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SIMULTANEOUS HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CATECHOLAMINE-RELATED COMPOUNDS BY POST-COLUMN DERIVATIZATION INVOLVING COULOMETRIC OXIDATION FOLLOWED BY FLUORESCENCE REACTION

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SUMMARY

A highly selective and sensitive high-performance liquid chromatographic method for the determination of catecholamines (norepinephrine, epinephrine and dopamine) and related compounds (L-DOPA, normetanephrine, metanephrine, 3-methoxytyramine, 3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylacetic acid, homovanillic acid, vanillylmandelic acid, 3,4-dihydroxyphenylethylene glycol, 4-hydroxy-3-methoxyphenylethylene glycol and 4-hydroxy-3-methoxyphenylethanol) with a post-column technique involving coulometric oxidation followed by fluorescence derivatization is described. These compounds, 3,4-dihydroxybenzylamine and ferulic acid are separated within 35 min by ion-pair reversed-phase chromatography using acidic buffers (pH 3.1) with methanol–acetonitrile (3:2, v/v) gradient elution, and then oxidized by a commercial coulometric detector to the corresponding *o*-quinones, which are converted into fluorescent derivatives by reaction with 1,2-diphenylethylenediamine. The detection limits (signal-to-noise ratio = 3) on-column are 1.5–4 pmol for the two mandelic acids, 600 fmol for L-DOPA and 20–70 fmol for the others.

INTRODUCTION

The determination of catecholamine (CA)-related compounds (for abbreviations, see Table I) can afford much information on the sympathetic nerve functions and therefore it is useful in clinical and pharmacological studies (for reviews, see refs. 1–3). Many methods have been proposed, but only high-performance liquid chromatography (HPLC) with electrochemical detection (ED) permits the simultaneous determination of CA-related compounds[4–17].

We have developed an HPLC method for the simultaneous determination of CA-related compounds based on post-column derivatization involving electrochem-

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TABLE I

CATECHOLAMINE-RELATED COMPOUNDS AND THEIR ABBREVIATIONS

Compound	*Abbrevation*
Amino compounds:	
Norepinephrine[a] }	NE
Epineprine[a] } catecholamines	E
Dopamine[a] }	DA
Normetanephrine[b]	NM
Metanephrine[b]	M
3-Methoxytyramine[b]	3MT
L-DOPA[a]	L-DOPA
Acidic compounds:	
3,4-Dihydroxymandelic acid[a]	DOMA
3,4-Dihydroxyphenylacetic acid[a]	DOPAC
Vanillylmandelic acid[b]	VMA
Homovanillic acid[b]	HVA
Alcoholic compounds:	
3,4-Dihydroxyphenylethylene glycol[a]	DOPEG
4-Hydroxy-3-methoxyphenylethylene glycol[b]	MOPEG
4-Hydroxy-3-methoxyphenylethanol[b]	MOPET

[a] Catechol compounds.
[b] 4-Hydroxy-3-methoxyphenyl compounds.

ical oxidation followed by fluorescence reaction (Fig. 1). The CA-related compounds are separated by ion-pair reversed-phase chromatography with gradient elution using an acidic buffer and a buffer containing methanol and acetonitrile, and then electrochemically oxidized to the respective *o*-quinones by a coulometric technique. The *o*-quinones are converted into fluorescent derivatives with *meso*-1,2-diphenylethylenediamine (DPE)[18], a fluorogenic reagent for catechol compounds that has previously been applied to pre-column fluorescence derivatization HPLC for the determination of three CAs in plasma[19], platelets[20], erythrocytes[20] and urine[21,22]. 3,4-Dihydroxybenzylamine (DHBA) and 4-hydroxy-3-methoxycinnamic acid (ferulic acid) were also subjected to the investigation because they should be useful as internal standards when CA-related compounds are measured in biological samples.

