

# Determination of trace amino acids in human serum by a selective and sensitive pre-column derivatization method using HPLC-FLD-MS/MS and derivatization optimization by response surface methodology

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**Abstract** Analysis of trace amino acids (AA) in physiological fluids has received more attention, because the analysis of these compounds could provide fundamental and important information for medical, biological, and clinical researches. More accurate method for the determination of those compounds is highly desirable and valuable. In the present study, we developed a selective and sensitive method for trace AA determination in biological samples using 2-[2-(7H-dibenzo [a,g]carbazol-7-yl)-ethoxy] ethyl chloroformate (DBCEC) as labeling reagent by HPLC-FLD-MS/MS. Response surface methodology (RSM) was first employed to optimize the derivatization reaction between DBCEC and AA. Compared with traditional single-factor design, RSM was capable of lessening laborious, time and reagents consumption. The complete derivatization

can be achieved within 6.3 min at room temperature. In conjunction with a gradient elution, a baseline resolution of 20 AA containing acidic, neutral, and basic AA was achieved on a reversed-phase Hypersil BDS C<sub>18</sub> column. This method showed excellent reproducibility and correlation coefficient, and offered the exciting detection limits of 0.19–1.17 fmol/μL. The developed method was successfully applied to determinate AA in human serum. The sensitive and prognostic index of serum AA for liver diseases has also been discussed.

**Keywords** Trace amino acids analysis · Human serum · HPLC-FLD-MS/MS · Pre-column derivatization · Response surface methodology

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## Introduction

Amino acids (AA), the basic units of proteins, play an important role in the metabolic processes of a living organism. Recently, analysis of trace AA in physiological fluids have received more attention (Badawy et al. 2008; Suryawan et al. 2009; Gowacki et al. 2010a) because the analysis of these compounds could provide fundamental and important information for medical, biological, and clinical fields. For example, the determination of physiological AA is of importance not only to the clinical diagnosis and treatment of hereditary diseases (Berson et al. 1976), liver diseases (Cascino et al. 1978), neuropathy (Zeman et al. 2008), diabetes (Carlsten et al. 2009), atherothrombotic disease (Gowacki et al. 2010a), etc., but also to the evaluation of the nutritive status and normal reference of the patient and breast-fed infants (Lindblad et al. 2008). Thus, the accurate trace AA determination in complex physiological fluids, and human or plant tissue is

reckoned to be one of the most important applications in medical, clinical, and biological fields.

Most AA show neither natural UV absorption nor fluorescence; thus, the detection of them at trace levels using absorptiometry is fairly difficult. Therefore, chemical derivatization is necessary to increase detection sensitivity and improve selectivity by means of pre-column or post-column high-performance liquid chromatography (HPLC) (Gatti et al. 2010) and electrophoretic microchips (Munro et al. 2000) or CE (Lin et al. 2007) separation. These techniques have been widely used in food, chemical, and biological sciences for AA analysis. Although many different types of labeling reagents have been developed, such as *o*-phthalaldehyde (OPA) (Gowacki et al. 2010b), 9-fluorenyl methyl chloroformate (FMOC) (Meng and Peng 2009), 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (Hou et al. 2009), phenyl isothiocyanate ester (PITC) (Molnar-Perl 1994) and so on, a variety of shortcomings in their applications have also been reported. For example, the OPA was only limited to primary AA. 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. For FMOC, after derivatization the excess FMOC reagent should be removed by a fussy extraction with some loss of hydrophobic derivatives. AQC has been successfully developed as a popular pre-column derivatization reagent for AA; 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 AA derivatives were usually higher than those for the later ones. An improved method for trace AA determination in biological samples capable of providing high selectivity, sensitivity, and reproducibility of analysis, with short derivatization time and minimal sample preparation is often required and meaningful. In our previous studies, we described the synthesis of 2-[2-(7H-dibenzo[a,g]carbazol-7-yl)-ethoxy] ethyl chloroformate (DBCEC) and its applications to analyze trace aliphatic amines and aldehydes (You et al. 2009b; You et al. 2009a). In this paper, we described a selective and sensitive HPLC-FLD method using DBCEC as pre-column derivatization reagent for the trace AA determination in biological samples. DBCEC is first studied for the determination of AA in serum samples, and showed some advantages including mild derivatization conditions, ease-of-handling, selective, and high detection sensitivity. The AA in human serum containing 22 healthy subjects and 27 hepatitis patients were determined by the developed method. The sensitive and prognostic index of serum AA for liver diseases was also discussed.

The derivatization is of great importance for the sufficient labeling of the analyzed components. Traditionally,

derivatization optimization in analytical chemistry has been carried out by monitoring the influence of one factor at a time on an experimental response (Yoshitake et al. 2003; Sotgia et al. 2008; Gatti et al. 2010). This optimization procedure is troublesome, reagents and time-consuming as well as ignoring the interaction effect of parameters. Compared to this classical method, response surface methodology (RSM) is more efficient, requires fewer data and provides interaction effects on the response besides factor effects (Bezerra et al. 2008). In this study, RSM was first applied to optimize the main derivatization parameters including derivatization time, pH, and concentration of DBCEC.

