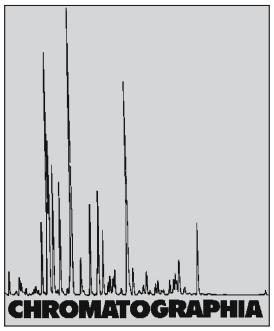
LC Determination of Amino Acids in Rat Plasma with Fluorescence Detection: Application to Exercise Physiology



2008, *67*, 375–382

Xianen Zhao[1,2], Yourui Suo[1,✉]

[1] Northwest Institute of Plateau Biology, Chinese Academy of Sciences, No. 59 Xiguan Street, Xining 810001, People's Republic of China; E-Mail: yrsuo@163.com
[2] Graduate School of Chinese Academy of Sciences, Beijing 100039, People's Republic of China

Received: 31 August 2007 / Revised: 11 December 2007 / Accepted: 20 December 2007
Online publication: 25 January 2008

Abstract

An LC method for the determination of 20 amino acids (AAs), using 1,2-Benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC) as fluorescent labeling reagent, has been validated and applied for the analysis of AAs in rat plasma at three different states concerning exercise physiology. Identification of AA derivatives was carried out by LC-MS with electrospray ion (ESI), and the MS-MS cleavage mode of the representative tyrosine (Tyr) derivative was analyzed. Gradient elution on a Hypersil BDS C_{18} column gave good separation of the derivatives. Excellent linear responses were observed and good compositional data could be obtained from as little as 50–200 μL of plasma samples. The contents of 20 AAs in rat plasma of three groups (24 rats, group A: quiet state, group B: at exercising exhaust, group C: 12 h after exercising exhaust) exhibited evident difference corresponding to the physiological states. Facile BCEOC derivatization coupled with LC-FLD-ESI-MS analysis allowed the development of a highly sensitive method for the quantitative analysis of trace level of AAs from plasma or other biochemical samples.

Keywords

Column liquid chromatography-mass spectrometry
Amino acids in rat plasma
1,2-Benzo-3,4-dihydrocarbazole-9-ethyl chloroformate

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. Studies indicated that excitatory and inhibitory amino acids had important function in exercise physiology [1], pathophysiological process of cerebral ischemia [2]. The change of the amino acid content in the human body has a close relation to various diseases, and the ratio of branched chain amino acids (BCAA) and aromatic amino acids (AAA) in plasma have been widely applied as the judgment standard of liver diseases [3, 4]. Therefore, the determination of trace level amino acids has important significance for exercise physiology, clinical medicine, neurophysiology, food science, disease diagnosis and control, and so on.

Recently, LC fluorescence detection (FLD) with pre-column derivatization has been widely applied for the determination of amino acids. Although a number of different types of reagents have been developed, such as *o*-phthaldialdehyde (OPA) [5, 6], 9-fluorenyl methyl chloroformate (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

DOI: 10.1365/s10337-008-0522-9
0009-5893/08/03

Table 1. Gradient elution program

Time (min)	A (%)[a]	B (%)	C (%)	Time (min)	A (%)	B (%)	C (%)
0	70	30	0	40	0	80	20
15	55	45	0	50	0	20	80
20	2	98	0	55	0	5	95
28	2	96	2	57	0	0	100
28.3	2	88	10	65	0	0	100
30	2	83	15				

[a] The composition of mobile phase A, B and C was described in Sect. 2.3

popular pre-column derivatization reagent for amino acids with satisfactory results, however, only 10% of the fluorescent intensity in aqueous solution compared to that in pure acetonitrile solution was observed for its derivatives. Thus the limits of detection (LOD) for the early-eluted amino acid derivatives were usually higher than those for the later ones. PITC method had a fast derivatization rate and created single and stable derivatives, but its derivatives were merely detected by UV with poor detection limits about pmol levels. 1,2-Benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC) has been used as pre-column derivatization reagent to determine amino acids in bovine serum albumin, melon seeds and bryophyte plants [10–12] in our laboratory. In this study, AAs of rat plasma at three states (quiet state, at exercising exhaust, 12 h after exercising exhaust) were determined using BCEOC as pre-column derivatization reagent, and the relationship of exercise physiology and AA contents in rat plasma was discussed by comparing the contents of excitatory and inhibitory amino acids, branched chain and AAA in different states.

Experimental

Chemicals and Reagents

1,2-Benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC) was synthesized in our laboratory [10–12]. Twenty amino acid standards were purchased from Sigma (St. Louis, MO, USA). Spectroscopically pure acetonitrile was purchased from Merck (Darmstadt, Germany). Plasma samples of Wistar male rats (purchased from experimental animal center of Lu Nan Pharmacy Group Joint-stock, 180–220 g) were collected by Prof. Hongzhen Liu (Sports College of Qufu Normal University). Formic acid, sodium hydroxide and boric acid were purchased from Beijing Chemical Reagent Co. (Beijing, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used were also of analytical grade unless otherwise stated.

