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Determination of amino acids in rat brain microdialysate with 1,2,5,6-dibenzocarbazole-9-ethyl chloroformate as labeling reagent by high performance liquid chromatographic fluorescence detection and mass spectrometric identification☆

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ABSTRACT

A high performance liquid chromatography method for the determination of 20 amino acids (AAs), using 1,2,5,6-dibenzocarbazole-9-ethyl chloroformate (DBCEOC–Cl) as a novel fluorescent labeling reagent, has been developed and applied for the analysis of AAs in rat brain microdialysate. The simultaneous separation of 20 AA derivatives was achieved on a Hypersil BDS C_{18} column with gradient elution. And the identification of AA derivatives was carried out by on-line electrospray ionization mass spectrometry in positive ion mode. The AA derivatives were detected with excitation and emission at 300 nm and 395 nm, respectively. Excellent linear responses were observed with coefficients of >0.9988. The detection limits ranged from 0.217 to 4.75 nmol/L, at a signal-to-noise ratio of 3. The intra-day and inter-day precision for each AA was <3.2% and <4.3%, respectively. The mean recoveries for all AAs studied were in the range of 92.5–105.6%. Good compositional data could be obtained from as little as 15 μL of microdialysate. Facile DBCEOC–Cl derivatization coupled with high performance liquid chromatography and fluorescence detection allowed the development of a highly sensitive method for the quantitative analysis of trace levels of AAs from microdialysate.

1. Introduction

Amino acids (AAs) are not only the basic structural units of proteins; they are also a source of energy and serve as precursors for the biosynthesis of neurotransmitters, porphyrins, polyamines, and nitric oxide. Further, glutamate and the nonprotein amino acid γ-aminobutyric acid play important roles as neurotransmitters, and carnitine is used in intracellular lipid transport. Quantitative analysis of AAs is required in several fields, including clinical diagnostics of inborn errors of AA metabolism, biomedical research, bioengineering, and so on. Also, more and more interests shifted from the analysis of protein hydrolysates to the analysis of free AAs in various biological matrices [1]. In vivo microdialysis offers a unique approach to monitor biochemical events related to brain function and metabolism, and has been used extensively in many systems to measure the release of endogenous transmitters and other neuroactive substances during normal and pathological conditions. However, basal levels of AAs in microdialysates are typically in the low picogram per milliliter range, which makes analytical measurement a challenge [2].

Different methods for analysis of AAs have been developed and commercialized. Still, efforts to improve existing methodology with regard to robustness, reproducibility, and sensitivity are ongoing. Diverse analytical methods have been proposed for the analysis of AAs including gas chromatography (GC) [3,4], high-performance liquid chromatography (HPLC) [5,6] and capillary electrophoresis (CE) [7,8]. Traditionally, the determination of AAs has been conducted by ion-exchange chromatography, followed by post-column derivatization with ninhydrin. So far, the analysis of AAs using pre-column derivatization and reversed-phase HPLC separation has become widely accepted. Typical derivatization reagents include 9-fluorenylmethyl chloroformate (FMOC–Cl) [9,10], 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [11,12], *N*-(9-fluorenylmethoxycarbonyloxy) succinimide (FMOC–OSu) [13], orthophthalaldeyde (OPA) [14,15], phenyl

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isothiocyanate (PITC) [16,17], dabsyl chloride [18,19] and dansyl chloride [20,21]. However, a number of reports describe various shortcomings in the application of these reagents to analysis. These include short detection wavelengths, bad reproducibility, poor stability, and so on [1,22–24].

In our previous studies, we described the synthesis of some fluorescence labeling reagents and its applications for the analysis of AAs by HPLC with fluorescence detector (FLD), such as carbazole-9-ethyl chloroformate (CEOC–Cl) [25] and 1,2-benzocarbazole-9-ethyl chloroformate (BCEC–Cl) [26]. In this work, we report the synthesis of a novel reagent 1,2,5,6-dibenzocarbazole-9-ethyl chloroformate (DBCEOC–Cl). With DBCEOC–Cl as labeling reagent, a HPLC–FLD method for the simultaneous determination of 20 AAs has been developed and validated. At the same time, the proposed method has also been applied for the determination of AAs in rat brain microdialysate from a salicylate-induced tinnitus animal model. The results demonstrate that the method is suitable for quantitative analysis of trace levels of AAs from microdialysate.

2. Experimental

2.1. Instruments

Experiments were performed using Agilent 1100 Series HPLC (Agilent, USA). The HPLC 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 mass spectrometer (MSD Trap SL) from Bruker Daltonik (Bremen, Germany) was equipped with an electrospray ionization (ESI) source (model G1948A). The HPLC-MSD system was controlled by Agilent Chemstation software (version B.01.01). Derivatives were separated on Hypersil BDS C_{18} column (200 × 4.6 mm, 5 μm i.d., Dalian Elite Co., China). 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). Fluorescence excitation and emission spectra were obtained on a F7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), and the ultraviolet spectra were recorded by CARY 300 UV–vis spectrometry (Varian, Australia).

2.2. Chemicals

1,2,5,6-Dibenzocarbazole-9-ethyl chloroformate (DBCEOC–Cl) was synthesized in authors' laboratory as described in Section 2.3 and Fig. 1. Twenty AA standards were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile (CH_3CN) was purchased from Yucheng Chemical Reagent Co. (Shandong Province, China). Acetic 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.