## Experimental

### Instrumentation and chemicals

HPLC separation, MS identification and sample analysis were carried out using Agilent 1100 Series high-performance liquid chromatography/mass spectrometry (HPLC-MSD ion Trap SL, a complete LC-MS/MS). The mass spectrometer 1100 Series LC-MSD Trap-SL (ion trap) from Bruker Daltonik (Bremen, Germany) was equipped with an electrospray ionization (ESI) source. Ion source conditions: ESI in positive ion mode, nebulizer pressure 241.3 kPa, dry gas temperature 350°C, dry gas flow 9.0 l/min and capillary voltage-3500 V. Derivatives were separated on Hypersil BDS C18 column (200 mm × 4.6 mm, 5 μm, Yilite Co Dalian, China). Fluorescence excitation and emission spectra were obtained on a 650-10S fluorescence spectrophotometer (Hitachi, Japan). The mobile phase was filtered through a 0.2-μm nylon membrane filter (Alltech, Deerfield, IL, USA).

Twenty amino acid standards including alanine (Ala), arginine (Arg), aspartic acid (Asp), cysteine (Cys), glutamic acid (Glu), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), Ornithine (Orn), Tryptophan (Trp), Cystine ((Cys)<sub>2</sub>), glycine (Gly), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr), valine (Val) and γ-aminobutyric acid (GABA), were obtained from Sigma Corporation. Other reagents such as acetic acid were all analytically pure. Pure water was prepared by Milli-Q super pure water system. DBCEC was synthesized in our laboratory (You et al. 2009b).

### Preparation of standard solutions and samples

Individual stock solutions ( $1 \times 10^{-2}$  M) of 20 AA were prepared in water. The mixed standard solutions for HPLC analysis were prepared by diluting the stock solutions with 0.2 M sodium borate buffer (pH 9.0). The DBCEC solution

(0.01 mol/L) was prepared by dissolving 32.6 mg of DBCEC in 10 mL of acetonitrile and the corresponding low concentration of reagent was diluted using anhydrous acetonitrile.

#### Derivatization procedure

80  $\mu\text{L}$  of acetonitrile, 100  $\mu\text{L}$  of 0.2 M sodium borate buffer (pH 9.46), 10  $\mu\text{L}$  of AA solution, and 110  $\mu\text{L}$  DBCEC were added into a 2.0-ml vial successively, The vial was sealed at room temperature for about 6.3 min, and then, 100  $\mu\text{L}$  of 50% acetic acid was added into the solution until the final pH was in the range of 6.0–6.5. Finally, the derivatized sample was directly injected into the HPLC system for analysis. The derivatization scheme of typical Glu with DBCEC is shown in Fig. 1a.

#### Extraction and derivatization procedure for serum samples

The blood samples were obtained from 88 military hospital of China, and taken from 27 hepatitis patients (ages 45–55). The control group was from 22 healthy volunteers.

The blood samples were taken from the veins by means of plastic syringes, and after 1 h at room temperature, centrifuged at 2,500g for 15 min. Then the serum were immediately frozen and stored at  $-70^\circ\text{C}$  until analysis. An aliquot of 100  $\mu\text{L}$  of serum was added into a 2 mL Eppendorf tube. Protein precipitation was carried out by adding 1 mL of acetonitrile. Eppendorf tubes were vortex-mixed for 30 s and after standing for 15 min, the tubes were centrifuged at 15,000g for 15 min. The supernatant was transferred into another tube.

The derivatization of extracted serum samples proceeded as follows: 50  $\mu\text{L}$  of serum extraction solutions was added into a 2-mL vial, 200  $\mu\text{L}$  acetonitrile, 120  $\mu\text{L}$  of borate buffer and 50  $\mu\text{L}$  of DBCEC solution were then added. The following analysis procedure was the same as the derivatization procedure of AA standards described above.

#### HPLC

Derivatives were separated on a reversed-phase Hypersil BDS  $\text{C}_{18}$  column (200 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ ), in conjunction with a gradient elution. Eluent A was 30% acetonitrile (containing 30 mM, pH 3.7 ammonium/formic acid buffer); B was 50% acetonitrile (containing 30 mM, pH 3.7 ammonium/formic acid buffer), and C was 95% acetonitrile. The flow rate was constant at 1.0 mL  $\text{min}^{-1}$  and the column temperature was set at  $35^\circ\text{C}$ . The fluorescence excitation and emission wavelengths were set at

$\lambda_{\text{ex}} = 300$  and  $\lambda_{\text{em}} = 395$  nm. The gradient elution program was presented in Table 1.