Instrumentations

Experiments were performed using Agilent HP 1100 Series high-performance liquid chromatography and mass spectrometry (LC-MSD Trap SL, a complete LC-MS-MS, Agilent, USA). The LC system consisted of an online vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), and a fluorescence detector (FLD) (model G1321A). The LC system was controlled by HP Chemstation software (version B.01.01, Agilent). The mass spectrometer 1100 Series LC-MSD Trap-SL (ion trap) from Bruker Daltonik (Bremen, Germany) was equipped with an electrospray ionization (ESI) source. The mass spectrometer system was controlled by Esquire-LC NT software (version 5.3). Derivatives were separated on Hypersil BDS C_{18} column (200 × 4.6 mm, 5 μm i.d., Dalian Elite Co., China). Fluorescence excitation and emission spectra were obtained on a 650-10S fluorescence spectrophotometer (Hitachi, Japan). A Paratherm U2 electronic water-bath (Hitachi, Tokyo, Japan) was used to control the temperature. The mobile phase was filtered through a 0.2-μm nylon membrane filter (Alltech, Deerfiled, IL, USA).

Liquid Chromatographic Conditions

LC separation of amino acid derivatives was carried out on a Hypersil BDS C_{18} column by gradient elution. Eluent A was 30% of acetonitrile/water solution (30:70, *v*/*v*) consisting of 30 mmol L^{-1} formic acid buffer (pH 3.7); B was 50% of acetonitrile/water solution (50:50, *v*/*v*); C was acetonitrile/water (95:5, *v*/*v*). The flow rate was constant at 1.0 mL min^{-1} and the column temperature was set at 30 °C. The fluorescence excitation and emission wavelengths were set at λ_{ex} 333 nm and λ_{em} 390 nm, respectively. The gradient elution conditions used for the separation of amino acid derivatives are shown in Table 1.

Mass Spectrometer Conditions

The electrospray ionization-mass spectrometer (ESI-MS) was operated in positive ion mode. The electrospray capillary voltage was set to 3,500 V [13] and the nebulizer pressure was set to 0.24 MPa. Nitrogen was used as a drying gas for solvent evaporation. The drying gas temperatures were kept at 350 °C with flow rate of 9.0 L min^{-1}. Protonated analyte molecules were subjected to collision induced dissociation using helium as the collision gas to yield product ions for each analyte.

Preparation of Standard Solutions

Individual stock solutions (1.0×10^{-2} mol L^{-1}) of the amino acids were prepared in water, and if necessary, 6 mol L^{-1} HCl or 6 mol L^{-1} NaOH were added until the compound dissolved. The standard amino acids for LC analysis at individual concentrations of 5.0×10^{-5} mol L^{-1} were prepared by diluting the corresponding stock solutions (1.0×10^{-2} mol L^{-1}) of each amino acid with acetonitrile. The BCEOC solution (1.0×10^{-2} mol L^{-1}) was prepared by dissolving 32.6 mg BCEOC in 10 mL of acetonitrile, and low concentration BCEOC solution (1.0×10^{-3} mol L^{-1})

was obtained by diluting it using acetonitrile. When not in use, all solutions were stored at 4 °C.

Derivatization Procedure

The BCEOC-amino acids derivatization proceeded in a water/acetonitrile solution with alkaline medium. 15 μL amino acids were transferred into a 2-mL vial, 160 μL acetonitrile, 300 μL of 0.2 mol L^{-1} borate buffer (pH 9.0) and 60 μL of BCEOC solution (1.0×10^{-3} mol L^{-1}) were then added. The solution was shaken for 1 min and allowed to stand for 10 min at 40 °C. After derivatization, to the solution 10 μL 30% formic acid were added until the final pH value at the range of 5–7. Then the derivatized sample solution was directly injected into the LC system for analysis. The derivatization process is shown in Fig. 1.