2.3. Synthesis of the derivatization reagent (DBCEOC–Cl)

2.3.1. Synthesis of 1,2,5,6-dibenzocarbazole-9-ethanol (DBEEOC–OH)

1,2,5,6-Dibenzocarbazole [27] (20 g), KOH (7.0 g), and 2-butanone (200 mL) were mixed and rapidly cooled to 0 °C with ice water along with vigorous stirring. A cooled mixture of epoxyethane (6.2 g) in 50 mL of 2-butanone solution was added dropwise within 1 h. The contents were kept at ambient temperature for another 2 h with stirring. The solution was then heated to 50 °C for 2 h and concentrated by a rotary evaporator. After cooling, the residue was transferred into 200 mL of ice water with vigorous stirring for 0.5 h; the precipitated solid was recovered by filtration, washed with water and 30% ethanol solution, and dried at room temperature for 48 h. The crude products were recrystallized three times from methanol (200 mL × 3); affording a white crystal, yield (67.4%). Found: C, 84.84; H, 5.50; N, 4.49; calculated: C, 84.86; H, 5.50; N, 4.50; MS: m/z: 313.2 $[M+H]^+$.

2.3.2. Preparation of 1,2,5,6-dibenzocarbazole-9-ethyl chloroformate (DBCEOC–Cl)

To a solution containing 4.0 g solid phosgene and 100 mL dichloromethane (0 °C) in a 500-mL round-bottomed flask, a mixture of DBCEOC–OH (5.0 g) and pyridine (2 mL catalyst) in 150 mL dichloromethane solution was added dropwise within 2 h with stirring. After stirring at 0 °C for 4 h, the contents were kept at ambient temperature for another 6 h with vigorous stirring. Then the solution was concentrated using a rotary evaporator. The residue was extracted four times with warm diethyl ether; the combined diethyl ether layers were concentrated in vacuum to yield a white crystal. The crude products were recrystallized twice from diethyl ether to give the white powder, yield (56.4%). Found: C, 73.87; H, 4.32; N, 3.75; Cl, 9.46; calculated: C, 73.90; H, 4.31; N, 3.75; Cl, 9.48.

2.4. Preparation of solutions

Individual stock solutions (1.0×10^{-2} mol/L) of the AAs were prepared in water, and if necessary, 6 mol/L HCl or 6 mol/L NaOH was added until the compound dissolved completely. The standard AAs for HPLC analysis at individual concentrations of 5.0×10^{-5} mol/L were prepared by diluting the corresponding stock solutions (1.0×10^{-2} mol/L) of each AA with acetonitrile. The DBCEOC–Cl solution (5.0×10^{-3} mol/L) was prepared by dissolving 19.2 mg DBCEOC–Cl in 10 mL of acetonitrile. When not in use, all solutions were stored at 4 °C.

2.5. Surgery, microdialysis and drug treatment

Rats were anesthetized with Nembutal (44 mg/kg, i.p., 1.5% (g/mL) sodium pentobarbital solution). Rats were placed in a stereotaxic frame and a homemade heating pad was used to maintain body temperature at 37 °C. The surface of the brain was exposed through a hole drilled through the skull. Concentric design microdialysis probes (MD-2200, probe tip 2 mm, BAS) were implanted into the left inferior colliculus (AP = 8.8; ML = 2.0; DV = 3.5) and were secured permanently in position with skull screws and dental cement. After surgery, rats were replaced into BAS Raturn System (MD-1401).

The second day after surgery, microdialysis probes were connected to a perfusion pump (CMA100, Stockholm, Sweden) and to a refrigerated fraction collector (BAS). The probes were perfused in freely moving animal at a flow rate of 1 μL per minute with artificial cerebrospinal fluid (147 mmol/L NaCl, 4.0 mmol/L KCl, 1.2 mmol/L $CaCl_2$, 1.0 mmol/L $MgCl_2$). Microdialysates were collected at 30-min intervals. After 2-h stabilization, three fractions were collected to obtain basal values for self-control before drug application. The rats were injected with sodium salicylate (350 mg/kg, i.p., in a 100 mg/mL solution of saline) to make salicylate-induced tinnitus animal models. When not in use, all microdialysate samples were stored at −20 °C.

2.6. Derivatization procedure

The derivatization proceeded in a water/acetonitrile solution with alkaline medium. 15 μL AA standards or microdialysate were transferred into a 2-mL vial, 200 μL acetonitrile, 225 μL of 0.1 mol/L borate buffer (pH 9) and 50 μL of DBCEOC–Cl solution (5.0×10^{-3} mol/L) were then added. The solution was shaken for

