Simultaneous determination of urinary catecholamines and 5-hydroxyindoleamines by high-performance liquid chromatography with fluorescence detection

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A simple, selective and sensitive high-performance liquid chromatographic method with fluorescence detection is described for the simultaneous determination of catecholamines and 5-hydroxyindoleamines. The method is based on the derivatization of the amines with benzylamine in the presence of potassium hexacyanoferrate(III) under mild conditions. The resulting fluorescent derivatives are separated on a reversed-phase column, Cosmosil 5C18, using a mixture of acetonitrile and 10 mmol l^{-1} acetate buffer (pH 6.0) (35 + 65 v/v) as mobile phase, and are detected spectrofluorimetrically at 480 nm with excitation at 345 nm. The detection limits (signal-to-noise ratio = 3) for the amines are 2.8–236.0 fmol for an injection volume of 50 µl. The method was successfully applied to the determination of 5-hydroxyindol-3-ylacetic acid, serotonin, norepinephrine and epinephrine in human urine. The method is simple and does not require clean-up of urine samples.

Keywords: *Catecholamines; 5-hydroxyindoleamines; benzylamine; fluorescence derivatization; high-performance liquid chromatography; urine*

Catecholamines (CAs) and 5-hydroxyindoleamines (5-HIAs) are widely distributed in biological materials and play physiologically important roles in the body. In order to evaluate the physiological investigations, a simple, selective and highly sensitive method for the simultaneous determination of CAs and 5-HIAs is required.

High-performance liquid chromatographic (HPLC) methods with conventional electrochemical detection (ECD) have been widely used for the simultaneous determination of the amines.[1–5] However, the methods have limited sensitivity and selectivity. Thus, an improved HPLC method has been developed using three-potential electrochemical detection (ECD).[6] Even using this method, however, an inherent problem due to the short lifetime of electrochemical cells in the detector is still encountered. On the other hand, fluorescence detection has been focused upon because of its high sensitivity. Although both amines have native fluorescence, the fluorescence is very weak. Therefore, HPLC methods with native fluorescence have been employed for the determination of only a few amines, such as 5-hydroxyindoleacetic acid, which are present in large amounts in biological materials.[7] Furthermore, the methods require complicated clean-up procedures. Recently, a fairly sensitive post-column HPLC method with fluorescence detection has been reported for measuring CAs and 5HIAs simultaneously.[8] The method is based on the simultaneous derivatization of the amines with *meso*-1,2-diphenylethylenediamine as the fluorescence derivatization reagent. However, the reagent is highly sensitive for CAs, but not for 5HIAs.

We have previously developed a highly sensitive fluorimetric HPLC method for the determination of 5HIAs in human urine and plasma, based on the fluorescent reaction of 5HIAs with benzylamine.[9–11] Furthermore, recently we have reported that benzylamine also reacts with CAs under conditions different from those for 5HIAs to give highly fluorescent derivatives.[12] In this study, the optimum derivatization conditions for the simultaneous determination of CAs and 5HIAs were examined (Fig. 1),[13] and we have developed a highly sensitive and selective HPLC method with fluorescence detection for the simultaneous quantification of CAs and 5HIAs. In order to evaluate the usefulness of the method, it was applied to the HPLC determination of urinary CAs and 5HIAs.

Experimental

Reagents, solutions and apparatus

De-ionized, distilled water, purified with a Milli-Q II system (Millipore, Milford, MA, USA), was used for all aqueous solutions. Norepinephrine hydrogentartrate and dopamine hy-

