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Fluorescence derivatizing procedure for 5-hydroxytryptamine and 5-hydroxyindoleacetic acid using 1,2-diphenylethylenediamine reagent and their sensitive liquid chromatographic determination

Masaaki Kai[a,*], Hiroko Iida[b], Hitoshi Nohta[b], Myung Koo Lee[c], Kazuko Ohta[a]

[a]*School of Pharmaceutical Sciences, Nagasaki University, Bunkyo-Machi, Nagasaki 852-8521, Japan*
[b]*Faculty of Pharmaceutical Sciences, Kyushu University, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*
[c]*College of Pharmacy, Chungbuk National University, Kaeshin-Dong, Cheongju 361-763, South Korea*

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Abstract

A pre-column derivatization method using a fluorogenic reagent, 1,2-diphenylethylenediamine (DPE) was studied for the sensitive HPLC determination of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA), which are biosubstances used in the diagnosis of several diseases. For the quantitative determination, the biogenic indole compounds were converted to their corresponding fluorescent derivatives with DPE in the presence of potassium hexacyanoferrate (III) at room temperature, and then the derivatives were separated by reversed-phase liquid chromatography with fluorescence detection. The chromatographic detection limits of the fluorescent peaks at a signal-to-noise ratio of 3 were 0.3 fmol for 5-HT and 0.2 fmol for 5-HIAA. The proposed method permits the simultaneous quantification of 5-HT and 5-HIAA at concentrations higher than 2.4 n*M* in human urine without a clean-up procedure.

Keywords: 5-Hydroxytryptamine; 5-Hydroxyindoleacetic acid; 1,2-Diphenylethylenediamine

1. Introduction

An increased concentration of 5-hydroxytryptamine (5-HT), one of the metabolites of tryptophan, has been recognized in the physiological fluids of patients with carcinoid syndrome [1], migraine [2] and schizophrenia [3]. 5-Hydroxyindoleacetic acid (5-HIAA), the major metabolite of 5-HT, is also an active indicator of the diagnosis of carcinoid tumor [4, 5]. Therefore the measurement of 5-HT and 5-HIAA in human body fluids is necessary for clinical and pathological investigations.

Several methods have been reported for the quantification of 5-HT and 5-HIAA in human biological specimens. The most common methods are by high-performance liquid chromatography (HPLC) with either fluorescence detection without derivatization [4–6] or electrochemical detection [1, 7–10]. Indole compounds emit a weak native fluorescence at emission wavelengths (λ_{em}) of 320~350 nm with irradiation at excitation wavelengths (λ_{ex}) of 280~300 nm, and they are electrochemically oxidized at a

*Corresponding author.

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potential of +0.5~0.8 V vs. a Ag/AgCl electrode. These detection methods are simple and permit the detection of picomole quantities of the indole compounds. They are not selective for 5-hydroxyindole compounds, however, and biological specimens occasionally require a clean-up procedure such as liquid–liquid or liquid–solid phase extraction in order to avoid interference from biological matrices.

Recently, a fluorescence derivatization reaction has become a viable choice for the sensitive and selective HPLC detection of biogenic compounds [11]. An organic reagent, 1,2-diphenylethylenediamine (DPE), produces fluorescent derivatives of catecholamines (Fig. 1A) [12–14] and it also yields unknown fluorescent derivatives with 5-hydroxyindole compounds [15]. Therefore, we studied the ability of the DPE reaction to detect 5-HT and 5-HIAA. It has recently been reported that benzylamine and its analogues react with 5-hydroxyindole compounds and produce fluorescent or chemiluminescent derivatives [16–19]. The derivatives of 5-hydroxyindole compounds with DPE (Fig. 1B) may be the same as those (Fig. 1C) [16, 17] produced by the benzylamine reaction on the basis of the chromatographic separation of the fluorescent derivatives.

In this paper, we report on the optimum conditions for the pre-column derivatization of 5-hydroxyindole compounds with DPE, in order to sensitively quantify 5-HT and 5-HIAA in human urine by HPLC with fluorescence detection. For the chromatographic quantification, 5-hydroxyindole (5-HI) was used as an internal standard (I.S.). The fluorescent product of 5-HT was analyzed by liquid chromatography–mass spectrometry (LC–MS) to determine its molecular mass.

