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A simple liquid chromatographic method based on intramolecular excimer-forming derivatization and fluorescence detection for the determination of tyrosine and tyramine in urine

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Abstract

A liquid chromatographic (LC) method for sensitive and selective fluorometric determination of *p*-hydroxyphenylethylamino group containing compounds is described. This method is based on an intramolecular excimer-forming fluorescence derivatization with a pyrene reagent, 4-(1-pyrene)butanoyl chloride, followed by reversed-phase LC. The analytes, containing an amino moiety and a phenolic hydroxyl moiety in a molecule, were converted to the corresponding dipyrene-labeled derivatives by one-step derivatization. The dipyrene-labeled derivatives afforded intramolecular excimer fluorescence (440–540 nm), which can clearly be discriminated from the normal fluorescence (360–420 nm) emitted from reagent blanks. The derivatives of tyrosine and tyramine could be separated by reversed-phase LC on ODS column under conditions of isocratic elution. The detection limits (signal-to-noise ratio = 3) for tyrosine and tyramine were 4.5 and 2.6 fmol per 20 μL injection, which corresponded to analyte concentrations of 0.9 and 0.5 nM, respectively.

Keywords: *p*-Hydroxyphenylethylamino group containing compounds; Tyrosine; Tyramine; Liquid chromatography; Derivatization; Urine; Excimer fluorescence

1. Introduction

Tyrosine has *p*-hydroxyphenylethylamino group structure in a molecule and is one of the most important amino acids functioned as precursors of catecholamines, melanin, thyroid hormones, and several opiate peptides that have a tyrosyl residue at the terminal amino group of the amino acid sequence [1–3]. Analysis of tyrosine related compounds is of continued interest due to the role they play in the central nervous system of numerous organisms [4,5].

There are many determination methods for *p*-hydroxyphenylethylamino group containing compounds including tyrosine by liquid chromatography (LC) with several detection systems [6–16]. In general, UV detection is not sensitive and selective, and electrochemical detection tends to lack reproducibility mainly due to hysteretic degradation of the electrode. Although the LC with mass spectrometry (MS) method is sensitive and highly reliable, its apparatus and operating cost are too expensive for routine analyses. On the other hand, some tyrosine related compounds have native fluorescence themselves but the fluorescent intensity is not so strong. Thus, fluorescence derivatization procedure to convert weakly fluorescent compounds into the strong fluorescent derivatives is needed for highly sensitive analysis [7–9,14]. However, the major defect of these methods in the highly sensitive assay is that other compounds are also derivatized to afford the same fluorescent spectra as those of tyrosine related compounds, which cause interfering peaks in the chromatogram. Therefore, they require highly sophisticated LC separation conditions and/or sample clean-

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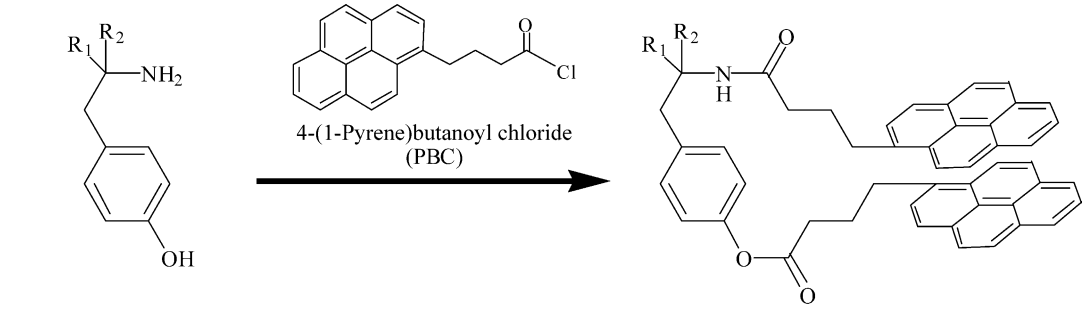

Compound	R_1	R_2
Tyrosine	H	COOH
2-Amino-3-(4-hydroxyphenyl)-2-methylpropanoic acid	CH_3	COOH
Tyramine	H	H

Fig. 1. Intramolecular excimer-forming fluorescence derivatization of tyrosine related compounds with 4-(1-pyrene)butanoyl chloride.

up procedures are inevitable to eliminate the interferences from other contaminated compounds. Whereas, only a few selective fluorescent derivatization methods for tyrosine related compounds have been introduced [17,18].