#### Data statistics

The response surface graphs of predicted values by models were plotted using a software Design-Expert 7.1.3 Trial (State-Ease, Inc., Minneapolis, MN, USA), meanwhile, the coefficients of the quadratic polynomial model were also obtained by this software. The quality of the fitted model was expressed by the determined coefficient ( $R^2$ ), and its statistical significance was checked by an  $F$  test. The analysis of AA data statistic work was accomplished with SPSS 13.0. The results were expressed in the form of “average  $\pm$  standard deviation” ( $\bar{X} \pm S$ ). Data were analyzed with independent sample  $T$  test and one-way analysis of variance.

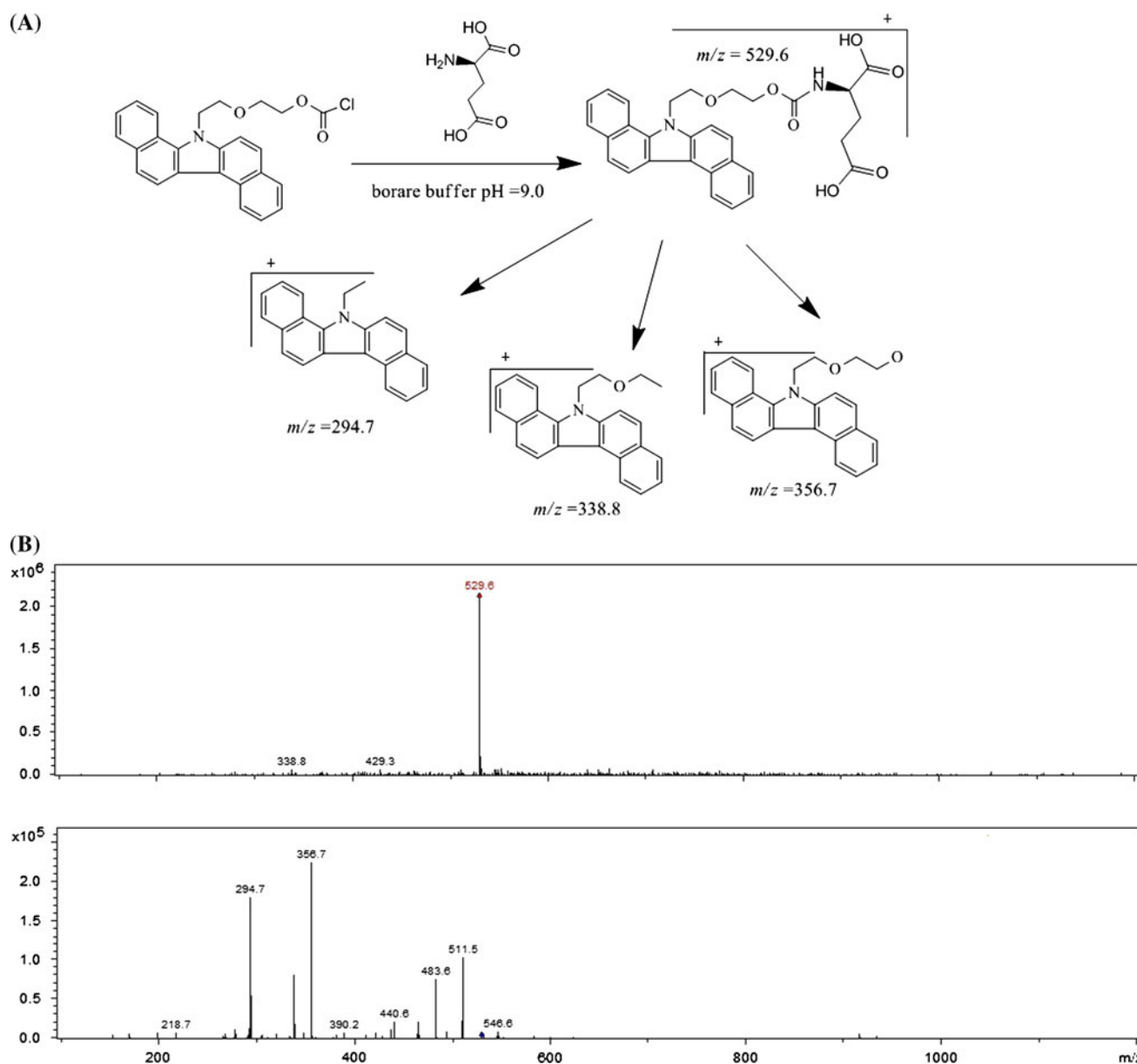
## Results and discussion

#### Optimization of derivatization parameters

Derivatization time, pH, and concentration of DBCEC were the main derivatization parameters affecting the derivatization yield, and chosen for further optimization by employing a three-level, three-variable Box-Behnken design (BBD) from RSM. Glu and Trp were used as standards of the tested compounds. The average peak area of Glu and Trp derivatization was taken as the response,  $Y$ , which reflects the detector response. A total of 17 experiments were designed (Table 2), and the peak areas of each experiment were presented in Table 2. Regression analysis was performed, based on the experimental data, and the predicted second-order polynomial model was as follows:

$$Y = 2566.80 + 148.25X_1 - 55X_2 + 200.5X_3 + 44X_1^2 - 22X_2^2 - 33X_3^2 - 136.15X_1X_2 - 1246.65X_1X_3 - 297.15X_2X_3$$