2. Experimental

2.1. Chemicals and solutions

5-HT, 5-HIAA, and 5-HI were purchased from Nakarai Chemicals (Kyoto, Japan). DPE was purchased from Tosoh (Tokyo, Japan). Other chemicals were of the highest purity available and were used as received. Doubly distilled water was used. A stan-

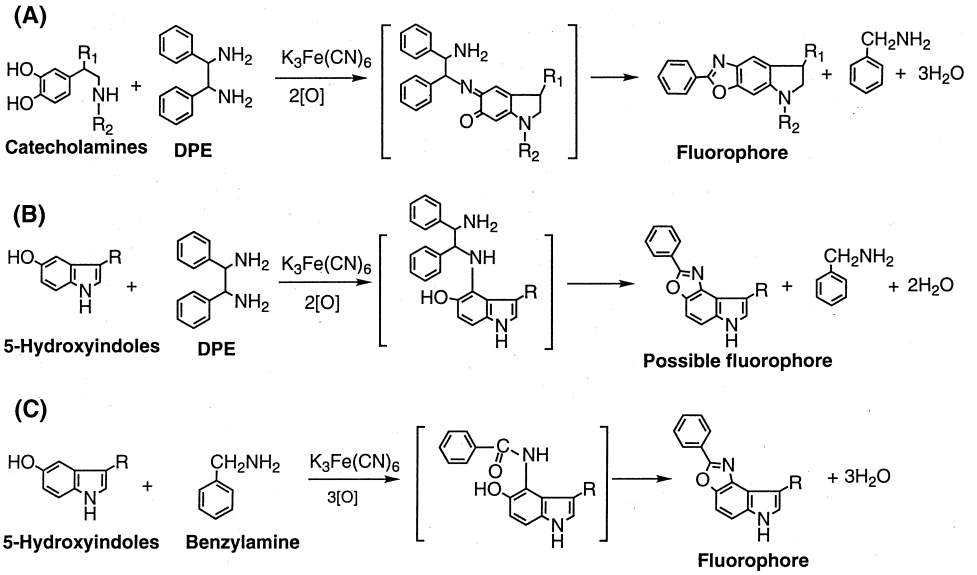

Fig. 1. Reaction schemes for fluorescence derivatizations of (A) catecholamines with DPE, (B) 5-hydroxyindole compounds with DPE, and (C) 5-hydroxyindole compounds with benzylamine.

dard solution of 5-hydroxyindole compounds was prepared with water.

2.2. Preparation of the urine sample and fluorescence derivatization

A 24-h urine sample was collected from a healthy volunteer, and then diluted 20 times with water. The diluted urine was passed through a disposable filter (pore size 0.45 μm) before use. Fifty μl of 5.0 *M* 5-HI (I.S.) and 150 μl of H_2O (or a standard mixture of 5-HT and 5-HIAA for the calibration curve) were added to 800 μl of the diluted urine. This mixture was used for the fluorescence derivatization.

Successively, 10 μl of 0.3 *M* sodium borate buffer (pH 8.0), 50 μl of 30 m*M* $K_3Fe(CN)_6$, and 240 μl of 40 m*M* DPE dissolved in dioxane were added to 100 μl of the urine mixture or a standard mixture of 5-HT, 5-HIAA and 5-HI. The mixture was allowed to stand for 20 min at ambient temperature (26°C). After the reaction, the mixture (10–100 μl) was analyzed by HPLC with fluorescence detection.

2.3. Liquid chromatography and spectrofluorimetry

The HPLC system consisted of a PU-980 pump (Jasco, Tokyo, Japan), an LG-980-02 ternary gradient unit (Jasco) equipped with a low-pressure degasser, a 7725 injector (Rheodyne, Cotati, CA, USA), and an FP-920 fluorescence detector (Jasco). For the quantitative analysis of 5-HT and 5-HIAA, the fluorescent derivatives in the reaction mixture were separated on a TSKgel ODS-80TM reversed-phase column (250×4.6 mm I.D., 5-μm particle size, Tosoh, Tokyo, Japan) by linear gradient elution with 55 to 68% (v/v) acetonitrile in the mobile phase containing 10% 0.2 *M* sodium acetate buffer (pH 5.0) for 26 min. The column was washed with a high concentration (80%) of the organic modifier for 15 min to remove additional hydrophobic substances after separation of the urine sample, and then reconstituted with the initial mobile phase for at least 15 min. The flow-rate of the mobile phase was 0.7 ml min^{-1}. The fluorescent peaks were monitored at λ_{ex} 330 nm and λ_{em} 455 nm.