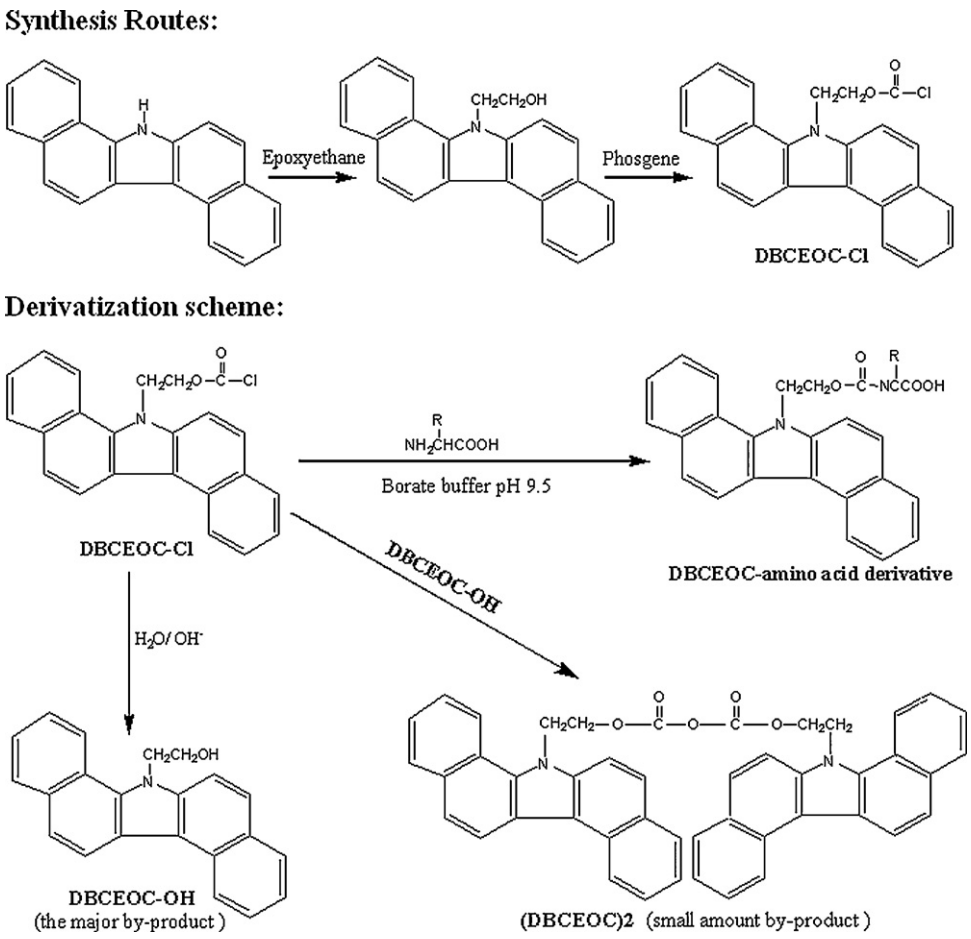

Fig. 1. Synthesis routes of DBCEOC–Cl and derivatization scheme of DBCEOC–Cl with amino acids.

1 min and allowed to stand for 15 min at 30 °C. After shaking for 1 min, 10 μL 36% acetic acid was added to the vial until the final pH value at the range of 5–7. Then the derivatized sample solution was directly injected into the HPLC system for analysis. The derivatization process is shown in Fig. 1.

2.7. HPLC and ESI-MS conditions

HPLC separation of DBCEOC–AA derivatives was carried out on a Hypersil BDS C_{18} column by gradient elution. Eluent A was 30% aqueous acetonitrile solution (H_2O/CH_3CN, 70/30, *v*/*v*) containing 30 mmol/L formic acid buffer (pH 3.5); Eluent B was 50% aqueous acetonitrile solution (H_2O/CH_3CN, 50/50, *v*/*v*) containing 30 mmol/L formic acid buffer (pH 3.5); Eluent C was 95% aqueous acetonitrile solution (H_2O/CH_3CN, 5/95, *v*/*v*). The gradient conditions: initial = 30% A and 70% B, 15 min = 100% B, 20–26 min = 95% B and 5% C, 35 min = 75% B and 25% C, 40 min = 65% B and 35% C, 55 min = 30% B and 70% C, 60 min = 100% C, followed by a wash with 100% C for 10 min. The flow rate was constant at 1.0 mL/min and the column temperature was set at 30 °C. The fluorescence excitation wavelengths (λ_{ex}) and emission wavelengths (λ_{em}) were set at 300 nm and 395 nm, respectively.

Chromatographic peaks were identified by spiking the working standard with each individual AA in turn, and simultaneously confirmed by on-line mass spectrometry. Ion source conditions: ESI in positive ion detection mode; nebulizer pressure 35 psi; dry gas temperature 350 °C; dry gas 9.0 L/min; capillary voltage 3500 V.

2.8. Quantification

The calibration curves were constructed for each DBCEOC–AA derivative by plotting peak area versus concentration. All AAs in rat brain microdialysate were quantified using the external standard method with fluorescence detection at $\lambda_{ex}/\lambda_{em}$ = 300/395 nm.

3. Results and discussion

3.1. Spectral properties of DBCEOC-derivatives

Carbazole derivatives are one of the most studied and important classes of photochromic molecules, and they exhibit interesting photochromic properties. DBCEOC–Cl, BCEC–Cl and CEOC–Cl are all chloroformate reagents based on the fluorescence core structure of carbazole. In our experiments, the spectral properties of DBCEOC–Cl derivatives were investigated in comparison with those of CEOC–Cl and BCEC–Cl. The solutions of the hydroxyl precursor for each chloroformate were individually prepared and used to determine the ultraviolet absorbance and relative fluorescence intensity, and the obtained ultraviolet and fluorescence spectra are presented in Fig. 2.

