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Short communication

# Liquid chromatography method for detecting native fluorescent bioamines in urine using post-column derivatization and intramolecular FRET detection

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**Abstract**

Liquid chromatography (LC) with fluorescence detection is described for simultaneous determination of native fluorescent bioamines (indoleamines and catecholamines). This is based on intramolecular fluorescence resonance energy transfer (FRET) in an LC system following post-column derivatization of native fluorescent bioamines' amino groups with *o*-phthalaldehyde (OPA). OPA fluorescence was achieved through an intramolecular FRET process when the molecules were excited at maximum excitation wavelength of the native fluorescent bioamines. Bioamines separated by reversed-phase LC on ODS column were derivatized with OPA and 2-mercaptoethanol. This method provides sufficient selectivity and sensitivity for the determination of normetanephrine, dopamine, tyrosine, 5-hydroxytryptamine, tryptamine, and tryptophan in healthy human urine without prior sample purification.


*Keywords:* FRET; Liquid chromatography; Post-column derivatization; Indoleamines; Catecholamines; *o*-Phthalaldehyde

## 1. Introduction

Native fluorescent bioamines, indoleamines and catecholamines, play physiologically important roles in the body. Biological levels of these amines are related to various diseases, such as Parkinson disease [1], hypertension [2], neuroblastoma [3], and pheochromocytoma [3,4], and are useful analytical tools in the diagnosis of these diseases. Thus, a simple method for the simultaneous determination of indoleamines and catecholamines is needed.

A number of methods have been reported for the determination of these monoamines. The most widely used methods are based on liquid chromatography (LC) using native fluorescence detection [5], electrochemical detection (ECD) [6], and mass spectrometry (MS) [7]. Although native fluorescence detection provides reproducible results and requires simpler analytical systems, it is neither sensitive nor selective. Only a few fluorogenic derivatization methods have been reported for amino moieties [8–10]. These methods, however, are not selective for indoleamines and catecholamines. On the other hand, highly selective LC methods based on specific fluorescence derivatizations have been developed [11–13]. Although these approaches are sensitive and specific for their respective target amines, they cannot be applied to the simultaneous determination of a number of bioamines. ECD is also used most widely, but it tends to lack reproducibility—mainly because of hysteretic degradation of the electrode. MS detection is selective, sensitive, and highly reliable, but its apparatus and operating cost are too expensive for routine analyses.

In our previous work, we developed a highly selective determination method for indoleamines and catecholamines through pre-column derivatization with *o*-phthalaldehyde (OPA) and LC using intramolecular fluorescence resonance energy transfer (FRET) detection (Fig. 1) [14]. The fluorescence intensities of indoleamines and catecholamines, except for those of tyrosine (Tyr) and normetanephrine (NM), were more intense at excita-

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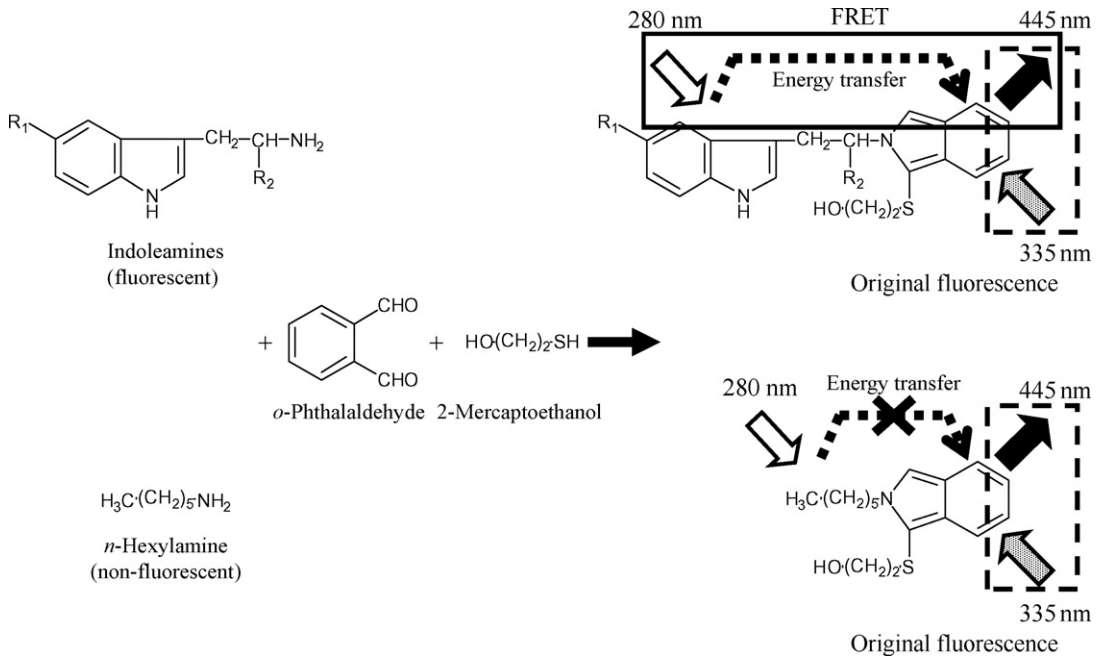