The uncorrected fluorescence spectra of the peak fractions were measured with an F-2000 spectrofluorometer (Hitachi, Tokyo, Japan). Spectral bandwidths of 5 nm were used for both the excitation and emission monochromators.

2.4. LC–MS

The column effluent corresponding to the fluorescent peak of 5-HT (50 m*M* used) was collected after isocratic separation with a mobile phase composed of acetonitrile–0.2 *M* triethylamine acetate (pH 5.0)–water (57:10:33). The fraction was concentrated by reduced pressure. The concentrated fraction was rechromatographed and then the column effluent was introduced into a Hitachi M1000 mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) interface. The mass detection conditions of the fluorescent product were almost the same as those reported previously for the LC–MS determination of a cysteine protease inhibitor [20].

3. Results and discussion

3.1. Chromatographic detection

First, we determined whether DPE gives a single fluorescent derivative for 5-HT under the reaction conditions previously optimized for catecholamines [12, 13]. When an aqueous solution (300 μl) of 5-HT and epinephrine (250 n*M* each) was warmed at 37°C for 40 min with 100 μl of 0.1 *M* DPE (in acetonitrile), 10 μl of 36 m*M* potassium hexacyanoferrate(III), and 300 μl of acetonitrile [12, 13], the derivatives of 5-HT and epinephrine produced were separated by reversed-phase LC and each fluorescent peak was detected by fluorimetry (Fig. 2A).

The fluorescence spectra of the peaks corresponding to 5-HT and epinephrine were measured individually; the maximum wavelengths of λ_{ex} and λ_{em} were 330 nm and 455 nm for the 5-HT peak, and 345 nm and 485 nm for the epinephrine peak, respectively. The spectra and the retention time of the fluorescent derivative of 5-HT with DPE were identical to those [16] produced by the benzylamine reaction. The molecular mass of the 5-HT product was measured by LC–MS. The protonated molecular ion $[M+H]^+$ of the product was observed at m/z

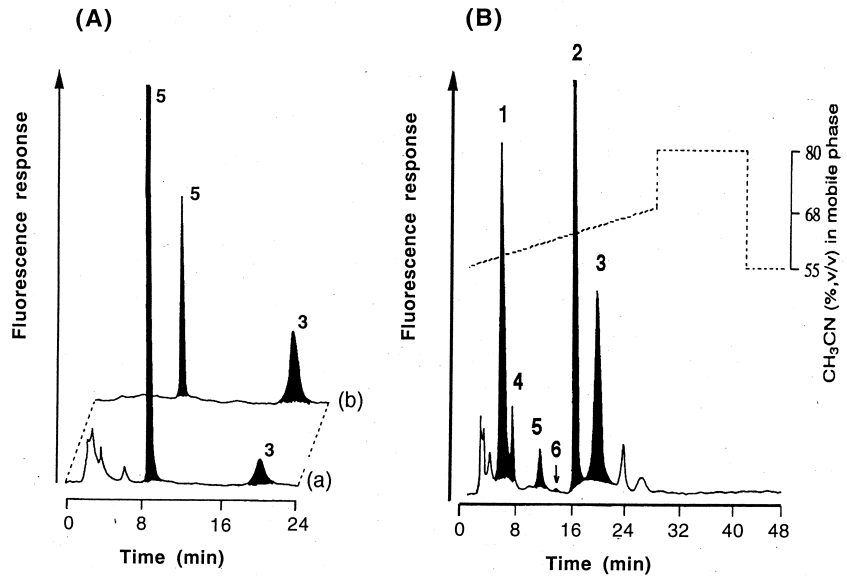

Fig. 2. Comparison of the chromatograms of (A) 5-HT and epinephrine (250 n*M* each) obtained by a previous derivatization method [12, 13] for catecholamines, and of (B) 5-HIAA, 5-HI, 5-HT, epinephrine, norepinephrine and dopamine (200 n*M* each) obtained by the present derivatization method. Peaks: 1=5-HIAA; 2=5-HI; 3=5-HT; 4=norepinephrine; 5=epinephrine; 6=dopamine; others=reagent blank. Detection wavelengths: chromatogram (A)–(a), λ_{ex} 345 nm and λ_{em} 485 nm; chromatograms (A)–(b) and (B), λ_{ex} 330 nm and λ_{em} 455 nm. HPLC conditions: chromatogram (A), isocratic elution of a mobile phase composed of acetonitrile–50 m*M* Tris–hydrochloric acid buffer (pH 7.0) (60:40, v/v) and other conditions were the same as those in Section 2; chromatogram (B), see Section 2.