Recently, we developed several highly selective and sensitive methods for determination of polyamines by LC with fluorescence detection as their intramolecular excimers formed derivatization [19–24]. The derivatization reagent, 4-(1-pyrene)butanoic acid *N*-hydroxysuccinimide ester, was shown to react with primary and secondary amino moieties of polyamine molecules. The resulting polypyrene-labeled derivatives of polyamines provided intramolecular excimer fluorescence at the wavelength region of 440–520 nm, which was shifted markedly to the higher emission wavelengths as compared to the wavelengths of the non-derivatized pyrene monomers themselves and monopyrene-labeled monoamines (360–420 nm). This chemistry allowed to analyze selectively polyamines even in the complex samples containing monoaminergic compounds. More recently, we have found that 4-(1-pyrene)butanoyl chloride (PBC) reacts with not only polyamines, but also phenol compounds such as bisphenols [25–28].

In the present paper, we describe an intramolecular excimer-forming derivatization method for fluorometric determination of *p*-hydroxyphenylethylamino group containing compounds, which have an amino moiety and a phenolic hydroxyl group in a molecule, based on their one-step derivatization with PBC (Fig. 1). The new method allows a highly sensitive and selective determination of tyrosine related compounds. We confirmed the structure of dipyrene-labeled derivative by LC–MS. Furthermore, this fluorometric method was successfully applied to the determination of tyrosine and tyramine in normal human urine.

2. Experimental

2.1. Reagents and solutions

All chemicals and solvents were of the highest purity available, and were used as received. Distilled water, purified using a Milli-QII system (Millipore, Milford, MA, USA), was used for all aqueous solutions. Tyrosine and tyramine hydrochloride were purchased from Wako Pure Chemicals (Osaka, Japan), and DL-2-amino-3-(4-hydroxyphenyl)-2-methylpropanoic acid (methyltyrosine) was from ICN (Irvine, CA, USA). PBC was obtained from Toronto Research Chemicals (North York, Ontario, Canada) and used without further purification.

Stock solutions (1.0 mM) of tyrosine related compounds were prepared in water and stored at −20 °C. These solutions were stable for at least 1 week and diluted further with acetonitrile to the required concentrations before use. The 5 mM solution of PBC dissolved in acetonitrile was usable for at least 3 days when stored at −20 °C. Aqueous solutions of potassium carbonate (1 M) and taurine (10 mM) were stored at room temperature and used up for 3 days.

2.2. Urine sample

Normal urine samples were obtained from healthy volunteers in our laboratory. The urine samples (ca. 5 mL) collected from single-morning urination of the volunteer were frozen at −20 °C and stored until analysis. The urines were diluted 100-fold with acetonitrile before analysis, and the diluted urine samples were cooled in ice-water and passed through a disposable filter (0.45 μm, 13 mm i.d., cellulose acetate; Millipore). The filtrate was subjected to the derivatization.

2.3. Derivatization procedure

To a 200-μL aliquot of standard solution or diluted urine sample placed in a 3.5-mL Reacti-vial (Pierce, Rockford, IL, USA), 10 μL of 1 M potassium carbonate and 200 μL of 5 mM PBC solution were added. The vial was sealed tightly and heated at 60 °C for 60 min. After cooling in ice-water, 390 μL of 10 mM taurine was added to stop the derivatization and to decompose the PBC, and a 20-μL portion of the reaction mixture was injected into the chromatograph.