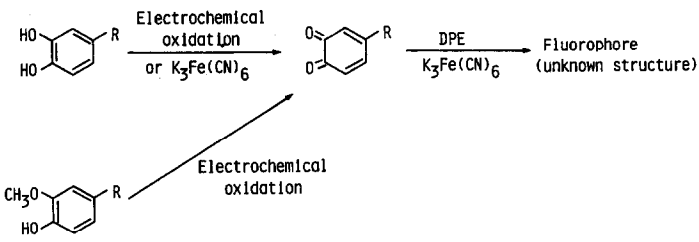

Fig. 1. Fluorescence derivatization of catechol and 4-hydroxy-3-methoxyphenyl compounds with DPE.

EXPERIMENTAL

Reagents, solutions and apparatus

NE hydrogentartrate and DA hydrochloride were purchased from Wako (Osaka, Japan), NM hydrochloride, M hydrochloride, DOMA, VMA and HVA from Nacalai Tesque (Kyoto, Japan), E hydrogentartrate, 3MT hydrochloride and L-DOPA from Sigma (St.Louis, MO, U.S.A.) and DHBA hydrochloride, DOPAC, DOPEG, MOPEG piperazine salt and MOPET from Aldrich (Milwaukee, WI, U.S.A.). Ferulic acid was obtained from Wako and recrystallized twice from water. Their standard solutions were prepared in 50 m*M* hydrochloric acid and stored at −20°C. Highly purified glycine for electrophoresis and sodium hexanesulphonate were purchased from Nacalai Tesque. All other chemicals were of analytical-reagent grade. Deionized, distilled water was used. DPE was synthesized by the method of Irving and Parkins[23] with minor modifications[18]. Dopamine 3- and 4-O-sulphates were prepared according to the method of Jenner and Rose[24].

Uncorrected fluorescence spectra were measured with a Hitachi MPF-4 spectrofluorimeter using semimicro quartz cells (10 mm width parallel to the excitation beam, 3 mm width parallel to the emission beam; 1 ml).

HPLC system and conditions

Fig. 2 shows a schematic diagram of the HPLC system. A Hitachi Model 655A-12 liquid chromatograph and a Model 655-66 controller were used for gradient elution. The HPLC column (150 × 4.6 mm I.D.) contained 5-μm TSK-gel ODS-80TM (Tosoh, Tokyo, Japan). The column temperature was ambient. A Hitachi 655A coulometric monitor was employed for the electrochemical oxidation of CA-related compounds. A Hitachi F1000 spectrofluorimeter fitted with a 20-μl flow cell was used.

The mobile phase consisted of two eluents, an aqueous solution containing 60 m*M* citric acid, 32 m*M* disodium hydrogenphosphate, 1.7 m*M* sodium hexanesulphonate as ion-pairing reagent and 0.1 m*M* disodium EDTA (pH 3.1, eluent A) and

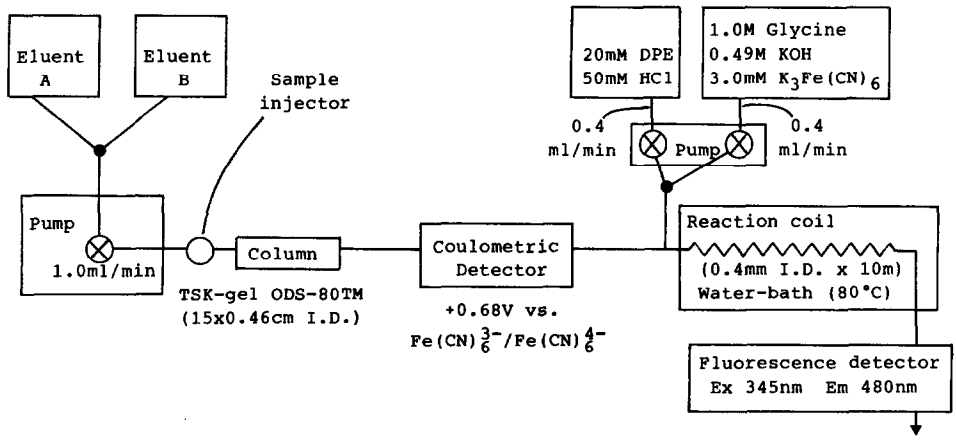

Fig. 2. Schematic diagram of post-column derivatization HPLC of catecholamine-related compounds. Ex = Exitation wavelength; Em = emission wavelength.

