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Enantiomeric separation of amino acids derivatized with 7-fluoro-4-nitrobenzoxadiazole by capillary liquid chromatography/tandem mass spectrometry

Yaru Song [a], Ming Shenwu [a], Shulin Zhao [b], Dongyan Hou [c], Yi-Ming Liu [a,*]

[a] *Department of Chemistry, Jackson State University, 1400 J.R. Lynch Street, Jackson, MS 39217-0510, USA*
[b] *College of Chemistry and Chemical Engineering, Guangxi Normal University, Guilin 51004, China*
[c] *Department of Chemistry, Anshan Normal University, Anshan 114005, China*

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Abstract

Pre-column derivatization allowed stacking amino acid enantiomers on C_{18} reversed-phase micro extraction columns, thus facilitating sample loading in capillary HPLC/tandem mass spectrometry. Two tagging reagents, i.e. 7-fluoro-4-nitrobenzoxadiazole (NBD-F) and 1-fluoro-2,4-dinitrobenzene (DNB-F) were evaluated. Both of them reacted readily with amino acids at an elevated temperature, resulting in derivatives that were effectively stacked and suitable for a sensitive MS/MS detection as well. Separation of the tagged enantiomers on a teicoplanin chiral stationary phase (CSP) with mobile phases compatible with MS detection was investigated. NBD-amino acid enantiomers (12 pairs) tested were all base-line resolved. However, the efforts to separate DNB-F tagged amino acid enantiomers on this CSP were not successful. Separation conditions including pH, organic modifiers, and column dimension were studied. All the NBD-amino acids studied could be sensitively detected by MS/MS detection set in the negative ion mode, but only a few including NBD-Asp, BND-Glu, NBD-Ser, and NBD-Thr were detected in the positive ion mode. Thus, the selectivity for enantiomeric determination of excitatory amino acids (e.g. Asp and Glu) was further improved by choosing MS/MS detection in the positive ion mode.

Keywords: Amino acids; Enantiomer; Chiral separation; HPLC–MS/MS; Teicoplanin chiral stationary phase; NBD-F

1. Introduction

High levels of D-amino acids have been recently detected both free and bound in peptides in a variety of living systems [1–4]. In search for a better understanding of the biological significance of D-amino acids, enantiomeric determination of amino acids in biological samples has become highly desirable. Analytical methods based on high performance liquid chromatography (HPLC), capillary electrophoresis, enzymatic and immunochemical biosensors, etc. were reported. Comprehensive reviews on analysis of amino acid enantiomers were given [4–6]. Recently, HPLC/mass spectrometry (MS) based methods have been developed for chiral analysis of amino acids aiming for improved method selectivity and detection sensitivity [7–9].

HPLC coupled to MS via an electrospray ionization (ESI) source has become a popular analytical technique. However, the sensitivity of an HPLC–MS combination is often lackluster compared with those of HPLC/fluorescence or electrochemical detection, particularly when standard-size HPLC columns (e.g. 4 mm i.d.) are used. This problem is partly caused by sample dilution within the relatively large volume comprising the column and tubing [10]. A direct approach to improve the sensitivity is to reduce the column/emitter dimensions and thus the separation/electrospray flow rate. Therefore, capillary or nanoHPLC–MS regime is gaining research interest [11–14]. Unfortunately, sample loading can be a problem due to a very low flow rate of the mobile phase used in such miniaturized separation schemes. One

* Corresponding author. Tel.: +1 601 9793491; fax: +1 601 9793674.
E-mail address: yiming.liu@jsums.edu (Y.-M. Liu).

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can overcome this problem by reducing the sample volume. Various nanoliter sample injection valves are commercially available. However, the method sensitivity is significantly compromised with such nanoliter sampling loops. An elegant approach was described by Haskins et al. [15], where multiple pumps and valves were used so that the sample was loaded at a high flow rate and then eluted out at nL/min. This approach works well only when the analytes can be stacked on the column. We previously reported that pre-column derivatization of a sample with an appropriate tagging reagent not only facilitates loading/stacking the sample on an extraction micro-column, but also enhances the MS/MS detection sensitivity [16–18].