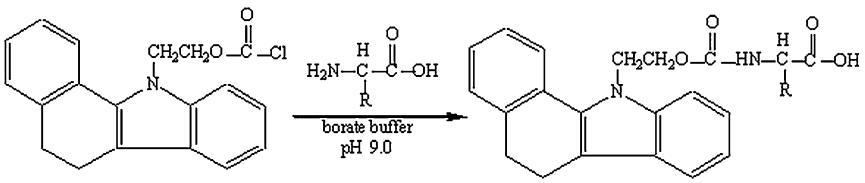


Fig. 1. Derivatization scheme of BCEOC with amino acids

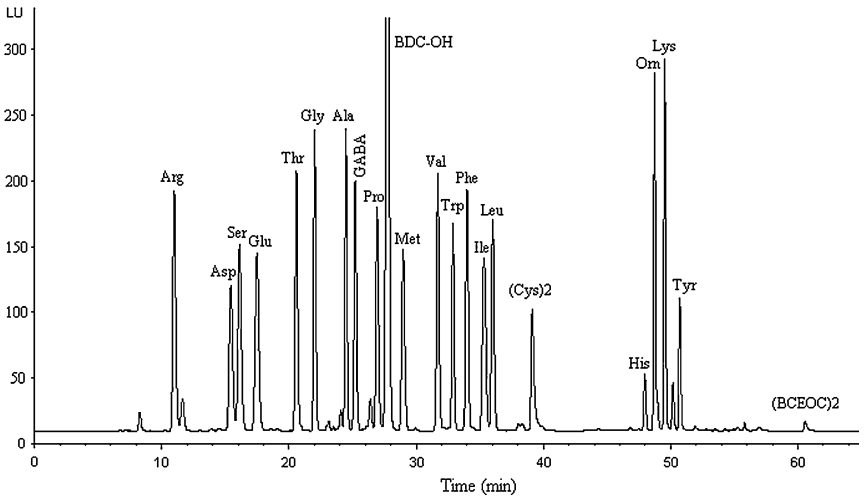


Fig. 2. Chromatogram of 20 standard amino acid derivatives. The concentration of each amino acid was all 5.0 μmol L^{-1}. Chromatographic conditions: Column temperature at 30 °C; Excitation wavelength λ_{ex} 333 nm, emission wavelength λ_{em}390 nm; Hypersil BDS C_{18} column (200 × 4.6 mm, 5 μm i.d.); Flow rate = 1.0 mL min^{-1}; Peaks: **Arg** = Arginine; **Asp** = Aspartic acid; **Ser** = Serine; **Glu** = Glutamic acid; **Thr** = Threonine; **Gly** = Glycine; **Ala** = Alanine; **GABA** = 4-Amino-butyric acid; **Pro** = Proline; **Met** = Methionine; **Val** = Valine; **Phe** = *β*-Phenylalanine; **Trp** = Tryptophan; **Ile** = *iso*-Leucine; **Leu** = *nor*-Leucine; **$(Cys)_2$** = Cystine; **His** = Histidine; **Orn** = Ornithine; **Lys** = Lysine; **Tyr** = Tyrosine; **BDC-OH** = 1,2-Benzo-3,4-dihydrocarbazole-9-ethanol; **$(BCEOC)_2$** = Bis(1,2-Benzo-3,4-dihydrocarbazole-9-ethyl) carbonate

Grouping and Exercise Project of Rats

Twenty-four rats were adaptively trained for 1 week on the electric drive treadmills (made in China, rate was 10 m min^{-1}, training time 20 min d^{-1}). After one week, they were separated stochastically into three groups (eight rats for one group) by avoirdupois for three sampling states: group A (quiet state), group B (at exercising exhaust), group C (12 h after exercising exhaust). When the electric drive treadmills were constant at the rate of 10 m min^{-1}, rats of B group and C group were exercised to be exhausted (judgment standard: rats could not hold up to exercise, stimulation and drive were of no effect; when the rats were taken down from electric drive treadmills and their abdomen were upturned, they could not turn over by themselves; responses were slow). Blood samples of group B were immediately collected after they were taken down from electric drive treadmills when they were exhausted. Blood samples of group C were collected 12 h after exercising exhaust. Blood samples of group A were collected at their quiet state without exercise.

Extraction and Derivatization Procedure for Plasma Sample

To the blood samples of three groups heparin sodium as anticoagulant was added and left to stand for 10 min at room temperature (20 °C), and then centrifuged (3,000 rpm for 15 min at 4 °C). Plasma was gained from suspension. To a hard glass tube, 200 μL 5% ice-cooled $HClO_4$ solution and 200 μL plasma were added and vortexed for 30 s. After waiting for 10 min at room temperature for the deposition of protein, the solutions were centrifuged (18,000 rpm for 5 min at 4 °C). The supernatants were separated and 20% NaOH added to neutralize the excess amount of $HClO_4$ to pH 8. The last plasma extraction solutions were stored at −80 °C until LC analysis. It was proved that the volume of plasma in the range of 50–200 microlitres could give satisfactory accuracy when adjustment with the dosage of $HClO_4$ and NaOH solutions was relevant with the above mentioned. The derivatization of extracted plasma samples proceeded as follows: 60 μL of plasma extraction solutions was added into a 2-mL vial, 150 μL acetonitrile, 300 μL of 0.2 mol L^{-1} borate buffer (pH 9.0) and 50 μL of BCEOC solution (1.0×10^{-2} mol L^{-1}) were then added. The following analysis procedure was the same as the derivatization procedure of amino acid standards described above.

Table 2. Linear regression equations, correlation coefficients, LOD, LOQ, MS, and reproducibility for retention times and peak areas ($n = 6$) of amino acid derivatives