The molecular structures in Fig. 2(a) indicate that BCEC–OH and DBCEOC–OH are derived from CEOC–OH by fusing benzo groups to the carbazole moiety, and the three compounds can be deemed as a series of carbazole derivatives. As can be seen from ultraviolet spectra in Fig. 2(b), in the series of CEOC–OH, BCEC–OH and DBCEOC–OH, the maximum absorption wavelengths exhibit obvious red-shift (for CEOC–OH, 206 nm; for BCEC–OH,

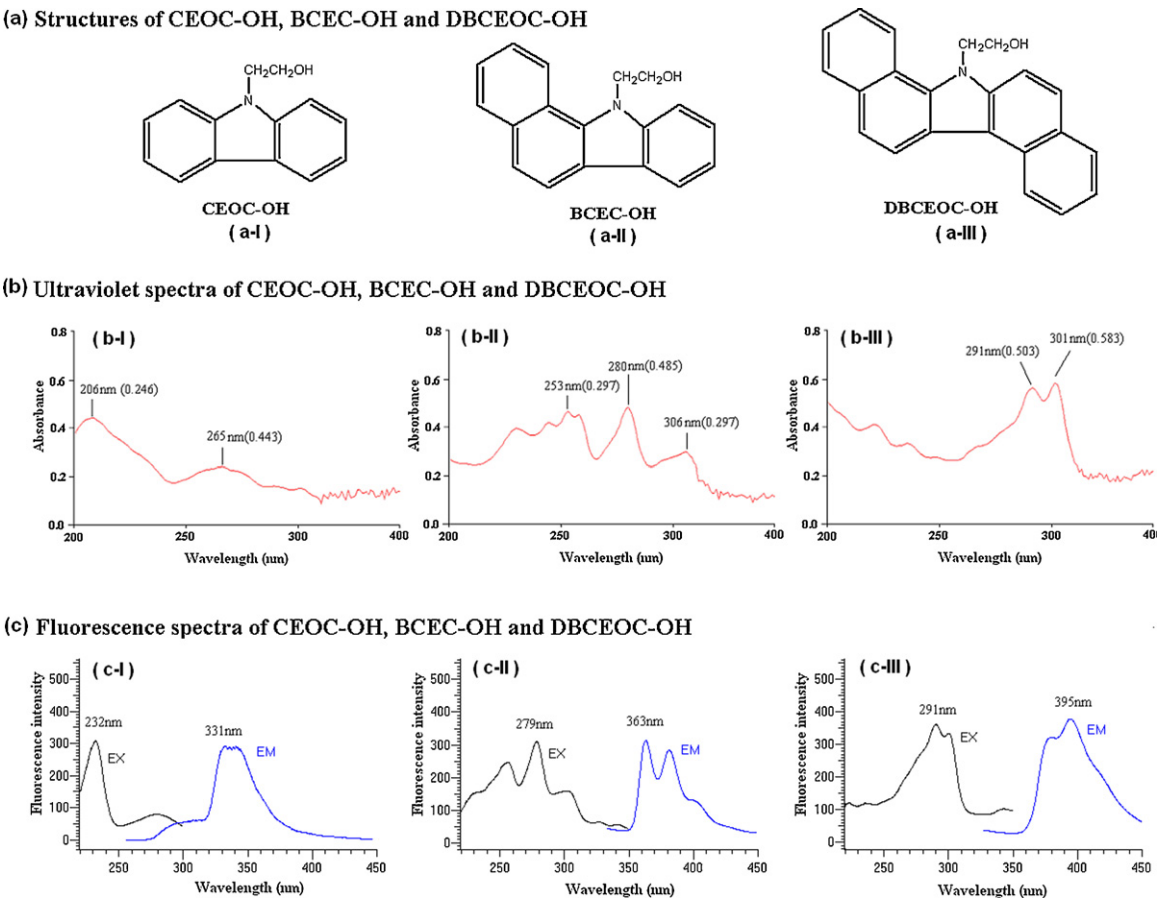

Fig. 2. Structural schemes (a), ultraviolet spectra (b) and fluorescence spectra (c) for the hydroxyl precursors of CEOC–Cl (I), BCEC–Cl (II) and DBCEOC–Cl (III). CEOC–OH, BCEC–OH and DBCEOC–OH are the hydroxyl precursors of CEOC–Cl, BCEC–Cl and DBCEOC–Cl, respectively. CEOC–OH, BCEC–OH and DBCEOC–OH solutions were individually prepared at concentration of 1.0×10^{-5} mol/L (used to test ultraviolet absorption) and 1.0×10^{-7} mol/L (used to test fluorescence properties) in acetonitrile.

280 nm; for DBCEOC–OH, 301 nm), and the corresponding molar absorption coefficients (ε) increase remarkably (for CEOC–OH, 2.46×10^4 L moL^{-1} cm^{-1}; for BCEC–OH, 4.85×10^4 L moL^{-1} cm^{-1}; for DBCEOC–OH, 5.83×10^4 L moL^{-1} cm^{-1}). Fluorescence spectra in Fig. 2(c) show that the maximum excitation and emission also exhibit obvious red-shift in this series (for CEOC–OH, $\lambda_{ex}/\lambda_{em}$ = 232/331 nm; for BCEC–OH, $\lambda_{ex}/\lambda_{em}$ = 279/363 nm; for DBCEOC–OH, $\lambda_{ex}/\lambda_{em}$ = 291/395 nm), and DBCEOC–OH give the highest fluorescence intensity among the three compounds. The outstanding spectral properties of DBCEOC–OH are probably due to the fact that dibenzocarbazole has the most rigid structural plane and highly conjugated π–π system. The superior spectral properties would make DBCEOC–Cl a more sensitive labeling reagent for AAs than BCEC–Cl and CEOC–Cl.

