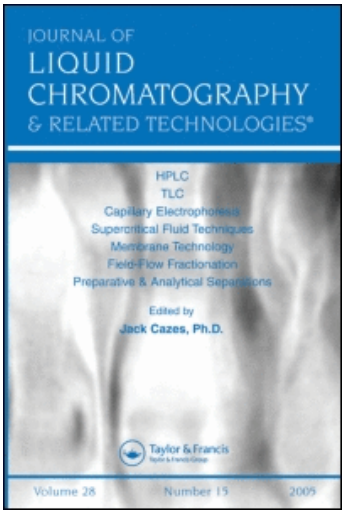

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Separation of Twelve Catecholamine Metabolites and Determination of Some of the Metabolites in Human Urine by Liquid Chromatography with Electrochemical Detection

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SEPARATION OF TWELVE CATECHOLAMINE METABOLITES AND DETERMINATION OF SOME OF THE METABOLITES IN HUMAN URINE BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

Separation of twelve catecholamine metabolites on a reversed-phase column was achieved, when eluate from the column was amperometrically monitored at +800 mV vs. Ag/AgCl. The mobile phase was a 0.1 M acetate buffer (pH 5.0). The flow rate and column temperature were 1.5 ml/min and 25 ± 1°C, respectively. Ethyl acetate extraction of the catecholamine metabolites from acidified human urine was studied and optimized. We

demonstrate the feasibility of the method showing some liquid chromatographic results.

INTRODUCTION

It has generally been recognized that the easily oxidizable and/or reducible compounds play various important roles in living bodies. Therefore, mumerous methods for the determination of those redox compounds in biological samples have been developed. The coupling of liquid chromatography (LC) with electrochemical detection (ED) for the determination of biogenic amines via anodic oxidation was first accomplished by Kissinger et al. [1]. This initial work demonstrated detection limits of approximately 10 pg of catecholamines (CA's). Since then tremendous publications have been appeared detailing many modifications and applications of the LCED technique. Recent advances in electrochemical detectors make them logical choice for easily oxidizable substances [2].

CA's and their metabolites are electroactive compounds of biological and clinical interests. Recently, Hadfield et al. have developed a method for the determination of nine CA metabolites during a single LCEC chromatogram [3]. In a previous report [4], we described an attempt at the diagnosis of neuroblastoma by reversed-phase LCEC determination of urinary CA metabolites such as 4-hydroxy-3-methoxymandelic acid (VMA) and 4-hydroxy-3-methoxyphenylacetic acid (HVA). In addition, we showed the chromatograms of ethyl acetate extracts from urine samples of a patient with neuroblastoma before and after operation with and without postoperative Endoxan and Vincristine treatment [4]. We also found [4] that many unidentified chromatographic peaks were diminished after the operation. The

unidentified peak components in the urine are potentially useful as biological markers for diagnosis of neuroblastoma. Therefore, we aimed to identify those components.

In this paper, we have re-examined the extraction procedure for CA metabolites from human urine and developed a method for the quantitative determination of them by LCEC. In addition, we have identified some of the previously unknown components.

MATERIALS AND METHODS

All the experiments were conducted at 25 ± 1°C, unless otherwise stated.

Separation of twelve CA metabolites on a reversed-phase column was examined. The column was a 150 mm x 4.6 mm I.D. stainless steel tube packed with Chemcosorb 5-ODS-H (particle size, 5 μm; Chemco Sci., Ltd., Osaka, Japan). Eluate from the column was monitored by aid of an electrochemical detector (Model ECP-1, Kotaki Co., Ltd., Funabashi, Chiba, Japan), set at +800 mV *vs.* Ag/AgCl. A constant flow pump (Model 655, Hitachi, Tokyo, Japan) were used with a 0.1 M acetate buffer (CH_3COOH-CH_3COONa, pH 5.0) at a flow rate of 1.5 ml/min. The column temperature was 25 ± 1°C.

3-Hydroxy-4-methoxymandelic acid (isoVMA), 4-hydroxy-3-methoxyphenyllactic acid (VLA), 3,4-dihydroxyphenylglycol (DHPG), 4-hydroxy-3-methoxyphenylglycol (MHPG), normetanephrine (NM), and 3-methoxytyramine (3-MT) were purchased from Sigma, St. Louis, MO, U.S.A. and VMA, HVA, vanillic acid (VA), and metanephrine (M) were obtained from Nakarai Chemical Co., Ltd., Tokyo, Japan. 3,4-Dihydroxyphenylacetic acid (DOPAC) and 3,4-dihydroxymandelic acid (DOMA) were supplied by Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A. and Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan,

respectively. Any other chemicals used in this study were supplied by Wako Pure Chemicals Ind., Ltd., Osaka, Japan. All the stock solutions were prepared in 0.1 N hydrochloric acid. All the buffers and aqueous solutions were prepared with glass-distilled deionized water.