an aqueous solution containing 20% methanol–acetonitrile (3:2, v/v) as an organic modifier (pH 3.1, eluent B). Gradient elution using eluents A and B [0–20% (v/v) of the organic modifier] was applied for the separation, as shown in Fig. 3. The flow-rate was 1.0 ml/min. After a single run, eluent A (organic modifier concentration 0%) was passed through the column for 20 min to establish complete equilibrium for the subsequent sample injection.

The eluate from the column was introduced into the coulometric detector to oxidize catechol and 4-hydroxy-3-methoxyphenyl compounds to *o*-quinones (Fig. 1). The applied oxidation potential was the potential difference between the working and reference electrodes in the coulometric detector, and a potential of +0.68 V was usually used. Both electrodes were made of carbon cloth. The electrolyte for the reference electrode was an equimolar (200 m*M*) mixture of potassium hexacyanoferrates (II and III) containing potassium nitrate and potassium hydroxide (200 m*M* each), and the reference electrode also served as a counter electrode.

The effluent from the coulometric detector was then mixed with a stream of a mixture of two reagent solutions, 20 m*M* DPE in 50 m*M* hydrochloric acid and an aqueous solution of 1 *M* glycine – 0.49 *M* potassium hydroxide – 3 m*M* potassium hexacyanoferrate (III). Both reagent solutions delivered at a flow-rate of 0.4 ml/min by an SSP DM2M-1026 pump (Sanuki Kogyo, Tokyo, Japan). The fluorescence derivatization reaction was performed in a reaction coil (Tough tubing, 10 m × 0.47 mm I.D.; Gasukuro Kogyo, Tokyo, Japan) immersed in a water-bath (80°C). The resulting fluorescence was monitored at 480 nm with an excitation wavelength of 345 nm.

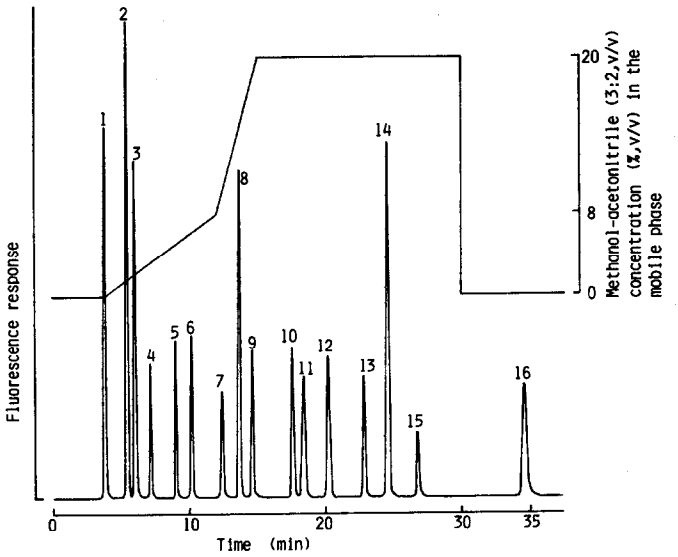

Fig. 3. Chromatogram of a standard mixture of catecholamine-related compounds. Peaks: 1 = DOMA; 2 = DOPEG; 3 = NE; 4 = VMA; 5 = L-DOPA; 6 = E; 7 = DHBA (internal standard); 8 = MOPEG; 9 = NM; 10 = DA; 11 = M; 12 = DOPAC; 13 = 3MT; 14 = MOPET; 15 = HVA; 16 = ferulic acid. Amounts (on-column): VMA and HVA, 500 pmol; L-DOPA and ferulic acid, 100 pmol; others, 10 pmol.

