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MEASUREMENT OF CATECHOLAMINES, THEIR PRECURSOR AND METABOLITES IN HUMAN URINE AND PLASMA BY SOLID-PHASE EXTRACTION FOLLOWED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DERIVATIZATION

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SUMMARY

A high-performance liquid chromatographic method is described for the determination in human urine and plasma of catecholamines, their precursor and metabolites [amino compounds (norepinephrine, epinephrine, dopamine, normetanephrine, metanephrine, 3-methoxytyramine and L-DOPA), acidic compounds (3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylacetic acid, vanillylmandelic acid and homovanillic acid) and alcoholic compounds (3,4-dihydroxyphenylethyleneglycol and 4-hydroxy-3-methoxyphenylethyleneglycol)]. Urine (0.5 ml) containing 3,4-dihydroxybenzylamine and 4-hydroxy-3-methoxycinnamic acid (internal standards) is deproteinized with perchloric acid, and the resulting solution is fractionated by solid-phase extraction on a strong cation-exchange resin cartridge (Toyopak IC-SP S) into two fractions (amine fraction and acid–alcohol fraction), which include 3,4-dihydroxybenzylamine and 4-hydroxy-3-methoxycinnamic acid, respectively. Plasma (0.7 ml) is deproteinized in the presence of 3,4-dihydroxybenzylamine (internal standard) in the same manner, and the resulting solution is directly used as an acid–alcohol fraction, while an amine fraction is obtained as for urine. Each fraction is subjected to the previously established ion-pair reversed-phase chromatography with post-column derivatization involving coulometric oxidation followed by fluorescence reaction with 1,2-diphenylethylenediamine. The detection limits, at a signal-to-noise ratio of 5, of the compounds measured in urine are 300 pmol/ml for the two mandelic acids, 2–7 pmol/ml for the other acidic and alcoholic compounds, 12 pmol/ml for L-DOPA and 0.6–2 pmol/ml for the other amino compounds; the corresponding values for plasma samples are 80, 0.5–3, 10 and 0.6–3 pmol/ml, respectively.

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INTRODUCTION

Since the concentrations of catecholamines (CAs) [norepinephrine (NE), epinephrine (E) and dopamine (DA)], their precursor (L-DOPA) and metabolites [normetanephrine (NM), metanephrine (M), 3-methoxytyramine (3MT), 3,4-dihydroxymandelic acid (DOMA), 3,4-dihydroxyphenylacetic acid (DOPAC), vanillylmandelic acid (VMA), homovanillic acid (HVA), 3,4-dihydroxyphenylethyleneglycol (DOPEG), 4-hydroxy-3-methoxyphenylethyleneglycol (MOPEG) and 4-hydroxy-3-methoxyphenylethanol (MOPET)] are closely related to the sympathetic nerve function, their determination in biological fluids has been useful for biomedical and clinical investigations of CA-related diseases and also for diagnosis and follow-up of these diseases (for review, see refs. 1–3). Many assay methods have been reported: radioenzymic methods [4–6], gas chromatography–mass spectrometry (GC–MS) [7–11] and high-performance liquid chromatography (HPLC) with fluorescence [12,13] or electrochemical detection (ED) [12–17]. The radioenzymic methods are highly sensitive and the GC–MS methods give the most reliable results, but these methods cannot measure these compounds simultaneously. The HPLC–ED methods are sensitive and permit the simultaneous determination of the compounds in rat brain, but they are not selective enough to be applied to complex biological samples, such as urine and plasma, even when the samples are purified by extraction with an organic solvent [18,19], acid-washed alumina [20] or boric acid gel [21].