Human urine samples were obtained from healthy male volunteers and a patient with neuroblastoma before and after surgery. The urine samples from the patient were provided by Dr. Toshiharu Tokoro (The Jikei University School of Medicine, Tokyo, Japan). Ethyl acetate (EA) was used to extract HVA and VMA from human urine. A flow diagram of the extraction procedure is illustrated in Fig. 1. An aliquot (0.5 ml) of urine (or an artificially prepared test solution) was mixed with 0.5 ml of 1 M Walpole buffer (CH_3COONa-HCl, pH 0.65) containing 4 M sodium chloride to acidify the urine and 5 ml of EA was added to the acidified urine. The mixture was vigorously shaken on a thermomixer for 1 min and was then centrifuged at 2,000 g for 5 min. The upper layer (Layer I) was found to contain CA metabolites. The LCEC examinations, however, revealed that the lower layer (Layer II) also contained significant amounts of urinary CA metabolits. The Layer II afforded another extraction of CA metabolites with EA. The Layer II was mixed with 5 ml of EA on a thermomixer, and once more centrifuged at 2,000 g for 5 min. The lower layer (Layer IV) was discarded. The upper layer (Layer III) was mixed with the Layer I and the mixture was vacuum-evaporated to dryness. The resultant thus obtained was dissolved in 0.5 ml of 0.1 N hydrochloric acid and an aliquot (10 µl) of the resulting acidic solution (EA-extract) was injected onto the column. The recoveries of twelve CA metabolites from 0.5 ml of an artificially prepared test solution with EA were estimated to the same procedure illustrated in Fig. 1.

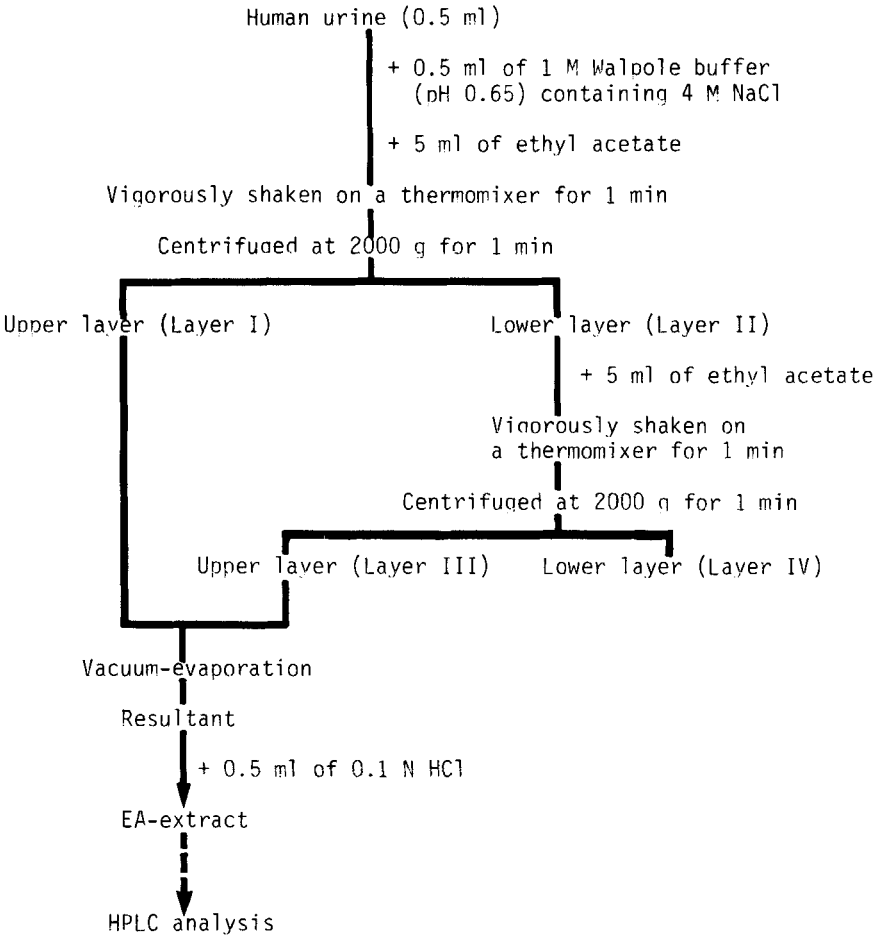

Fig. 1 Flow sheet of ethyl acetate extraction procedure for catecholamine metabolites.

