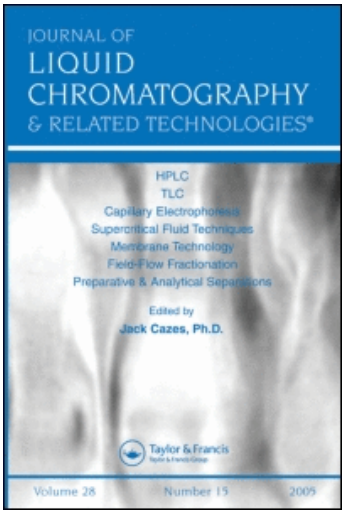

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LIQUID CHROMATOGRAPHIC IDENTIFICATI-FICATION OF URINARY CATHECHOLAMINE METABOLITES ADSORBED ON ALUMINA

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ABSTRACT

Human urinary catecholamine metabolites adsorbed on alumina was identified by high-performance liquid chromatography with electrochemical detection. Since we found that some catecholamine metabolites interfered with the quantitative determination of other catecholamines such as dopamine, norepinephrine, and epinephrine in human urine samples by the liquid chromatographic method combined with the so-called alumina pretreatment procedure. It was found that alumina could adsorb urinary unidentified electroactive components as well as catecholamines. Some of the unidentified components were found to be uric acid, ascorbic acid, 3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylglycol, 3-methoxy-4-hydroxymandelic acid, and 3,4-dihydroxyphenylacetic acid. On the basis of the above information, we have developed a method for the determination of catecholamines in human urine samples by high-performance liquid chromatography with electrochemical detection.

INTRODUCTION

Recent progress in high-performance liquid chromatography (HPLC) with electrochemical detection (ED) has allowed us to determine physiological catecholamines (CA's) with high sensitivity as well as with good selectivity (1,2). A commmon method for the extraction, preconcentration and preliminary purification of CA's in biological samples such as body fluids and mammalian tissues is based on the alumina procedure developed by Anton ans Sayre (3). If a constant recovery of each CA can be obtained after the alumina pretreatment of biological samples, the quantitative determination of the biogenic amines can be achieved with good reproducibility. As pointed out by several investigators (4-6), the alumina pretreatment procedure has disadvantages. One of them is that alumina is difficult to standardize. Thus, no two batches can be relied upon to give the same performance. Moreover,adsorption and subsequent desorption of CA's to and from alumina require careful manipulation for yielding a constant recovery. Nevertheless, the alumina pretreatment procedure has been widely used as a routine method because it is easy to perform in a short period of time and has relatively high recoveries of CA's from phsiologic materials. Recently, we have re-examined and determined the optimum condition for extracting and preliminary purifying CA's from biological materials according to the so-called alumina procedure (7-9). During the course of the CA study, as described in our previous study (10), we have recognized that some unidentified peaks appeared in the chromatogram monitored by aid of an electrochemical detector tend to interfere with the quantitative determination of CA's in biological samples. Therefore, we aimed to identify the

unidentified peak components. It is known that the levels of ascorbic acid (AA) and uric acid (UA) in body fluids are extremely high compared to any other naturally occuring electroactive components such as CA's and recognized (10,11) that the AA and UA peaks which appear in a chromatogram monitored amperometrically tend to interfere with the quantitative determination of CA's in biological samples. Rossetti et al. (12) found that UA interfered in the determination of epinephrine (E). Mckay et al. (13) used AA oxidase to avoid the interference by the AA peak in determining norepinephrine (NE) in brain tissue.

On the basis of the above information, we have used an artificially prepared solution containing dopamine (DM), NE, E, and AA as a test solution in optimizing the chromatographic conditions for the determination of CA's by HPLC-ECD (10,11). In addition, we have developed a method for the determination of AA and UA in biological samples by HPLC-ECD (10, 11, 14-16).

In this paper, we have identified urinary electroactive components. In addition, we have developed a method for the determination of CA's in human urine by HPLC-ECD.