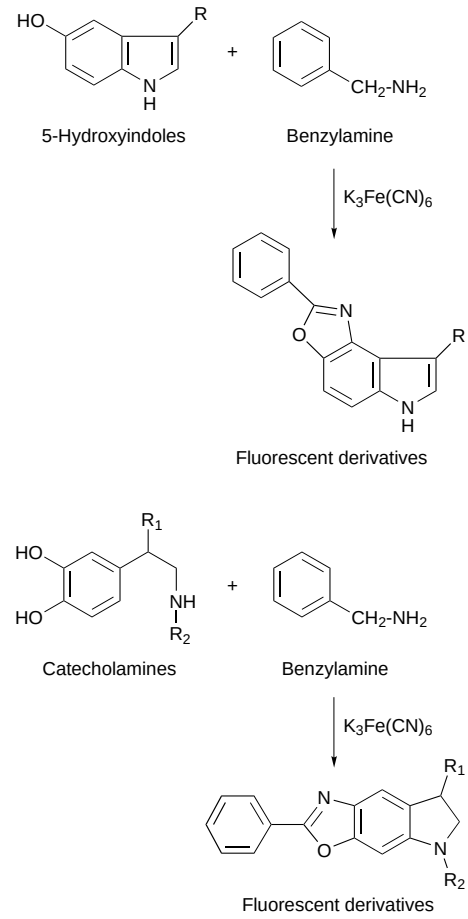

Fig. 1 Fluorescence derivatization of CAs and 5HIAs with benzylamine.

drochloride were purchased from Wako (Osaka, Japan). Isoproterenol hydrochloride was obtained from Nacalai Tesque (Kyoto, Japan) and epinephrine hydrogentartrate E from Sigma (St. Louis, MO, USA). 5-Hydroxytryptophan, 5-hydroxytryptamine (5HT), 5-hydroxyindol-3-ylacetic acid (5HIAA), 5-hydroxyindole-3-acetamide (5HA), 5-hydroxytryptophol and *N*-acetyl-5-hydroxytryptamine were obtained from Sigma. Standard solutions of these compounds were prepared in 0.1 mmol l^{-1} hydrochloric acid and kept frozen (−20 °C) in amber-coloured test-tubes. Benzylamine hydrochloride (Wako) was recrystallized twice from methanol. Benzylamine solution (0.15 mol l^{-1}) was prepared in aqueous 90% methanol and used within 1 week. Potassium hexacyanoferrate(III) (Wako) solution (50 mmol l^{-1}) was prepared in aqueous 50% methanol and used within 1 week. All other chemicals were of the highest purity available and were used as received.

Derivatization procedure

To a 200 μl portion of an aqueous test solution of CAs and 5HIAs, 200 μl of the benzylamine solution, 100 μl of a mixture of 0.1 mol l^{-1} CAPS buffer (pH 11.0) and methanol (3 + 7 v/v) and 100 μl of the potassium hexacyanoferrate(III) solution were added successively. The mixture was allowed to stand at 50 °C for 20 min. A 50 μl portion of the final reaction mixture was injected into the chromatograph.

For the reagent blank, 200 μl of water in place of the test solution were subjected to the same procedure.

Procedure for the determination of CAs and 5HIAs in human urine

Urine samples were obtained from healthy volunteers in our laboratories. Aliquots of about 20 ml from a single morning urine sample of the volunteers were stored and frozen at −20 °C until analysis. The urine was diluted 20-fold with water before analysis and passed through a disposable filter (0.45 μm, 13 mm id, cellulose acetate) (Millipore).

To 100 μl of the diluted urine sample, 100 μl of 100 nmol l^{-1} 5HA solution as an internal standard was added. The mixture (200 μl) was treated according to the derivatization procedures. A portion (50 μl) of the resulting mixture was injected into the chromatograph.

The amounts of 5HIAA, NE, 5HT and E in urine were calibrated by means of the internal standard method; 100 μl of of the internal standard solution added to the diluted normal urine sample in the derivatization procedure were replaced with 100 μl of the internal standard solution containing 13 fmol–2.0 nmol per 100 μl^{-1}. The urinary values of the amines were read from a calibration graph constructed using compounds added in various amounts to the urine.

Urinary creatinine concentrations were determined according to the Jaffe reaction, using a Wako creatinine test.

LC system and operating conditions

A Jasco (Tokyo, Japan) Model 880-PU high-performance liquid chromatograph equipped with an AS-4000 intelligent autosampler (50 μl loop) and a Jasco FP-920 spectrofluorimeter fitted with a 16 μl flow cell were used. The benzylamine derivatives of CAs and 5HIAs were separated on a Cosmosil 5C18 (5 μm) reversed-phase column (250 × 4.6 mm id) (Shiseido, Tokyo, Japan) by isocratic elution with a mixture of acetonitrile and 10 mM acetate buffer (pH 6.0) (35 + 65 v/v). It was operated at an excitation wavelength of 345 nm and an emission wavelength of 480 nm. The flow rate of the mobile phase was 1.0 ml min^{-1}. The column temperature was ambient (23 ± 2 °C).