Fluorescence spectra of DBCEOC–AA derivatives were also obtained by on-line spectra scanning with FLD (model G1321A, Agilent, USA), while derivatives were eluted in HPLC system with aqueous acetonitrile mobile phase. The results showed that, for most DBCEOC–AA derivatives, the maximum excitation and emission wavelengths were at 300 nm and 395 nm, respectively. Therefore, the λ_{ex} 300 nm and λ_{em} 395 nm were used in the subsequent experiments.

3.2. Stability of DBCEOC–Cl and its derivatives

After a solution of DBCEOC–Cl in anhydrous acetonitrile was hermetically stored at 4 °C for one week, derivatization yields for AAs were not obviously different from those obtained with newly prepared DBCEOC–Cl solution. The stabilities of the corresponding derivatives were also investigated. A standard solution containing 50 pmol AAs was derivatized and neutralized according to the procedure described in Section 2.6. This solution was repeatedly analyzed by HPLC–FLD after being placed at room temperature for 0, 1, 2, 4, 8, 16, 24 and 48 h, respectively. The relative standard deviations (RSD) of peak area were <3.4%. Thus it can be seen that the stability of DBCEOC–AA derivatives is suitable for chromatographic analysis.

3.3. Chromatographic separation and ESI-MS identification

3.3.1. HPLC separation of 20 DBCECOC–AA derivatives

The side chains of the tested 20 AAs varied substantially, which made these DBCEOC–AA derivatives very different from each other. Most AAs were labeled with one moiety of dibenzocarbazole; some were tagged with two moieties of dibenzocarbazole, like tyrosine, lysine, ornithine, histidine and cystine; some still had polar groups on side chain after derivatization, such as guanidyl (arginine), hydroxyl (serine and threonine), carboxyl (aspartic acid and glutamic acid) and so on. These derivatives had very different retention behavior on reversed phase columns. To achieve simultaneous separation of this complex mixture, a ternary elution program (see Section 2.7) was carried out on a Hypersil BDS C_{18} column. The chromatograms obtained under these conditions are shown in Fig. 3. Most DBCEOC–AA derivatives are separated with satisfactory resolution, and by-products and impurities from reagent do not produce any interference to the determination of the DBCEOC–AA derivatives.

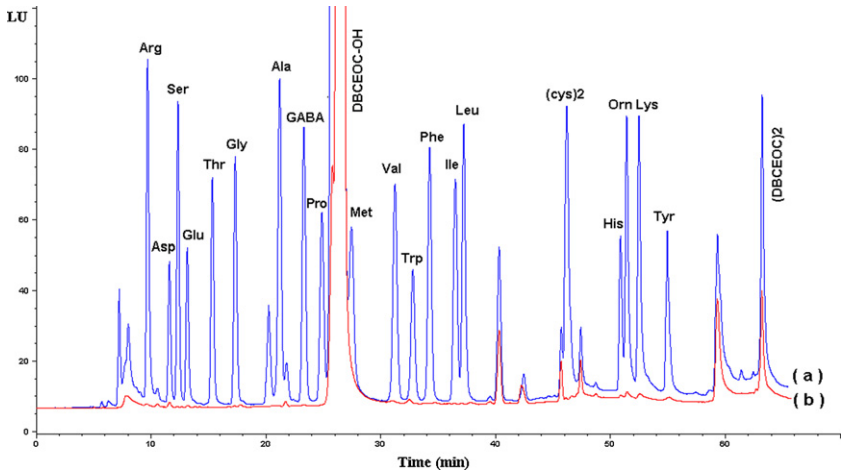

Fig. 3. Chromatograms for standard amino acids derivatives. Chromatographic conditions: Hypersil BDS C_{18} column (4.6 mm × 200 mm, 5 μm), column temperature 30 °C; flow rate 1.0 mL/min; excitation and emission: $\lambda_{ex}/\lambda_{em}$ = 300/395 nm. Peaks are labeled with abbreviations for all amino acids: Arg (arginine), Asp (aspartic acid), Ser (serine), Glu (glutamic acid), Thr (threonine), Gly (glycine), Ala (alanine), GABA (4-aminobutanoic acid), Pro (proline), Met (methionine), Val (valine), Trp (tryptophan), Phe (phenylalanine), Ile (isoleucine), Leu (leucine), $(Cys)_2$ (cystine), His (histidine), Orn (ornithine), Lys (lysine), Tyr (tyrosine), DBCEOC–OH (1,2,5,6-dibenzocarbazole-9-ethanol), $(DBCEOC)_2$ (bis-(1,2,5,6-dibenzocarbazole-9-ethyl)-carbonate).

3.3.2. Separation of critical pairs (proline, DBCECOC–OH and methionine)

To achieve simultaneous separation of the 20 DBCEOC–AA derivatives, the most troublesome problem lay in the separation of DBCEOC–OH with adjacent peaks (methionine and proline). Excess of DBCEOC–Cl reagent was necessary to ensure the complete derivatization. However, the excess reagents would cause a broad by-product peak of DBCEOC–OH in chromatogram, which often partially or completely bury the peaks of adjacent AA derivatives. We overcame this problem by taking measures in two aspects: improving derivatization procedure and controlling mobile phase pH.