MATERIALS AND METHODS

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

3-Hydroxy-4-methoxymandelic acid (V'MA), 3-mthoxy-4-hydroxyphenylacetic acid (VLA), 3,4-dihydroxyphenylglycol (DHPG), 3-methoxy-4-hydroxyphenylglycol (MHPG), normeta nephrin (NM), and 3-mthoxytyramine (3-MT) were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.) and 3-methoxy-4-hydroxymandelic acid (VMA), homovanillic acid

(HVA), vanillic acid (VA), and metanephrine (M) were obtained from Nakarai Chemicals Ltd. (Tokyo, Japan). 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxymandelic acid (DOMA) were from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan), respectively. Alumina of analytical reagent grade (70-230 mesh ASTM, Merck & Co. Inc. (Darmstadt, W-Germany) was used without any further treatments. DM, NE, E, UA, AA, and any other chemicals were also purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). All the stock solutions were prepared in 0.1 M hydrochloric acid. All buffers and aqueous solutions were prepared with glass-distilled-deionized water.

A constant volume pump (Model 655, Hitachi, [Tokyo, Japan]) pushed mobile phase through a reversed-phase high-performance liquid chromatographic column system. In this study, two different column systems were used for the identification of the unidentified electroactive components adsorbed on alumina. Column system I was a stainless-steel tube (150 mm x 4.6 mm i.d.) packed with Chemcosorb 5-ODS-H (particle size; 5 μm, Chemco Science Co. Ltd.[Osaka, Japan]). Column system II was the combination of a 50 mm x 4.6 mm i.d. stainless-steel precolumn packed with Fine SIL C_{18} (particle size; 5 μm; Jasco [Tokyo, Japan]) and a 150 mm x 4.6 mm i.d. stainless-steel tube packed with #3057 octadecyl silica gel (particle size; 3 μm, Hitahi [Tokyo, Japan]). When column system I was used, the mobile phase was a 0.1 M acetate buffer (CH_3COOH-CH_3COONa, pH 5.0) and the flow rate was 1.5 ml/min. Column system I is useful for the complete separation of the above described CA metabolites (17). When column system II was employed, a 0.2 M phosphate buffer (KH_2PO_4-H_3PO_4, pH 2.0) was used as a developing solvent and the flow rate was 1.0 ml/min. Column system

II seems to be useful for the determination of CA's by HPLC-ECD (10). Eluate from each column system was monitored by aid of an electrochemical detector (Model; ECP-1, Kotaki [Funabashi, Chiba, Japan]) set at +800 mV vs. Ag/AgCl. In the electrochemical detector, a glassy carbon plate (GC-20, Tokai Carbon Co. Ltd. [Tokyo, Japan]) was used as a working electrode and electrochemical potential was always positive at the glassy carbon electrode (oxidation mode) relative to the Ag/AgCl. A stainless steel wire was used as a counter electrode. The glassy carbon electrode was hand-polished according to the procedure previously described (18). The column temperature was always 25 ± 1°C.

Human urine samples were obtained from healthy male volunteers and a patient with pheochromocytoma after surgery. The urine sample from the patient was provided by Dr. Masato Kumagai (The Jikei University School of Medicine, Tokyo, Japan). The alumina pretreatment procedure was conducted according to the method of Anton and Sayre (3) with some modifications. Th typical alumina procedure, which was established for this study after the re-examination, is represented in Fig. 1. An aliquot of the HCl layer was injected onto the column system for the identification of the unidentified peak components adsorbed on alumina as well as for the determination of CA's in human urine samples. The recoveries of NE, E, and DM after the alumina treatment were estimated to be 84.0 ± 1.0%, 60.4 ± 1.0%, and 60.6 ± 1.0%, respectively. The recovery of UA was found to be 20%. The recovery of AA did not show any constant value, probably due to the fact that the adsorption of AA on alumina is strongly dependent on the concentrations of the vitamin. When column system II was used for the determination of CA's, the minimum detectable quantity of each CA was found to be about 10 pg.