Tentative identification of the chromatographic peaks was performed on the basis of retention time and co-chromatography with the reference compounds.

RESULTS AND DISCUSSION

Figure 2 shows a typical reversed-phase high-performance liquid chromatogram obtained by injecting an

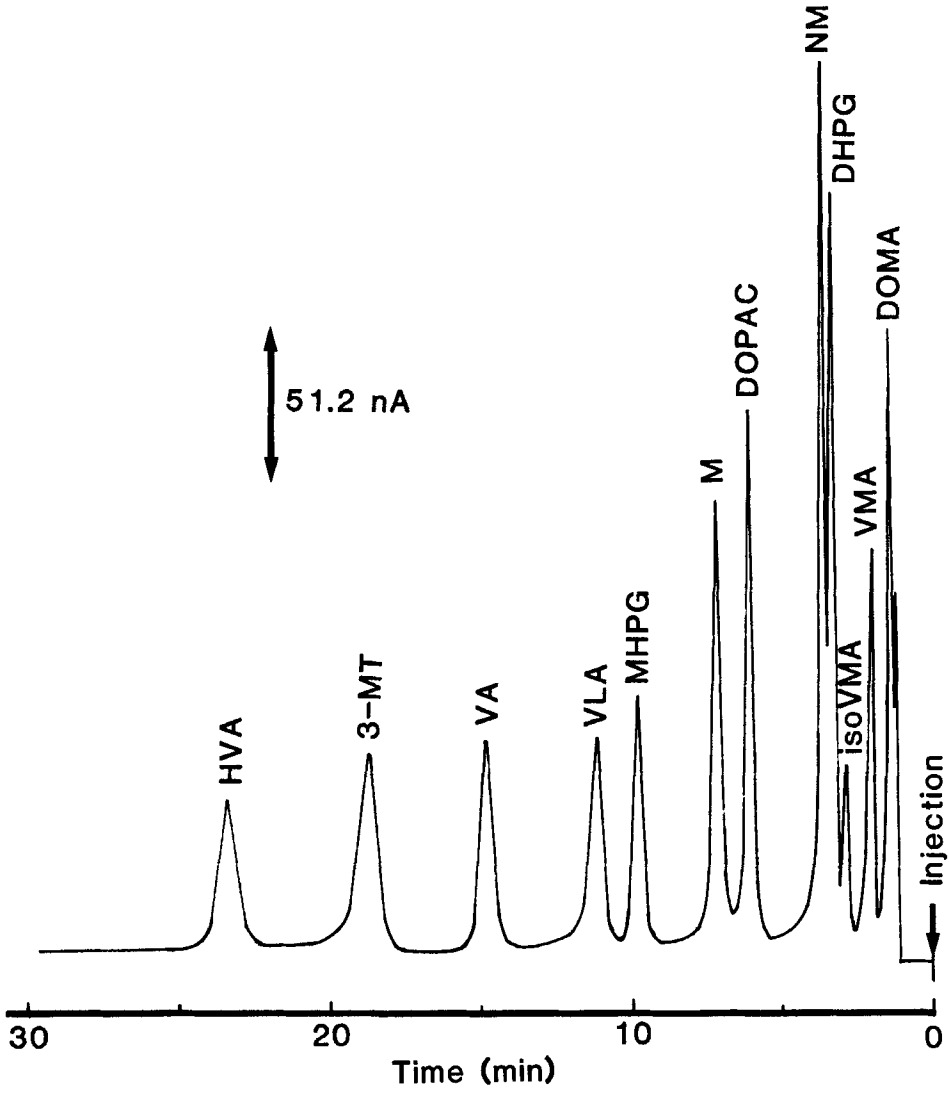

Fig. 2 A typical chromatogram of twelve catecholamine metabolites test solution containing 50 ng of each compounds. Chromatographic conditions: column, Chemcosorb-5-ODS-H (150 mm x 4.6 mm I.D.); eluent, 0.1 M acetate buffer (CH_3COOH-CH_3COONa, pH 5.0); flow rate, 1.5 ml/min; column temperature, 25 $\pm 1^{\circ}$ C; glassy carbon electrode set at +800 mV vs. Ag/AgCl.