RESULTS AND DISCUSSION

Fig. 3 shows the chromatogram of a standard mixture of fourteen CA-related compounds, DHBA and ferulic acid. These compounds could be separated within 35 min under the recommended conditions. Table II shows the retention times and relative peak heights for the CA-related compounds and the fluorescence excitation and emission spectra of the corresponding eluates from the fluorescence detector. The fluorescence excitation and emission maxima are around 340 and 475 nm, respectively. With the present HPLC system, the fluorescence was monitored at 480 nm emission with excitation at 345 nm for the highly sensitive determination of CAs, the concentrations of which in biological samples are the lowest of CA-related compounds.

Chromatographic separation

The investigations of the HPLC separation were mainly carried out by monitoring the oxidation current in the coulometric detector. The proposed HPLC method permitted the complete separation of fourteen CA-related compounds, DHBA and ferulic acid with a linear gradient using a citrate–phosphate buffer and the buffer containing methanol–acetonitrile (3:2, v/v) (Fig. 3). Methanol, acetonitrile, ethanol

TABLE II

RETENTION TIMES, FLUORESCENCE EXCITATION AND EMISSION MAXIMA AND RELATIVE PEAK HEIGHTS OF THE DPE DERIVATIVES

Portions (50 μl) of 5 nmol/ml CA-related compounds, DHBA, ferulic acid and dopamine 3- and 4-O-sulphates were injected into the chromatograph.

Compound	*Retention time (min)*	*Excitation maximum (nm)*	*Emission maximum (nm)*	*Relative peak height*[a]
NE	6.1	340	480	100
E	10.2	350	495	49
DA	17.7	345	475	45
NM	14.7	340	480	44
M	18.5	350	495	36
3MT	22.9	345	475	42
L-DOPA	9.1	340	470	4.7
DOMA	3.8	340	480	2.2
DOPAC	20.3	340	470	42
VMA	7.2	340	480	0.8
HVA	26.8	340	470	19
DOPEG	5.5	340	475	141
MOPEG	13.7	340	475	97
MOPET	24.7	340	475	105
DHBA	12.5	345	480	31
Ferulic acid	34.7	365	500	3.3
Dopamine 3-O-sulphate	8.1	345	475	48
Dopamine 4-O-sulphate	7.9	345	475	51

[a] The peak height for NE was taken as 100.

and their mixtures were examined as organic modifiers in gradient elution; a mixture of methanol and acetonitrile (2:3, v/v) gave the most satisfactory separation.

The CA-related compounds contain an amino, carboxyl or alcoholic moiety (Table I). For the simultaneous separation of such compounds, ion-pair reversed-phase HPLC is the most suitable technique.

As catechol compounds are fairly stable in acidic media, an acidic buffer, consisting of a mixture of citric acid and disodium hydrogenphosphate (pH 3.1), was used as the mobile phase. However, the amino compounds were all in cationic form in this buffer and were not retained well on the reversed-phase HPLC column. By adding an anionic ion-pairing reagent, the amino compounds were retained on the column and thus the simultaneous separation of the CA-related compounds was achieved. As ion-pairing reagents, benzenesulphonate, pentanesulphonate, hexanesulphonate, heptanesulphonate, octanesulphonate, octanesulphate and lauryl sulphate (all sodium salts) were examined. Although these reagents resulted in longer retention times for the amino compounds in that order, the HPLC column could not be equilibrated with octanesulphonate, octanesulphate and lauryl sulphate, even after passing the mobile phase containing one of them through it for 3 h at a flow-rate of 1.0 ml/min. Therefore, hexanesulphonate was selected as the optimum reagent; with increasing concentration of the sulphonate, the retention times for the amino compounds became longer; 1.7 m*M* was used for rapid and complete separation. Hexanesulphonate did not affect the retention times of the acidic and alcoholic compounds. The pH of the mobile phase, which was adjusted by using citric acid – disodium hydrogenphosphate buffer, citrate buffer or perchlorate buffer, affected the retention times of the acidic compounds in the pH range 2.5–5.0; higher pHs made the retention times shorter. Citric acid – disodium hydrogenphosphate buffer of pH 3.1 afforded a complete separation of the CA-related compounds. The concentrations of citric acid and the phosphate in the mobile phase affected the retention times of the amino compounds and the efficiency of the coulometric oxidation. With increasing concentrations of the acid and the phosphate, the retention times decreased. A constant and stable efficiency of the oxidation in the coulometric cell was obtained with 20 m*M* citric acid – 10 m*M* disodium hydrogenphosphate buffer or more concentrated buffers; a 60 m*M* citric acid – 32 m*M* phosphate buffer is recommended. EDTA was added to the mobile phase to protect the coulometric cell and the HPLC column from adhesion of metal ions.