Fig. 1. FRET-inducing derivatization of indoleamines with OPA.

tion/emission wavelengths (nm) of 280/445 (FRET detection) than at 335/445 (conventional fluorescence detection). On the other hand, the fluorescence intensities of native nonfluorescent amines in the sample were weaker when monitored with FRET detection than when monitored with conventional fluorescence detection. Thus, this method could be used to the determination of indoleamines in human urine without prior sample purification. It was, however, difficult to apply this derivatization method to routine analysis of simultaneous and continuous determination of indoleamines and catecholamines because their pre-labeled OPA derivatives were unstable in the reaction mixture. Recently, however, an online pre-column derivatization method with OPA using a programmable autosampler was reported for amino acid analysis [15].

In the present work, we report a selective LC method for the simultaneous determination of indoleamines [tryptophan (Trp), 5-hydroxytryptophan (5-HTP), tryptamine (TA), and 5-hydroxytryptamine (5-HT)] and catecholamines [Tyr, 3,4-dihydroxyphenylalanine (DOPA), dopamine (DA), norepinephrine (NE), and NM] by online post-column derivatization with OPA and intramolecular FRET detection. The present method is validated with respect to selectivity, sensitivity, and precision. Furthermore, we applied this method to the simultaneous determination of NM, DA, Tyr, 5-HT, TA, and Trp in human urine without prior sample purification. The method presented here is the first report of FRET detection with post-column derivatization.

## 2. Experimental

### 2.1. Reagents and solutions

5-HTP, 5-HT creatinine sulfate complex, TA hydrochloride, NM hydrochloride, and 2-mercaptoethanol (2-ME) were purchased from Sigma (St. Louis, MO, USA). DOPA, DA hydrochloride, NE hydrogen tartrate, OPA, and trifluoroacetic acid (TFA) were obtained from Wako (Osaka, Japan). Trp, Tyr, and *n*-hexylamine (HA) were purchased from Kishida (Osaka, Japan). All organic solvents were of LC grade. Other chemicals were of the highest purity available and were used as received. These reagents and solvents are toxic if eyes, lungs or skin are exposed to them, and therefore, they should be carefully handled in accordance with the most current material safety data sheets. Distilled water, which was further purified with a MilliQ gradient system (Millipore, Milford, MA, USA), was used to prepare all aqueous solutions.

Stock solutions (1.0 mM) of the indoleamines, catecholamines, and HA were prepared in water and were stored at 4 °C. These solutions were stable for at least 1 month and were diluted further with water to the required concentrations before use. The post-column reagent solution was 5 mM OPA and 10 mM 2-ME in a mixture of methanol, 50 mM sodium hydroxide, and 0.1 M sodium tetraborate (50:41:9, v/v). This post-column solution was used within 24 h.

### 2.2. Urine samples

Urine samples were obtained from healthy volunteers ($n = 7$) in our laboratory. Aliquots of *ca*. 10 mL from single-morning samples were stored and frozen at −20 °C until analyzed. Amines in urine samples were stable for at least 1 week. Before analysis, a 20 μL aliquot of human urine and 280 μL of water were pipetted successively into an Ultrafree-MC cartridge (10000 NMWL, cellulose, 45 mm × 10.6 mm O.D., Millipore). The cartridge was centrifuged at 2500 × *g* for 10 min, and the filtrate (20 μL) from the cartridge was injected into the LC system.