On one hand, we tried to improve the derivatization procedure to produce less amount of DBCEOC–OH, including three specific attempts. Firstly, heptyl amine was added to the derivatization solution as a quenching reagent. Thus, quite a few excess reagents were changed to DBCEOC–heptyl amine instead of DBCEOC–OH. And the resulted narrow DBCEOC–OH peak facilitated the separation of these critical pairs. It was found that this measure worked well when AAs were at large concentration levels. However, no obvious improvement was observed when trace amount of AAs were tested. So this measure was not used in our subsequent experiments. Secondly, we added hexane to the derivatization solution to extract DBCEOC–OH before HPLC injection. The results indicated that the organic solvents extraction also resulted in serious loss of hydrophobic AA derivatives. Therefore, this measure was not adopted in subsequent experiments. Thirdly, the DBCEOC–Cl concentration used in derivatization procedure was optimized to minimize the DBCEOC–OH peak. Under the premise of quantitative derivatization, a low concentration of DBCEOC–Cl at 0.5 mmol/L was used in our experiments, which gave a relative narrow DBCEOC–OH peak in chromatograms (described detailedly in Section 3.4.3).

On the other hand, we optimized the mobile phase pH to achieve satisfactory resolution of DBCEOC–OH with methionine or proline derivative. The choice of mobile phase pH value was tested on the Hypersil BDS C_{18} column with formic ammonium buffers in the range of 3.0–6.0. It was found that the retention of DBCEOC–AA derivatives was more susceptible to mobile phase pH than that of DBCEOC–OH, and the resolution of these critical pairs was significantly dependent on the mobile phase pH. With mobile phase pH > 4.0, the peak of proline derivative was separated, but the peak of methionine derivative was embedded in the peak of DBCEOC–OH. As mobile phase pH decreased from 4.0 to 3.5, the retention of DBCEOC–AA derivatives increased obviously, whereas the retention of DBCEOC–OH remained unchanged. As a consequence, the peak of methionine derivative gradually came out of the DBCEOC–OH peak, and the peak of proline derivative moved close to DBCEOC–OH. With the mobile phase pH further decreasing from

Table 1
The ESI-MS/MS data for DBCEOC–AA derivatives.

DBCEOC–AA derivatives	$[M+H]^+$ and $[M+NH_4]^+$	MS/MS data
Mono-labeled AAs[a]		
Arg	511.7	294.1; 312.5; 337.9; 451.1; 494.3
Asp	470.3	203.9; 293.8; 311.9; 423.8; 452.2
Ser	442.2	175.7; 293.7; 311.3; 395.8; 422.8
Glu	484.1	199.8; 293.9; 437.8; 465.5
Thr	456.2	189.6; 293.7; 311.6; 409.6; 437.6
Gly	412.1	145.6; 293.8; 311.6;
Ala	426.3	159.8; 293.8; 311.8; 380.0; 407.1
GABA	440.2	293.7; 311.0; 421.3
Pro	452.2	185.6; 293.6; 311.1; 405.3
DBCEOC–OH	311.6; 355.4	266.6; 293.7
Met	485.8	219.2; 293.9; 311.8; 391.7; 439.8; 467.0
Val	454.1	187.6; 293.4; 311.5; 407.7; 434.5
Trp	541.2; 558.1	274.5; 293.7; 311.6; 495.4; 522.9
Phe	501.7; 518.5	235.4; 293.8; 311.5; 455.3; 482.8
ILe	467.4	201.2; 293.5; 421.5; 449.6
Leu	468.0	201.5; 293.7; 420.7; 450.7
Double-labeled AAs[b]		
(Cys)2	912.9; 930.4	293.6; 311.8; 913.5
His	829.1	293.6; 311.8; 562.3
Orn	805.2; 822.6	293.6; 311.8; 495.0
Lys	818.4; 836.7	293.6; 311.8; 508.1
Tyr	853.1; 870.7	293.6; 311.5; 544.2
$(DBCEOC)_2$	647.5; 664.7	293.7; 647.5

[a] Mono-labeled AAs, amino acid derivatives labeled with one moiety of dibenzocarbazole.
[b] Double-labeled AAs, amino acid derivatives labeled with two moieties of dibenzocarbazole.

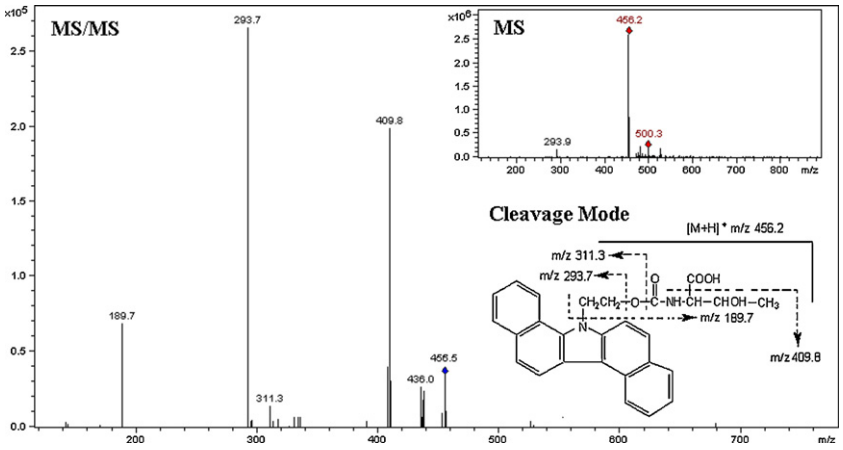

Fig. 4. The representative MS and MS/MS profiles for DBCEOC-threonine derivative.