The analysis of variance (ANOVA) for the experimental results of BBD was presented in Table 3. In the selected experimental range, the derivatization yield mostly depended on the concentration of DBCEC, followed by derivatization time and pH. The  $P$ -value of the model was  $<0.0001$  (Table 3), which indicated that the model fitness was significant. The value of  $R^2$  (0.9845) revealed that the experimental data were in good agreement with the predicted values of peak area.  $F$  value for the lack of fit in Table 3 was insignificant ( $P > 0.05$ ), meaning that this model was sufficiently accurate for predicting the relevant responses.



**Fig. 1** Scheme of derivatization reaction of DBCEC with Glutamic (a); the cleavage mode of protonated molecular ion (a); MS spectra of Glutamic derivative: molecular ion MS, and MS/MS (b), Scanning

range from 100 to 1,000 amu under ESI positive ion mode; derivatives were isolated from a Hypersil BDS-C18 column and into the on-line mass spectrometer

**Table 1** Gradient elution program

Time (min)	A (%)	B (%)	C (%)
0	30	70	0
15	0	100	0
20	0	95	5
40	0	65	35
45	0	35	70
55	0	30	70

The 3D response surface in Fig. 2 provided a method to visualize the relationship between responses and experimental levels of each variable and the type of interactions between two test variables. Figure 2a depicted the effect of derivatization time and pH on the peak area. With a given derivatization time, peak area increased rapidly with the increasing derivatization pH and reached a maximum value, followed by a decline with its further increase. Figure 2b described the interaction between the concentration of DBCEC and derivatization time. With a definite

**Table 2** The Box-Behnken design matrix of three test variables in actual and coded level along with the observed responses (peak area)

Run	Independent variable			Y (Peak area)
	X <sub>1</sub> Derivatization time (min)	X <sub>2</sub> Derivatization pH	X <sub>3</sub> Concentration of DBCEC	
1	4.5 (0) <sup>a</sup>	9.50 (0) <sup>a</sup>	3.50 (0) <sup>a</sup>	2,508
2	4.5 (0)	9.50 (0)	3.50 (0)	2,480
3	4.5 (0)	12.00 (+1)	1.00 (−1)	907
4	4.5 (0)	9.50 (0)	3.50 (0)	2,533
5	1.00 (−1)	7.00 (−1)	3.50 (0)	1,149
6	4.5 (0)	12.00 (+1)	6.00 (+1)	1,100
7	4.5 (0)	9.50 (0)	3.50 (0)	2,513
8	1.00 (−1)	9.50 (0)	6.00 (+1)	2,300
9	1.00 (−1)	12.00 (+1)	3.50 (0)	880
10	1.00 (−1)	9.50 (0)	1.00 (−1)	1,713
11	4.5 (0)	7.00 (−1)	6.00 (+1)	1,205
12	4.5 (0)	9.50 (0)	3.50 (0)	2,800
13	4.5 (0)	7.00 (−1)	1.00 (−1)	880
14	8.00 (+1)	9.50 (0)	1.00 (−1)	2,011
15	8.00 (+1)	12.00 (+1)	3.50 (0)	1,307
16	8.00 (+1)	7.00 (−1)	3.50 (0)	1,400
17	8.00 (+1)	9.50 (0)	6.00 (+1)	2,510

<sup>a</sup> actual level (coded level)