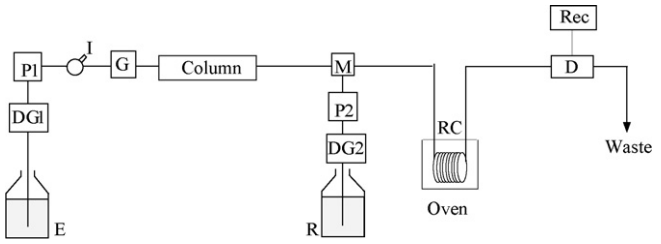


Fig. 2. Schematic flow diagram of the post-column LC-fluorescence system for the determination of indoleamines and catecholamines. $P_1$ and $P_2$, LC pumps; $DG_1$ and $DG_2$, degassers; I, injector (20 μL); G, guard column; Column, analytical column; M, mixing device; RC, reaction coil (10 m × 0.5 mm I.D., PEEK; 60 °C); D, fluorescence detector; Rec, integrator; E, mobile phase (1.0 mL/min) = (A) 0.04% TFA–methanol (99:1, v/v) and (B) 0.04% TFA–methanol (90:10, v/v) [0 → 12 min, 0% (B); 12.0 → 12.1 min, 0 → 100% (B); 12.1 → 60.0 min, 100% (B)]; R, fluorogenic reagent solution (0.5 mL/min) = 5 mM OPA and 10 mM 2-ME in methanol–50 mM NaOH–0.1 M $Na_2B_4O_7$ (50:41:9, v/v).

### 2.3. LC system and conditions

#### 2.3.1. Conditions for the detection of OPA derivatives

Fig. 2 shows a schematic representation of an LC post-column fluorescence system. The LC system comprised two Jasco (Tokyo, Japan) PU-980 chromatograph pumps, a Rheodyne (Cotati, CA, USA) model 7125 syringe-loading sample injector equipped with a 20-μL sample loop, a Jasco DG-980-50 online degasser, a Jasco LG-980-02 low-pressure gradient unit, and a Hitachi (Tokyo, Japan) L-7485 spectrofluorometer fitted with a 12-μL flow-cell. The slit-widths of the excitation and emission monochromators were set at 18 nm. The native fluorescent bioamines were separated on a reversed-phase YMC-Pack ODS-AQ (250 mm × 4.6 mm I.D.; particle size: 5 μm; YMC, Kyoto, Japan) equipped with a guard-column ODS-AM (23 mm × 4.0 mm I.D.; YMC) by gradient elution with (A) 0.04% TFA–methanol (99:1, v/v) and (B) 0.04% TFA–methanol (90:10, v/v) as the mobile phase. Gradient elution began at 0% B, was kept constant at 0% for 12.0 min, was increased to 100% over 0.1 min, was kept constant at 100% for 47.9 min, and was then reduced to 0% over 1 min. The total analysis time was 75 min. The flow rate of the mobile phase was set at 1.0 mL/min, and the column temperature was ambient (23 ± 2 °C).

The effluent from the LC column was mixed with a post-column reagent solution containing 5 mM OPA and 10 mM 2-ME in the mixture of methanol, 50 mM sodium hydroxide, and 0.1 M sodium tetraborate (50:41:9, v/v) using a T-type mixing device at a flow rate of 0.5 mL/min. The mixture was then passed through a reaction coil (10 m × 0.5 mm I.D., PTFE tubing, GL Science, Tokyo, Japan) immersed in a 60 °C column oven (860-CO, Jasco). The resulting fluorescence was monitored at excitation and emission wavelengths of 280 and 445 nm, respectively. For comparative studies, the conventional fluorescence of OPA derivatives was monitored at excitation and emission wavelengths of 335 and 445 nm, respectively. Chromatograms were recorded on a Hitachi D-2500 integrator, and all peaks were quantified by the valley-to-valley and valley-to-baseline method.

#### 2.3.2. Conditions for native fluorescence detection

The indoleamines and catecholamines standards were injected into LC system described in Section 2.3.1 without post-column reagent solution and reaction coil. The fluorescence detector was operated at the excitation and emission wavelengths of 280 and 335 nm, respectively.

### 2.4. Quantification and validation

The native fluorescent bioamines amounts were calculated using the calibration curve by the peak area of OPA-labeled bioamines. The linearity of the method for native fluorescent bioamines was checked by preparing a calibration graph at four different concentrations: 0–100 nmol/mL urine for NM, DA, 5-HT, and TA, and 0–500 nmol/mL urine for Tyr and Trp. The equations of the calibration curves were calculated by least-squares analysis. The method was validated using the urine samples (four levels) used for calibration graph.