Coulometric oxidation

In the reaction of DPE with catechol compounds[18,19], it is presumed that catechol compounds are converted into *o*-quinones in the first stage by hexacyanoferrate (III) and then react with DPE to produce fluorescence, because catechol compounds are readily and rapidly oxidized into *o*-quinones by oxidizing reagents such as oxygen, potassium hexacyanoferrate(III) and iodine. Therefore, 4-hydroxy-3-methoxyphenyl compounds also have the possibility of reacting with DPE after conversion to the respective *o*-quinone compounds by electrochemical oxidation (Fig. 1). Based on this assumption, an electrochemical detector was incorporated in the post-column derivatization HPLC of the CA-related compounds. Two types of electrochemical detectors, amperometric and coulometric, have been utilized in the HPLC analysis of CAs and their metabolites. In the present method, quantitative oxidation of 4-hy-

droxy-3-methoxyphenyl compounds to *o*-quinones is required and hence a coulometric detector was employed.

Fig. 4 shows hydrodynamic voltammograms of the CA-related compounds and dopamine 3-O-sulphate in this HPLC system; their half-wave potentials obtained from the voltammograms varied, depending on the compounds (Table III). The potentials of 4-hydroxy-3-methoxyphenyl compounds were higher than those of the catechol compounds by 0.2–0.3 V. In this system, a potential of +0.68 V was tentatively selected for complete oxidation and reproducible results.

The fluorescence peak heights for 4-hydroxy-3-methoxyphenyl compounds were proportional to the respective responses in the coulometric detector with changing applied potential in the range 0.2–0.68 V. However, those of catechol compounds were independent of the applied potential. When an amperometric detector (Yanagimoto VDM 101) was used in place of the coulometric detector, the resulting fluorescence peak heights for 4-hydroxy-3-methoxyphenyl compounds were 2% or less of those obtained with the coulometric detector.

When the eluate for M was applied in the reversed-phase mode used in the pre-column derivatization HPLC of CAs[19], it gave the same chromatogram as that obtained for its demethylated compound, E, and the same was found with the other pairs of CA-related compounds (NE with NM, DA with 3MT, DOMA with VMA, DOPAC with HVA and DOPEG with MOPEG). The eluates from the fluorescence detector corresponding to the catechol compounds showed the same fluorescence excitation and emission maxima as those for the respective 3-O-methylated (3-methoxy-4-hydroxyphenyl) compounds (Table II).

The above observations strongly support the presumption that 4-hydroxy-3-methoxyphenyl compounds are oxidatively demethylated to *o*-quinones by coulometric oxidation and then react with DPE to produce fluorescence (Fig. 1).

In fact, the eluate for each CA-related compound gave multiple (1–4) peaks in addition to the DPE derivative peak when it was subjected to reversed-phase HPLC, although a single peak was always obtained for each CA in the pre-column deri-

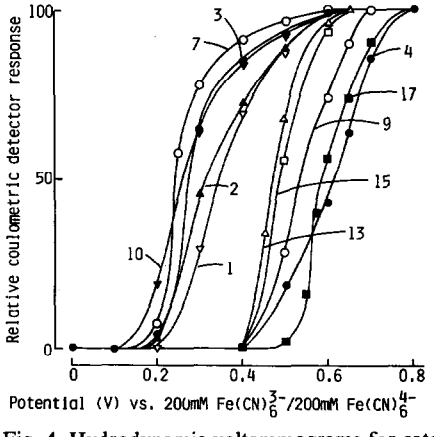

Fig. 4. Hydrodynamic voltammograms for catecholamine-related compounds. Peaks and amounts as in Fig. 3; 17, dopamine 3-O-sulphate and 10 pmol on-column. For the relative coulometric detector response, the oxidative current at 0.8 V for each compound was taken as 100.