2.4. LC with fluorescence detection system

An isocratic LC system consisted of a Jasco (Tokyo, Japan) PU-1580 liquid chromatograph pump, a Rheodyne (Cotati, CA, USA) Model 7725i syringe-loading sample injector equipped with a 20-μL sample loop, a Jasco DG-1580-53 on-line degasser, a reversed-phase TSKgel Super-ODS (100 mm × 4.6 mm i.d.; particle size, 2 μm; Tosoh, Tokyo, Japan), and a Hitachi (Tokyo, Japan) L-7485 spectrofluorometer fitted with a 12-μL flow-cell. A mixture of acetonitrile–water–acetic acid (70:30:1, v/v) was used as a mobile phase. The flow-rate of the mobile phase was set at 1.0 mL/min, and the column temperature was ambient (23 ± 3 °C). The fluorescence detector was operated at excitation and emission wavelengths of 345 and 475 nm, respectively, and the slit-widths of both monochromators were set at 15 nm.

In the assay of urine sample, the column was washed at the end of each measurement by passing 95% (v/v) acetonitrile through them at a flow rate of 1.0 mL/min for 15 min and reconditioned to initial conditions for additional 30 min.

2.5. LC–MS system

A Finnigan (San Jose, CA, USA) LCQ, ion-trap mass spectrometer equipped with an electrospray ionization (ESI) interface, was used in place of a fluorescence detector. Other separation conditions were the same as described in the previous section. The effluent from the LC column was directly introduced to the LC–MS interface without splitting. The ion source voltage and temperature of the heated capillary were at −3.5 kV and 280 °C, respectively. For the MS/MS analysis of peak component, collision energy and precursor ion were set at 35 V and 720.8 ($[M-H]^-$ of dipyrene-labeled tyrosine), respectively.

3. Results and discussion

3.1. LC separation

We used tyrosine, methyltyrosine, and tyramine as the model compounds of *p*-hydroxyphenylethylamino group containing compounds. Under the LC conditions with the mobile phase at neutral pH, the derivatives of tyrosine and methyltyrosine were co-eluted with the early-eluting reagent blanks. They were moderately retained on a reversed-phase column using the acidic mobile phase by suppressing ionization of the carboxylic group. The best separation of the PBC derivatives of tyrosine, methyltyrosine, and tyramine, and reagent blank components (intermolecular excimer fluorescence peaks of PBC and its hydrolysate, 4-(1-pyrene)butanoic acid) was achieved within 45 min on an ODS column using acetonitrile–water–acetic acid (70:30:1, v/v) as the mobile phase. Acetic acid in the mobile phase did not affect the fluorescence properties (shape of spectra and intensity) of the dipyrene-labeled derivatives. A typical chromatogram obtained with a standard mixture of tyrosine, methyltyrosine, and tyramine is illustrated in Fig. 2.

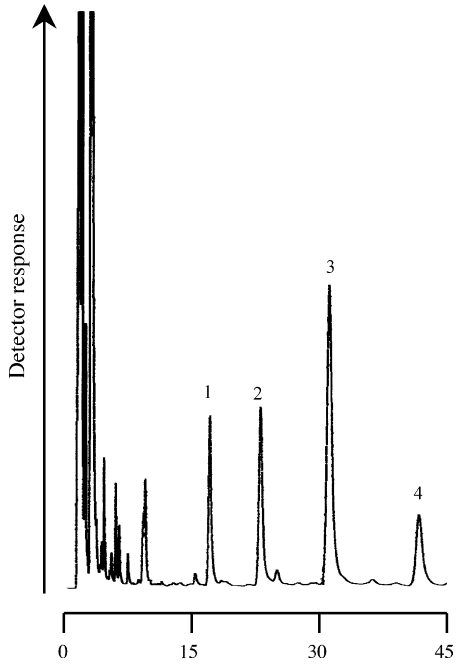

Fig. 2. Chromatogram obtained with the pyrene-labeled tyrosine related compounds (5 pmol each on column). Peaks: 1, tyrosine; 2, DL-2-amino-3-(4-hydroxyphenyl)-2-methylpropanoic acid; 3, tyramine; 4, PBC; others, reagent blanks.