We have developed an ion-pair reversed-phase HPLC method [22] for the determination of the catechol and 4-hydroxy-3-methoxyphenyl compounds by post-column derivatization, involving coulometric oxidation of the compounds to the corresponding *o*-quinones followed by fluorescence derivatization. The derivatization was carried out with 1,2-diphenylethylenediamine (DPE), a fluorogenic reagent for catechol compounds [23], in the presence of glycine as an accelerator and potassium hexacyanoferrate(III) as an oxidant. The compounds were separated using citrate buffers (pH 3.1) with methanol–acetonitrile (3:2, v/v) gradient elution. The aim of this study was to establish a selective and sensitive method for the determination of CAs, their precursor and metabolites in human urine and plasma. The compounds in samples are fractionated into two fractions that contain amino compounds (NE, E, DA, NM, M, 3MT and L-DOPA), and acidic (DOMA, DOPAC, VMA and HVA) and alcoholic (DOPEG, MOPEG and MOPET) compounds by solid-phase extraction on a strong cation-exchange cartridge, and then separated and determined by the above-mentioned HPLC method. 3,4-Dihydroxybenzylamine (DHBA) and 4-hydroxy-3-methoxycinnamic acid (ferulic acid) were used as internal standards.

EXPERIMENTAL

Reagents, solutions and apparatus

DHBA, ferulic acid, sodium hexanesulphonate and standard samples of CAs, their precursor and metabolites were obtained as described previously [22]. DPE was synthesized as described previously [23,24]. All other chemicals were of reagent grade. Deionized and distilled water was used. The standard solutions of CAs, their precursor and metabolites were prepared in 50 m*M* hydrochloric acid and stored at −20°C. Toyopak IC-SP S cartridge (sulphopropyl resin, 0.15 ml, H^+ form; Tosoh, Tokyo, Japan) was washed with 10 ml of water before use.

Human urine (24-h) was collected in the presence of 10 ml of 6 *M* hydrochloric acid and stored at −20°C until assay. Heparinized blood (2 ml) was centrifuged at 1000 *g* at 4°C for 15 min, and the plasma was stored at −70°C until assay.

Uncorrected fluorescence spectra were measured with a Hitachi MPF-4 spectrofluorimeter using semimicro quartz cells (1 ml). The pH was measured with a Hitachi-Horiba M-7 pH meter at 25°C.

HPLC system and conditions

The HPLC system was essentially the same as mentioned in the previous paper [22]; the applied potential in the coulometric device and the sensitivity in the fluorescence detector were modified depending on the compounds to be detected, and a gradient elution was carried out by using two eluents, an aqueous solution containing 60 m*M* citric acid, 32 m*M* disodium hydrogenphosphate, 1.7 m*M* sodium hexanesulphonate and 0.1 m*M* disodium EDTA, and the solution containing additionally 20% of a methanol–acetonitrile mixture (3:2, v/v) (see Figs. 1 and 2).

Sample preparation for HPLC

Urine samples. To 0.5 ml of urine sample (temporary or 24-h), were added 25 μl each of 10 nmol/ml DHBA and 40 nmol/ml ferulic acid and 0.5 ml of 1.0 *M* perchloric acid, and the mixture was centrifuged at 1000 *g* at 4°C for 15 min. To the supernatant (0.7 ml), 30 μl of 2.0 *M* potassium carbonate were added to adjust the pH to 1.0–1.5, and the mixture was centrifuged at 1000 *g* at 4°C for 5 min. The resulting supernatant (0.5 ml) was poured into a Toyopak IC-SP S cartridge, and the cartridge was washed with 1.5 ml of water. The effluent and the washings were combined and used for the measurement of acidic and alcoholic compounds including ferulic acid (acid–alcohol fraction). The cartridge was again washed successively with 0.5 ml of aqueous 50% (v/v) ethanol and 5 ml of water. The amino compounds, including DHBA, in the cartridge were eluted with 0.5 ml of 2.0 *M* sodium perchlorate. The eluate was used as an HPLC sample for the amino compounds (amine fraction). Each fraction