**Table 3** Estimated regression coefficients for the quadratic polynomial model and ANOVA for the experimental results

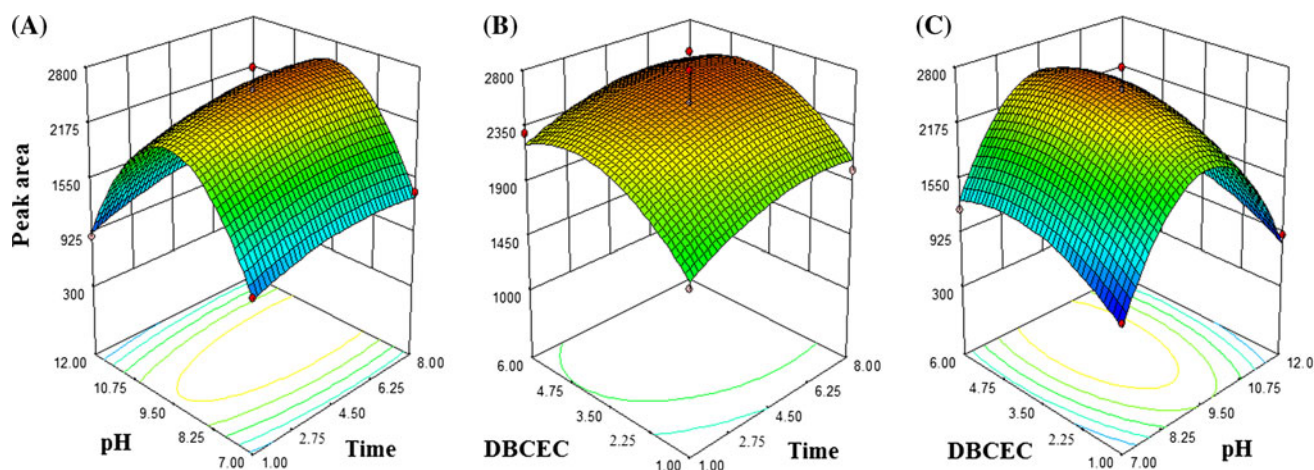
Regression coefficient	Estimated coefficients	Degree of freedom	F value	P value
$\beta_0$	2566.80	1	49.42	<0.0001
<b>Linear</b>				
$\beta_1$	148.25	1	9.97	0.0160
$\beta_2$	−55.00	1	1.37	0.2797
$\beta_3$	200.50	1	18.24	0.0037
<b>Interaction</b>				
$\beta_{12}$	44.00	1	0.44	0.5287
$\beta_{13}$	−22.00	1	0.11	0.7501
$\beta_{23}$	−33.00	1	0.25	0.6344
<b>Quadratic</b>				
$\beta_{11}$	−136.15	1	4.43	0.0735
$\beta_{22}$	−1246.65	1	371.10	<0.0001
$\beta_{33}$	−297.15	1	21.08	0.0025
Lack of fit		3	1.04	0.4662
Pure error		4		
$R^2$	0.9845	Adjusted $R^2$	0.8646	

derivatization time, the peak area increased rapidly with the increase of the concentration of DBCEC and reached the highest value, and then no obvious variation for peak areas were observed with the further increasing concentration of DBCEC. Figure 2c showed the interaction between derivatization pH and concentration of DBCEC.

The optimal conditions obtained by calculating the regression equation were as follows: derivatization time, 6.3 min; derivatization pH 9.46; concentration of DBCEC, 4.3-fold molar excess to total molar AA.

The concentration of the labeling reagent can significantly affect the form of the monosubstituted derivatives for diamino acids. Several experiments were carried out to further verify the concentration of DBCEC. For this experiment, a solution of diamino acids containing Lys, Orn, and Glu, was prepared. The range of the labeling reagent concentration was 1.0- to 4.3-fold molar excess to total molar AA. Results indicated that with as little as a 3.0-fold molar excess of labeling reagent, the derivatization of AA was incomplete and resulted in monosubstituted derivatives. The fluorescence intensity was constant when the labeling reagent concentration was controlled at 3- to 4.3-fold molar excess to total molar AA; increasing the reagent beyond this level had no significant effect on derivatization yields. Thus, 4.3-fold molar excess of labeling reagent can avoid the form of monosubstituted derivatives and ensure the sufficient labeling of the analyzed components.

In the present study, RSM exhibited great superiority compared to traditional optimization methods based on single-factor design (Yoshitake et al. 2003; You et al. 2003; Sotgia et al. 2008; Gatti et al. 2010). Less laborious, time and reagents-consuming could result in sufficient labeling of the analyzed AA components, which is an added benefit of this method.



**Fig. 2** The 3D response surface of detection responses (peak area) affected by derivatization pH, derivatization time and DBCEC concentration: the effect of derivatization time and pH on the peak

area (a); the effect of derivatization time and DBCEC concentration on the peak area (b) and the effect of derivatization pH and DBCEC concentration on the peak area (c)

### HPLC separation and MS identification

The mobile phase pH can significantly affect the resolution. The pH value of the mobile phase in RP separation of AA were often adjusted by boric or phosphate buffer. In the present study, formic acid/ammonia buffer was used to control mobile phase pH, which could reduce the metal salt pollution on the mass ion chamber. Furthermore, the acidic eluents could provide hydrogen proton to enhance the ESI ionization efficiency of AA derivatives. We also found when the pH value of mobile phase A was 3.7, a complete baseline resolution for 20 AA derivatives was obtained within the shortest time and good peak shape (Fig. 3). Thus, pH = 3.7 was chosen as the mobile phase pH.