## 3. Results and discussion

### 3.1. LC separation

Typical chromatograms obtained with a standard mixture of indoleamines (TA, Trp, 5-HT, and 5-HTP), catecholamine-related compounds (Tyr, DOPA, DA, NE, and NM), and HA are shown in Fig. 3. Good separations of the amines were achieved on a YMC-Pack ODS-AQ column using gradient elution from aqueous 1% methanol to 10% in 60 min. The fluorescence intensities of the indoleamines and catecholamines, except for those of Tyr and NM, were more intense at excitation/emission wavelengths (nm) of 280/445 (FRET detection, Fig. 3A) than at 335/445 (conventional fluorescence detection, Fig. 3B) (Table 1). On the other hand, the fluorescence intensities of HA and other native nonfluorescent amines in the sample were weaker when monitored with FRET detection than when monitored with conventional fluorescence detection. Thus, this post-column intramolecular FRET-inducing derivatization method permits the selective and sensitive determination of indoleamines and catecholamines in samples that also con-

Table 1
Detection limits of OPA derivatives and non-derivatized amines

| Compound | OPA derivative | | Non-derivatized amine |
|---|---|---|---|
| | FRET[a] (nM) | Conventional fluorescence[a] (nM) | Native fluorescence[a] (nM) |
| Trp | 1.5 | 3.8 | 60 |
| 5-HTP | 1.3 | 3.1 | 17 |
| 5-HT | 1.4 | 2.9 | 8.5 |
| TA | 1.6 | 3.9 | 34 |
| Tyr | 1.9 | 1.8 | 42 |
| DOPA | 1.7 | 2.9 | 30 |
| DA | 3.5 | 4.0 | 28 |
| NE | 1.9 | 2.9 | 24 |
| NM | 1.5 | 1.5 | 15 |
| HA | 5.9 | 2.9 | – |

[a] Signal-to-noise ratio of 3.

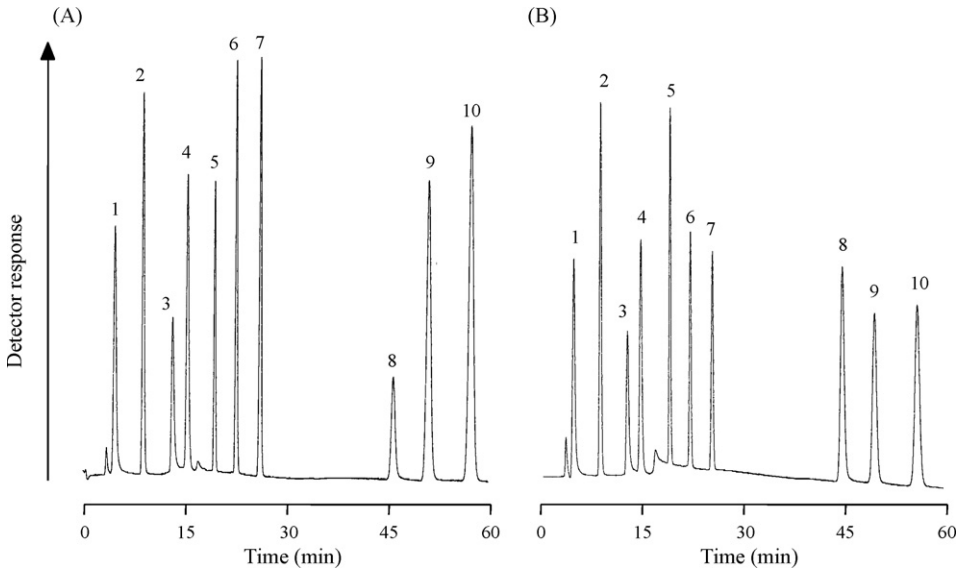


Fig. 3. Chromatograms obtained using a standard mixture of indoleamines and catecholamines (50 pmol each on the column). (A) FRET and (B) conventional fluorescence detections monitored at excitation wavelengths of 280 and 335 nm, respectively; emission wavelength: 445 nm. Peaks: 1, NE; 2, NM; 3, DA, 4, DOPA; 5, Tyr; 6, 5-HT; 7, 5-HTP; 8, HA; 9, TA; and 10, Trp.

tain nonfluorescent amines, as is the case with the pre-column derivatization method [14].

### 3.2. Optimum conditions for OPA derivatization

The concentrations of OPA and 2-ME had effects on fluorescent derivatization. Concentrations of OPA in the range of 5–10 mM provided maximum and constant peak areas; thus, a 5-mM solution was used. 2-ME afforded maximum and constant peak areas at concentrations of 5–15 mM in the reagent solution; thus, a concentration of 10 mM was adopted for the derivatization reagent.