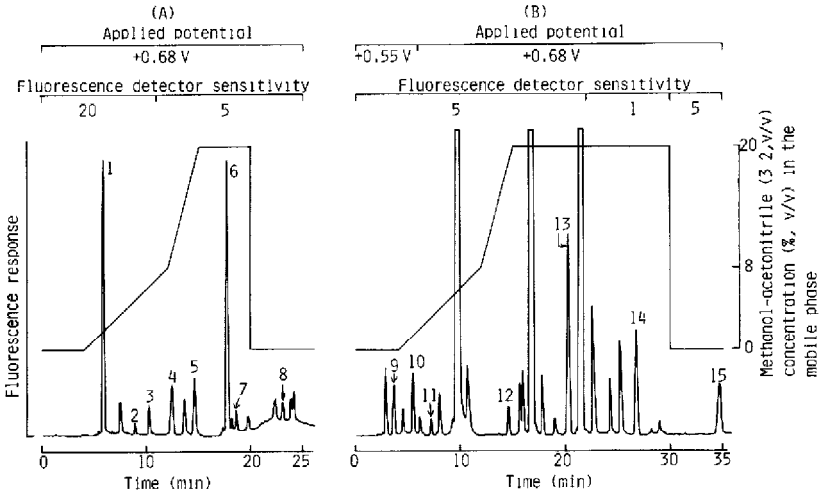

Fig. 1. Chromatograms of (A) amine and (B) acid-alcohol fractions obtained by treatment of a human urine sample according to the standard procedure. Peaks and concentrations (pmol/ml) in parentheses: 1 = NE (211); 2 = L-DOPA (208); 3 = E (46), 4 = DHBA (internal standard, 500); 5 = NM (401); 6 = DA (1820); 7 = M (117); 8 = 3MT (111); 9 = DOMA (21 200); 10 = DOPEG (418); 11 = VMA (18 900); 12 = MOPEG (282); 13 = DOPAC (4590); 14 = HVA (26 600); 15 = ferulic acid (internal standard, 20 000).

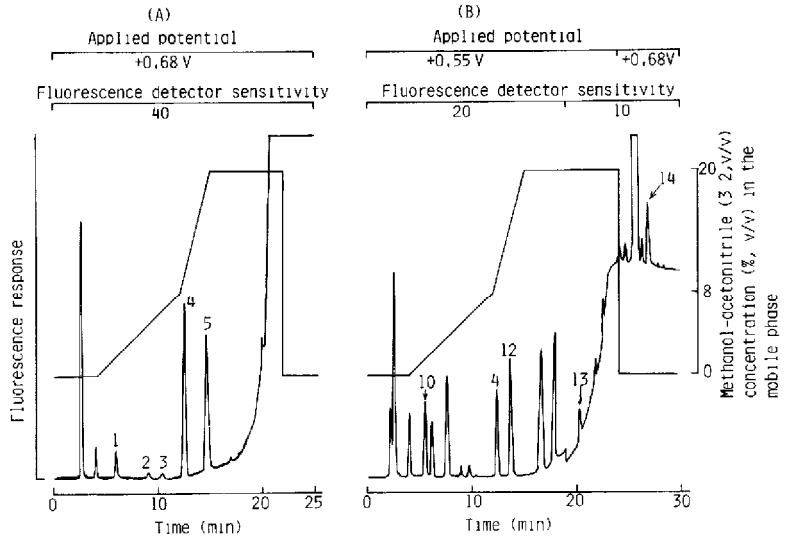

Fig. 2. Chromatograms of (A) amine and (B) acid-alcohol fractions obtained by treatment of a human plasma sample according to the standard procedure. Peaks as in Fig. 1. Concentrations (pmol/ml): 1, 2.4; 2, 12; 3, 0.5; 4, 50; 5, 28; 10, 9.3; 12, 22; 13, 20; 14, 123.

was passed through a 0.2-μm Millipore filter (Nihon Millipore, Tokyo, Japan) and then a 50-μl aliquot was injected into the chromatograph.