3.5 to 3.0, the peak of methionine derivative was separated, but the peak of proline derivative gradually moved into the DBCEOC–OH peak. Therefore, the mobile phase pH of 3.5 was adopted to achieve simultaneous separation of these AA derivatives.

In brief, use of 0.5 mmol/L DBCEOC–Cl for derivatization produced a narrow DBCEOC–OH peak when the pH of the mobile phase was 3.5. This greatly enhanced the resolution of the critical pairs.

3.3.3. ESI-MS identification

Chromatographic peaks were identified by spiking the working standard with each individual AA in turn, and simultaneously confirmed by online post-column ESI-MS in positive mode. Data from the MS and MS/MS spectra for all of the AAs derivatives are displayed in Table 1. These data indicate that all DBCEOC–AA derivatives exhibit intense quasi-molecular ion of $[M+H]^+$ in MS, and give abundant fragment ions at m/z 294 and m/z 311 in MS/MS. The representative MS, MS/MS profiles and cleavage mode are shown in Fig. 4. The characteristic fragment ion at m/z 311 derives from the cleavage of the CH_2O–CO bond, and the fragment ion at m/z 294 comes from the cleavage of the CH_2–OCO bond. The selected reaction monitoring based on the m/z $[M+H]^+ \rightarrow m/z$ 294 and m/z 311 transitions is specific for the DBCEOC–AA derivatives.

3.4. Optimization of derivatization

3.4.1. General considerations

For the tested 20 AAs, there were four types of active function groups which react with the chloroformate reagent, namely primary amine (in most AA), secondary amine (proline), imidazole (histidine bares both primary amino and imidazole group) and phenol (tyrosine bares both primary amino and phenol group). Besides, it has been reported that aspartic acid and glutamic acid were out of ordinary because of their less reactivities [28]. Therefore, glycine, proline, histidine, tyrosine, aspartic acid and glutamic acid were selected as representatives to optimize the derivatization conditions in our experiments.

DBCEOC–Cl has the same active function group of chloroformate with CEOC–Cl, BCEC–Cl and FOMC–Cl. These chloroformate reagents are active enough to react with amino compounds at room temperature in basic medium. In our experiments, all derivatizations were carried out at 30 °C in the mixed solvent of borate buffer and acetonitrile (50/50, v/v). On this basis, the optimal parameters for the derivatization were investigated, including buffer concentration, buffer pH, DBCEOC–Cl concentration and reaction time.

3.4.2. Effect of borate buffer on derivatization

The derivatization procedures were carried out in the mixed medium of borate buffer and acetonitrile. The precipitate phenomena must be avoided, because either borate or reagent separating out from the derivatization system would significantly affect the reproducibility of quantitative derivatization. A mixture of 0.1 mol/L borate and acetonitrile permitted smooth derivatization of AAs even when the concentration of DBCEOC–Cl was 2 mmol/L. Effects of borate buffer pH on derivatization were also investigated in the range of 7–11. The results indicated that, the responses of DBCEOC–AA derivatives were very low when the buffer pH <8.5 or >10, and the pH values of borate buffer for maximum derivatization yield fell into the range of 8.5–9.5 (specifically, glycine and proline, 8.5; histidine, 9.5; tyrosine, 9.5; aspartic acid and glutamic acid, 9.0). Therefore, 0.1 mol/L borate buffer with pH 9.0, a compromised result, was used in subsequent derivatization procedure.

3.4.3. Effect of DBCEOC–Cl concentration and time on derivatization

The effects of DBCEOC–Cl concentrations (expressed in mmol/L, related to the total volume of the reaction media) on derivatization were also studied. The derivatization procedures were carried out over various periods of time by using DBCEOC–Cl at 0.05, 0.1, 0.5, 1 and 2 mmol/L five concentration levels. As observed in all cases, the fluorescence responses of DBCEOC–AA derivatives initially underwent an increase and then a plateau as the reaction time increased. Using 0.1 mmol/L or lower concentration of DBCEOC–Cl, the maximum derivatization could not be achieved until 30 min; using 0.5 mmol/L DBCEOC–Cl, the fluorescence responses of most DBCEOC–AA derivatives reached the maximum and constant value at about 10 min (for the less active aspartic acid and glutamic acid, it was about 15 min); using 1 mmol/L or 2 mmol/L DBCEOC–Cl, the maximum and constant derivatization was achieved within 5 min. These results indicated that the larger concentration of DBCEOC–Cl used for derivatization would result in the shorter reaction time for maximum derivatization. However, excess of DBCEOC–Cl would result in a very broad by-product peak of DBCEOC–OH which masks the adjacent peaks. To achieve a rapid quantitative derivatization with small DBCEOC–OH peak, the DBCEOC–Cl concentration of 0.5 mmol/L and 15 min reaction time, as the best compromise, was selected as optimum derivatization condition.