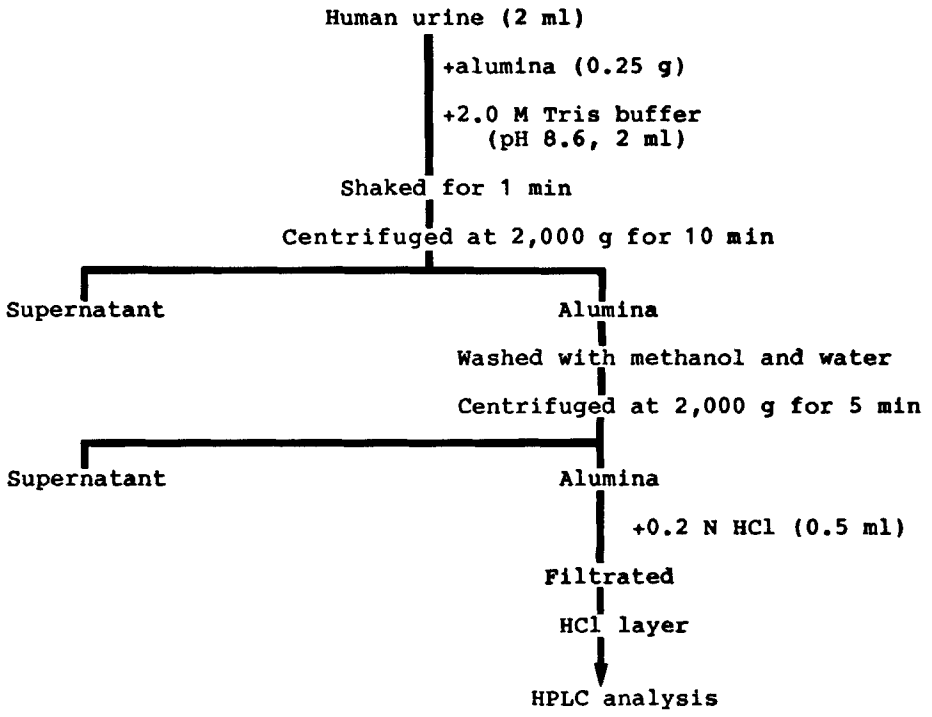

Figure 1. Flow sheet of the alumina treatment procedure for extraction, concentration and preliminary purification of catecholamines. Ten microliters of the HCl layer were injected onto a column. The alumina procedure was conducted at room temperature (25 $\pm$ 1°C), unless otherwise stated.

Tentative identification of the chromatographic peak components was performed on the basis of retention behavior and co-chromatography with the reference compounds.

RESULTS AND DISCUSSION

Recently, we have developed a method for the determination of CA metabolites in various biological

samples by HPLC-ECD (17). As an example, Fig. 2 shows a liquid chromatogram of an artificially prepared sample containing DOMA, VMA, V'MA, DHPG, NM, DOPAC, M, MHPG, VLA, VA, 3-MT, and HVA, when 10 μl of the test solution was injected onto the column system I. The mobile phase was the acetate buffer (pH 5.0) and the flow rate was 1.5 ml/min. Fig. 3 shows the typical chromatogram obtained by injecting 10 μl of the HCl layer (see Fig.1) prepared from the urine sample obtained from the patient with pheochromocytoma after operation onto column system I under the same chromatographic conditions as used in Fig. 2. As seen in FIg. 3, the peaks of VMA, DHPG, UA, and DOPAC were identified. This observation indicates that alumina can adsorb VMA, DHPG, UA, and DOPAC.

On the basis of the above information, we assumed that some peaks of CA metabolites might interfere with the quantitative determination of DM, NE, and E in human urine samples by HPLC-ECD. Fig. 4 shows a liquid chromatogram of the same HCl layer as used in Fig. 3, when 10 μl of the sample was injected onto the column system II. Fig. 5 shows a liquid chromatogram of a standard solution containing 250 ng/ml of each CA recorded under the same chromatographic conditions as used in Fig. 4. As seen in the figure, the peak of each CA was well separated and allowed easy determination of CA's in human urine samples. The amounts of NE, E, and DM in the urine obtained from the patient were estimated to be 391 ng/ml. 33.2 ng/ml and 212 ng/ml, respectively. As indicated in Fig. 4, some of the previously unidentified peak components have been identified as DOMA, DHPG, VMA, and DOPAC. The AA peak has been found between the paeks of NE and E. When the urine sample was stored for several days and aliquot of the sample was injected, the AA peak could not be observed. The peak X located between the peaks of DM and UA has not been identified. The peak X

