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Simultaneous determination of primary and secondary phenethylamines in biological samples by high-performance liquid chromatographic method with fluorescence detection

Xiao-Feng Guo[a,b], Jie-Yu Wang[a], Hong Wang[a,*], Hua-Shan Zhang[a]

[a] Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), Department of Chemistry, Wuhan University, Wuhan 430072, China
[b] Suzhou Institute of Wuhan University, Suzhou, China

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ABSTRACT

Phenylalanine is an essential amino acid and its metabolites relate to various physiological and immune functions of living organisms. To monitor the alteration of concentration of primary and secondary phenethylamines including *N*-methyltyramine, octopamine, tyramine, tyrosine and phenylalanine in the metabolic pathway of phenylalanine, a sensitive and selective reversed-phase high-performance liquid chromatographic method has been developed in this study. The identification and quantification of phenethylamines were performed by fluorescent detection after pre-column derivatization with 1,3,5,7-tetramethyl-8-(*N*-hydroxysuccinimidyl butyric ester)difluoroboradiaza-*s*-indacene, an excellent fluorescent probe which could react with both primary and secondary amino groups simultaneously. The derivatization was carried out at 25 °C for 25 min, and the separation was performed on a C18 column within 20 min. The linear ranges were from 2.0 to 100 nM for phenylalanine and tyramine to 5.0 to 250 for tyrosine and octopamine, with the detection limits of 0.1 nM for octopamine, tyramine, tyrosine and phenylalanine and 0.2 nM for *N*-methyltyramine (signal-to-noise ratio = 3), which allowed for the sure determination of phenethylamines at trace levels in the real samples without complex pretreatment or enrichment during multitudinous samples analysis. The proposed method has been validated by the analysis of the five target compounds in biological samples with spiked recoveries of 96.4–104.4% and the relative standard deviation of 1.0 and 4.4%.

1. Introduction

Phenethylamines, such as phenylalanine (Phe), tyrosine (Tyr), tyramine (Tyra), *N*-methyltyramine (NMT) and octopamine (Oct) (Fig. 1), are important biological compounds. In these compounds, phenylalanine is an essential amino acid and could only be acquired from food, while the others are nonessential and could be both acquired from food and converted from phenylalanine. These phenethylamines relate to various physiological and immune functions of living organisms. Phenylalanine and tyrosine have several metabolites including octopamine and tyramine, which have been considered as neurotransmitters in invertebrates to regulate many actions such as growth, foraging, moving, learning and so on [1–3]. Tyrosine is also the precursor of catecholamines which are major brain and peripheral neurotransmitters in mammals. While for human, octopamine and tyramine used to be considered functionally unimportant in the central nervous system in a long period because of their low physiological concentrations and the absence of specific receptors. However, two G protein-coupled receptors of octopamine were discovered recently in brains and tonsils of mammals, which suggest that octopamine may play important roles in several physiological and pathological procedures of human like cirrhosis, schizophrenia and Parkinson's disease [4–6]. Moreover, octopamine could also be applied for the prophylaxis and treatment of adiposis and Type II diabetic nephropathy [7–9]. Besides, *N*-methyltyramine is a metabolite of tyramine and has many physiological functions including stimulates gastric and pancreatic secretions [10], and it is also a molecule which has a similar function to ephedrine alkaloids as dietary supplements but more safe [11]. Therefore, a quantification method of phenethylamines with high sensitivity and selectivity is always desired.

By far, several analytical methods have been developed for the quantitative analysis of phenethylamines in biological samples, including high-performance liquid chromatography (HPLC)

* Corresponding author. Tel.: +86 27 87218924; fax: +86 27 68754067.
E-mail address: hongwang@whu.edu.cn (H. Wang).

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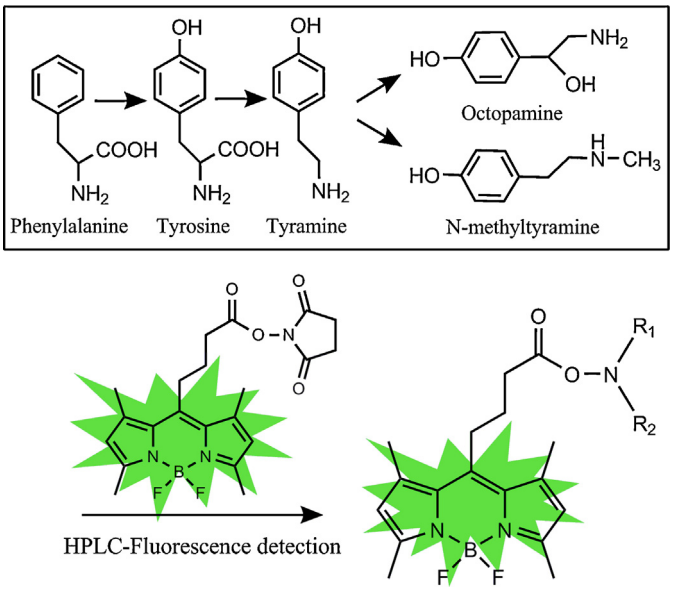

Fig. 1. Structures of phenethylamines and their derivatization using TMBB-Su.