Amino acid	$Y = AX \pm B$[a]	Correlation coefficients	LOD (fmol)[b]		LOQ[c] (nmol L^{-1})	MS $[M+H]^+$	RSD (%)	
			BCEOC	AQC[d]			Retention time	Peak area
Arg	$Y = 28.07X + 3.85$	0.9997	12.2	119	5.40	464	0.009	1.32
Asp	$Y = 17.15X + 0.47$	0.9999	27.2	311	5.97	423	0.012	1.47
Ser	$Y = 25.30X + 3.89$	0.9995	17.2	237	5.76	395	0.010	1.28
Glu	$Y = 24.03X-3.77$	0.9997	19.8	315	5.90	437	0.011	1.32
Thr	$Y = 24.86X + 3.86$	0.9999	12.7	162	5.43	409	0.006	1.18
Gly	$Y = 27.09X + 4.75$	0.9999	9.3	293	5.35	365	0.005	1.29
Ala	$Y = 26.01X + 3.94$	0.9999	10.4	155	5.37	379	0.004	1.11
GABA	$Y = 25.48X + 1.51$	0.9999	11.8	#	5.38	393	0.005	1.20
Pro	$Y = 25.77X + 3.14$	0.9999	13.1	278	5.44	405	0.004	1.04
Met	$Y = 25.95X + 2.48$	0.9999	13.0	74	5.44	439	0.007	1.48
Val	$Y = 28.04X + 9.39$	0.9999	13.6	57	5.46	407	0.006	1.02
Try	$Y = 25.90X + 9.28$	0.9999	9.2	#	5.33	494	0.004	0.86
Phe	$Y = 27.01X + 9.45$	0.9999	8.3	38	5.27	455	0.007	0.96
Ile	$Y = 26.68X + 4.84$	0.9999	19.4	47	5.86	421	0.005	1.08
Leu	$Y = 24.90X + 5.46$	0.9999	10.4	48	5.37	421	0.006	1.07
$(Cys)_2$	$Y = 19.66X-6.20$	0.9998	19.4	794	5.87	ND	0.045	2.29
His	$Y = 3.60X + 1.63$	0.9998	36.8	188	6.05	734	0.018	1.51
Orn	$Y = 32.79X + 4.08$	0.9999	7.7	#	5.24	711	0.013	1.32
Lys	$Y = 34.91X + 6.24$	0.9998	6.3	112	5.16	723[e]	0.009	1.54
Tyr	$Y = 15.40X + 2.16$	0.9999	15.8	93	5.78	758[e]	0.009	1.43

[a] Y peak area; X injected amount (pmol)
[b] LODs were calculated at S/N = 3:1, the injection volume was 10 μL
[c] LOQs were obtained when S/N = 10:1, the injection volume was 10 μL
[d] Data was from [15]
[e] Detected with negative ion mode
Detection limits were not reported in reference [15]
ND Not detected

Quantitative Analysis

All amino acids in rat plasma were quantified using the external standard method with FLD at 390 nm. The calibration curves for each BCEOC-amino acid derivatives were obtained by linear regression plotting peak area versus injection amounts of amino acid standards according to the optimum derivatization and separation conditions. Quantitative conversion of amino acids in rat plasma to their BCEOC derivatives was guaranteed by using an excess of BCEOC.

Statistic Method

All the statistic work was accomplished with SPSS software. The results were expressed in the form of "average ± standard deviation" ($\overline{X} \pm S$). Data were analyzed with independent sample t test, and significant difference level was $P < 0.05$.

Results and Discussion

Derivatization Reaction

The main factors affecting the derivatization yields were pH value of buffer, reaction time, temperature and concentration of derivatization reagent. A complete derivatization was achieved with the addition of 4-fold molar reagent excess to the total amino acids at 40 °C for 10 min with a pH value of borate buffer at 9.0. Detailed description about how to optimize BCEOC derivatization reaction conditions was reported in our work [10, 12].

Particularly, in 20 kinds of amino acids, just Ser and Thr have an alcoholic hydroxyl group and Tyr has a phenolic hydroxyl group, but only the phenolic hydroxyl group in Tyr was derivatized by BCEOC. This might be attributed to the fact that the electron density of H–O bond of the phenolic hydroxyl group is lower than that of the alcoholic hydroxyl group which came from the formation of p-π conjugated system of benzene ring and p orbit of phenolic hydroxyl group. The binding force of H–O bond of the phenolic hydroxyl group is weaker than that of the alcoholic hydroxyl group. Thus, the hydrogen of the phenolic hydroxyl group is easier to be dissociated as H^+ than that of the alcoholic hydroxyl group [14] so as to create phenolic hydroxy anion which react with the acyl chloride active group of BCEOC through nucleophilic substitution reaction in borate buffer (pH = 9.0).

LC Separation and MS Identification

Based on the combination of short retention time and adequate resolution for the accurate quantitative analysis, a good baseline resolution for 20 amino acids was achieved by adjusting the gradient elution conditions and the pH of eluent A. Because of the two alkaline nitrogen atoms in the molecular core

structure of BCEOC and BCEOC-amino acid derivatives, by controlling the pH of eluent A at 3.7 with ammonia formate, the simultaneous separation of 20 amino acid derivatives was achieved with the shortest retention times and the sharpest peaks especially for Asp and Ser which were difficult to separate. Additionally, an acidic mobile phase would be useful to the ionization of amino acid derivatives. When the pH value of eluent A was less than 3.5 or more than 3.8, Asp and Ser, BDC-OH and Met, Ile and Leu co-eluted.

With optimum conditions, 20 amino acid derivatives were separated with a good baseline resolution as shown in Fig. 2. In our previous study [12], after derivatization, the derivatized solution was extracted using hexane/ethyl acetate (10:1, *v*/*v*) to remove excess reagent hydrolysate (BDC-OH). But our further studies show that the organic solvents extraction could result in some loss of hydrophobic amino acid derivatives (e.g. Val, Trp, Phe, Ile, Leu, $(cys)_2$). So we tried to find a way to overcome it. Finally, by appropriate dilution within the analytical linear range, the chromatographic separation (Fig. 2) was satisfactory when the derivatization solution was injected directly without any extraction as described in "Derivatization procedure". The identification of amino acid derivatives was carried out by on-line mass spectrometry with ESI source. MS data of all amino acid derivatives are shown in Table 2. MS and MS-MS spectra of the representative BCEOC-Tyrosine derivative are shown in Fig. 3, and its MS cleavage mode is analyzed in Fig. 4.

