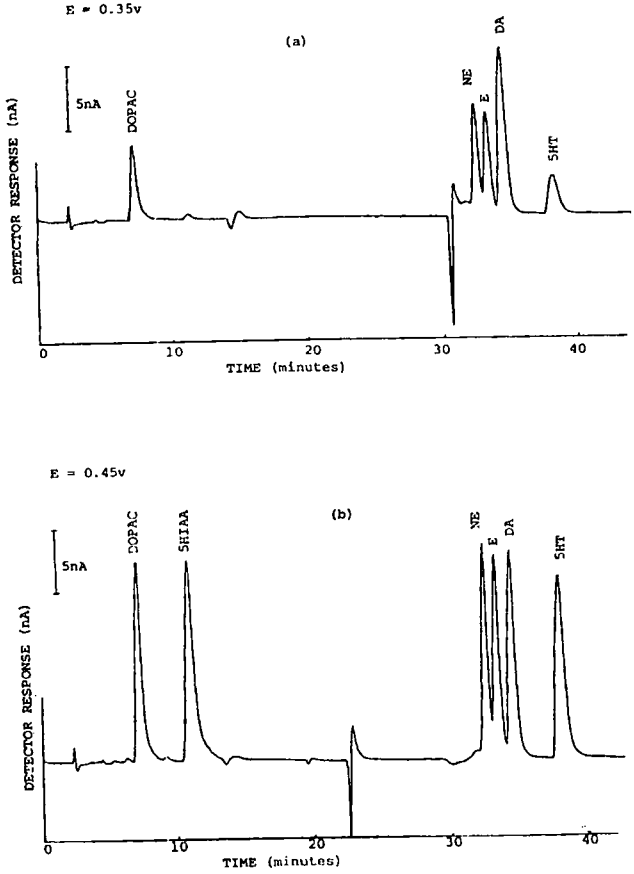

Figure 4: Chromatograms of 10^{-5} M standard mixture at different applied potentials. Chromatographic conditions as described in Experimental. Sensitivity is 50 nA f.s.. (a) 0.35 V; (b) 0.45 V; (c) 0.55 V; (d) 0.65 V; (e) 0.85 V; (f) 0.95 V.