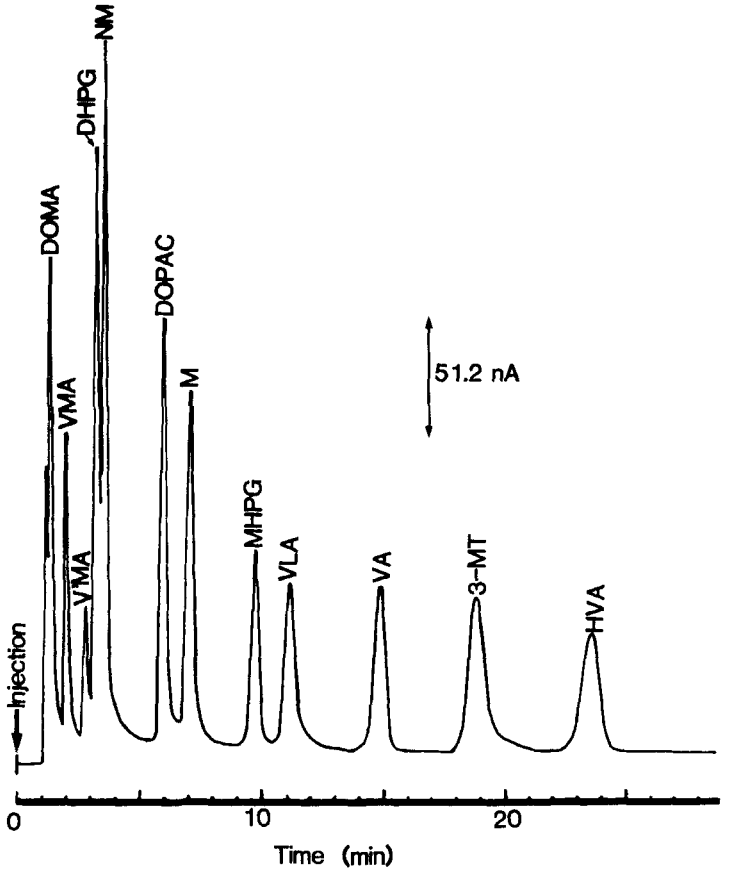

Figure 2. A revesed-phase high-performance liquid chromatogram obtained by injecting 10 µl of a test solution containing DOMA, VMA, V'MA, DHPG, NM, DOPAC, M, MHPG, VLA, VA, 3-MT, and HVA onto column system I. Column system I was a stainless-steel tube (150 mm x 4.6 mm i.d.) packed with Chemcosorb 5-ODS-H (particle size, 5 µm). 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. Abbreviations are defined in the text.

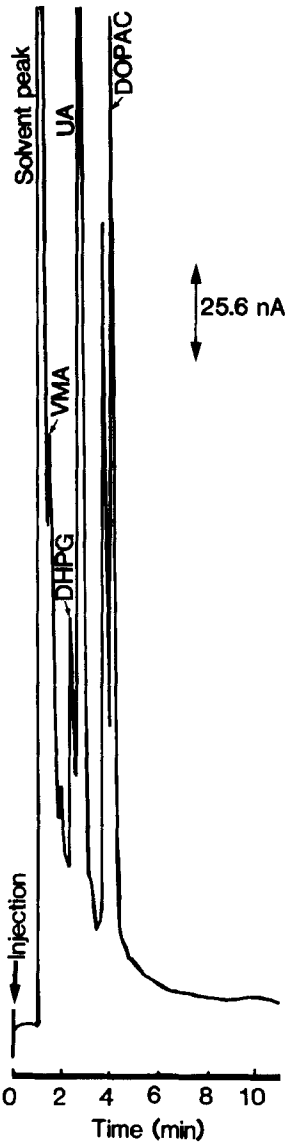

Figure 3. The typical chromatogram obtained by injecting 10 µl of the HCl layer (see Fig. 1) prepared from the urine sample, which was obtained from the patient with pheochromocytoma, under the same chromatographic conditions as used in Fig. 2.