It is well known that the derivatization reaction between primary amines and OPA is accelerated in the presence of a weakly alkaline solution. We observed maximum peak areas when sodium hydroxide (50 mM)–sodium tetraborate (0.1 M) (82:18, v/v) was used as the alkaline solution. Water-soluble organic solvents such as dimethylsulfoxide, tetrahydrofuran, methanol, 2-propanol, and dioxane also accelerated the derivatization of native fluorescent bioamines with OPA. Of these solvents, dimethylsulfoxide and methanol gave the most intense peak in the chromatogram. However, dimethylsulfoxide caused an increase in the number of background noises in the chromatogram. Hence, we selected methanol. The methanol concentration affected the peak responses of OPA derivatives, and 40–60% of methanol in the post-column reagent solution provided maximum and constant peak areas. Therefore, a mixture of methanol, 50 mM sodium hydroxide, and 0.1 M sodium tetraborate (50:41:9, v/v) was used for the post-column derivatization reagent solution.

The fluorescence reactions of the native fluorescent bioamines with OPA proceeded fairly rapidly, even at 0 °C. Higher temperatures allow the fluorescence peak areas to develop faster. However, temperatures higher than 80 °C caused the peak areas to decrease. Hence, 60 °C was selected for the post-column derivatization. The effect of the length of the reaction coil (0.5 mm I.D.) on fluorescence development was examined in the range 5–15 m; a 10-m coil gave the highest and most reproducible peak responses.

### 3.3. Method validation

#### 3.3.1. Specificity

Many nonfluorescent amines reacted with OPA under the present conditions to give the corresponding fluorescence derivatives. However, the compounds that we tested – ammonia and amino acids other than Trp and Tyr – had retention times that were different from those of the indoleamines and catecholamines; thus, they did not interfere with the analysis. Moreover, the signals of these nonfluorescent amines under FRET detection decreased less than 50% in comparison with their signals obtained under conventional fluorescence detection.

Other biologically important substance or environmental compounds did not give any peaks under the present conditions at a concentration of 10 μM. The compounds tested were α-keto acids (α-ketoglutaric acid and phenylpyruvic acid), other acids (acetic acid, palmitic acid, stearic acid, homovanillic acid, and 5-hydroxyindole-3-acetic acid), sugars (D-glucose, D-fructose, D-galactose, D-ribose, *N*-acetyl-D-glucosamine, maltose, and sucrose), nucleic acid bases (adenine, guanine, thymine, cytosine, and uracil), and other compounds (methanol, acetone, phenol, cholesterol, and urea).

#### 3.3.2. Linearity

The relations between the amounts of individual native fluorescent bioamines and their peak areas were linear over the concentration range of 20–40,000 nM. The linear correlation coefficients ($n = 3$) were >0.997 for all the native fluorescent bioamines.

### 3.3.3. Precision

The within-day precision was established by repeated determinations ($n = 6$) using standard mixtures of the native fluorescent bioamines (1 μM). The relative standard deviations of the peak areas did not exceed 5.8% for any of the bioamines that we examined.

### 3.3.4. Detection limits

Table 1 presents the detection limits we obtained for the OPA derivatives detected by using FRET wavelengths and conventional wavelengths and for the underivatized bioamines detected by native fluorescence. The detection limits (signal-to-noise ratio = 3) of the native fluorescent bioamines using FRET derivatization were in the range of 1.3–3.5 nM; this range is 1–3 times lower than that obtained with the use of conventional fluorescence detection. Sensitivity of this method was almost similar to that obtained when using the pre-column derivatization method [14] and other published method [7]. Thus, our method enabled the highly sensitive determination of native fluorescent bioamines upon their derivatization with OPA and subsequent FRET detection.

## 3.4. Application to human urine

To investigate the practicality of the post-column FRET derivatization method for use in biological analysis, the method was applied to the determination of native fluorescent bioamines in human urine. Fig. 4 presents the typical chromatograms obtained with human urine from healthy subject. Peaks 1–6 in Fig. 4 were identified as the OPA derivatives of NM, DA, Tyr, 5-HT, TA, and Trp, based on (a) their retention times compared with those of the standard compounds and (b) co-elution of the standard and the sample with the use of various gradient patterns. The fluorescence intensities of six native fluorescent bioamines, except for those of Tyr and NM, were more intense when monitored at an excitation wavelength of 280 nm (Fig. 4A) than when monitored at 335 nm (Fig. 4B). On the other hand, other amines present in urine gave weaker peaks with FRET detection than with conventional fluorescence detection. Thus, the FRET detection method provided a simple chromatogram for biological analyses.