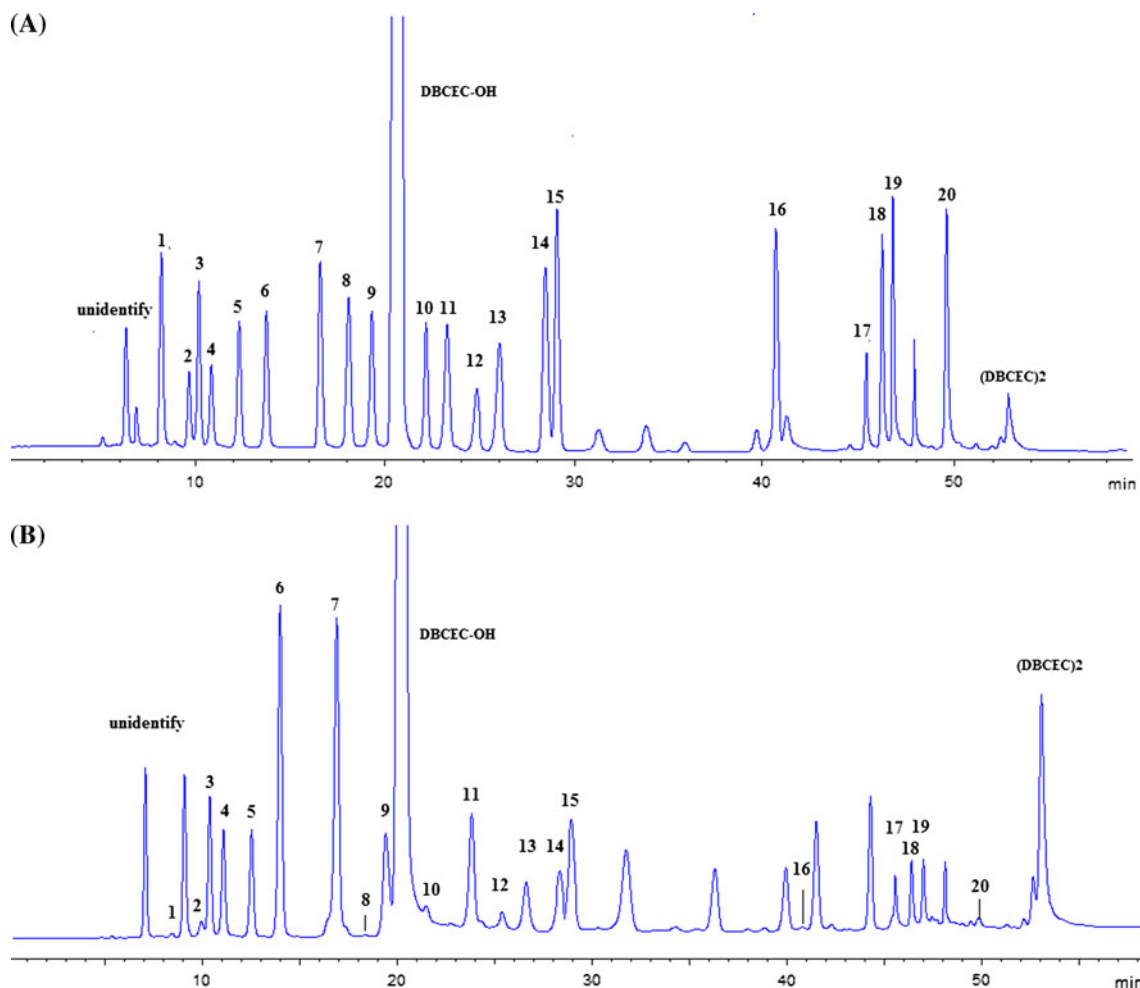
The structural identification of AA derivatives was carried out by on-line MSD with ESI source in positive ion mode. The MS, MS/MS spectra and the cleavage mode of representative Glu derivative were shown in Fig. 1. The molecular ions (MS,  $[M + H]^+$  ion) and specific fragment ions (MS/MS) of AA derivatives were listed in Table 4. All derivatives showed an intense molecular ion peaks at  $[M + H]^+$  ions. The collision-induced dissociation spectra (MS/MS) of molecular ions (MS,  $[M + H]^+$  ion) produced intense and stable fragment ions at  $m/z$  294.7,  $m/z$  338.8 and  $m/z$  356.7 (Fig. 1), which was specific fragment ions for DBCEC-labeled AA derivatives. DBCEC can specifically react with AA or other amino compounds. Other compounds with amino group may be present in real samples, and were presumably co-extracted and derivatized by DBCEC; but this interference can be monitored by the molecular ions and the characteristic fragment ions, and can also be avoided by adjusting the mobile phase gradient.

### Stability of DBCEC and AA derivatives

After the anhydrous acetonitrile solution of DBCEC had been stored at 4°C in darkness for 2 weeks, the AA derivatization yields obtained with the little change (<1.0%). The corresponding neutral solution of derivatives was stored at 4°C and at room temperature in darkness as well as in daylight for a period of 1 week, during which time they were analyzed six times. Results indicated that these AA derivatives were stable enough to be efficiently analyzed by HPLC analysis at least 48 h later, with the normalized peak areas varying by <2.32%.