aliquot (10 µl) of an artificially prepared test solution containing 5 µg/ml of each CA metabolite into the LCEC system under the present chromatographic conditions. Figure 3 shows the hydrodynamic voltammograms for twelve CA metabolites obtained using the present chromatographic system by repeated injections of 50 ng of each CA metabolite at different electrochemical detector potentials. When eluate from the column was monitored by aid of the electrochemical detector set at +500 mV vs. Ag/AgCl, the peaks of DHPG, DOPAC, and DOMA were appeared and any other CA metabolites contained in the test solution could not be oxidized to form their respective chromatographic peaks in a chromatogram. When the detector was set at +800 mV vs. Ag/AgCl, twelve CA metabolites could be detected in the same test solution as used in Fig. 2. Therefore, the detector was always set at +800 mV vs. Ag/AgCl throughout this study, unless otherwise stated. Triplicate injections gave standard deviations of peak height and retention times of 0.5% and 1%, respectively. More than 500 time injections of the different artificially prepared test solutions did not diminished the efficiency of the separation of CA metabolites. When the detector sensitivity was lowered, the working electrode was re-polished according the procedure previously described [6]. In Fig. 2, separation among the peaks of isoVMA, DHPG, and NM seems to be not perfect. As will be described later, the liquid chromatograms examinations revealed that the EA-extract did not contain NM. We have found (unpublished result) that human urine samples do not contain isoVMA. It was shown by perfusion experiments on isolated rat livers [7] that DOMA can be methylated not only to VMA but, to lesser extent, to isoVMA as well. Therefore, NM and isoVMA do not interfere with the quantitative determination of DHPG in human urine samples.

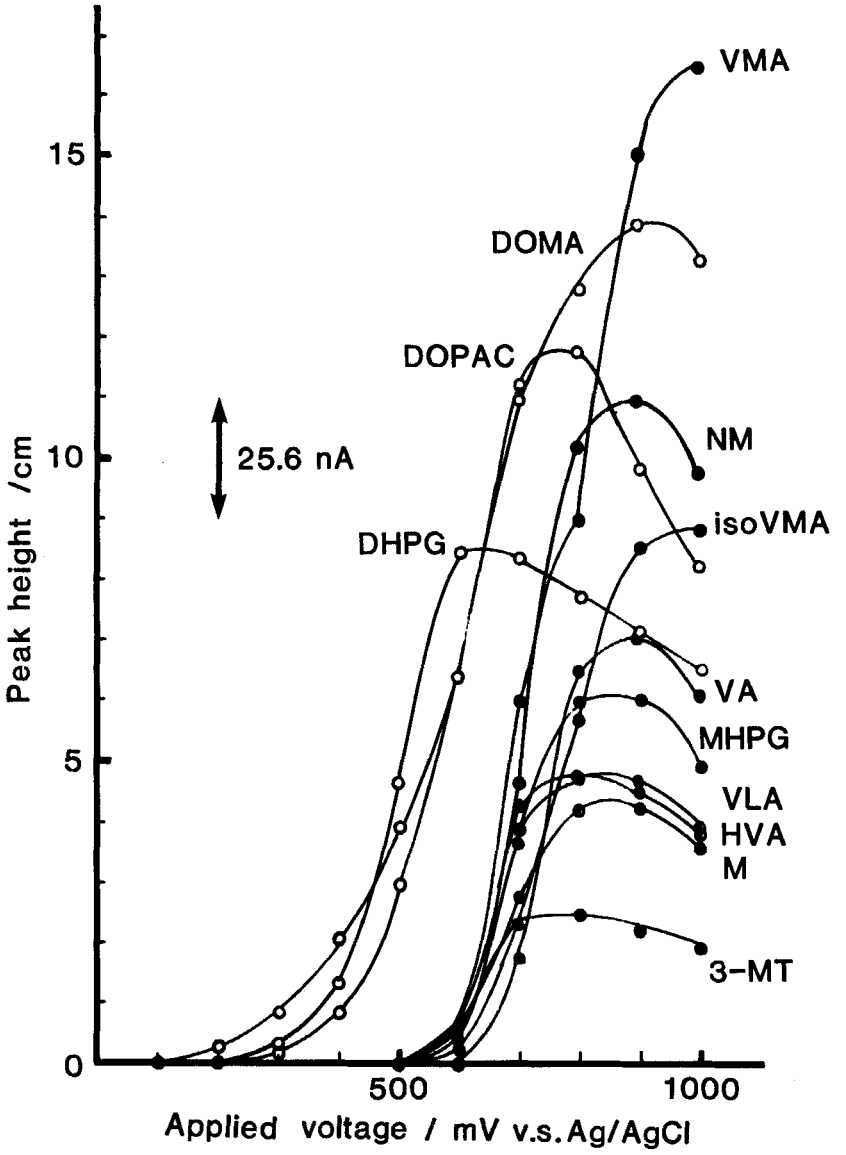

Fig. 3 Hydrodynamic voltammograms for twelve catecholamine metabolites (50 ng) in 0.1 M acetate buffer pH 5.0 at a flow rate of 1.5 ml/min.