[12–15], capillary electrophoresis [16,17], molecularly imprinted solid-phase extraction (MISPE) [18] and electrochemical detection (ECD) [19]. Among these methods, HPLC followed by fluorescence detection is one of frequently used analytical techniques owing to its high sensitivity and selectivity. Since phenethylamines have intrinsic fluorescence, HPLC with fluorescence detection method can be used to analyze them directly without chemical derivatization. However, short absorbance and emission wavelength, small molar absorption coefficient, low fluorescence quantum yield and background interference from complex matrix [14] which could be resolved using fluorescent labeling reagents. Hence, pre-column derivatization combined with HPLC-fluorescence detection is regarded as an ideal analytical method for trace amounts of phenethylamines in complex matrix of bio-samples.

Since there are both primary and secondary amines in phenethylamines, the fluorescent reagent used for phenethylamines analysis should label all of them. However, the reports for determination of primary and secondary phenethylamines simultaneously using HPLC-fluorescence detection were not many. Till now, typical fluorescent derivatization reagents which can label primary and secondary aliphatic amines simultaneously are 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) [20], fluorescein isothiocyanate (FITC) [21], 4-fluoro-7-nitrobenzofurazan (NBD-F) [22], 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [23] and *N*-hydroxysuccinimidyl 4,3,2′-naphthapyrone-4-acetate (NPA-OSu) [24]. However, all of these reagents have a variety of drawbacks. For example, long derivatization time is need for FITC and high temperature (60 °C) is required for NBD-F, and operation procedure will be more complex than operating at room temperature. The excess FMOC-Cl in the derivatization procedure has to be removed by extraction or derivatization with a specific amine to form derivatives having no interference to the analysis of target compounds in order to prevent the generation of derivative with hydroxyl group of methanol. AQC and NPA-OSu have short absorbance and emission wavelengths which cannot avoid background interference from complex matrix.

1,3,5,7-Tetramethyl-8-(*N*-hydroxy-succinimidyl butyric ester)difluoroboradiaza -*s*-indacene (TMBB-Su) has been synthesized and applied for amine determination in our group [25] and has high fluorescence quantum yield, long emission wavelength, shorter reaction time, milder derivatization conditions (like lower temperature and weaker alkaline environment) when deriving with primary and secondary amines [26]. In the present work, to verify the applicability of TMBB-Su for the determination primary and secondary phenethylamines, a new HPLC method with fluorescence detection was developed using TMBB-Su as pre-column derivatization reagent and the results indicated that the proposed method has obvious advantages in sensitivity and selectivity with mild derivatization conditions.

2. Experimental

2.1. Apparatus

An LC-20A HPLC system (Shimadzu, Tokyo, Japan) with RF-10AXL fluorescence detector (Shimadzu, Tokyo, Japan) and Lab Solutions/LC Solution Lite chromatography chemstation (Shimadzu, Tokyo, Japan) were used in the experiments. Sample injection volume was 20 μL. The separation was performed on an ODS column (5 μm, 150 mm × 4.6 mm, i.d., Inertsil, GL Sciences, Tokyo, Japan). The pH values of solutions were measured using Mettler Toledo Delta 320 pH meter (Mettler-Toledo, Shanghai, China).

2.2. Chemicals and reagents

TMBB-Su was synthesized in our laboratory [23]. Phenylalanine, tyrosine, tyramine and *N*-methyltyramine were purchased from Biochemical Reagents Company (Shanghai, China). Octopamine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Unless otherwise specified, all other reagents were of analytical grade. Water used for preparing solutions was purified by a Milli-Q system (Millipore, Bedford, MA, USA).

The TMBB-Su stock solution was prepared by dissolving TMBB-Su in acetonitrile to give a concentration of 1.0×10^{-4} M. The stock solutions of the analytes (1.0×10^{-4} M) were prepared by dissolving phenethylamines in water. Dilution of these stock solutions to appropriate concentrations was performed before use. H_3BO_3–$Na_2B_4O_7$ buffers were prepared by mixing 0.025 M $Na_2B_4O_7$ solution with 0.10 M H_3BO_3 solution to the required pH value. Citric acid (H_3Cit)–Na_2HPO_4 buffers were prepared by mixing 0.10 M H_3Cit solution with 0.10 M Na_2HPO_4 solution to the required pH value. When not in use, all standards were stored at 4 °C in a refrigerator.

2.3. Derivatization procedure

To a 0.5 mL of Eppendorf tube containing 175 μL of mixed amines and 125 μL of H_3BO_3–$Na_2B_4O_7$ buffer (pH 7.8), 135 μL 1.0×10^{-4} M TMBB-Su and 65 μL acetonitrile were added. The whole solution was diluted to the mark with water and kept at 25 °C for 25 min. Then, an aliquot (20 μL) of the reaction mixture was diluted with mobile phase and injected into the chromatographic system.

2.4. Chromatographic separation

The chromatographic separations were performed at ambient temperature on an Inertsil ODS-SP column with gradient elution. Eluent A was methanol and eluent B was 10 mM, pH 4.5, citric acid–Na_2HPO_4 buffer solution. Before the analysis, the column was pre-equilibrated for 30 min with mobile phase (A:B = 62:38, v/v). Then 20 μL of the prepared sample solution was injected and the derivatives were eluted at a flow rate of 0.7 mL/min. The gradient elution program was used as following: 0–9 min, 62% A; 10–25 min, 75% A. The chromatographic system was re-equilibrated with mobile phase (A:B = 62:38, v/v) for 20 min between runs. The