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Analytical Methods

Enantiomeric purity determination of L-amino acids with pre-column derivatization and chiral stationary phase: Development and validation of the method

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ABSTRACT

A simple, efficient and general HPLC method for the determination of enantiomeric purity of a series of L-amino acids was developed. In order to improve the detection sensitivity, pre-column derivatization was adopted and 7-chloro-4-nitrobenzoxadiazole (NBD-Cl) was selected as derivatization reagent. NBD-amino acid enantiomers were then enantioseparated on a Pirkle-type chiral stationary phase, Sumichiral OA-2500S (250 mm × 4.6 mm, 5 μm), using a mobile phase composed of acetonitrile–methanol (50:50, v/v) containing 5 mmol L^{-1} citric acid at the flow rate of 0.5 mL min^{-1}. The detection wavelength was 470 nm. All the eleven pairs of tested amino acid enantiomers were well separated, and trace amounts of D-amino acids (0.5%) in the presence of a large excess of corresponding L-enantiomers could be quantified. The proposed method was validated in terms of selectivity, precision, linearity range, LOD, LOQ and accuracy, and then successfully applied to the determination of enantiomeric purity in bulk samples of L-amino acids.

1. Introduction

Amino acids are essential for life. The 20 genetically encoded amino acids comprise the building blocks of proteins and have many important functions in metabolism. With the exception of glycine, all the 20 amino acids have chiral centers and have L-enantiomer and D-enantiomer, respectively. Since the physiological environment within a living organism is chiral, the biological activities of enantiomeric forms of amino acids differ dramatically (Ilisz, Berkecz, & Péter, 2008). L-amino acids are exclusively used as substrates for the polymerization and formation of peptides and proteins in living systems (Fujii, Kaji, & Fujii, 2011). While D-amino acids, which were recently detected in various living organisms in the form of free D-amino acids and D-amino acid residues in peptides and proteins, are increasingly being recognized as important signaling molecules in mammals, including humans (Brückner & Fujii, 2010; Friedman, 2010; Fuchs, Berger, Klomp, & de Koning, 2005; Martinez-Rodriguez, Martinez-Gómez, Rodriguez-Vico, Clemente-Jimenez, & Heras-Vázquez, 2010). For example, D-serine was found to play a significant role in the central nervous system, and D-aspartate was found to have a role in endocrine and/or neuroendocrine organs (Fuchs et al., 2006; Snyder & Kim, 2000; Topo et al., 2010; Yamamoto, Tanaka, Ishida, & Horiike, 2010).

The importance of separating enantiomers is increasing with the increasing need for single enantiomer in the food and medical fields. It is crucial to identify enantiomers in the medicine field to avoid toxic effects (Aboul-Enein & Ali, 2003). The Food and Drug Administration (FDA) and the European Medicines Evaluation Agency (EMEA) recommend the use of validated methods to evaluate the enantiomeric purity of single-isomers. Although various pharmacopoeias (e.g. USP 34-NF 29, BP 2010, ChP 2010, and JECFA) provide titration methods to assay L-amino acid content, none of these methods distinguish between L- and D-amino acids and do not consider the presence of enantiomeric impurities. It was proved that there was poor correlation between amino acid titration data and specific rotation data or content data (Lee et al., 2012). Consequently, a more accurate and sensitive enantioseparation method for determination of the enantiomeric purity is urgently needed for better quality control of L-amino acids.

There have been a large number of publications concerning the separation of enantiomeric isomers of amino acids for the past 20 years. Analytical methods based on high performance liquid chromatography (HPLC), capillary electrophoresis, enzymatic and immunochemical biosensors were reported (Frattini, Rosini, Pollegioni, & Pilone, 2011; Gogami, Okada, & Oikawa, 2011; Mello

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& Kubota, 2002; Miyoshi et al., 2012; Song, Shenwu, Zhao, Hou, & Liu, 2005; Yoshikawa, Ashida, Hamase, & Abe, 2011). The general method that is widely used is still HPLC method, and pre- or post column derivatization step is usually adopted to improve the sensitivity of detection (Ilisz et al., 2008). However, as far as we are aware, most of these developed methods are dealing with enantioseparation of certain amino acids or single-enantiomer content determinations, and there have been still few reports exclusively on the enantiomeric purity determination.

In this study, a sensitive and efficient HPLC method coupled with pre-column derivatization was developed for the determination of enantiomeric purity of L-amino acids. Pre-column derivatization of sample with NBD-Cl, a widely used tagging reagent for HPLC analysis of amino acids (Imai & Watanabe, 1981; Toyo'oka, 2005; Toyo'oka, Jin, Tomoi, Oe, & Hiranuma, 2001), improved the detection sensitivity and made the method generally suitable for enantiomeric purity determination of a series of free amino acids. The method was fully validated and successfully applied to the determination of the enantiomeric purity in bulk samples of several L-amino acids.