Figure 4 is the calibration graphs of peak height against amounts of the twelve CA metabolites. The graph is linear in three plots beyond the concentration range of concern in the present study. The low limit was found to be lower than 500 pg for each compound.

There appeared numerous unidentified peaks which interfered with the quantitative determination of urinary CA metabolites under the LCEC conditions. Levels of HVA and VMA in human urine have been regarded in the diagnosis in the treatment of neuroblastoma [8,9]. Therefore, we aimed to look for an appropriate procedure for extracting, pre-concentrating and preliminary purifying urinary CA metabolites with good recoveries as well as with high selectivity.

After a comparative study [4], it has been found that extraction of HVA and VMA with EA from human urine samples is one of the best procedures. The recoveries of twelve CA metabolites from the test solution are tabulated in Table 1. As seen in the table, the highest recovery after the first extraction was obtained for HVA. NM and 3-MT, however, could not be transfered from the test solution to the Layer I. After the twice extractions of the CA metabolites, the recoveries of DOMA, VMA, isoVMA, DOPAC, VLA, VA, and HVA were estimated to be greater than 90%.

Fig. 5 shows the partition constant of each CA metabolite estimated between EA and the test solutions, with various acidic pH values and various concentrations. It has generally been recognized that CA metabolites tend to decompose at pH higher than 6.0. In addition, the increase of pH introduced the decrease of the partition constants of HVA, VA, VLA, VMA, and isoVMA. We, therefore, did not make the partition study at higher pH than 5.0 As seen in the figure, the partition constants of NM and 3-MT were found to be zero. The