Plasma samples. To 0.7 ml of plasma, 50 μl of 700 pmol/ml DHBA and 350 μl of 2.0 *M* perchloric acid were added, and the mixture was centrifuged at 1000 *g* at 4°C for 15 min. The pH of the supernatant (0.7 ml) was adjusted to 1.0–1.5 by addition of ca. 30 μl of 2.0 *M* potassium carbonate, and the mixture was centrifuged at 1000 *g* at 4°C for 5 min. The supernatant was analysed (acid–alcohol fraction) without fractionation. The remaining supernatant (0.5 ml) was poured onto a Toyopak IC-SP S cartridge. The cartridge was washed successively with 1 ml of water, 0.5 ml of aqueous 50% ethanol and 5 ml of water.

TABLE I

EFFECT OF VARIOUS MODIFICATIONS[a] OF THE SAMPLE PREPARATION OR THE POST-COLUMN DERIVATIZATION ON THE PEAKS FOR CATECHOLAMINES, THEIR PRECURSOR AND METABOLITES[b]

Peak	Modification[c]					
	A	B	C	D	E	F
NE	–	+	–	+	+	–
E	–	+	–	+	+	–
DA	–	+	–	+	+	–
NM	–	–	+	–	+	–
M	–	–	+	–	+	–
3MT	–	–	+	–	+	–
L-DOPA	–	+	–	+	+	–
DOMA	–	+	–	+	–	+
DOPAC	–	+	–	+	–	+
VMA	–	–	+	–	–	+
HVA	–	–	+	–	–	+
DOPEG	–	+	–	+	+	–
MOPEG	–	–	+	–	+	–

[a](A) DPE was omitted from the reagent solution for the post-column derivatization; peaks for catechol and 4-hydroxy-3-methoxyphenyl compounds were not observed in the chromatograms. (B) No potential was applied in the coulometric device; peaks for catechol compounds were observed, but those for 4-hydroxy-3-methoxyphenyl compounds were not. (C) An alumina-unextracted sample was treated according to the solid-phase extraction procedure followed by the HPLC, peaks for 4-hydroxy-3-methoxyphenyl compounds were observed, but those for catechol compounds were not. (D) An alumina-extracted sample was treated as in (C); peaks for catechol compounds were observed, but those for 4-hydroxy-3-methoxyphenyl compounds were not. (E) A DEAE effluent was treated as in (C); peaks for acidic compounds were not observed. (F) A DEAE-extracted sample was directly injected into the chromatograph; peaks for acidic compounds were observed.

[b]In urine samples, the peaks were all confirmed, and in plasma samples, the peaks for NE, E, NM, L-DOPA, DOPAC, HVA, DOPEG and MOPEG were confirmed.

[c]+, a peak was observed; –, no peak was observed.

The amino compounds in the cartridge were eluted with 0.5 ml of 2 *M* sodium perchlorate. The eluate contained the amine fraction. Each fraction was filtered and subjected to HPLC as for urine samples.

Procedures for alumina treatment and solid-phase extraction using anion-exchange resin cartridge

The following sample treatments were carried out in the peak identification (see Table I).