Uncorrected fluorescence excitation and emission spectra of the eluate from the column were measured with a Hitachi Model 650-60 spectrofluorimeter fitted with a 20 μl flow cell; the spectral bandwidths were 5 nm in both the excitation and emission monochromators.

Results and discussion

HPLC and derivatization conditions

The optimum HPLC and derivatization conditions were examined by using a mixture of 5HIAA, 5HT, 5HA, NE and E (20 nmol l^{-1} each).

A typical chromatogram obtained with a mixture of the amines is shown in Fig. 2. A good separation was achieved on a Cosmosil 5C18 (5 μm) reversed-phase column (250 × 4.6 mm) by isocratic elution with a mixture of acetonitrile and 10 mM acetate buffer (pH 6.0) (35 + 65 v/v); the individual compounds gave single peaks.

The effect of the pH of the reaction mixture on fluorescence development was examined by using a 40 mmol l^{-1} Britton–Robinson buffer solution at various pH values [Fig. 3(A)]. The highest peaks were obtained at pH 7–8 for NE and at pH 8–12 for all 5HIAs tested. On the other hand, E afforded a larger peak height with increasing pH. Since E is present in human urine at an extremely low concentration, pH 11.0 , which is the optimum pH for E, was employed for the procedure. CAPS buffer (0.1 mol l^{-1}) solutions afforded peak heights about 1.2 times larger than the Britton–Robinson buffer (40 mmol l^{-1}). Water-soluble organic solvents such as methanol, acetonitrile, ethanol and dimethyl sulfoxide accelerated the derivatization of CAs and 5HIAs with benzylamine (Table 1). Of these solvents, methanol and acetonitrile were the most effective for all the CAs and 5HIAs tested; methanol was employed tentatively in the procedure. The methanol concentration affected the peak heights of the benzylamine derivatives; for the CAs and 5HIAs tested, 60–70 and 30–40% of methanol respectively, in the buffer solutions provided almost maximum peak heights [Fig. 3(B)]. Therefore, 0.1 M CAPS buffer (pH 11.0)– methanol (3 + 7 v/v), was used as the buffer solution for the simultaneous determination of the amines.

Concentrations of benzylamine of 0.05–0.20 and at 0.125 –0.25 mmol l^{-1} in the reagent solution provided almost maximum and constant peak heights for the CAs and 5HIAs tested, respectively [Fig. 3(C)]; a 0.15 mmol l^{-1} solution was adopted in the recommended procedure.

Oxidizing agents [sodium periodate, potassium hexacyanoferrate(III) and hydrogen peroxide] were used as accelerators

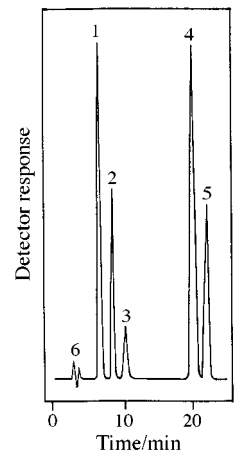

Fig. 2 Chromatograms obtained with a standard mixture of CAs and 5HIAs. A portion (200 μl) of a standard solution of 5HIAA, NE, 5HT, 5HA and E (16 pmol ml^{-1} each) was treated according to the derivatization procedure and the resulting mixture was subjected to HPLC. Peaks: 1 = 5HIAA; 2 = NE; 3 = 5HT; 4 = 5HA; 5 = E; 6 = reagent blank.

in the reaction between the amines and benzylamine. Of the three agents, potassium hexacyanoferrate(III) gave the most intense fluorescence for both CAs and 5HIAs. The largest peak heights were obtained at concentrations >50 mmol l^{-1} potassium hexacyanoferrate(III) in the solution for all amines tested except NE [Fig. (3D)]. NE gave the highest peak at 2 mmol l^{-1} potassium hexacyanoferrate(III) and the peak height was decreased at 5–40 mmol l^{-1}. The concentration range 50–150 mmol l^{-1} potassium hexacyanoferrate(III) in the solution provided almost constant peak heights for NE, and 50 mmol l^{-1} was employed in the procedure.