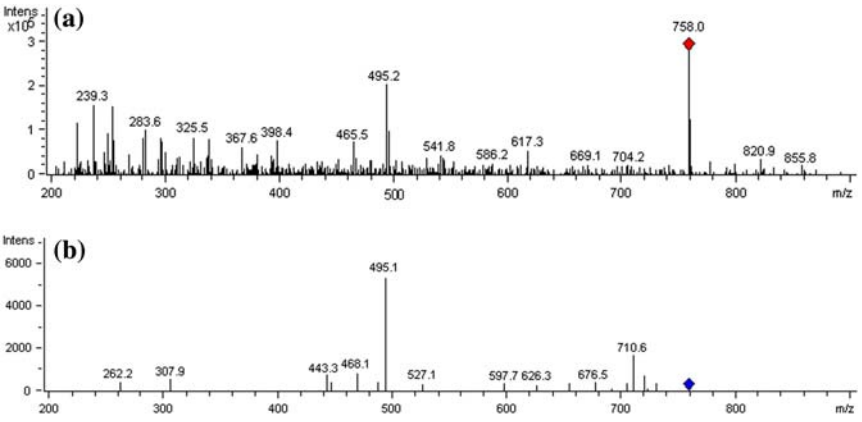


Fig. 3. Molecular ion MS spectra (**a**) and MS-MS (**b**) of representative tyrosine derivative (negative ion mode)

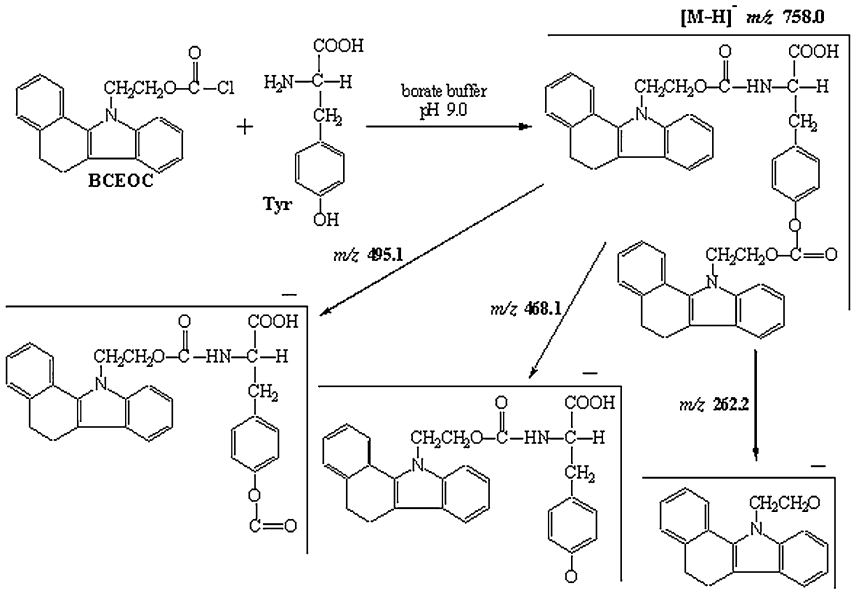


Fig. 4. The scheme of MS cleavage mode of tyrosine derivative (negative ion mode)

Method Validation

Linearity and Detection Limits

On the basis of the optimum derivatization conditions, the calibration graphs were established with the peak area (*Y*) versus amino acid injection amounts (*X*). Injected amounts were from 51.6 fmol to 105.6 pmol with an injection volume of 10 μL (the corresponding linear range was from 5.16 to 10560 nmol L^{-1} with a 2,046-fold concentration range). Linear regression equations, correlation coefficients and LODs for all amino acid derivatives are shown in Table 2. All amino acid derivatives are found to give excellent linear responses over this range with correlation coefficients of 0.9995–0.9999. The calculated LODs of each amino acid (at a signal-to-noise ratio of 3:1) were 6.3–36.8 fmol (0.63–3.68 nmol L^{-1}). The limits of quantitation (LOQs, shown in Table 2) of 20 amino acids were also obtained from derivatized standards when S/N = 10:1 and ranged from 5.16 nmol L^{-1} of Lys to 6.05 nmol L^{-1} of His (injection volume 10 μL). AQC has been developed as a popular pre-column derivatization reagent for amino acids since as reported in [15]. In this study, the LODs of BCEOC and AQC for amino acids were compared (Table 2), and it was seen that the LODs of BCEOC for amino acids are about 3–15 times lower than those of AQC. Thus, BCEOC had higher detection sensitivity.