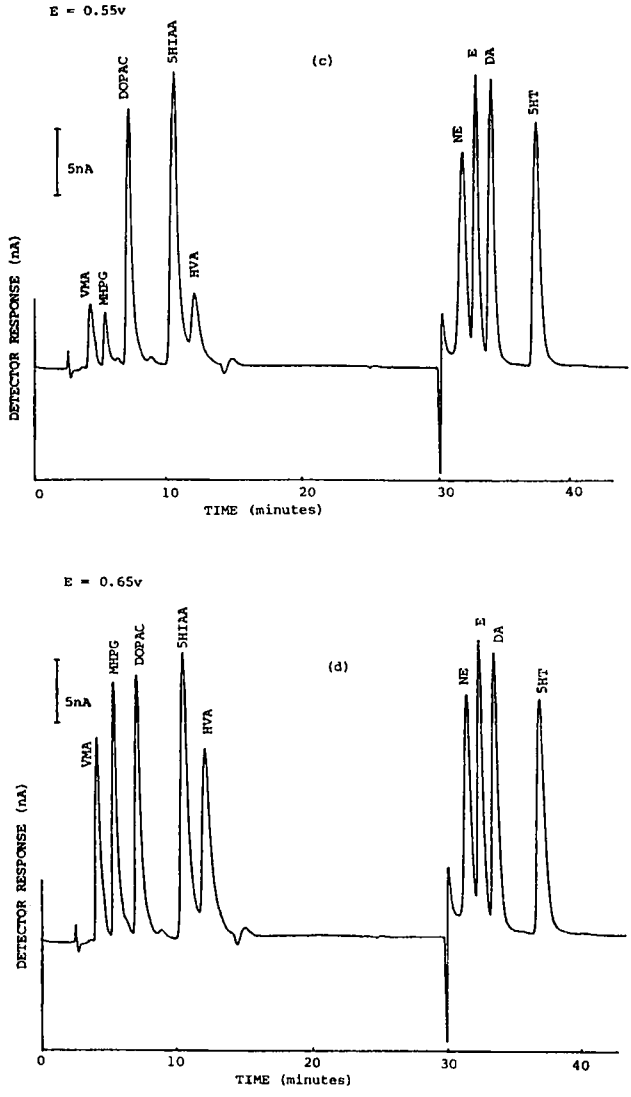

Figure 4C and 4D

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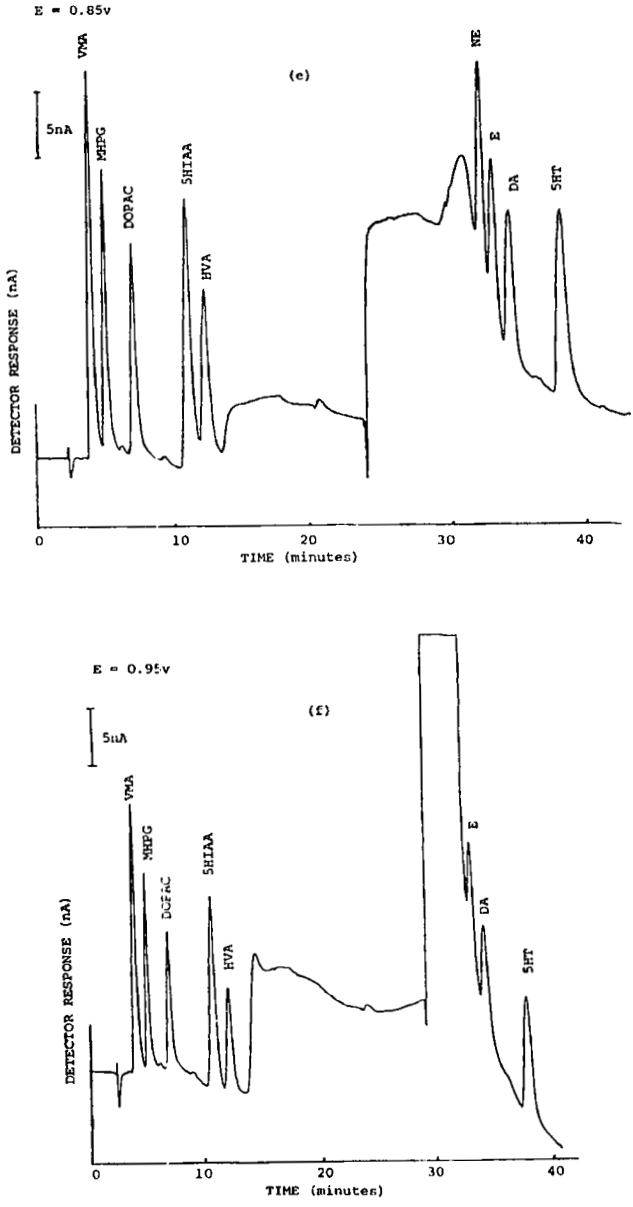

Figure 4E and 4F

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TABLE 5(a)
Peak Height H in nA of the Nine Compounds at Different Potentials E (V)*

E(V)	VMA	MHPG	DOPAC	5-HIAA	HVA	NE	E	DA	5HT
0.75	18.6	22.4	19.3	20.6	16.2	22.6	22.1	21.6	20
0.35	0	0	5.8	0	0	8.5	7.8	12.8	3.1
0.45	0	0	15.0	15.0	0	16.6	15.8	16.2	14.3
0.55	4.8	4.0	19.8	22.7	5.6	15.9	22.6	22.3	19.6
0.65	15.7	20.0	20.6	22.3	14.8	18.7	22.9	21.9	18.4
0.85	29.1	22.5	16.8	20.2	13.2	14.8	10.6	10.7	14.1
0.95	25.1	18.6	13.0	17.6	9.5	8.5	8.3		11.1

* Concentrations of each compound were 10^{-5} M.