2. Experiments

2.1. Materials and chemicals

D-, L-alanine (Ala), D-, L-methionine (Met), D-, L- leucine (Leu), D-, L-tryptophan (Trp), D-, L-asparagine (Asn), D-, L-valine (Val), D-, L- lysine (Lys), D-, L-phenylalanine (Phe), D-, L-serine (Ser), D-, L-threonine (Thr) and D-, L-cysteine (Cys) were purchased from Sigma Chemical Co. (St Louis, MO, USA). L-amino acid bulk drugs were provided by Jirong Pharmaceutical Co., Ltd., (Shijiazhuang, China). 7-chloro-4-nitrobenzoxadiazole (NBD-Cl) and chiral derivatization reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) were obtained from ACROS ORGANICS (Belgium). Methanol and acetonitrile of HPLC grade were got from Concord Tech. Co., (Tianjin, China). All the other reagents were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the experiments.

2.2. Instruments

2.2.1. Analysis with NBD-Cl as derivatization reagent

The assay was performed on Jasco HPLC system and Sepu 3000 Chemstation software was used for system control and data acquisition. Enantioseparation was achieved on a chiral column, Sumichiral OA-2500S (250 mm × 4.6 mm, 5 μm), which was kept at 25 °C. As the mobile phase, methanol–acetonitrile (50:50, v/v) containing 5 mmol L^{-1} citric acid was used at the flow rate of 0.5 mL min^{-1}. The detection wavelength was set at 470 nm. 20 μL of reaction mixture was subjected to HPLC.

2.2.2. Analysis with FDAA as derivatization reagent

The diastereoisomers were separated on an achiral column, Kromasil C_{18} (150 mm × 4.6 mm, 5 μm), using the Jasco HPLC system. The column temperature was kept constant at 25 °C. The mobile phase was composed of acetonitrile-0.2% acetic acid (35:65, v/v) at the flow rate of 1.0 mL min^{-1}. The detection wavelength was 340 nm and the injection volume was 20 μL.

2.3. Preparation of solutions

Different stock solutions of eleven tested D-, L-amino acid enantiomers were prepared by dissolving accurately weighed amounts of approximately 8 mg of each compound in 10 mL of demineralized water (800.00 μg ml^{-1}). Sample stock solutions of the eleven tested L-amino acid bulk samples were prepared by dissolving 8 mg of each analyte in 10 mL demineralized water (800.00 μg ml^{-1}). All the stock solutions were stored in a refrigerator (4 °C) protected from light.

2.4. Derivatization

2.4.1. Pre-column derivatization with NBD-Cl

To 200 μL sample solution, 200 μL borate buffer (100 mmol L^{-1} at pH 8.5) and 200 μL NBD-Cl (4 mmol L^{-1}) were added. The mixture was vortexed and heated at 60 °C in the water bath for 1 h protected from light. After heating, the mixture was evaporated by heating to 50 °C under a stream of nitrogen. The residue was dissolved in 1 mL mobile phase. For blank sample, 200 μL water was used instead of sample solution.

2.4.2. Pre-column derivatization with FDAA

200 μL of sample solution was mixed with 40 μL $NaHCO_3$ (1 mol L^{-1}) and 200 μL FDAA (1%). The mixture was vortexed and incubated at 40 °C in water bath for 1 h away from light. After cooling down, 20 μL HCl (2 mol L^{-1}) was added. The reaction mixture was centrifuged (5 min, 11873 × g) and then injected for HPLC analysis.

3. Results and discussion

3.1. Development of the method

3.1.1. The comparison of derivatization reagents

Two main strategies have evolved for the enantioseparation of amino acids by HPLC: an indirect method, based on the formation of diastereomers by the reactions of amino acids with a chiral derivatization agent and separation of the diastereomeric derivatives on an achiral stationary phase; and a direct method, based on the formation of diastereomers on a chiral stationary phase (Ilisz et al., 2008). To enantioseparate the amino acids effectively and efficiently, both of the two strategies were adopted and two kinds of commonly used derivatization reagents were compared including achiral derivatization reagent (NBD-Cl) (Imai & Watanabe, 1981; Toyo'oka, 2005; Toyo'oka, Jin, Tomoi, Oe, & Hiranuma, 2001; Toyo'oka, Tomoi, Oe, & Miyahara, 1999) and chiral derivatization reagent (FDAA) (Bhushan & Agarwal, 2010; Bhushan & Brückner, 2004; Bhushan & Brückner, 2011). Three pairs of amino acid enantiomers (Ser, Val and Phe) were chosen for the comparison study.

For the derivatization reaction process and the composition of HPLC mobile phase might affect the sensitivity and resolution, the conditions that were found to yield best enantioseparation results in pilot experiments were used for comparing the two kinds of derivatization reagents. The results were summarised in Table 1. LOD and LOQ indicated the lowest detectable and quantitative amount for the method respectively, which were especially important for the determination of impurity. Much lower LODs and LOQs were obtained when using NBD-Cl as derivatization reagent for three tested amino acids. But the differences in enantiomeric

Table 1
Enantioseparation results of three selected amino acid using two derivation reagents.

	FDAA			NBD-Cl		
	Ser	Val	Phe	Ser	Val	Phe
Detection limit (μg·mL^{-1})	0.75	0.50	0.75	0.05	0.12	0.04
Quantitative limit (μg·mL^{-1})	1.50	1.50	2.00	0.20	0.40	0.10
Resolution	1.6	3.9	3.0	2.0	1.8	2.6