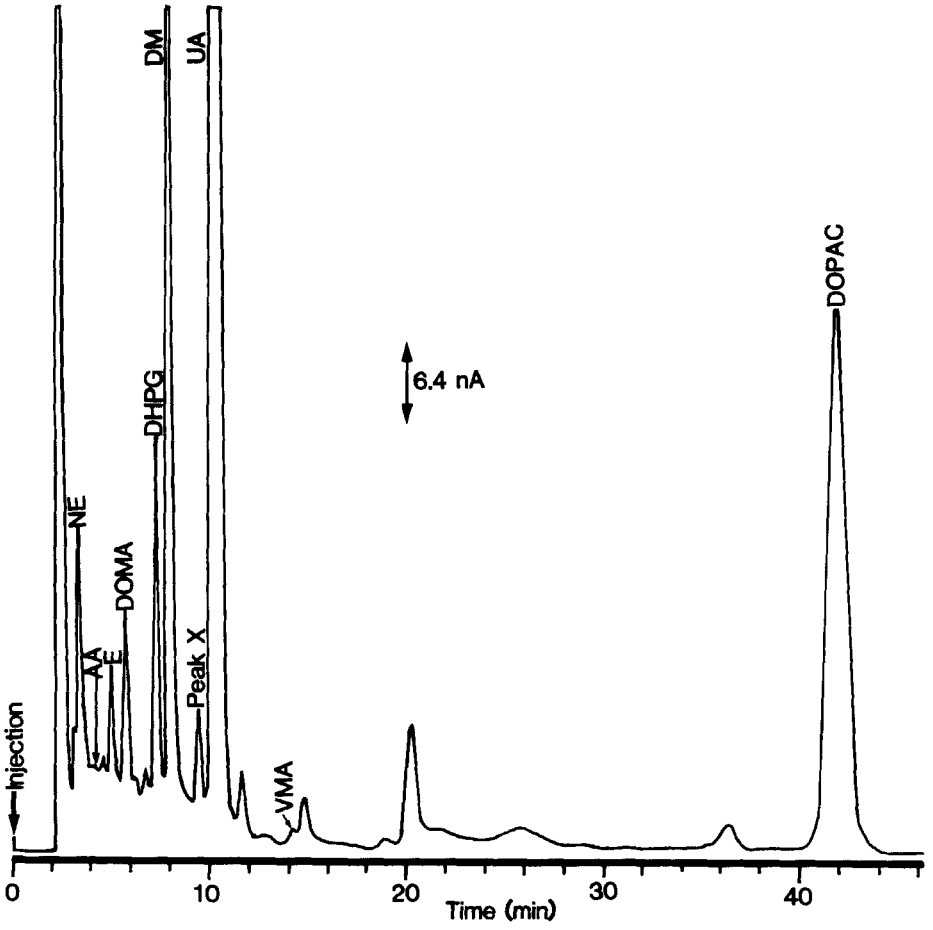

Figure 4. The typical chromatogram obtained by injecting 10 µl of the HCl layer preparation obtained from a healthy male volunteer onto column system II. The mobile phase was a 0.2 M phosphate buffer (pH 2.0). The flow rate and column temperature were 1.0 ml/min and 25 ± 1°C, respectively. For further expalnation of the chromatographic conditions, see the text.

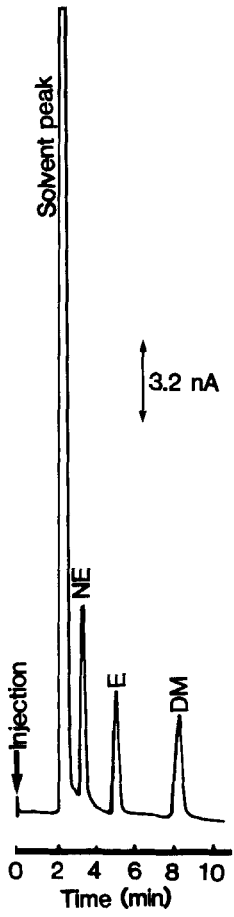

Figure 5. A reversed-phase liquid chromatogram of a test solution containing NE, E, and DM obtained under the same chromatographic conditions as used in Fig. 4.

component(s) may interfere with the quantitative determination of DM by HPLC-ECD, probably due to the so-called column-to-column and lot-to-lot variations. Therefore, we are now aiming to identify the peak X component(s).