Alumina treatment (Fig. 3A). An aliquot (1.0 ml) of urine or plasma sample

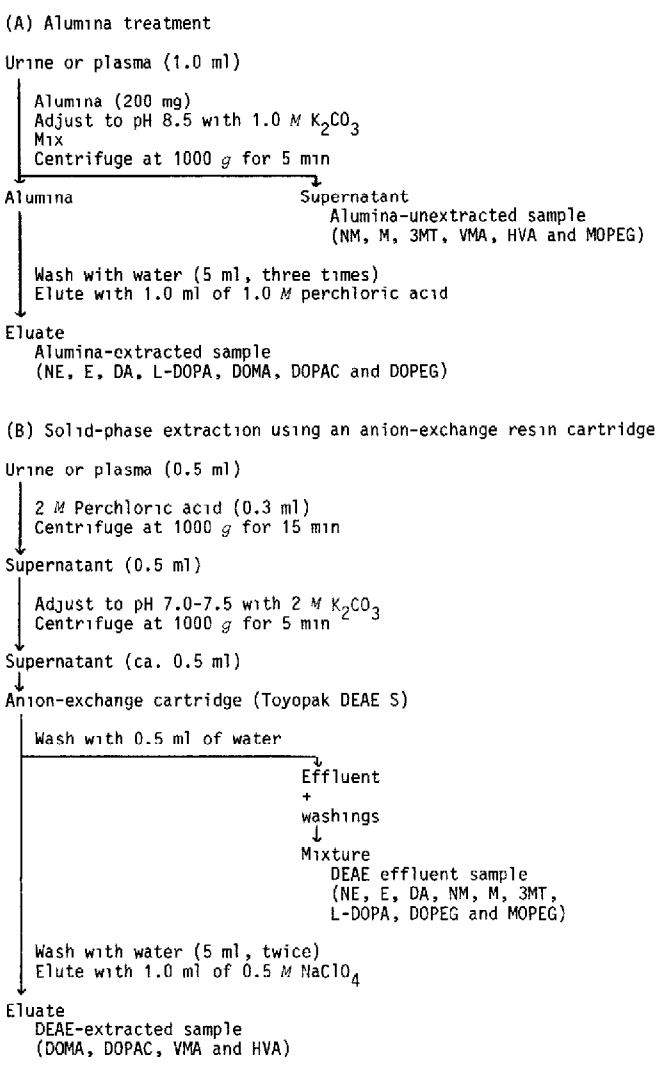

Fig. 3. Sample treatments used in peak identification.

was added to 200 mg of acid-washed alumina [20], and the pH of the mixture was adjusted to 8.5 with 1.0 *M* potassium carbonate. The mixture was thoroughly mixed and then centrifuged at 1000 *g* for 5 min. The supernatant was used as an alumina-unextracted sample. The alumina was washed with 5 ml of water (three times). The adsorbed catechol compounds were eluted with 1.0 ml of 0.1 *M* perchloric acid. The eluate was used as an alumina-extracted sample.

Solid-phase extraction using anion-exchange resin cartridge (Fig. 3B). To 0.5 ml of urine or plasma sample, 0.3 ml of 2 *M* perchloric acid was added and the mixture was centrifuged at 1000 *g* for 15 min. The supernatant (0.5 ml) was neutralized (pH 7.0–7.5) by the addition of 2 *M* potassium carbonate. The mixture was centrifuged at 1000 *g* for 5 min, and the resulting supernatant was poured onto a Toyopak DEAE S cartridge (DEAE resin, 0.15 ml, Cl^- form; Tosoh). The cartridge was washed with 0.5 ml of water. The effluent and the washings were combined (DEAE effluent). The cartridge was again washed with 5 ml of water (twice), and acidic compounds were eluted with 1.0 ml of 0.5 *M* sodium perchlorate. The eluate was used as a DEAE-extracted sample.

RESULTS AND DISCUSSION

Typical chromatograms of the amine and acid–alcohol fractions, obtained from human urine and plasma samples according to the procedure, are shown in Figs. 1 and 2, respectively. When the samples were analysed by monitoring the oxidative current in the coulometric device, the peaks of NE, L-DOPA, E, DHBA, M, 3MT, DA, DOMA, DOPEG, VMA, MOPEG and HVA were partially or wholly overlapped by unidentified peaks that were attributable to endogenous reducing compounds present in the samples. Most of the endogenous compounds did not fluoresce in the present post-column derivatization.

In both urine and plasma samples, the concentrations of the amino compounds are generally much lower than those of the acidic and alcoholic compounds (see Tables II and III), and there are many unknown compounds that fluoresce under the present HPLC conditions. Therefore, the amino compounds could not be determined when the deproteinized samples were directly subjected to HPLC. The extraction of amino compounds by solid-phase extraction on a cation-exchange resin (Toyopak IC-SP S) cartridge is the most appropriate for the HPLC sample preparation of the amino compounds in both urine and plasma samples. For the determination of the acidic and alcoholic compounds in urine samples, the effluent from the cartridge could be used as acid–alcohol fraction. On the other hand, in plasma samples, the deproteinized sample was directly used as an acid–alcohol fraction to avoid dilution of the sample in the solid-phase extraction procedure, because the amino compounds in the deproteinized sample did not interfere with the determination of the acidic and alcoholic compounds.