The fluorescence reactions of CAs and 5HIAs with benzylamine proceeded fairly rapidly even at 0 °C. Higher temperatures allowed the fluorescence peak heights to develop faster. Maximum and constant peak heights were achieved by heating for 20–60 min for CAs and for 5–60 min for 5HIAs at 50 °C (Fig. 4). The peak heights decreased on heating at temperatures >60 °C. Hence a temperature of 50 °C and a reaction time of 20 min were selected for simultaneous and reproducible determinations.

The benzylamine derivatives in the final solutions were stable and gave constant peak heights for at least 10 h in daylight at room temperature.

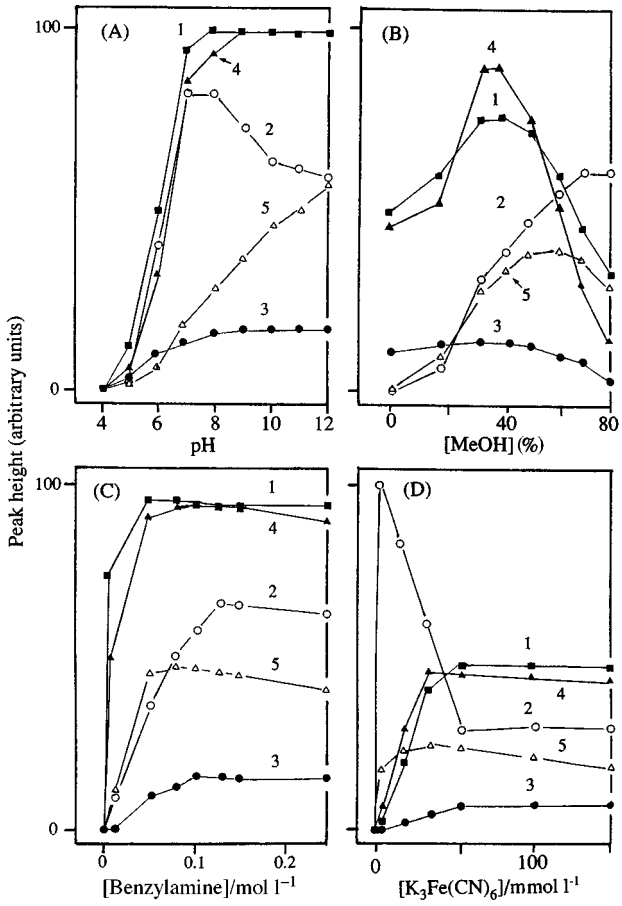

Fig. 3 Effects of (A) pH, (B) methanol, (C) benzylamine and (D) potassium hexacyanoferrate(III) concentrations in their solutions on the fluorescence derivatization. 1, 5HIAA; 2, NE; 3, 5HT; 4, 5HA; and 5, E.

Table1 Effect of organic solvents (70% v/v) in the buffer solution on the fluorescence peak height. The fluorescent peak height from *E* (methanol) was taken as 100

Organic solvent	5HIAA	5HT	5HA	NE	E
Methanol	198	32	198	123	100
Acetonitrile	209	43	211	253	91
Ethanol	73	35	148	405	75
Dimethyl sulfoxide	177	28	211	130	86
H_2O	136	11	118	0	0

Calibration graph, precision and detection limit

The calibration graphs were linear over the concentration range 2 fmol–250 pmol in a 50 μl injection volume for all CAs and 5HIAs tested. The precision was established by repeating 10 analyses concurrently on 20 pmol each of 200 μl^{-1}. The RSDs did not exceed 2.9% for all CAs and 5HIAs tested. The detection limits (signal-to-noise ratio = 3) are 2.8 (5HIAA), 18.0 (5HT), 2.8 (5HA), 4.8 (NE) and 5.6 fmol (E) per 50 μl (injection volume).

Reaction of other CAs and 5HIAs

Many CAs and 5HIAs reacted with benzylamine under the derivatization conditions to give the corresponding peaks in the chromatogram; the compounds and their retention times are given in Table 2. The excitation and emission spectra of the benzylamine derivatives eluted from the column were measured. The excitation and emission maxima were around 350 and 470 nm, respectively, independent of the CAs and 5HIAs tested. All CAs and 5HIAs except dopamine could be detected at high sensitivity (detection limits for the amines are 2.8–39.2 fmol on-column). The reason for this low sensitivity for dopamine remains unknown.