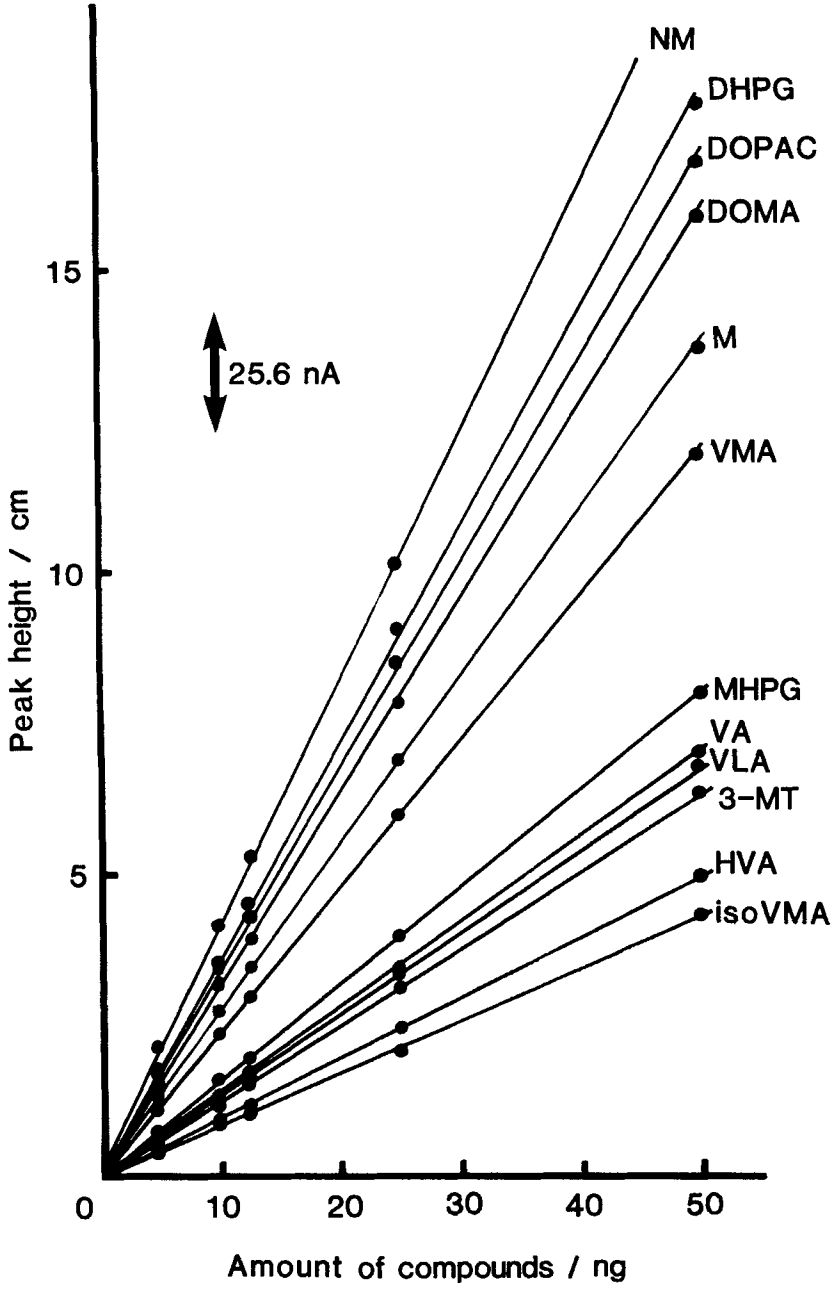

Fig. 4 Calibration graph of peak height against amounts of twelve catecholamine metabolites. Chromatographic conditions were the same as in Fig. 2.

TABLE 1

Percent Recovery of Catecholamine Metabolites from Test Solution with Ethyl Acetate

	RECOVERY			
	V=2 ml x 1	V=2 ml x 2	V=5 ml x 1	V=5 ml x 2
DOMA	75.8	94.1	88.8	98.8
VMA	69.7	90.8	85.4	97.9
isoVMA	69.2	90.5	85.1	97.9
DHPG	13.8	25.7	29.1	49.7
NM	-	-	-	-
DOPAC	86.3	98.1	94.2	99.7
M	0.90	1.97	2.90	5.74
MHPG	30.6	51.8	52.8	77.8
VLA	81.0	96.4	91.5	99.3
VA	88.4	98.6	95.1	99.8
3-MT	-	-	-	-
HVA	89.6	98.9	95.6	99.8

partition constants of M, MHPG, and DHPG were not great compared with those of HVA, VA, DOPAC, and VLA. Repeated extraction also increased the recovered amounts of them, although much time was consumed for EA evaporation..

Extraction of the CA metabolites from urine was also studied and good similar results to the aqueous standards.

As described above, we have optimized the procedure for extracting twelve CA metabolites from human urine sample. We, therefore, applied the method to the urine samples obtained from a patient with neuroblastoma

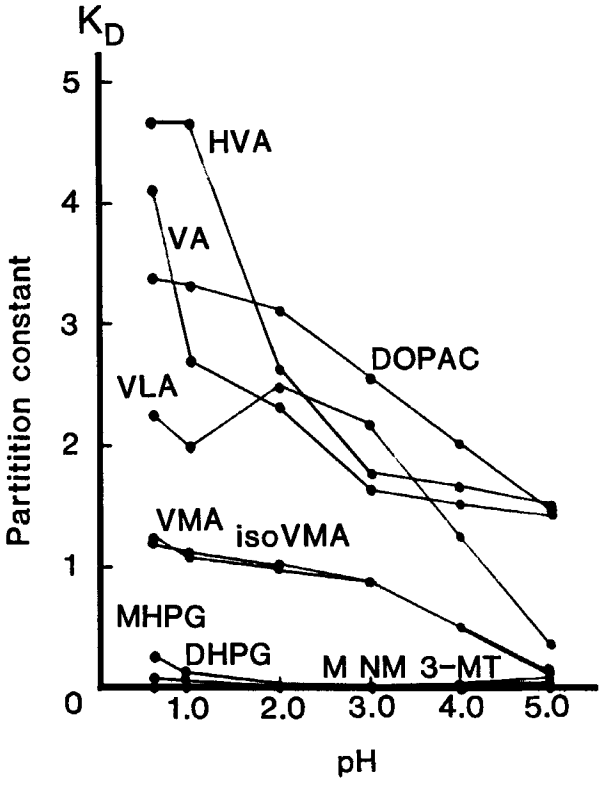

Fig. 5 Partition constants of twelve cetecholamine metabolites between ethyl acetate and acidic solutions (5 μg/ml).

before and after surgery. Fig. 6 shows the chromatogarm obtained by injecting an aliquot(2.5 μl) of the EA-extract prepared from the patient before surgery. As seen in the figure, the peaks of DOMA, VMA, DOPAC, VLA, VA, and HVA were identified. The amounts of those CA metabolites in the urine samples were estimated and tabulated in Table 2. As showed in the table, the levels of VLA and HVA were higher before the surgery than those after it, whereas the levels of DOMA and VMA did not