The peaks corresponding to CAs, their precursor and metabolites could be

TABLE II

AMOUNTS OF CATECHOLAMINES, THEIR PRECURSOR AND METABOLITES IN 24-H URINES FROM HEALTHY PERSONS

Sex	Age	Amount (nmol per 24 h)									Amount (μmol per 24 h)			
		Amino compounds							Alcoholic compounds		Acidic compounds			
		NE	E	DA	NM	M	3MT	L-DOPA	DOPEG	MOPEG	DOPAC	DOMA	HVA	VMA
M	56	189	34	967	520	77	283	537	461	197	3.28	38.4	13.5	12.3
M	40	504	43	1458	815	172	237	368	752	390	5.27	67.2	24.4	19.2
M	35	447	64	1396	709	220	56	370	723	100	4.12	8.1	23.9	22 4
M	32	324	80	2292	655	184	341	1530	657	292	7.48	26.6	32.2	21 4
M	29	380	62	1519	469	124	365	660	457	265	4.50	16.2	27.0	22 5
M	28	513	74	1180	112	168	219	737	377	213	3.37	33.9	29.1	15.5
M	28	784	56	2128	103	122	495	1118	855	263	6.45	31.1	35.1	36.6
M	26	294	20	1829	312	123	133	983	417	210	5.28	28.1	26.1	20.7
M	24	221	46	1824	401	117	111	208	418	282	4.59	21.2	26.6	18.9
F	23	621	43	1498	128	184	193	523	634	232	3.40	44.5	18 8	21.9
F	23	279	27	1915	222	94	249	1339	663	293	9.18	34.3	36.2	19.8
F	22	285	30	1501	328	119	277	640	207	172	5.15	75.6	27.9	14.9
F	22	200	79	1579	419	229	337	588	527	236	8.60	70.9	25.5	14.2
F	21	910	32	1752	128	87	191	347	591	301	4.15	124.0	26.1	17.4
F	21	485	74	1435	153	178	185	251	505	409	9.89	171.0	36.2	46.9
Mean		429	51	1618	365	147	245	680	550	264	5.65	52.0	27.1	21.6
S.D.		206	20	334	225	46	107	384	163	80	2.11	42.9	6.4	8.7

identified as follows: (1) their retention times and fluorescence excitation and emission spectra coincided with those of the standard compounds [22]; (2) an increase in peak heights was observed when they were co-chromatographed with the standards; (3) fluorescence peak heights for the 4-hydroxy-3-methoxyphenyl compounds were proportional to the respective responses in the coulometric device (hydrodynamic voltammograms) with changing applied potentials, as described previously [22]. Furthermore, the detector responses to applied potentials (from 0 to +0.68 V) were the same as those for the standards; (4) the presence of functional groups (catechol, 3-hydroxy-4-methoxyphenyl, amino and acidic moieties) was confirmed by various modifications of the sample preparation or the post-column derivatization, as shown in Table I.

The peaks all disappeared when DPE was omitted from the reagent solution for post-column derivatization (Table I, modification A). Therefore, the unidentified peaks in the chromatograms (Figs. 1 and 2) were attributable to the catechol and 4-hydroxy-3-methoxyphenyl compounds. DA-3- and -4-O-sulphates could be converted into *o*-quinones by electrochemical oxidation and gave peaks in the chromatogram. Their half-wave potentials were both 0.58 V. The values were higher than those of DA (0.26 V) and 3MT (0.48 V) [22].