Figure 6 shows a liquid chromatogram obtained by injecing 10 μl of the HCl layer prepared from a urine sample, which was obtained from a healthy male volunteer, onto column system II under the same chromatographic conditions as used in Figs. 4 and 5. As seen, the peaks of NE, AA, E, DOMA, DHPG, DM, UA, VMA, and DOPAC were identified and several unidentified peaks were observed between the peaks of solvent and UA. Those unidentified urinary electroactive peak components also seem to interfere with the quantitative determination of CA's. The amounts of NE, E, and DM in the urine sample obtained from the volunteer were estimated to be 47.8 ng/ml, 25.7 ng/ml, and 421 ng/ml, respectively.

As described above, we have demonstrated the fact that alumina can adsorb CA's as well as other urinary electroactive components such as UA, AA, DHPG, DOMA, VMA, and DOPAC. If the constant recoveries of UA, AA, DOMA, DHPG, VMA, and DOPAC had been able to be obtained after the alumina treatment of human urine samples, the quantitative determination of these compounds might be achieved with good reproducibility. In the cases of AA, DHPG, DOMA, VMA, and DOPAC, however, the constant recoveries have not been able to be obtained after the treatment. In the case of UA, the constant recovery has been obtained after the treatment, although it has not been high (about 20%) (19). Recently, we have determined the regional distribution of CA's and UA in pheochromocytoma excised from a patient with the tumor by the HPLC-ECD method coupled with the alumina treatment procedure (20). The chromatographic examinations have revealed (20)

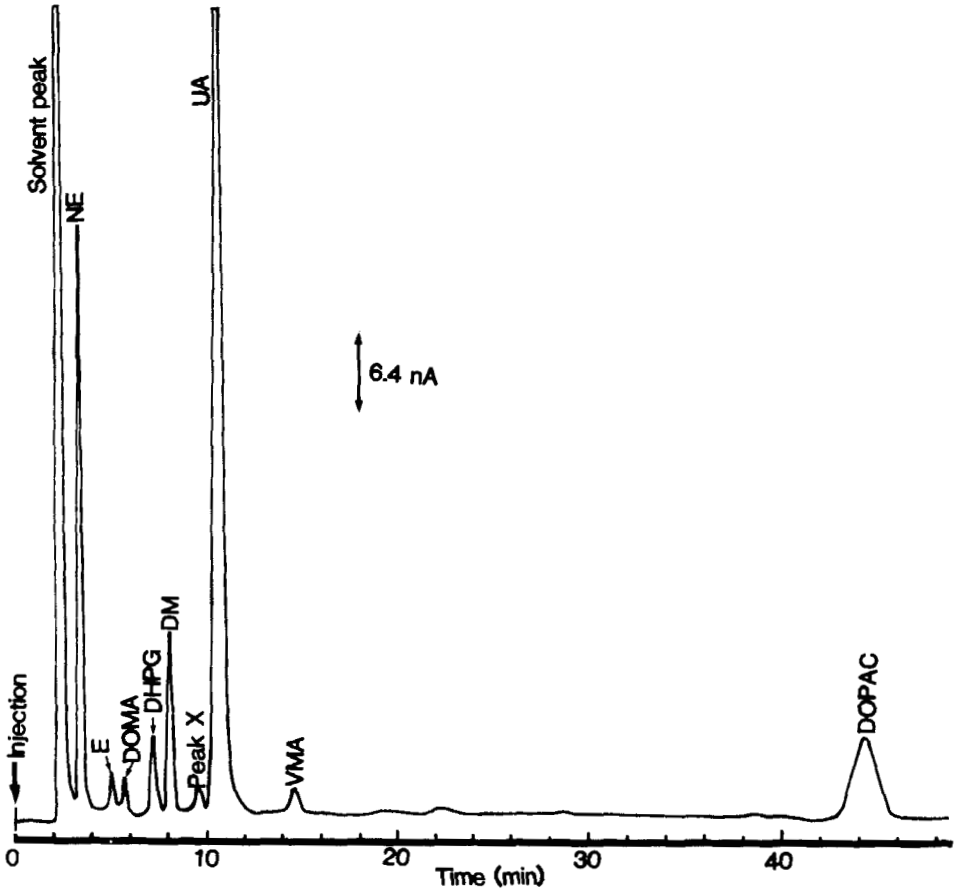

Figure 6. The typical reversed-phase liquid chromatogram of the same HCl preparation as used in Fig. 3. Ten microliters of the solution were injected onto the column system II under the same chromatographic conditions as used in Figs. 4 and 5. For further explanation, see the text.

that those, in which UA levels are high, also tend to contain high levels of E.