278 as a base peak in the mass range m/z 120–350. These results suggest that the fluorescent product of 5-HT produced by the DPE reaction was the same as the product of the benzylamine reaction, as shown in Fig. 1B and C.

The fluorescent derivatives of 5-hydroxyindole compounds produced by the DPE reaction were detected at λ_{ex} 330 nm and λ_{em} 455 nm (Fig. 2A–b). When the 5-HT product was detected at λ_{ex} 345 nm and λ_{em} 485 nm of the wavelengths optimized for the catecholamine derivative, the 5-HT peak was approximately three-times lower (Fig. 2A–a).

Fig. 2B shows a chromatogram of a standard mixture of 5-HT, 5-HIAA, 5-HI, epinephrine, norepinephrine and dopamine (200 n*M* each). Each fluorescent derivative was obtained under the optimum reaction and detection conditions recommended for 5-hydroxyindole compounds. Linear gradient elution with acetonitrile as an organic modifier in the mobile phase containing sodium acetate buffer (pH 5.0) gave satisfactory resolution of the derivatives produced by the DPE reaction. The peak heights for catecholamines in the chromatogram were 50- to 100-times lower than those previously reported [12, 13] for the sensitive determination of catecholamines by the DPE reaction, because the present method is optimized for 5-hydroxyindole compounds.

3.2. Reaction conditions

The derivatization reaction is performed with a buffer, an oxidant and DPE. Sodium borate buffer afforded the maximum production of the fluorescent derivative of 5-HT at pH 7.5–9.0 (Fig. 3A) in the concentration range of 0.1–0.4 *M* (Fig. 3B). A 0.3 *M* buffer (pH 8.0) is recommended for the derivatization reaction. The use of 0.3 *M* sodium phosphate buffer (pH 6–7) or 0.3 *M* sodium acetate buffer (pH 4–6) instead of the borate buffer resulted in reduced yields (60–70%). Of the oxidizing agents tested [sodium metaperiodate, hydrogen peroxide, and potassium hexacyanoferrate(III)], 35 m*M* hexacyanoferrate gave the highest production of the derivative (Fig. 3C). The derivative was not pro-

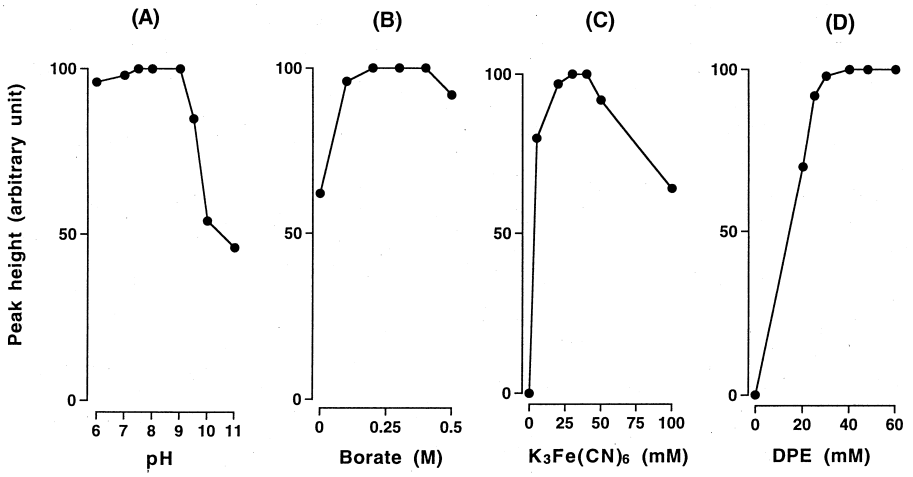

Fig. 3. Effects of (A) pH of sodium borate buffer, and concentrations of (B) borate buffer (pH 8.0), (C) potassium hexacyanoferrate(III) and (D) DPE on the fluorescence derivatization of 5-HT.

duced with the others (20 m*M*). DPE concentrations higher than 35 m*M* gave the maximum peak height (Fig. 3D); the recommended concentration is 40 m*M* DPE.