HALF-WAVE POTENTIALS [V, *vs.* 200 m*M* Fe $(CN)_6^{3-}$/200 m*M* Fe $(CN)_6^{4-}$] OF CATECHOLAMINE-RELATED COMPOUNDS, DHBA, FERULIC ACID AND DOPAMINE 3- AND 4-O-SULPHATES IN THE PRESENT HPLC CONDITIONS

Catechol compound	*Half-wave potential*	*3-Methoxy-4-hydroxyphenyl compound*	*Half-wave potential*
NE	0.27	NM	0.54
E	0.29	M	0.54
DA	0.26	3MT	0.48
L-DOPA	0.28	VMA	0.63
DOMA	0.35	HVA	0.48
DOPAC	0.28	MOPEG	0.49
DOPEG	0.32	MOPET	0.48
DHBA	0.23	Ferulic acid	0.49
Dopamine 3- and 4-O-sulphates			0.58

vatization HPLC[19]. This may be due to by-product(s) and/or degradation product(s) formed under the present derivatization conditions, where the reaction temperature (80°C) was much higher than that in the pre-column derivatization (37°C).

All catechol O-sulphate compounds are probably oxidized electrochemically to *o*-quinones and their half-wave potentials are suspected to be higher than those of catechol and 4-hydroxy-3-methoxyphenyl compounds, because dopamine 3- and 4-O-sulphates gave fluorescence peaks under the present HPLC conditions, both the effluents from the fluorescence detector showed the same fluorescence spectra as those for DA and 3MT (Table II) and the potentials of dopamine 3-O-sulphate, 3MT and DA increased in that order (Table III). Therefore, a chromatographic separation or a decrease in the applied potentials in the coulometric detector may be required for the determination of the CA-related compounds in biological samples containing catechol O-sulphate compounds.

Fluorescence derivatization reaction

In the manual spectrofluorimetric[18] and the pre-column HPLC[19–22] methods for the determination of CAs, glycine and a water-miscible organic solvent, respectively, were employed as accelerators of the DPE reaction. In the present system, the post-column derivatization reaction was promoted most effectively by the addition of glycine. The peak heights for the CA-related compounds increased with increasing glycine concentration up to 1.2 *M*; 1.0 *M* was selected. The peak heights also increased with increasing DPE concentration up to 25 m*M*, but at concentrations higher than 15 m*M*, they increased only slightly; 20 m*M* was therefore used. The optimum pH for the post-column derivatization reaction was 7.5–8.0 for all the CA-related compounds tested, and could be adjusted with potassium hydroxide in the reagent solution; pH 7.8 was selected as the optimum value.

The temperature in the fluorescence derivatization reaction affected the peak heights. On increasing the temperature, the peak height for MOPET increased and that for L-DOPA decreased only slightly. All the other CA-related compounds gave almost maximum peak heights at 70–80°C; 80°C was therefore selected as the optimum.

The concentration of potassium hexacyanoferrate(III) affected the peak heights (Fig. 5). Almost maximum peak heights were achieved at concentrations in the reagent solution ranging from 2 to 3 m*M* and a concentration higher than 5 m*M* caused a decrease in fluorescent peak heights, probably owing to the inner filter effect by hexacyanoferrate(III) and/or oxidative degradation of the fluorescent products, except that the peak height for MN increased only slightly on increasing the oxidant concentration even higher than 3 m*M*; 3 m*M* was selected as the optimum concentration.

Calibration graphs, detection limits and precision of the method

The calibration graphs were linear in the range (pmol per 50-μl injection volume) 5–1000 for DOMA, VMA and ferulic acid, 0.5–200 for L-DOPA and 0.1–100 for the other CA-related compounds and DHBA. The detection limits for the CA-related compounds, DHBA and ferulic acid and the precision of the method (repeatability) ($n = 5$) are shown in Table IV.