Reaction of substances other than CAs and 5-HIAs

The reactivity of other catechol compounds with benzylamine under the present derivatization conditions was studied. The compounds tested were 4-methylcatechol, 3,4-dihydroxycinnamic acid, catechol, pyrogallol, 3,4-dihydroxybenzylamine, 3,4-dihydroxybenzoic acid, L-dopa, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylethylene glycol, 2-hydroxyestrone and 4-hydroxyestrone. Benzylamine gave fluorescent peaks only for 4-methylcatechol, protocatechuic acid, protocatechualdehyde and 3,4-dihydroxycinnamic acid, and not for the other compounds, under the present derivatization conditions at a concentration of 10 μmol l^{-1}.

Other indoles (tryptophan, tryptamine, indole-3-ylacetic acid, 5-methoxy-L-tryptophan, 5-methoxytryptamine and 5-methoxyindol-3-ylacetic acid) exhibited no fluorescence under the derivatization conditions.

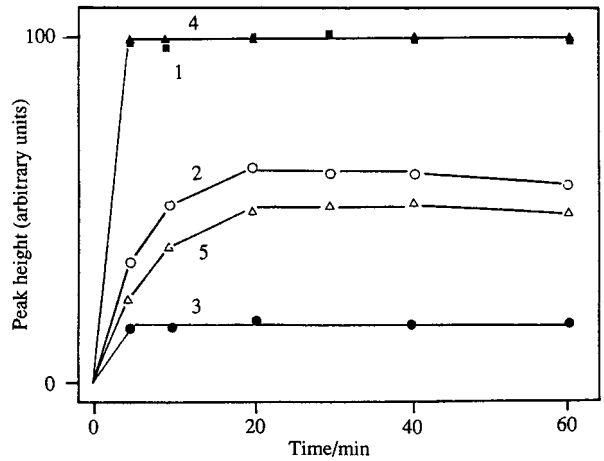

Fig. 4 Effect of reaction time at 50 °C on the fluorescence derivatization reaction. 1, 5HIAA; 2, NE; 3, 5HT; 4, 5HA; and 5, E.

Table 2 Excitation and emission maxima of the fluorescence, detection limits and retention times for catecholamines and 5-hydroxyindoles

Compound	Excitation maximum/nm	Emission maximum/nm	Detection limit/fmol[a]	Retention time/min
5-Hydroxyindole-3-acetic acid (5HIAA)	355	482	2.8	6.6
5-Hydroxytryptamine (serotonin, 5HT)	353	475	18.0	10.3
5-Hydroxyindole-3-acetamide (5HA)	353	479	2.8	20.3
5-Hydroxytryptophan (5HTP)	345	485	39.2	4.9
5-Hydroxyindole-2-carboxylic acid	339	458	16.8	5.5
N-Acetyl-5-hydroxytryptamine (*N*-Ac5HT)	345	485	4.8	32.7
5-Hydroxytryptophol (5HTOL)	341	487	4.4	34.5
Norepinephrine (NE)	355	481	4.8	8.8
Epinephrine (E)	356	485	5.6	21.4
Dopamine (DA)	354	479	236.0	39.8

[a] The amount in the injection volume (50 μl) giving a signal-to-noise ratio of 3.

Biologically important substances examined did not fluoresce at a concentration of 10 μmol l^{-1}. The compounds tested were CA metabolites (normetanephrine, metanephrine, 3-methoxytyramine, vanillylmandelic acid, homovanillic acid and 4-hydroxy-3-methoxyphenylethyleneglycol), kynurenine pathway compounds such as *N*-formyl-L-kynurenine, 3-hydroxy-L-kynurenine and 3-hydroxyanthranilic acid, 17 different L-α-amino acids, tryptamine, histamine, octopamine, creatine, creatinine, uric acid, putrescine, spermidine, spermine, acetone, formaldehyde, acetaldehyde, *p*-hydroxybenzaldehyde, lactic acid, pyruvic acid, α-ketoglutaric acid, phenylpyruvic acid, oxalic acid, homovanillic acid, acetic acid, D-glucose, D-fructose, d-galactose, D-ribose, D-glucosamine, maltose, sucrose, L-ascorbic acid, uracil, thymine, cytosine, adenine, guanine, cholesterol and cortisone.