As a preliminary study, we estimated the analytical recoveries of CA metabolites after the alumina treatment. Two milliliter of a test solution containing 5 μg each CA metabolite was treated according to the same procedure as illustrated in Fig. 1. The recovery was determined by the same HPLC-ECD method employed in Figs. 2 and 3. The recoveries of DOMA, VMA, V'MA, DHPG, NM, DOPAC, M, MHPG, VLA, VA, 3-MT, and HVA were estimated to be 20.8%, 36.8%, 34.2%, 2.40%, 0.00%, 11.5%, 0.00%, 0.00%, 45.3%, 18.9%, 0.00%, and 13.1%, respectively, after the alumina treatment under the present experimental conditions. On the basis of the above observations, we discuss the estimated recoveries after the alumina treatment in relation with the chemical structures of the CA metabolites. As pointed out by Anton and Sayre (3), the ability of alumina to bind substances with two vicinal phenolic hydroxyl groups, i.e. catechol and catechol derivatives, from weakly alkaline solution has been of particular important. However, the adsorption of catechol compounds by alumina is not completely specific. In this study, we have found that the adsorption of CA's by alumina is shared by DOMA, DHPG, VMA, DOPAC, UA, AA, and any other unidentified electroactive compounds in the HCl layer preparations obtained from human urine samples. This finding seems to give us a hint for the development of a method for preliminary purification and concentration of the electroactive components adsorbed on alumina in biological samples. But it has also found that the adsorption of the CA metabolites in biological samples onto alumina is not always quantitative. If the constant recoveries can be always obtained, the quantitative determination of DOMA, DHPG, VMA, and DOPAC would be

achieved with good reproducibility by a one-time chromatographic method. As demonstrated above, it has been found that NM, M, and 3-MT cannot be adsorbed on alumina under the present experimental conditions, whereas NE, E, and DM can be efficiently adsorbed on it. Compared with their chemical structures, NE, E, and DM have a hydroxyl group beside NM, M, and 3-MT have a methoxy group in place of a hydroxyl group (see Fig. 7). Figure 7 represents the chemical structures of CA's and thier metabolites. DOPAC, DOMA, and DHPG have two vicinal phenolic hydroxyl groups in their respective chemical structures. In fact, DOPAC and DHPG are relatively well adsorbed on alumina. However, the recovery of DHPG after the alumina treatment has been found to be small compared with those of DOPAC and DOMA. As seen in Fig. 7, DOPAC and DOMA have a carboxyl group in their respective chemical structures, whereas DHPG have no carboxyl group. The carboxyl group can be ionized to form COO^- and H^+ in neutral and alkaline media and the CA metabolites having a carboxyl group in their chemical structures can be efficiently adsorbed on alumina. We assumed that the CA metabolites, which have the m-hydroxyl and carboxyl groups in their respective chemical structures can be adsorbed on alumina with high efficiencies. In fact, HVA, VA, VLA, and VMA, which have both functional groups in their chemical structures, have found to be well adsorbed on alumina. On the contrary, MHPG, NM, M, N, and 3-MT, which have neither a m-hydroxyl group nor a carboxyl group in their chemical structure, have been scarcely adsorbed on alumina showing that the recoveries of these CA metabolites are negligible. On the basis of the above information, we speculate that the CA metabolite, which have at least two negatively charged functional groups such as $-COO^-$ and $-O^-$ in alkaline media can be adsorbed