Selectivity of the method

Biologically important substances having neither a catechol nor a 4-hydroxy-3-methoxyphenyl moiety did not fluoresce when they were injected directly into the post-column reaction system in an amount of 1.0 nmol per 50-μl injection volume. The compounds tested were seventeen different L-α-amino acids, tyramine, histamine, serotonin, octopamine, creatine, creatinine, uric acid, putrescine, spermidine, spermine, acetone, formaldehyde, acetaldehyde, *p*-hydroxybenzaldehyde, lactic acid, pyruvic acid, α-ketoglutaric acid, phenylpyruvic acid, oxalic acid, acetic acid, D-glucose,

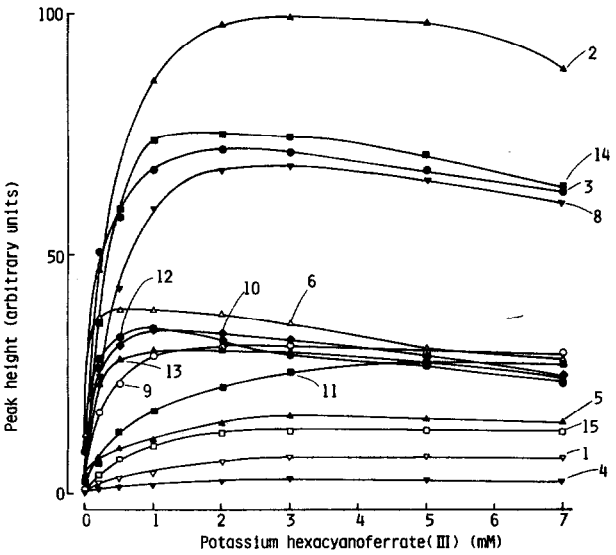

Fig. 5. Effect of potassium hexacyanoferrate(III) concentration in the reagent solution on the peak height. Portions (50 μl) of the same standard mixture as in Fig. 3 were subjected to HPLC. Peaks as in Fig. 3. Amounts (on-column); L-DOPA, DOMA and VMA, 50 pmol; others, 10 pmol.

TABLE IV

DETECTION LIMITS (SIGNAL-TO-NOISE RATIO = 3) FOR CATECHOLAMINE-RELATED COMPOUNDS, DHBA AND FERULIC ACID AND RELATIVE STANDARD DEVIATIONS (R.S.D.) OF THE PRESENT METHOD

Compound	*Detection limit (fmol on-column)*	*R.D.S.*[a] *(%)*
NE	30	0.9
E	60	2.2
DA	60	1.8
NM	60	2.1
M	60	1.5
3MT	70	1.1
L-DOPA	600	1.6
DOMA	1400	2.0
DOPAC	70	1.5
VMA	3900	1.6
HVA	120	1.7
DOPEG	20	1.2
MOPEG	30	0.7
MOPET	30	1.3
DHBA	70	1.3
Ferulic acid	900	1.8

[a] Portions (50 μl) of the same standard mixture as in Fig. 3 were subjected to HPLC.

D-fructose, D-galactose, D-ribose, D-glucosamine, maltose, sucrose, L-ascorbic acid, uracil, thymine, cytosine, adenine, guanine, cholesterol and cortisone. This suggests that the proposed method is selective for catechol and 4-hydroxy-3-methoxyphenyl compounds.

CONCLUSION

The highly selective and sensitive reaction of catechol compounds with DPE was successfully applied to the post-column derivatization of CA-related compounds, including 4-hydroxy-3-methoxyphenyl compounds, by introducing a coulometric oxidation technique. This HPLC method is comparable in terms of sensitivity to the HPLC–ED methods reported so far[4–17], and is has a higher selectivity, attributable to the highly selective fluorescence reaction. However, the sensitivity of the proposed method is lower than that of pre-column derivatization HPLC[19–22], which allows three CAs to be determined. The method should be applicable to the simultaneous determination of CA-related compounds in biological samples.

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