3.2. Structural analysis by LC–MS

The structure of pyrene-labeled tyrosine was confirmed by LC–MS with the ESI interface at the negative ion mode. The selected ion chromatogram suggests that dipyrene-labeled derivative was formed from tyrosine (M.W.; 721.8). Mass spectrum for the peak component also provided the corresponding quasi-molecular ion ($[M-H]^-$) as a base peak. When detected at *m*/*z* ($[M-H]^-$) corresponding to the monopyrene-labeled derivative of tyrosine and unlabeled tyrosine itself, no significant peak was observed in the respective ion chromatograms. Thus, we guessed that both of the amino moiety and phenolic hydroxyl group in tyrosine were derivatized quantitatively with PBC under the present derivatization conditions. The proposed derivatization reaction of

PBC with tyrosine related compounds yielding the excimer-forming fluorescence derivatives are presented in Fig. 1.

3.3. Derivatization conditions

Optimization studies for the derivatization were carried out to maximize the excimer fluorescence peak area for the tyrosine, methyltyrosine, and tyramine. The derivatization reaction was proceeded in the presence of organic solvents; methanol, ethanol, acetonitrile, dimethylsulfoxide, tetrahydrofuran, *N*,*N*-dimethylformamide or their mixtures. The best results were obtained when acetonitrile was used for the preparation of the PBC solution. A PBC concentration of 3–10 mM in the reagent solution provided maximal peak areas for all of them, therefore, a 5-mM PBC solution was chosen as optimal concentration and used in all further analyses. It is well known that the derivatization reactions of amines or phenols with carbonyl chloride proceed under alkaline conditions. Of the tested bases (pyridine, triethylamine, sodium acetate, sodium hydrogen carbonate, sodium carbonate and potassium carbonate), sodium and potassium carbonate gave maximum peak areas. PBC reacted with amino moieties at higher concentration of potassium carbonate [26,27], whereas with phenolic hydroxyl moieties at concentrations of 0.8–1.0 M [25,28]. Thus, a 1 M potassium carbonate was tentatively selected. The derivatization reaction proceeded more rapidly with increasing the reaction temperature in the range 20–80 °C, but a temperature higher than 80 °C caused decrease in the peak areas of the polyamines. The optimal conditions were selected at 60 °C and for the reaction times of 60 min.

After the derivatization procedure, taurine (monoamine) was added to stop the derivatization. In the reaction, a large excess of taurine reacted with the remaining reagent, PBC, to give monopyrene-labeled derivative, which fluoresced at 360–420 nm and was eluted at 2–5 min. In fact, the PBC peak (peak 4 in Fig. 2) was remarkably decreased in intensity by the addition of taurine. This addition did not cause the decrease of the stability of pyrene-labeled derivatives and the appearance of undesired peaks. The pyrene-labeled tyrosine, methyltyrosine, and tyramine in the final reaction mixture were stable, and still gave constant fluorescence intensities after standing for at least 1 week in the dark at room temperature.

3.4. Calibration graph, precision, and detection limit

The relationships between the amounts of individual *p*-hydroxyphenylethylamino group containing compounds and the peak heights were linear over the concentration range of 5 nM to 10 μM in the standard solution (25 fmol to 50 pmol per 20 μL injection volume); the linear correlation coefficients were more than 0.999 ($n = 3$) for tyrosine, methyltyrosine, and tyramine. The between-day precision values were established by repeated determinations ($n = 6$) using the mixtures of standard compounds (10 nM and 1.0 μM each in a sample solution, 50 fmol and 5 pmol, respectively, each per 20 μL injection volume); the relative standard deviations (R.S.D.s) were within 3.8%.