Various chiral stationary phases (CSP) have been developed for the separation of amino acid enantiomers [19]. Recently, teicoplanin (a macrocyclic glycopeptide) bonded silica particle was evaluated as a CSP for enantiomeric resolution of underivatized amino acids [7,20,21]. Separation of theanine enantiomers derivatized with either 9-fluorenylmethyloxycarbonyl chloride or 5-dimethylamino-1-naphthalenesulfonyl chloride was also achieved on a teicoplanin CSP [22]. The aim of the present work was to develop a sensitive capillary HPLC–MS/MS method for enantiomeric separation of amino acids. Pre-column derivatization of the sample with 7-fluoro-4-nitrobenzoxadiazole (NBD-F), a widely used tagging reagent for HPLC analysis of amino acids [23], and the subsequent stacking on C_{18} reversed-phase extraction columns, enantiomeric separation on a teicoplanin CSP, and MS/MS detection of the resulting derivatives were investigated.

2. Experimental

2.1. Chemicals and reagents

Amino acids, 7-fluoro-4-nitrobenzoxadiazole (NBD-F), 1-fluoro-2,4-dinitrobenzene (DNB-F), 2-propanol, formic acid, methanol (LC–MS grade), ethanol, sodium borate, and acetonitrile (LC–MS grade) were from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Artificial cerebrospinal fluid (aCSF) consisted of 148 mM NaCl, 3.0 mM KCl, 0.8 mM $MgCl_2$, and 1.4 mM $CaCl_2$ in water. Milli-Q water was used throughout the work.

2.2. Capillary LC–ESI–MS/MS

The capillary LC–ESI–MS/MS system consisted of two pumps (LC-10ADvp, Shimadzu, Kyoto, Japan), an on-line degasser (DGU-12A, Shimadzu), and an ion trap mass spectrometer with an ESI source (LCQ Deca, ThermoFinnigan, San Jose, CA, USA). A flow splitter (75 cm × 50 μm i.d.) was used to carry approximately 97% of the mobile phase delivered by the pumps to waste. Two capillary columns (a C_{18} reversed-phase extraction column and a teicolpanin CSP column) connected via a switching-valve were used in the system. The flow rate of mobile phase was adjusted to be at 28 μL/min for sample loading and at 4 μL/min for the separation by changing the pump's flow setting. Mobile phase A used for loading samples was water containing 0.1% formic acid. Mobile phase B used for the separation was a mixture of polar organic solvents and water. Isocratic elution was programmed as following: time 0.00–5.00 min, 100% mobile phase A was delivered through the extraction column to stack the sample with the eluent directed to waste; time 5.10–30.00 min, 100% mobile phase B was delivered to elute the analyte out of the extraction column and the eluent was directed onto the chiral column for the enantioseparation; time 30.00–5.00 min, 100% mobile phase A was delivered to equilibrate the extraction column and the eluent was directed to waste; time 35.10 min, stop. Injections were made by means of a Rheodyne 8125 injector equipped with a 20-μL sampling loop.

The capillary columns were prepared as described previously [16–18]. The teicoplanin CSP was obtained from a CHIROBIOTIC T™ column purchased from Astec (Whippany, NJ, USA). C_{18} reversed phase silica particles (5 μM) used for packing extraction columns were obtained from Restek (Bellefonte, PA, USA).

2.3. Pre-column derivatization with NBD-F or DNB-F

To 10 μL amino acid solution (0.25×10^{-5} to 5×10^{-5} M in water), 20 μL borate buffer (100 mM at pH 9.0) and 60 μL NBD-F or DNB-F (10 mM in acetonitrile) were added. The mixture was vortexed and heated at 65 °C for 20 min in a dry heating block. After heating, the solution was cooled down in running tap water and kept at 5 °C till use. Portions (20 μL each) of the derivative solution were injected into the HPLC–MS/MS system for analysis without further purification.

3. Results and discussion

3.1. Pre-column derivatization

Loading sample is a challenge in capillary or nano HPLC–MS separations because the flow rate of mobile phase is very low (typically in the range from 100 nL to 10 μL/min). For example, it takes several minutes to load a 20-μL sample, which is problematic if the analytes cannot be stacked on the column. Most amino acids are highly hydrophilic, which makes it difficult to stack them on reversed phase columns. Tagging amino acids with an appropriate reagent increases their hydrophobicity and molecular size so that they can be retained and stacked on C_{18} reversed phase extraction columns, thus facilitating sample loading in capillary HPLC separations. An additional advantage for the use of on-line column extraction technique is that pretreatment of biological samples can be minimized. The most widely used reagent for pre-column tagging of amino acids is *o*-phthaldialdehyde