### Reproducibility, correlation coefficient, recovery and LOD

The linearity was established by the analysis of AA standards concentration range of 5–12,500 nM, and all AA derivatives gave excellent linear responses with correlation coefficients >0.9992. The AA standard solution consisting of 30 pmol was analyzed to determine the reproducibility of the method. Relative standard deviations of retention time and peak area (RSD, %;  $n = 8$ ) were less than 0.55 and 1.42%, respectively. The recoveries were calculated as follows: recovery (%) =  $100(a - b)/c$ , where  $a$  was the measured concentration obtained from the extracted serum solutions which were spiked standard;  $b$  was the concentration of AA in the matrix and  $c$  was the added known concentration to the matrix. The analyses were carried out in duplicate, and the experimental recoveries obtained were ranged from 94 to 99%. Detection limits are an important consideration when the components of biological matrixes are analyzed, particularly when they are present at low or trace concentrations. The calculated detection limits of this



**Fig. 3** Representative chromatograms of AA standard derivatives (a) and AA derivatives in healthy subject serum (b). Hypersil BDS C<sub>18</sub> column: 4.6 mm × 200 mm × 5 m; column temperature 35°C; flow rate 1.0 ml/min; excitation and emission:  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 300/395$  nm

Peaks: 1 Arg, 2 Asp, 3 Ser, 4 Glu, 5 Thr, 6 Gly, 7 Ala, 8 GABA, 9 Pro, 10 Met, 11 Val, 12 Trp, 13 Phe, 14 Ile, 15 Leu, 16 (Cys)<sub>2</sub>, 17 His, 18 Orn, 19 Lys, 20 Tyr

method (at a signal-to-noise ratio of 3:1) were ranged from 0.19 to 1.17 fmol/ $\mu\text{L}$  (Table 4). These detection limits are significantly lower than those reported for AA determination. For example, Nagy et al. (2003) described a direct tandem mass spectrometric method for AA analysis in neonatal screening that generated 2–42 pmol/ $\mu\text{L}$  detection limits. Thiele et al. (2008) employed a derivatization-free method based on a strong cation exchange liquid chromatography coupled to tandem MSD for AA determination in plant tissues, and the detection limits for this method were from 0.1 to 3 pmol/ $\mu\text{L}$ . Jámbor et al. (Jámbor and Molnár-Perl 2009) developed an AA analysis method by HPLC using 9-fluorenylmethyloxycarbonyl chloride as pre-column derivatization reagent, and obtained the detection limits of 1–10 pmol/ $\mu\text{L}$ . Gatti et al. (2010) developed a pre-column derivatization reagent 2,5-Dimethyl-1H-pyrrole-3,4-dicarbaldehyde for HPLC/UV detection of AA. This method yielded the detection limits of 0.19–0.50 pmol/ $\mu\text{L}$ .

#### Trace AA analysis in human serum

Under the proposed conditions, the AA in human serum including 22 healthy subjects and 27 hepatitis patients were determined. The peaks were doubly identified by chromatographic retention time and on-line MS identification. As expected, 20 AA in serum samples could be simultaneously separated with a good baseline resolution. The representative chromatogram of AA in healthy subject is presented in Fig. 3b. Table 4 showed the compositional data of AA in serum samples. The levels of Lys, Glu, Gly, Ala, Met, Phe, His, Orn, and Ser in hepatitis patient serum were significantly higher than those of the healthy serum ( $P < 0.01$ ). However, the levels of Asp and Leu in hepatitis patient serum were significantly lower than those of the healthy subjects ( $P < 0.01$ ). GABA at trace levels, one of the chief inhibitory neurotransmitters, has been associated with alcohol addiction, depression, and sleep problems (Petty et al. 1999).

**Table 4** MS and MS/MS data of amino acid derivatives, detection limits, amino acids concentration in serum of 22 healthy subjects and 27 hepatitis patients ( $\mu\text{mol/L}$ , mean  $\pm$  SD), BCAA/AAA and BCAA/Tyr molar ratio