An organic water-miscible solvent is required to dissolve DPE. Dioxane, acetonitrile, and methanol all accelerated the derivatization reaction (Fig. 4A). A maximum peak height was obtained with 60% (v/v) dioxane in the reaction mixture. The fluorescence reaction appeared to proceed rapidly at 26°C (Fig. 4B). Therefore, the reaction was carried out for 20 min at an ambient temperature of 26±2°C.

At a concentration of 1.0 m*M*, the following biosubstances did not fluoresce under the recommended reaction conditions: L-ascorbic acid, L-alanine, L-glutamic acid, L-serine, L-threonine, L-tryptophan, creatinine, D-glucose, D-galactose, thymidine, cytidine, guanosine, 2-deoxyribose, choles-

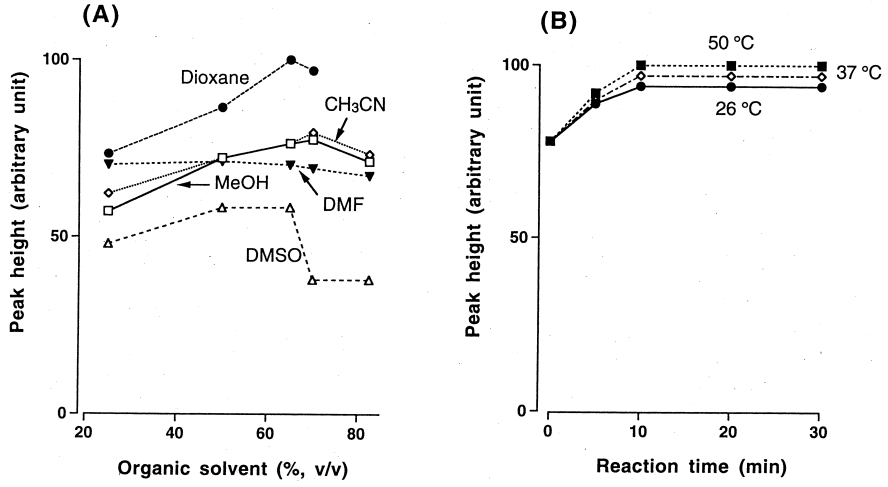

Fig. 4. Effects of (A) concentrations of water-miscible organic solvents, and of (B) reaction time and temperature on the fluorescence derivatization of 5-HT.

terol, *N*- acetylneuraminic acid and hippuric acid. Therefore, this derivatization method was selective for 5-hydroxyindole and catechol compounds.

3.3. Determination of 5-HT and 5-HIAA in human urine

Endogenous 5-HIAA and 5-HT in 20-fold diluted urine from a healthy person were detected fluorimetrically, as shown in Fig. 5A. 5-HI was used as an I.S. because it is not present in urine. The urinary concentration of 5-HT (peak 3) was very low compared to that of 5-HIAA (peak 1). Therefore, chromatographic separation was carried out twice for each sample. The chromatogram in Fig. 5A–a was obtained with the detector sensitivity increased 10-times and with 10-times the volume of the reaction mixture used for the chromatogram in Fig. 5A–b.

The conditions required to separate the 5-HIAA peak from large peaks with retention times of 2–6 min were investigated. Unknown fluorescent substances including the reagent blank produced these large peaks. The mobile phase used was a mixture of acetonitrile and sodium acetate buffer (pH 5.0). When Tris–hydrochloric acid buffer (pH 7 or 8) was used instead of the acetate buffer, the 5-HIAA peak was eluted early and overlapped with the peaks of the unknown fluorescent substances. However, the use of acidic acetate buffer (pH 4 or 5) in the mobile phase effectively separated the 5-HIAA peak from the large peaks, because the low pH increased its retention time. At pH 4.0, the 5-HT peak overlapped with a small peak due to the reagent blank of DPE. Therefore, we used acetate buffer with pH 5.0 in the mobile phase.