Table 1
Detection limits and retention times of biogenic amines and phenols

Compound	Detection limit[a] (fmol)	Retention time (min)
Tyrosine	4.5	15.4
Methyltyrosine	4.5	23.8
Tyramine	2.6	31.5
3,4-Dihydroxyphenylalanine	9.3	62.4
Dopamine	–	–
Norepinephrine	18	47.3
Epinephrine	12	38.9
3-Methoxytyramine	8.8	24.0
Normetanephrine	4.9	15.1
Metanephrine	8.9	22.1
3,4-Dihydroxyphenylacetic acid	0.5	17.9
3,4-Dihydroxyphenyl glycol	2.0	24.9
3,4-Dihydroxymandelic acid	22	16.3
5-Hydroxytryptophan	59	20.9
5-Hydroxytryptamine	200	24.2

[a] Defined as the amount per injection volume (20 μL) giving a signal-to-noise ratio of 3.

The detection limits (signal-to-noise ratio = 3) for tyrosine, methyltyrosine, and tyramine were 4.5, 4.5, and 2.6 fmol per 20 μL injection volume, respectively. These sensitivities are higher than those of LC methods with electrochemical detection and fluorescence derivatization [7–10,14,15].

3.5. Reaction of other substances

Some other biogenic polyamines, polyphenols, and aminophenols reacted with PBC under the proposed derivatization conditions to afford corresponded polypyrene-labeled derivatives. Table 1 lists the retention times and the detection limits for the pyrene-labeled derivatives of amines and phenols. Several compounds were detected with excimer fluorescence derivatization, and highly sensitive detection in the range of 0.5–200 fmol could be achieved. Some compounds, however, overlapped with each other under the proposed LC conditions.

The following biological compounds having only one amino (phenol) moiety or none in the molecule, at a concentration of 10 nmol/mL, did not afford any peak under the present conditions; the compounds tested were neutral and acidic amino acids, ammonia, acetylcholine, indoles (tryptamine, 5-hydroxyindole-3-acetic acid, *N*-acetylserotonin, 5-methoxytryptamine, and melatonin), α-keto acids (α-ketoglutaric acid and phenylpyruvic acid), other acids (acetic acid, palmitic acid, oxalic acid, uric acid, homovanilic acid, vanillylmandelic acid, and L-ascorbic acid), sugars (D-glucose, D-fructose, D-galactose, D-ribose, *N*-acetyl-D-glucosamine, maltose, and sucrose), nucleic acid bases (adenine, guanine, thymine, cytocine, and uracil), and other compounds (methanol, acetone, phenol, cholesterol, creatine, creatinine, and urea).

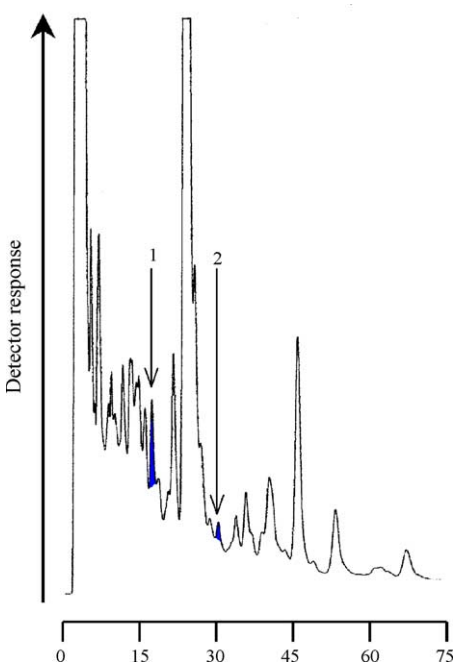

Fig. 3. Chromatogram obtained with the diluted urine sample. Peaks and concentrations (nmol/mL urine): 1, tyrosine (158); 2, tyramine (35); others, reagent blanks and biogenic amino(phenol) compounds.