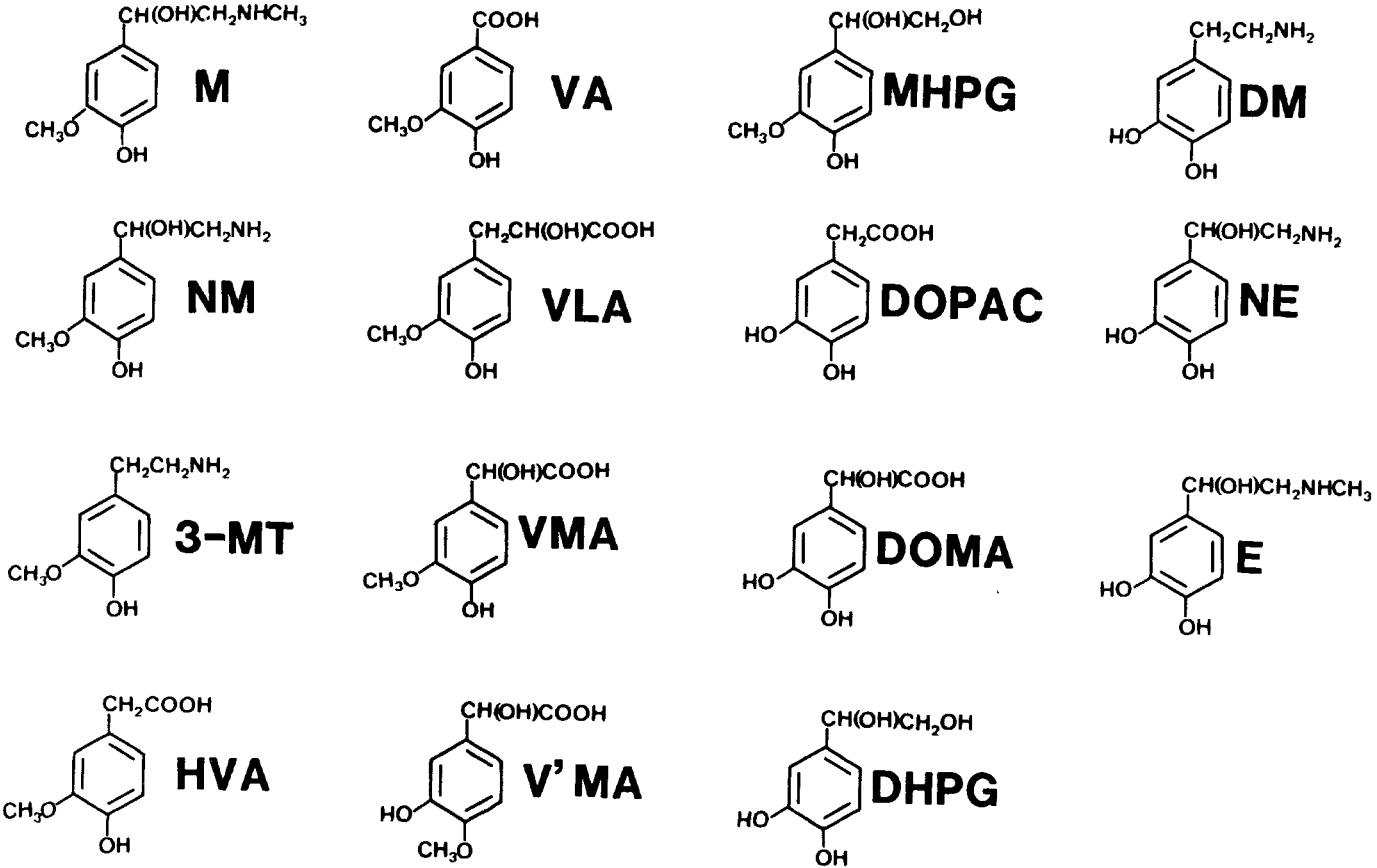

Figure 7. Chemical structures of catecholamines and their metabolites. Abbreviations are defined in the text.

on alumina. This speculation seems to be consistent with our experimental results on the recoveries of twelve CA metabolites after the alumina treatment demonstrated above.

As described above, DOPAC, DOMA, HVA, VA, VLA, VMA, and DHPG can be adsorbed on alumina in alkaline media. We are now aiming to optimaize the alumina treatment procedure for extracting, preliminary concentrating and purifying these CA metabolites in biological samples.

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