The calibration graphs of 5-HT and 5-HIAA in the urine sample were made by the standard addition method (Fig. 5B), plotting the ratios of their peak heights against the I.S. peak height ($n=2$ each). The two calibration curves showed a linear relationship between the ratios and concentrations of 10–60 μ*M* 5-HIAA and 20–80 n*M* 5-HT, respectively. The chromatographic fluorescence detection limits at a signal-to-noise ratio of 3 for 5-HT and 5-HIAA were 0.3 and 0.2 fmol per injected volume (10 μl), respectively. These amounts correspond to respective concentrations of 120 and 80 p*M* in the 20-fold diluted urine. Therefore, this method can measure urinary concentrations higher than 2.4 n*M*.

Intra-assay precision was established by repeated determinations ($n=10$) of 5-HIAA and 5-HT in pooled urine. The relative standard deviations were

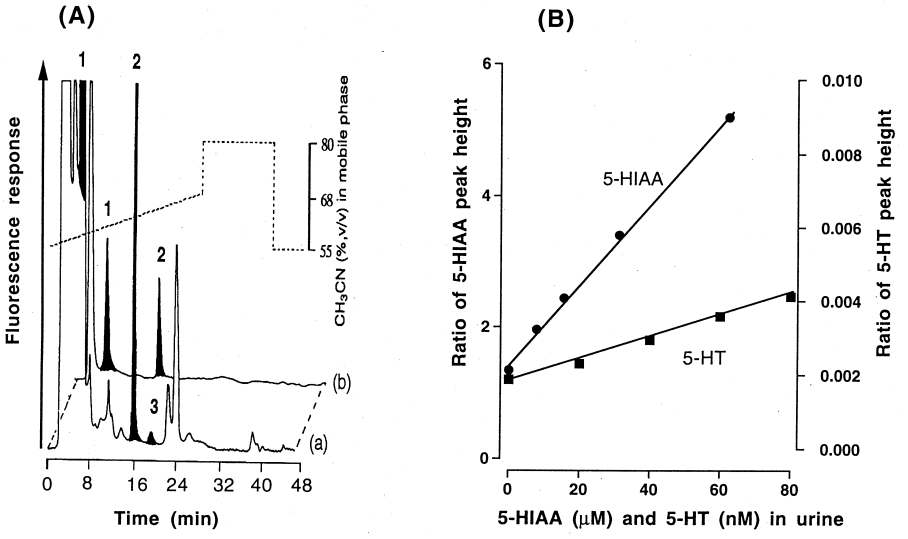

Fig. 5. (A) Typical chromatograms of healthy human urine, and (B) calibration graphs for 5-HT and 5-HIAA in the urine sample analyzed by standard addition method. Peaks: 1=5-HIAA; 2=5-HI (I.S.); 3=5-HT; 4=reagent blank. Detection conditions: chromatogram (A)–(a), ×10 of the detector response with 100 μl injection volume; chromatogram (A)–(b), ×1 of the detector response with 10 μl injection volume. HPLC conditions: see Section 2.

1.9% and 4.2% for the mean concentrations of 22 μ*M* 5-HIAA and 50 n*M* 5-HT, respectively. To evaluate the inter-assay precision on six consecutive days, we analyzed 18 samples from another pooled urine sample. The relative standard deviations were 2.4% and 4.9% for the mean concentrations of 38 μ*M* 5-HIAA and 78 n*M* 5-HT, respectively. The values for the endogenous 5-HIAA concentrations in the urine samples of healthy volunteers agree well with published data [4–6, 8]. The concentration of 5-HT is similar to that obtained by the electrochemical detection HPLC method [9], but slightly lower than that obtained by the radioenzymatic method [21].

In conclusion, the fluorogenic reagents of DPE (final 24 m*M*) produced fluorescent derivatives of 5-hydroxyindole compounds after 20 min in the presence of potassium hexacyanoferrate (final 3.75 m*M*), sodium borate (final 7.5 m*M*, pH 7–9), and dioxane (final 60%, v/v) at room temperature. The derivatives of 5-HIAA and 5-HT formed could be separated simultaneously by reversed-phase HPLC and selectively detected at λ_{ex} 330 nm and λ_{em} 455 nm. The proposed derivatization technique detects 5-HT and 5-HIAA at sub-femtomole levels. This high sensitivity permits the use of diluted urine specimens without the need for tedious clean-up procedures to reduce the interference from biological matrices needed to avoid damaging the column packing material. The selective detection method is unlikely to be affected by drugs administered to patients, since most drugs are not derivatized by DPE. This method should prove useful for both biological investigations such as microdialysis and for practical applications in medical diagnosis.

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