3.6. Determination of tyrosine and tyramine in human urine

In order to investigate the practicality of the intramolecular excimer-forming derivatization method in biological analysis, the present method was applied to the determination of tyrosine and tyramine in human urine. Fig. 3 illustrated a typical chromatogram obtained with the 100-fold diluted urine sample. The peak components of peaks 1 and 2 in Fig. 3 were identified as the PBC derivatives of tyrosine and tyramine, respectively, on the basis of their retention times by a comparison with those in Fig. 2, and also by co-chromatography using 60–80% (v/v) acetonitrile containing 0.5–2.0% (v/v) acetic acid as an eluent. The LC–MS/MS fragmentation pattern of peak 1 from urine sample was quite similar to that of the standard. When the urine sample was replaced with water, no peaks except for blank components were observed in the chromatogram. In addition, when PBC solution in acetonitrile was replaced with acetonitrile only, all peaks were not observed in the chromatogram. Furthermore, as described above, numerous biogenic compounds did not disturb the determination of tyrosine and tyramine. These observations support that peaks 1 and 2 in Fig. 3 have single components, the PBC derivatives of tyrosine and tyramine, respectively. Under the present LC conditions, because the peak of methyltyrosine was overlapped with those of other compounds (Table 1), we could not use methyltyrosine as an internal standard for the determination. Tyrosine and tyramine were determined by standard addition method.

When the same urine sample as that used in Fig. 3 was monitored at 375 nm (monomer fluorescence from pyrene), numerous large and broad peaks due to monopyrene compounds (4-(1-pyrene)butanoic acid, a hydrolysate of PBC, and the PBC derivatives of biogenic monoamines in urine) appeared in the chromatogram. On the contrary, when monitored at 475 nm (excimer fluorescence), simple chromatogram was obtained as shown in Fig. 3. This indicates that the present method is very useful for the quantification of tyrosine and tyramine in urine.

The calibration graphs of tyrosine and tyramine in urine were linear ($r = 0.999$) in the concentration range corresponding to 1–1000 nmol/mL urine. No significant changes in the slopes of the graphs were observed with urine used. The determinable concentrations of urinary tyrosine and tyramine were both ca. 1.0 nmol/mL urine (ca. 50 fmol on column). The recoveries of tyrosine and tyramine (100 nmol/mL urine) added to pooled urine were 98.3 ± 2.8 and $97.8 \pm 3.3\%$, respectively (mean $\pm$ S.D.; $n = 5$). The within-day precisions were established by repeated determination ($n = 5$) of tyrosine and tyramine concentration in urine (100 nmol/mL urine); the R.S.D.s were 4.3 and 3.8%, respectively.

The concentrations of tyrosine and tyramine in 11 normal human urine samples (male, seven; female, four) were determined by the proposed method. The mean values of tyrosine and tyramine were 133 ± 88 and 43 ± 18 nmol/mL urine, respectively, which are in good agreements with the reported data [16], and the proposed method has sufficient reproducibility. Furthermore, the present method does not require special pre-treatments.

4. Conclusion

By the present intramolecular excimer-forming derivatization, tyrosine related compounds were found to be converted to the respective polypyrene-labeled derivatives by one-step derivatization. The reaction conditions of *p*-hydroxyphenylethylamino group containing compounds with 4-(1-pyrene)butanoyl chloride were optimized in order to achieve maximal fluorescence yields of the resulting intramolecular excimers. They afforded intramolecular excimer fluorescence, which can be clearly discriminated from normal fluorescence of PBC and other pyrene concomitants, with irradiation of the pyrene excitation wavelength. This method might be useful for biomedical and clinical investigations of not only tyrosine and tyramine, but also other hydroxyphenylethylamino group containing compounds. Simultaneous determination of catecholamines, 5-hydroxyindoleamines, and their precursors and metabolites based on this chemistry are in progress.

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