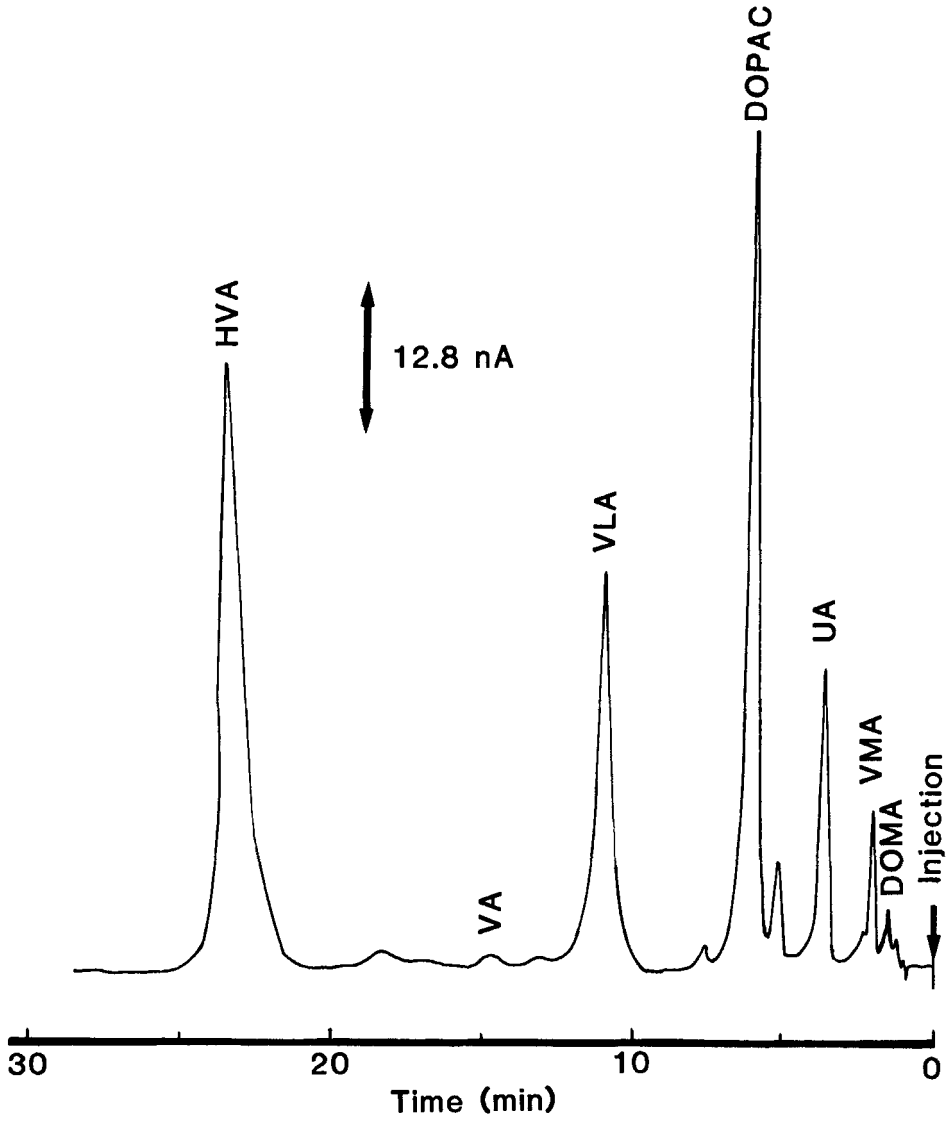

Fig. 6 Chromatogram of EA-extract from a patient with neuroblastoma before surgery. Chromatographic conditions were the same as in Fig. 2.

TABLE 2

Catecholamine Metabolites from a Patient with Neuroblastoma before and after Surgery

	Before Surgery	After Surgery
DOMA	1.09	1.09
VMA	4.89	4.71
DOPAC	23.7	0.68
VLA	31.1	10.6
VA	0.31	0.77
HVA	48.5	8.71

unit: μg/ml urine

significantly changed before and after the surgery. Fig. 7 shows a simplified model of CA metabolism in man. HVA, which is synthesized from dopamine (DM) and DOPA as the starting materials, is one of the end-product of the CA metabolism. VMA is synthesized from norepinephrine (NE) and epinephrine (E) and is also another end-product of the CA catabolism. As described above, the levels of DOPAC, VLA, and HVA in the urine sample before surgery were found to be significantly high compared with that after it. As shown in Fig. 7, VLA and DOPAC can be synthesized from DOPA and DM, respectively, to be degraded to HVA. DOMA, whose levels in the urine from the patient before and after surgery were found not to be different from each other, can be synthesized from NE and E to form VMA. Our observations suggest that disorder(s) might be introduced in the metabolic pathways of DM and/or DOPA in the patient.