TABLE 5(b)
Peak Height Ratios of Standard Compounds*

E (V)	VMA	MHPG	DOPAC	5-HIAA	HVA	NE	E	DA	5HT
0.35	0.00	0.00	0.30	0.00	0.00	0.38	0.35	0.59	0.16
0.45	0.00	0.00	0.78	0.73	0.00	0.73	0.71	0.75	0.72
0.55	0.26	0.19	1.03	1.10	0.35	0.70	1.02	1.03	0.98
0.65	0.84	0.89	1.07	1.08	0.91	0.83	1.04	1.01	0.92
0.85	1.56	1.00	0.87	0.98	0.81	0.65	0.48	0.50	0.71
0.95	1.35	0.83	0.67	0.85	0.59	0.38	0.38		0.56

*Ratios of peak heights at various voltages compared to peak heights at 0.75V, $R = H_{at\ x\ V}/H_{at\ 0.75\ V}$

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In previous work on polynuclear aromatic hydrocarbons using HPLC with an absorbance detector, peak identification was carried out by using different wavelengths [33]. With the EC detector, the standard mixture was chromatographed at different potentials instead of at different wavelengths (Fig. 4). Although the electrochemical characteristics of HVA have been investigated [34], the behavior of all the nine compounds of interest at potentials from 0.35 to 0.95 V has not been studied. Therefore, we investigated the electrochemical behavior of the biogenic amines and their metabolites, and used the peak height ratios at different potentials for peak identification. When the applied potential was 0.35 V, as compared to 0.75 V (Fig. 2), only DOPAC can be detected in the metabolite group and the peaks of all the amines decreased significantly (Fig. 4a). At 0.45 V (Fig. 4b), both DOPAC and 5HIAA can be detected in the metabolite group, and the peak height of 5HT increased. At 0.55 V (Fig.

TABLE 6
Comparison of Peak Height Ratios of Peaks in Urine Samples to Peak Height Ratios of Peaks in Standard Mixture

Compounds	Standard (RT* in min)	Urine Sample (RT)
VMA (0.35V/0.75V)	0.00 (4.3)	0.00 (4.4)
MHPG (0.35V/0.75V)	0.00 (5.5)	0.08 (5.5)
DOPAC (0.35V/0.75V)	0.30 (7.6)	0.31 (7.5)
5HIAA (0.35V/0.75V)	0.00 (7.6)	0.07 (7.6)
HVA (0.55V/0.75V)	0.35 (13.0)	0.43 (13.1)
NE (0.45V/0.75V)	0.73 (31.9)	0.70 (32.0)
E (0.35V/0.75V)	0.35 (32.8)	0.30 (33.0)
DA (0.35V/0.75V)	0.59 (33.8)	0.64 (34.0)
5HT (0.35V/0.75V)	0.16 (36.7)	0.21 (37.0)

*RT: Retention time

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4c), VMA, MHPG, HVA and NE can also be detected but their peak heights were much lower than those at potential 0.75 V. At 0.65 V (Fig. 4d), all peak heights were similar to those at potential 0.75 V. At 0.85 V and 0.95 V (Fig. 4e and f), the peak height of VMA increased compared with the peak at potential 0.75 V. However, potentials of 0.85 V and 0.95 V are not suitable for detecting compounds containing an amine group because there was a large baseline shift with mobile phase B. Therefore 0.75 V and 0.65 V were the best applied potentials for detecting all the compounds of interest. Table 5 (a and b) shows peak height data of the nine compounds at different potentials and the ratios of the peak heights at a different potential compared to the peak heights at 0.75 V. As can be seen from this table, it is possible to distinguish peaks of similar retention times by using peak height ratios at different potentials, e. g., 5HIAA which has a retention time at 11.6 has a ratio 1.10 at $H_{0.55V}/H_{0.75V}$ while the adjacent peak HVA at 13.0 minutes has a ratio of 0.35. Thus, peak height ratios in conjunction with retention times can be used in the identification of peaks. This method of peak identification was applied to urine samples, and the ratios of the peaks in the urine samples were compared to those of the standards (Table 6).

CONCLUSIONS

The RPLC-EC method described was a sensitive technique to determine concomitantly the basic biogenic amines serotonin, dopamine, norepinephrine, epinephrine and their five acidic metabolites in human urine. The separation was achieved using ion-paring and a step gradient. The method has the advantage over the method of Taylor et al. in that all nine compounds can be determined in one assay. In addition it provides high resolution and reproducibility. Satisfactory recoveries of the compounds of interest were obtained with a combination of extraction procedures. For the identification of these compounds in physiological fluids, peak height ratios at different potentials can be used. Although the analysis time is about 40 minutes, only one analysis is required to determine simultaneously the concentrations of these biogenic amines and their acidic metabolites. Therefore the method is suited for the determination and identification of the nine compounds in biological samples for investigations of mental illness and for studies of other diseases when it is desirable to monitor the concentrations of all nine compounds in one sample.

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