Amino acids	MS and MS/MS data	Detection limits (fmol/ $\mu\text{L}$ )	Control subjects	Hepatitis patients
Arg	556.8, 294.5, 338.5, 356.4	0.81	19.96 $\pm$ 6.3	24.49 $\pm$ 8.6
Asp	515.6, 294.5, 338.5, 356.4	0.70	34.80 $\pm$ 5.5	16.89 $\pm$ 4.1*
Ser	487.6, 294.5, 338.5, 356.4	0.97	163.79 $\pm$ 10.2	328.67 $\pm$ 25.2*
Glu	529.6, 294.5, 338.5, 356.4	0.28	204.29 $\pm$ 12.3	403.55 $\pm$ 19.2*
Thr	501.7, 294.5, 338.5, 356.4	0.73	142.17 $\pm$ 9.9	146.43 $\pm$ 8.7
Gly	457.6, 294.5, 338.5, 356.4	1.16	480.45 $\pm$ 41.3	1068.19 $\pm$ 89.5*
Ala	471.6, 294.5, 338.5, 356.4	0.54	409.80 $\pm$ 35.3	1118.86 $\pm$ 90.3*
GABA <sup>a</sup>	485.5, 294.5, 338.5, 356.4	0.30	0.43 $\pm$ 0.10	0.22 $\pm$ 0.06
Pro	497.5, 294.5, 338.5, 356.4	0.32	144.30 $\pm$ 10.3	261.89 $\pm$ 19.0
Met	531.3, 294.5, 338.5, 356.4	0.30	104.94 $\pm$ 7.7	317.06 $\pm$ 80.6*
Val	499.6, 294.5, 338.5, 356.4	0.41	150.53 $\pm$ 8.2	115.02 $\pm$ 17.5
Trp	585.9, 294.5, 338.5, 356.4	0.48	44.09 $\pm$ 3.4	46.22 $\pm$ 8.6
Phe	547.5, 294.5, 338.5, 356.4	0.88	75.86 $\pm$ 7.4	106.46 $\pm$ 10.3*
Ile	513.5, 294.5, 338.5, 356.4	0.70	94.54 $\pm$ 11.3	60.32 $\pm$ 10.5
Leu	513.5, 294.5, 338.5, 356.4	1.11	132.65 $\pm$ 8.7	78.31 $\pm$ 9.0*
(Cys) <sub>2</sub>	1004, 294.5, 338.5, 356.4	1.17	17.93 $\pm$ 5.6	16.74 $\pm$ 10.2
His	918.7, 294.5, 338.5, 356.4	1.13	73.60 $\pm$ 12.3	120.36 $\pm$ 20.7*
Orn	894.8, 294.5, 338.5, 356.4	0.30	62.59 $\pm$ 14.1	356.40 $\pm$ 40.6*
Lys	908.2, 294.5, 338.5, 356.4	0.43	42.75 $\pm$ 7.1	133.85 $\pm$ 13.7*
Tyr	944.6, 294.5, 338.5, 356.4	0.19	51.44 $\pm$ 3.4	43.37 $\pm$ 1.8
BCAA/AAA <sup>b</sup>			2.20 $\pm$ 0.3	1.29 $\pm$ 0.2*
BRT <sup>c</sup>			7.34 $\pm$ 0.4	5.85 $\pm$ 0.3*

BCAA is the summation of three branched-chain amino acids: Val, Ile, Leu; AAA is the summation of three aromatic amino acids: Phe, Trp, Tyr

\* Compared with the control subjects  $P < 0.01$

<sup>a</sup> GABA,  $\gamma$ -aminobutyric acid

<sup>b</sup> Fischer's ratio, (BCAA/AAA molar ratio)

<sup>c</sup> BRT, molar ratio of BCAA/Tyr

Patients with acute posttraumatic stress disorder (Vaiva et al. 2006), and depression (Petty et al. 1999; Sanacora and Saricicek 2007) presents lower serum GABA concentration than that of the healthy subjects. Hepatitis patients in this study also presented slight lower serum GABA concentration than the control group (Table 4). The molar ratio of branched chain amino acids (BCAA) to aromatic amino acids (AAA) (Kano et al. 1991) and BCAA to Tyr (Ninomiya et al. 1999) in serum have been used as a sensitive and prognostic index for liver diseases (BCAA was the summation of three branched-chain amino acids: Val, Ile, Leu, AAA was the summation of three aromatic amino acids: Phe, Trp, Tyr). As was shown in Table 4, BCAA/AAA and BCAA/Tyr molar ratio in hepatitis patient's serum were  $1.29 \pm 0.2$  and  $5.85 \pm 0.3$ , respectively, which were significant compared with those in the healthy subjects ( $P < 0.01$ ). These conclusions are consistent with the models established in previous studies (Kano et al. 1991; Ninomiya et al. 1999) and can validate the feasibility of the models using BCAA/AAA and BCAA/Tyr molar ratio as sensitive and prognostic index for liver diseases.

## Conclusion

A HPLC-FLD method for trace AA determination in biological samples was developed using DBCEC as

labeling reagent, this method was capable of providing high selectivity, sensitivity, and reproducibility of analysis, with short derivatization time and minimal sample. The derivatization conditions for AA derivatization were optimized by utilizing a three-level, three-variable BBD from RSM. Alternatively, traditional optimization method based on single-factor design can be laborious, time-consuming, and complicated. The complete derivatization reaction of DBCEC with AA could be achieved within 6.3 min at room temperature. This method offered the satisfactory LOD of 0.19–1.17 fmol/ $\mu\text{L}$  and was successfully applied to analyze the AA in human serum. Meanwhile, the method reported here shows great potential for trace AA determination in complex physiological fluids, human or plant tissue and other biological samples.

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