TABLE III

CONCENTRATIONS OF TWO CATECHOLAMINES, THEIR PRECURSOR AND SOME METABOLITES[a] IN PLASMA FROM HEALTHY PERSONS[b]

Sex	Age	Concentration (pmol/ml)							
		NE	E	NM	L-DOPA	DOPAC	HVA	DOPEG	MOPEG
M	56	5.0	1.2	24.8	11.0	16.4	172	11.1	38.2
M	41	3.2	1.0	29.5	8.0	—[c]	58	5.1	14.6
M	30	5.7	1.4	30.9	13.6	16.8	54	7.5	—
M	26	2.5	0.8	31.1	9.6	19.6	132	3.8	21.5
M	25	2.5	0.7	19.2	8.0	10.0	70	10.5	19.2
M	25	7.4	0.7	21.8	9.4	—	202	16.0	27.6
M	24	6.8	N.D.[d]	9.5	N.D.	9.4	60	10.3	—
M	24	2.9	N.D.	28.3	N.D.	22.8	118	1.1	27.1
F	25	2.4	0.5	27.5	11.8	20.2	123	9.3	22.0
F	24	3.8	1.3	28.8	11.2	22.4	170	7.0	30.2
F	23	3.7	1.2	18.1	14.0	35.2	414	13.9	14.2
Mean		4.2	1.0	24.5	10.7	19.2	143	8.7	23.8
S.D		1.7	0.3	6.4	2.1	7.3	99	4.2	7.3

[a]DA, M, 3MT, DOMA and VMA could not be determined because of their minute amounts.
[b]Blood samples were collected between 9:00 and 10:00 a.m. from healthy volunteers, in a sitting position, who had a small breakfast at ca. 7:30 a.m.
[c]Unknown peak(s) interfered with the determination of the compounds.
[d]Not detected.

Therefore such O-sulphate compounds may have half-wave potentials of ca. 0.6 V or higher and yield peaks. The potential was usually set at 0.68 V, as described previously [22]. For the acid–alcohol fraction from the urine sample, the applied potential was lowered to 0.55 V for the first 5 min (Fig. 1B), because the height of an unknown peak (peak a in Fig. 4) increased with increasing applied potentials. At 0.68 V it interfered with the determination of DOPEG (Fig. 4). This peak has a half-wave potential of more than 6.0 V, hence it may be ascribed to a catechol O-sulphate. The same was also true for the peaks for DOPEG and DOPAC in plasma samples; an applied potential of 0.55 V was used for the first 20 min (Fig. 2B).

A high background fluorescence at retention times of 20 min and longer in the chromatograms of plasma samples (Fig. 2A and B) remained unchanged even when water was injected instead of the sample, because the sensitivity of the fluorescence detector was kept high compared with that in the case of urine samples (Fig. 1A and B).

The concentrations of CAs, their precursor and metabolites in healthy persons (thirteen species in 24-h urine samples and eight species in plasma samples) could be determined by this method (Tables II and III). MOPET (reten-

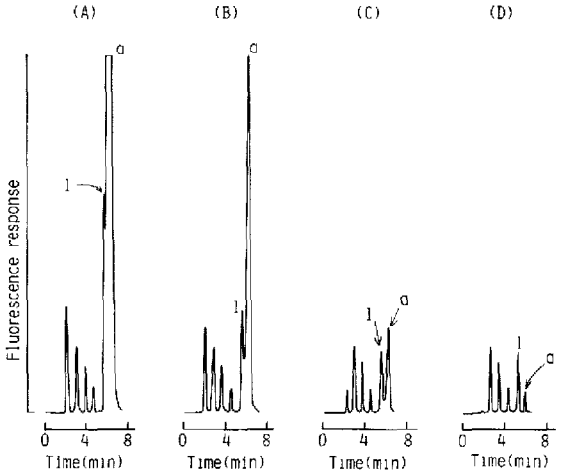

Fig. 4. Effect of applied potentials on the separation of DOPEG (peak 1) and the unknown peak a. A portion (0.5 ml) of a urine sample was treated according to the solid-phase extraction procedure, followed by HPLC at various applied potentials: (A) 0.68 V; (B) 0.60 V; (C) 0.56 V; (D) 0.55 V DOPEG concentration, 526 pmol/ml of urine.

tion time, 24.7 min) in both plasma and urine samples, and DA, M, 3MT, DOMA and VMA in plasma sample could not be measured at a signal-to-noise ratio of 5 because of their minute amounts and/or overlapping with unidentified peaks. The results obtained by this method agree with those previously reported [3].