The calibration curves of six native fluorescent bioamines in urine were linear ($r > 0.998$) over a concentration range of 0–100 nmol/mL urine for NM, DA, 5-HT, and TA, and 0–500 nmol/mL urine for Tyr and Trp. No significant changes in the slope of the curves were observed with urine used. The recoveries (mean ± S.D.) of NM, DA, 5-HT, and TA (3 nmol/mL urine), and of Tyr and Trp (200 nmol/mL urine) added to human urine were 90.2 ± 6.1, 102.1 ± 2.1, 101.3 ± 2.7, 100.4 ± 5.1, 82.3 ± 7.0, and 97.4 ± 1.2%, respectively ($n = 3$); the relative standard deviation of recovery were 6.8, 2.1, 2.7, 5.1, 8.5, and 1.2%, respectively. The concentrations (mean ± S.D.) of NM, DA, Tyr, 5-HT, TA, and Trp that we measured using the proposed method were 1.32 ± 0.40, 1.14 ± 0.24, 150 ± 38, 0.48 ± 0.11, 0.51 ± 0.28, and 106 ± 47 nmol/mL urine from healthy subjects ($n = 7$). These values agree well with reported data [14,16–21].

The intramolecular FRET-inducing post-column derivatization method permits the highly sensitive determination of the native fluorescent bioamines and can simplify the determination of NM, DA, Tyr, 5-HT, TA, and Trp in human urine because no special pretreatment procedures are needed.

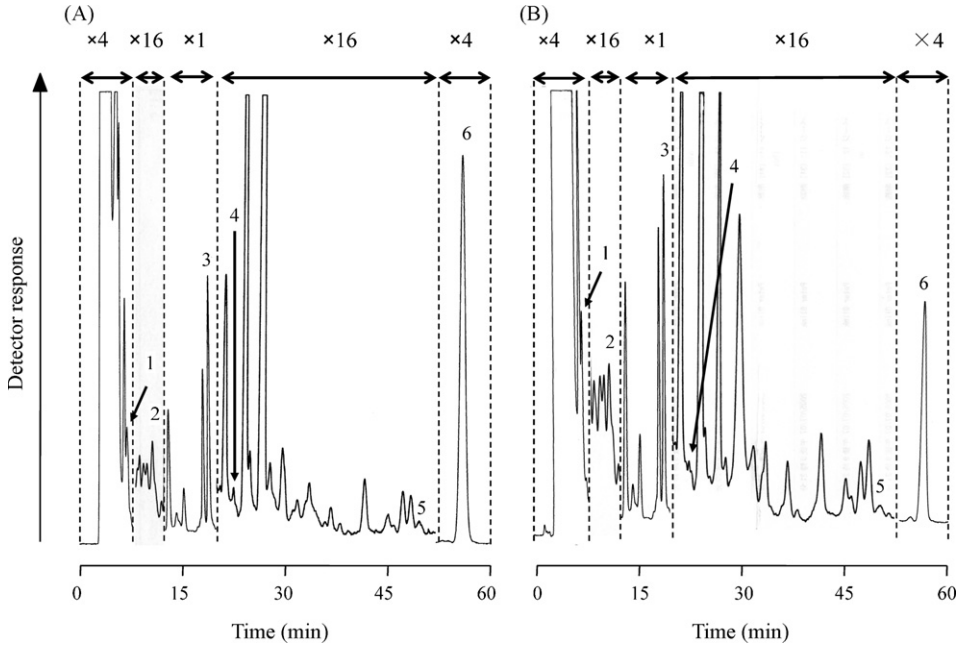


Fig. 4. Chromatograms obtained using a sample of urine from a healthy human. (A) FRET and (B) conventional fluorescence detections monitored at excitation wavelengths of 280 and 335 nm, respectively; emission wavelength: 445 nm. Peaks and concentrations (nmol/mL urine): 1, NM (1.08); 2, DA (1.06); 3, Tyr (214); 4, 5-HT (0.41); 5, TA (0.37); and 6, Trp (98.9).

## 4. Conclusions

We have developed a simple method for the simultaneous determination of indoleamines and catecholamines. This method is based on intramolecular FRET-induced derivatization and results in the highly selective determination of indoleamines and catecholamines. Moreover, FRET detection provides more intense signals than do conventional fluorescence detection and native fluorescence detection in the absence of derivatization. In fact, our method is sufficiently selective and sensitive to allow the simple assay of urine from healthy humans. Therefore, we believe that this method will be useful for biological and clinical investigations of native fluorescent bioamines.

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