Repeatability

Under the optimum chromatographic conditions, the method repeatability was examined by measuring peak areas and

Table 3. Recovery data for 20 amino acids (n = 3)

Amino acid	Added amount = 1 pmol (n = 3)		Added amount = 10 pmol (n = 3)		Added amount = 50 pmol (n = 3)		Average	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Arg	99.4	1.8	99.2	1.7	102.3	2.1	100.3	1.8
Asp	98.3	2.1	99.2	2.2	96.4	1.9	97.9	2.1
Ser	97.8	2.4	98.9	2.5	98.7	2.0	98.4	2.3
Glu	100.9	1.9	99.8	1.7	103.6	1.7	101.4	1.7
Thr	101.5	2.0	100.5	1.9	102.4	2.1	101.4	2
Gly	102.4	1.7	103.8	1.8	101.5	1.9	102.5	1.8
Ala	98.7	2.3	96.7	2.1	99.8	2.1	98.4	2.2
GABA	99.8	2.4	101.8	2.2	97.6	2.1	99.7	2.2
Pro	92.4	2.8	90.4	2.9	91.2	3.0	91.3	2.9
Met	88.4	2.3	87.6	2.5	87.4	2.7	87.8	2.5
Val	103.1	2.1	104.1	2.4	100.2	2.0	102.4	2.2
Trp	102.4	1.8	101.4	1.9	102.2	1.7	102.0	1.8
Phe	104.8	1.8	94.8	1.9	103.6	1.9	101.1	1.9
Ile	97.3	2.0	99.1	2.1	99.2	2.3	98.5	2.1
Leu	98.4	2.1	99.8	2.5	96.4	2.5	98.2	2.4
$(Cys)_2$	97.7	2.4	103.2	2.2	95.8	2.3	98.9	2.3
His	101.2	1.9	103.1	2.1	103.7	1.8	102.6	1.9
Orn	103.4	1.8	101.1	1.9	102.1	1.9	102.2	1.9
Lys	104.8	1.9	104.4	2.3	104.9	2.3	104.7	2.2
Tyr	102.8	2.0	103.2	2.4	103.5	2.4	103.2	2.3

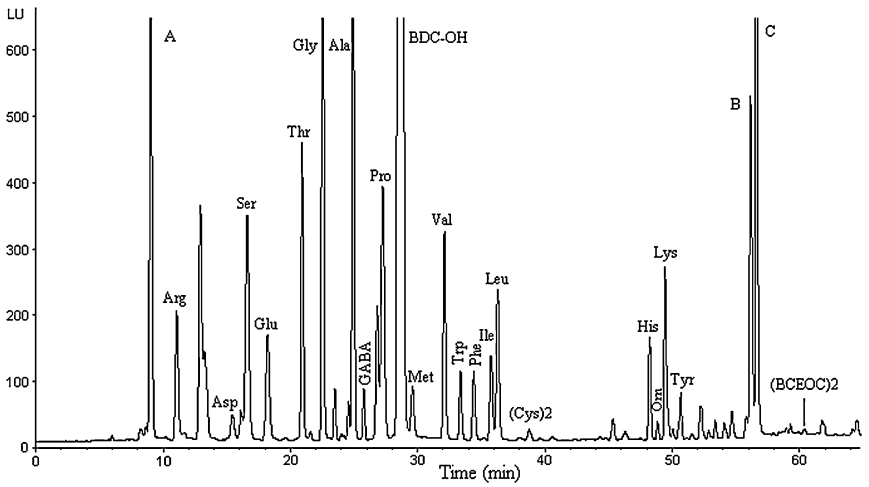


Fig. 5. Chromatogram of amino acid derivatives of Wistar rat plasma. Amino acid peaks and chromatographic conditions as Fig. 2. Peaks A, B and C were not identified (MS information for them was shown in part "Compositional Determination of Rat Plasma Samples")

retention times of six replicative injections of 50 pmol standard amino acid derivatives. From Table 2, it could be seen that relative standard deviations (RSDs) of the peak areas and retention times were from 0.86 to 2.29% and from 0.004 to 0.045%, respectively.

Stability

A solution at room temperature containing of 50 pmol standard amino acid derivatives was analyzed by LC at 0, 1, 2, 4, 8, 16, 24, 72 h for evaluating the stability of derivatives. The RSD values of all amino acid derivatives calculated by comparing peak area values with 0 h were all less than 3.0%, thus the stability of BCEOC-standard amino acid derivatives was satisfactory for the chromatographic analysis. Treated plasma samples (extracted plasma solutions) stored at −80 and 4 °C for 4 months were used for evaluating their stability. The RSD values of all amino acids in these samples calculated by comparing peak area values before 4 months (n = 3) with those after 4 months (n = 3) were all less than 5.2% for −80 °C and in the range of 21.5–30.6% for 4 °C, respectively. Therefore, for a long period storage of extracted plasma solution, −80 °C was necessary. In addition, the RSD values of all amino acids in the derivatized plasma samples at room temperature were all < 3.8% for 3 days (n = 6, respective three times analysis before or after 3 days) and < 8.5% for 1 week (n = 6, respective three times analysis before or after 1 week). Stability after three freeze (−80 °C) and thaw cycles of extracted plasma samples was also determined, and the RSD values of all amino acids were all < 3.2% (n = 6, respective three times analysis before or after cycles).

