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RESEARCH PAPER

Pre-column Derivatization High Performance Liquid Chromatography Tandem Mass Spectrometric Determination of Trace Level of Amino Acids in Rat Serum

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Abstract: A simple and sensitive method based on the derivatization of 20 amino acids with 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC) as derivatization reagent on a reversed-phase Hypersil BDS C_{18} column (4.6 mm × 200 mm, 5 μm) with a gradient elution followed by high performance liquid chromatography (HPLC) via fluorescence detection at 390 nm (excitation wavelength of 333 nm) and tandem mass spectrometric identification has been developed. Optimum derivatization, giving the corresponding stable fluorescent derivatives, was obtained by reacting amino acids with BCEOC at 40 ℃ for 10 min in borate buffer (pH 9.0) with four times excess of molar reagent. The linear range of 20 amino acids were 51.6 fmol–105.6 pmol, all correlation coefficients >0.9995, detection limits were 6.3–177.6 fmol (at signal to noise 3:1, *S/N*=3:1). The identification of amino acid derivatives was carried out by post-column tandem mass spectrometry with electrospray ion (ESI) source, and the MS/MS cleavage mode of representative tyrosine derivative was analyzed. Under all the above optimum experimental conditions, the contents of 20 amino acids in rat serum of 3 groups (A: Quiet, B: At exercising exhaust, C: 12 h after exercising exhaust) were determined. It was indicated that the contents of 20 amino acids in group B were obviously higher than those in group A, and the contents of amino acids of group C and group A were almost the same. The established method exhibited high sensitivity and excellent reproducibility, and provided a new technology for the determination of amino acids in rat serum.

Key Words: Rat serum; Amino acids; Pre-column derivatization; High performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS); Fluorescence detection

1 Introduction

Amino acids are not only a necessary nutrition source for mankind, animals and plants, but also an important substance for regulating organism's physiological function. Recently, studies indicated that excitatory and inhibitory amino acids had important functions in exercise physiology[1], pathological mechanism and physiological process[2] of cerebral ischemia. The contents change of amino acids in human body had close relation to many diseases, the ratio of branched chain amino acids and aromatic amino acids in serum (BCAA/AAA) had been widely applied as the judgment standard of liver diseases[3,4]. Therefore, the trace level determination of amino acids has important significance for exercise physiology, clinical medical science, neurophysiology, food science, disease diagnosis and control.

Most amino acids show neither natural UV absorption nor fluorescence, thus the detection of them at trace levels using absorptiometry is fairly difficult. Recently, high performance liquid chromatography (HPLC) fluorescence detection with pre-column derivatization had been widely applied to the determination of amino acids. Although a number of different

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types of fluorescent labeling reagents have been developed, such as *o*-phthaldialdehyde (OPA)[5–6], 9-fluorenyl methylchloroformate (FMOC-Cl)[7], 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC)[8], phenyl isothiocyanate ester (PITC)[9] and so on, a variety of shortcomings in their applications have also been reported. For example, the OPA was only limited to primary amino acids. For FMOC, after derivatization the excess FMOC reagent should be removed by a fussy extraction with some loss of hydrophobic derivatives. AQC has been developed as a popular pre-column derivatization reagent for amino acids with satisfactory results, however, only 10% of the fluorescent intensity in aqueous solution comparing with that in pure acetonitrile solution was observed for its derivatives. Thus, the detection limits for the early-eluted amino acids were usually higher than those for later ones. PITC method had fast derivatization rate and created single and stable derivatives, but its derivatives were merely detected by UV with poor detection limits about 1 pmol[10]. 1,2-Benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC) has been used as pre-column derivatization reagent to the determination of amino acids in bovine serum albumin, melon seeds and bryophyte plants[11,12] in the author's laboratory. In this study, amino acids of rat serum in three states (Quiet, at exercising exhaust, 12 hours after exercising exhaust) were determined using BCEOC as pre-column derivatization reagent, the relationship of exercise and amino acids in rat serum was discussed by comparing the contents of excitatory and inhibitory amino acids, branched chain and aromatic amino acids, respectively. It was indicated that BCEOC method had high sensitivity, good repeatability, and gave a new technique for the determination of amino acids in serum.

2 Experimental

2.1 Instruments and reagents

The HPLC system devices were from the HP 1100 series and consisted of vacuum degasser, quaternary pump, autosampler, thermostated column compartment, fluorescence detector (FLD) and electrospray ionization (ESI) source. Derivatives were separated on Hypersil BDS C_{18} column (200 mm × 4.6 mm 5 μM, Yilite Co. Dalian, China). Fluorescence excitation and emission spectra were obtained at a 650-10S fluorescence spectrophotometer (Hitachi, Japan).

1,2-Benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC) [11,12] was synthesized in our laboratory. 20 amino acids standard were purchased from Sigma Co. Wistar male rat (Experimental animal center of Lu Nan Pharmacy Group Joint-stock Ltd, 180–220 g) were provided by Prof. Liu Hongzhen. Spectroscopically pure acetonitrile was purchased from Merck Co. Formic Acid. The sodium hydroxide and boric acid were purchased from Beijing Chemical Reagent Co. (Beijng, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA).

2.2 Experimental

2.2.1 Preparation of standard solutions

Individual stock solutions (1.0×10^{-2} M) of the amino acids were prepared in water, and if necessary, 6 M HCl or 6 M NaOH was added until the compound dissolved. The standard amino acids for HPLC analysis at individual concentrations of 5.0×10^{-5} M were prepared by diluting the corresponding stock solutions (1.0×10^{-2} M) of each amino acid with acetonitrile. The derivatization reagent solution (1.0×10^{-3} M) was prepared by dissolving 3.26 mg BCEOC in 10 ml of acetonitrile. When not in use, all standards were stored at 4℃.

2.2.2 Derivatization

The BCEOC-amino acids derivatization was proceeded in water/acetonitrile solution in basic medium. 15 μl of amino acids was added into a 2-ml vial, 160 μl acetonitrile, 300 μl of 0.2 M borate buffer (pH 9.0) and 60 μl of BCEOC acetonitrile solution were then added, respectively. The solution was shaken for 1 min and allowed to stand for 10 min at 40℃. After derivatization, to the solution was added 10 μl 30% formic acid until the final pH range of 5–7. Then the derivatized sample solution was directly injected into the HPLC system for analysis. The derivatization process was shown in Fig.1.

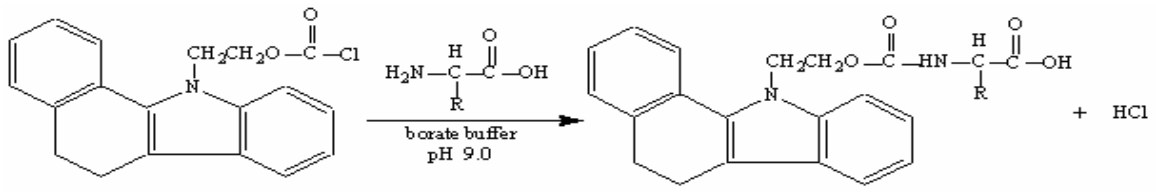

Fig.1 Derivatization scheme of BCEOC with amino acids

2.2.3 HPLC and MS conditions

High performance liquid chromatography separation of BCEOC derivatives was carried out on Hypersil BDS C_{18} column by a gradient elution. Eluent A was 30% of acetonitrile consisting of 30 mM formic acid buffer (pH 3.7);