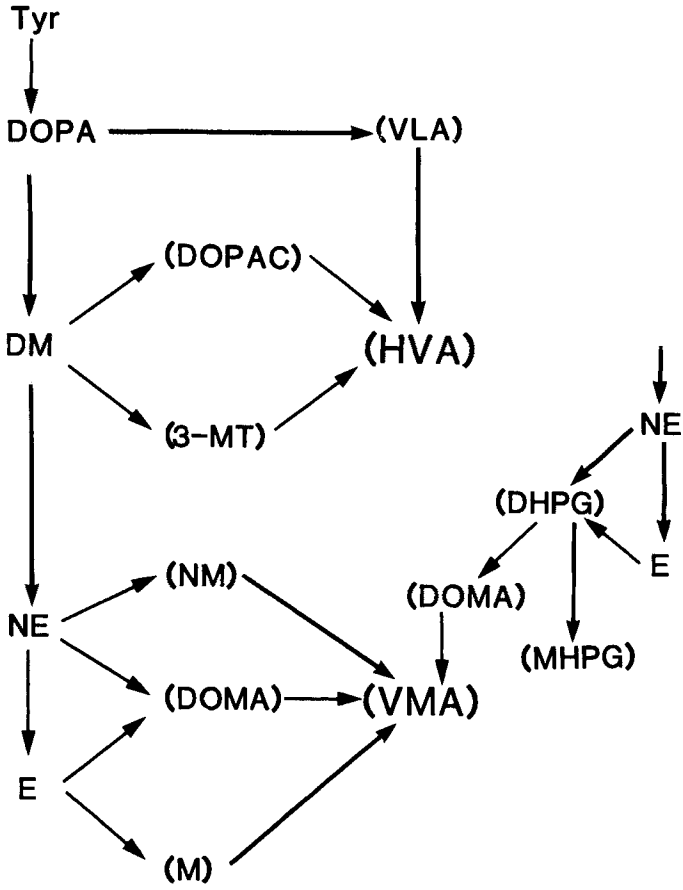

Fig. 7 A simplified model of catecholamine metabolism. Abbrevitions, see the text.

Measurement of VMA was advocated in the detection of nueroblastoma [10-12]. But high levels of urinary VMA were excreted in only 75% of patients with this disease [13]. When urinary HVA measurements along with VMA tests have been performed, approximately 95% of the patients with this tumor would be detected [13]. The patient with

neuroblastoma, whose urine samples were examined in this study, also did not show any significant change of VMA levels in his urine samples before and after surgery, although the levels of HVA were found to be different. Our finding also supports that the determination of urinary both HVA and VMA is essentially necessary in the detection of neuroblastoma. In addition, as demonstrated in this study, the determination of some other CA metabolites in urine seems to be helpful at early diagnosis of the tumor.

Reversed-phase LCEC combines speed, efficiency and high sensitivity and has been perfectly used as a diagnostic tool for neuroblastoma [14-16]. Since the discovery of the metabolic pathways of CA's [17], the determination of their urinary acidic and alcoholic catabolites has become an invaluable diagnostic aid for detection of CA-secreting tumors such as pheochromocytoma, neuroblastoma, and ganglioneuroma. The LCEC method developed in this study also seems to be a powerful tool for the detection of neuroblastoma as well as for understanding of the disorder(s) of CA metabolism in patients with the tumors. As pointed out by Newsome [18], there is a reason to doubt that the CA excretion in patients with pheochromocytoma is due to adrenergic neural stimulation of the tumor and consequent release of CA's. We, therefore, aimed to determine CA metabolites in the urine obtained from a patient with pheochromocytoma by the LCEC method coupled with the so-called alumina treatment procedure originally developed by Anton and Sayre [19]. We, however, could not carried out the determination of CA's and their metabolites during a single chromatogram [20]. It has been found [21] that when a stainless-steel tube (150 mm x 4.5 mm I.D.) packed with #3057 (particle size, 3 um; Hitachi, Tokyo, Japan), which is fitted with Fine

SIL C_{18} (particle size, 5 μm; Jasco, Tokyo, Japan) and the mobile phase is a 0.2 M phosphate buffer (KH_2PO_4-H_3PO_4, pH 2.0), the determination of NE, E, DM, ascorbic acid, uric acid, DOMA, DHPG, VMA, and DOPAC in human urine can be performed during a single chromatogram. Therefore, the LCEC method recently developed [20,21] is a powerful tool for a better understanding of disorder(s) of CA metabolic pathways in relation to some diseases.

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