Accuracy and Precision

Five replicates of each rat plasma sample (total 3 samples), which were spiked into standard amino acids at 0.1, 1.0 and 5.0 μmol L^{-1}, were analyzed for the determination of accuracy and precision. The intra-day and inter-day accuracy and precision were measured on the same day (n = 5) and on the sequential three days (n = 15), respectively. Accuracies were determined as the percentage ratios of the measured concentration to the spiked concentration and the coefficients of variation (CV%) were used to report the precision. The mean intra-day accuracy ranged from 92.6 to 104.8% with the mean CV% in the range of 2.4–7.6%. The mean inter-day accuracy ranged from 90.5 to 103.1% with the mean CV % in the range of 2.6–8.4%. These results indicate that quantification of amino acid in rat plasma can be carried out with acceptable accuracy and precision using this method.

Recovery

Blood samples were collected from the same species of Wistar male rats which were fed under the same environment and then the blood sample of one rat was divided into two equal parts. Only to one part 10 μL amino acid standard solution

were added (added amounts at three levels: 1, 10 and 50 pmol). Extraction and derivatization of all blood samples was the same as described earlier. The analyses were processed in three duplicates for each level ($n = 3$). After deducting the amino acids which were intrinsic in plasma, the average recoveries for 20 amino acids were between 87.8–104.7% with the largest mean RSD (%) <2.9% (Table 3).

Application to Exercise Physiology Study

Compositional Determination of Rat Plasma Samples

After the plasma samples were taken out from fridge and thawed, derivatization and chromatographic separation conditions were according to the optimum conditions mentioned earlier. The amino acid derivatives in rat plasma samples were doubly identified by comparing chromatographic retention time with standards and on-line mass spectrometry. A typical chromatogram of amino acids from rat plasma is shown in Fig. 5. Peaks A, B and C were not identified but their MS and MS-MS data in the positive ion mode were as follows: peak A, MS: *m/z* 415.1, MS-MS: *m/z* 370.1, 346.1, 329.1, 301.4, 264.1; peak B, MS: *m/z* 564.6, MS-MS: *m/z* 498.5, 355.4, 283.6, 264.1; peak C, MS: *m/z* 564.5, MS-MS: *m/z* 478.3, 439.2, 396.5, 356.7, 283.9, 264.1. The contents of amino acids from rat plasma of three groups are shown in Table 4. The ratios of Glu/GABA and BCAA/AAA in rat plasma at different states are shown in Table 5.

Table 4. Concentrations of amino acids in rat plasma at different states ($\overline{X} \pm S$, $n = 8$, nmol mL^{-1})

Amino acid	Quiet	At exercising exhaust	12 h After exercising exhaust
Arg	229.18 ± 8.46	281.32 ± 8.48*	230. 75 ± 10.65
Asp	184.54 ± 6.65	223.29 ± 10.66*	189.26 ± 11.38
Ser	540.49 ± 9.74	608.33 ± 12.18	549.33 ± 14.75
Glu	281.65 ± 10.31	346.78 ± 10.26*	290.46 ± 8.78
Thr	474.40 ± 11.26	538.65 ± 13.34	476.37 ± 7.58
Gly	749. 08 ± 7.83	832.47 ± 12.56	740.12 ± 12.91
Ala	822.49 ± 12.69	904.56 ± 10.73	831.38 ± 13.46
GABA	101.06 ± 5.24	182.46 ± 10.46*	115.87 ± 7.83
Pro	581.77 ± 13.98	664.46 ± 7.65	590.28 ± 8.74
Met	133.53 ± 8.36	150.69 ± 8.43	136.27 ± 6.56
Val	323.94 ± 6.52	371.22 ± 12.81*	334.65 ± 9.64
Trp	109.83 ± 0.27	186.73 ± 7.19**	118.24 ± 6.95
Phe	117.57 ± 5.76	166.32 ± 7.62*	125.23 ± 4.46
Ile	157.85 ± 3.48	180.79 ± 10.34*	148.96 ± 3.88
Leu	305.96 ± 9.46	352.18 ± 10.77*	314.37 ± 8.76
$(Cys)_2$	75.09 ± 4.23	86.67 ± 4.57	79.64 ± 12.96
His	876.68 ± 14.82	978.28 ± 8.38	880.62 ± 14.84
Orn	89.15 ± 6.92	102.49 ± 11.45	86.73 ± 6.58
Lys	213.32 ± 8.56	266.72 ± 10.86*	221.72 ± 7.46
Tyr	132.36 ± 6.74	188.58 ± 6.34*	140.38 ± 4.36

* Compared with the control $P < 0.05$
** Compared with the control $P < 0.01$

Table 5. Ratio of corresponding amino acids in rat plasma at different states

Ratio	Quiet	At exercising exhaust	12 h After exercising exhaust
Glu/GABA	2.79 ± 0.38	1.90 ± 0.34*	2.51 ± 0.35
BCAA/AAA**	2.19 ± 0.16	1.67 ± 0.28*	2.07 ± 0.32

* Compared with the control $P < 0.05$
** BCAA is the summation of three branched-chain amino acids: Leu, Ile, Val, AAA is the summation of three aromatic amino acids: Phe, Tyr, Trp