Determination of CAs and 5HIAs in human urine

The proposed HPLC method was applied to the determination of CAs and 5HIAs in human urine. Fig. 5 shows a typical chromatogram obtained from human urine. The peaks for 5HIAA, 5HT, 5HA, NE and E were attributed on the basis of their retention times and fluorescence excitation and emission spectra of the eluates in comparison with the standard compounds, and also by co-chromatography of the standards and urine sample. The peaks for all amines tested were well separated within 25 min. Other peaks appeared in the chromatogram only when the urine sample was treated with benzylamine.

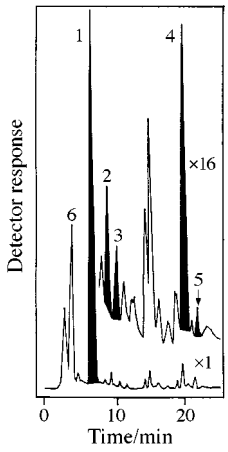

Fig. 5 Chromatograms obtained with a human urine sample. A portion (100 μl) of a diluted urine sample containing 5HIAA (45.8 nmol l^{-1}), 5HT (2.25 nmol l^{-1}), NE (442 pmol l^{-1}) and E (55 pmol l^{-1}) was treated according to the procedure, and the resulting mixture was subjected to HPLC. Peaks 1–6 as in Fig. 2; other peaks are unknown endogenous substances in human urine.

This indicates that there are a number of compounds in urine which react with the reagent which we were unable to identify. Linear relationships were observed between the peak height ratios and the concentrations of CAs and 5HIAs added to urine, up to at least 400 nmol ml^{-1} each; the linear correlation coefficients of the calibration graphs were 0.999 for all the amines. The linear ranges, the coefficients and the slopes of the calibration graphs were independent of the urine samples used. The within-day and between-day precision for NE, E and DA was examined by using a normal urine containing 5HIAA (45.8 nmol ml^{-1}), 5HT (2.25 nmol ml^{-1}), NE (442 pmol ml^{-1}) and E (55 pmol ml^{-1}). The RSDs did not exceed 3.0% for within-day determinations (n = 10) and 4.2% for between-day determinations (n = 8). The detection limits (signal-to-noise raitio = 3) for 5HIAA, 5HT, NE and E were 6.8, 43.2, 11.6 and 13.4 pmol ml^{-1} urine, respectively.

The free 5HIAA, 5HT, NE and E in urine samples from healthy volunteers were determined by the proposed method (Table 3). The mean values and their standard deviations for the amines from healthy subjects were in good agreement with those obtained by other workers.[14–17] In comparison with HPLC–ECD methods, the proposed method has sufficient selectivity and sensitivity for the determination of the amines in human urine.

Conclusion

Both CAs and 5HIAs react readily with benzylamine under mild conditions to form strongly fluorescent compounds, that can be

Table 3 Urinary excretion of 5-hydroxyindoles and catecholamines from healthy volunteers

Subject	Age	Sex	5HIAA[a]	5HT[a]	NE[a]	E[b]
1	22	Male	38.7	1.53	0.48	23.1
2	22	Male	51.3	0.67	0.36	25.4
3	22	Male	48.2	0.82	0.20	16.6
4	23	Male	34.0	1.60	0.55	32.5
5	23	Male	43.5	2.14	0.45	54.4
6	24	Male	42.4	2.70	0.46	25.4
7	24	Male	49.2	1.12	0.47	27.8
8	24	Male	45.0	0.91	0.23	25.4
9	38	Male	48.7	3.49	0.27	16.0
10	47	Male	53.9	3.82	0.27	20.7
11	69	Male	58.6	2.04	0.44	17.8
12	21	Female	64.4	2.33	0.35	20.1
13	21	Female	66.0	2.53	0.37	34.9
14	21	Female	66.0	3.01	0.27	17.8
15	22	Female	57.1	2.20	0.29	21.3
Mean			51.3	2.06	0.36	25.4
SD			10.0	0.96	0.11	10.1

[a] μmol per gram creatinine. [b] nmol per gram creatinine.

separated by reversed-phase HPLC. In order to examine the suitability of the method, it was applied to the HPLC determination of some CAs and 5HIAs in human urine. Five CAs and 5HIAs were quantified successfully by using the HPLC method. The HPLC methods permits the direct and simultaneous determination of CAs and 5HIAs in human urine without any clean-up procedures. The method should be useful for biological and clinical investigations of CAs and 5HIAs.

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