Linear relationships were obtained between the ratios of the peak heights of the amino compounds to that of DHBA and amounts of the compounds added to 0.5 ml of urine in the range 2–1000 pmol for L-DOPA and 0.2–200 pmol for the others, and between the ratios of the peak heights of the acidic and alcoholic compounds to that of ferulic acid and amounts of the compounds added to 0.5 ml of urine in the range 10–3000 pmol for DOMA and VMA and 0.2–200 pmol for the others. When the standard compounds were added to 0.7 ml of plasma in the range 10–3000 pmol for DOMA and VMA, 2–1000 pmol for L-DOPA and 0.2–200 pmol for the others, the ratios of the peak heights of the compounds to that of DHBA were also in direct proportion to the amounts of the compounds added.

The recoveries of the standard compounds added to 0.5 ml of urine in amounts of 10 nmol for DOPAC, DOMA, HVA, VMA and ferulic acid, and 0.1 nmol for the others, were 60% for L-DOPA, 64% for ferulic acid, 93% for DOPEG and MOPEG, and 85–90% for the others and DHBA, and each relative standard deviation (R.S.D., $n=5$) was less than 2.5%. When the compounds were added to 0.7 ml of plasma in amounts of 2 nmol for DOMA and VMA and 10 pmol for the others and DHBA, the recoveries were 64% for L-DOPA, 90–93% for

the other amino compounds and DHBA, and 98–101% for the acidic and alcoholic compounds with maximum R.S.D. of 3.0%.

The detection limits (pmol/ml) at a signal-to-noise ratio of 5 for urine samples were 0.6 (NE), 1 (E and NM), 2 (DA, M, 3MT and DOPEG), 12 (L-DOPA), 300 (DOMA and VMA), 7 (DOPAC and MOPET), 10 (HVA) and 3 (MOPEG). The corresponding values for the plasma sample were 0.6 (NE), 1 (E, NM and MOPEG), 3 (DA, M, 3MT, DOPAC, MOPET and HVA), 10 (L-DOPA), 100 (DOMA and VMA) and 0.5 (DOPEG). The present method seems to be one of the most sensitive HPLC methods reported so far [3,12–17].

The precision of the method was established by repeated determinations ($n=5$) of pooled urine and plasma. The R.S.D. values [the compounds and concentrations (pmol/ml) in parentheses] in urine were 2.1% (NE, 475), 1.3% (E, 75), 3.5% (DA, 1740), 8.8% (NM, 416), 2.5% (M, 63), 4.9% (3MT, 292), 6.3% (L-DOPA, 771), 4.1% (DOMA, 59 900), 1.7% (DOPAC, 6150), 7.5% (VMA, 2440), 2.9% (HVA, 30 200), 3.0% (DOPEG, 579) and 3.61% (MOPEG, 277), and those in plasma were 4.2% (NE, 4.4), 3.8% (E, 1.1), 6.4% (NM, 27.1), 7.1% (L-DOPA, 11.6), 8.5% (DOPAC, 20.2), 5.8% (HVA, 158), 2.2% (DOPEG, 10.3) and 6.5% (MOPEG, 23.9).

In conclusion, this HPLC method is more selective than the widely used HPLC–ED methods [12–17], so it permits the quantification of CAs, their precursor and a variety of their metabolites in urine and plasma samples with very simple clean-up of the samples. This method is sensitive enough for urine samples and therefore should be useful for biomedical and clinical investigations of CAs and their metabolites in urine. However, the sensitivity is generally the same as that of the HPLC–ED methods and is still unsatisfactory for DA, M, 3MT, DOMA and VMA in normal plasma samples.

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