Data Analysis and Discussion

Table 4 and 5 show that: (1) In Table 4, the amino acid contents in rat plasma of Group B were significantly higher than those of Group A, which indicated that exhausting exercise enhanced the metabolism of amino acids in the rat body. There was no significant difference of amino acid contents between Group C and A, which indicated that amino acids almost came back to the levels of the quiet state (Group A) after 12 h of exercising exhaust; (2) Comparing Group B with Group A, amino acids whose contents in rat plasma rose with significant differences ($P < 0.05$, Table 4) after exhausting exercise were as follows: Arg, Lys, excitatory amino acid neurotransmitters Glu and Asp, inhibitory amino acid neurotransmitter GABA, BCAAVal, Ile and Leu, AAA Phe and Tyr. Amino acid whose content in rat plasma rose with remarkable difference ($P < 0.01$, Table 4) after exhausting exercise was aromatic amino acid Trp. Trp was the synthetic precursor of 5-hydroxytryptamine (5-HT) which brought exercise-induced central fatigue, content of Trp in plasma rose significantly in exhaustive exercise and this impelled more Trp to enter blood–brain barrier so as to cause exhaustive exercise fatigue. That was in accordance with the report of Blomstrand [16]. Amino acids whose contents had elevatory trend but no statistical significance were as follows: Ser, Thr, Gly, Ala, Pro, Met, $(Cys)_2$, His, Orn; (3) Comparing Group B with Group A, although the contents of excitatory amino acids Glu and Asp, inhibitory amino acid GABA rose with significant difference ($P < 0.05$, Table 4), enhancement degree of Glu and Asp was less than GABA. As a result, the ratio of Glu/GABA declined with significant difference ($P < 0.05$, Table 5). The enhancement of the GABA content had a general inhibitory effect to central neuron, when at exercising exhaust state (Group B), rats were in a central inhibitory state, that was in accordance with the development of exercise-induced central fatigue [17]; (4) Comparing Group B with A, although the contents of BCAA Val, Ile and Leu, and AAA

Phe, Tyr and Trp rose with significant difference ($P < 0.05$, Table 4), enhancement degree of BCAA was less than AAA. As a result, the ratio of BCAA/AAA declined with significant difference ($P < 0.05$, Table 5). That impelled some precursors of inhibitory monoamine neurotransmitters, such as Phe, Tyr, Trp, to enter into the brain, and the contents of 5-hydroxytryptamine in rat brain rose obviously, that was in accordance with the development of exercise-induced central fatigue [18].

Conclusions

An LC-FLD-ESI-MS method has been validated for the analysis of amino acids in rat plasma and applied for studying the relationship of exercise physiology and AA contents in rat plasma by contrasting the contents of excitatory and inhibitory amino acids, BCAA and AAA in different states. It was indicated that the BCEOC method had high sensitivity, excellent accuracy and precision, and provided a good technique for the determination of amino acids in plasma.

Acknowledgments

The authors thank Prof. Jinmao You (College of Chemical Science, Qufu Normal University, Qufu 273165, China) for technical assistance and the valuable suggestions. This work was supported by the National Natural Science Foundation of China (Beijing, No. 20075016).

References

1. Aguiló A, Castaño E, Tauler P, Guix M, Serra N, Pons A (2000) J Nutr Biochem 11(2):81–86
2. Li H, Wang H, Chen J, Wang L, Zhang H, Fan Y (2003) J Chromatogr B Analyt Technol Biomed Life Sci 788(1):93–101
3. Kano T, Nagaki M, Takahashi T, Ohnishi H, Saitoh K, Kimura K, Muto Y (1991) Gastroenterol JPN 26(3):344–349
4. Shiels M, Czaja A, Ludwing J (1985) Dig Dis Sci 30(9):819–824
5. Dobashi Y, Santa T, Nakagomi K, Imai K (2002) Analyst 127(1):54–59
6. Nojiri S, Kamata K, Nishijima M (1998) J Pharm Biomed Anal 16(8):1357–1362
7. Bauza T, Blaise A, Daumass F, Cabanis J (1995) J Chromatogr A 707(2):373–379
8. Cohen S, Deantonis K (1994) J Chromatogr A 661:25–34
9. Molnar P (1994) J Chromatogr A 661:43–50
10. Zhao X, You J, Suo Y (2007) J Liq Chromatogr Rel Technol 30:1963–1979
11. Shi Y, Ming Y, Wang C, Zhao X, Zhang R, You J (2006) Chin J Anal Chem 34(4):503–507
12. You J, Ming Y, Shi Y, Zhao X, Suo Y (2005) Talanta 68:448–458
13. Simonetta F, Sang W, Gregory G, Dolnikowski, Jacob S (2002) Anal Chem 74:4526–4531
14. Wang XL (1997) Organic Chemistry, 3rd edn. High Education Press, Beijing, pp 112–113
15. Cohen S, Michaud D (1993) Anal Biochem 211(2):279–287
16. Blomstrand E, Celsing F, Newsholme E (1988) Acta physiol Scand 133:115–121
17. Davis J, Bailey S (1997) Med Sci Sports Exerc 29(1):45–57
18. Lehmann M, Huonker M, Dimeo F, Heinz N, Gastmann U, Treis N, Steinacker J, Keul J, Häussinger D (1995) Int J Sports Med 16(3):155–159