3.5. Method validation

The validation parameters studied were selectivity, response linearity, detection limits (LOD), precision (repeatability and intermediate precision) and accuracy (recovery studies).

Table 2
Linearity, detection limits, precision and accuracy data for DBCEOC–AA derivatives.

Amino acids	Linearity r^2	LOD (nmol/L (fmol))[a]	Precision (%)[b]		Accuracy (%)[c]	
			Intraday ($n=3$)	Interday ($n=9$)	Mean recovery	RSD ($n=3$)
Arg	0.9990	0.866 (8.66)	2.2	3.1	95.7	3.5
Asp	0.9988	1.45 (14.5)	2.0	2.9	94.2	3.7
Ser	0.9998	0.907 (9.07)	1.8	2.3	96.3	2.9
Glu	0.9988	1.56 (15.6)	1.7	3.0	93.5	3.5
Thr	0.9994	0.681 (6.81)	1.5	2.6	95.6	2.9
Gly	0.9996	0.533 (5.33)	1.4	2.2	96.5	2.6
Ala	0.9994	0.525 (5.25)	1.9	2.4	104.7	2.8
GABA	0.9988	0.821 (8.21)	2.6	3.0	103.1	3.8
Pro	0.9996	2.87 (28.7)	2.1	4.2	98.3	2.9
Met	0.9990	4.75 (47.5)	2.3	3.0	95.7	3.1
Val	0.9996	0.402 (4.02)	1.5	2.0	97.8	2.6
Trp	0.9998	0.217 (2.17)	2.3	2.8	97.7	2.7
Phe	0.9998	0.353 (3.53)	1.6	2.1	96.5	2.9
Ile	0.9996	0.679 (6.79)	1.8	2.5	95.4	2.6
Leu	0.9996	0.710 (7.10)	1.9	2.4	98.6	2.7
$(Cys)_2$	0.9990	0.536 (5.36)	2.8	3.2	103.7	3.1
His	0.9990	1.58 (15.8)	3.2	3.4	95.3	3.4
Orn	0.9988	0.462 (4.62)	2.1	3.2	104.2	2.9
Lys	0.9990	0.359 (3.59)	1.7	2.4	105.6	3.1
Tyr	0.9990	1.92 (19.2)	3.1	3.7	97.7	4.3

[a] LODs were calculated at a signal to noise of 3.
[b] Precision values expressed in relative standard derivation.
[c] Accuracy values expressed with recoveries.

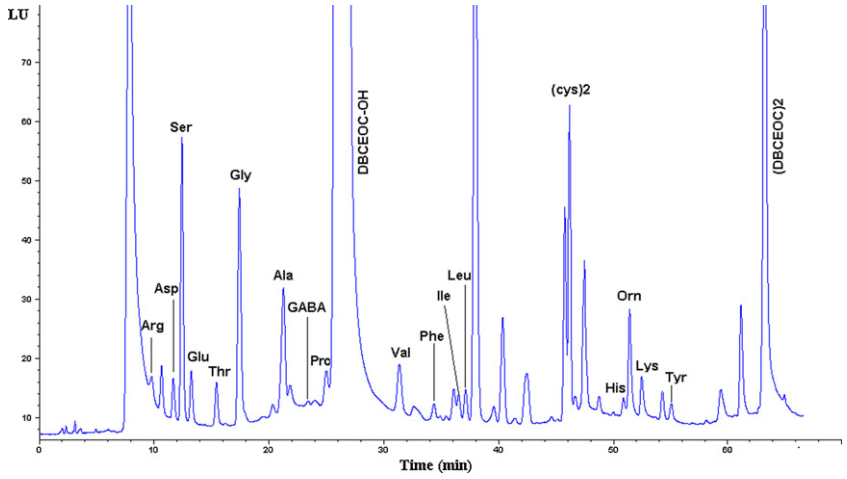

Fig. 5. The representative chromatogram for amino acids profile in rat brain microdialysate. Chromatographic conditions and peak labels were the same with Fig. 3.

The selectivity was tested using a microdialysate sample spiked with each standard at 5 μmol/L, and the selectivity criterion was that resolution between adjacent peaks (R) was at least 1.5. In this sample R values <1.5 were obtained for the following peak pairs: proline with DBCEOC–OH, DBCEOC–OH with methionine, and ornithine with histidine. Therefore, acceptable peak resolutions for most of AA derivatives were achieved.

The relationships between the peak areas and the amounts of the individual AAs were all linear (correlation coefficients > 0.9988; $n=5$) over the concentration ranges from 5 to 5×10^3 nmol/L (except for proline and methionine) and 10 to 5×10^3 nmol/L (proline and methionine). The LODs were established as the concentration of compound whose peak area gave a signal-to-noise ratio of 3, and the LODs were in the range from 0.217 to 4.75 nmol/L (Table 2). DBCEOC–Cl provides lower LOD values than other derivatizing agents used in ultraviolet or fluorescence detection, such as FMOC–Cl (0.11–0.38 μmol/L) [10], AQC (0.026–0.36 μmol/L) [11], OPA (0.092–0.51 μmol/L) [14], Dabsyl–Cl (12–52 nmol/L) [18] and Dansyl–Cl (0.1–2 μmol/L) [21], which permits us to determine lower content of AAs than methods involving other reagents.

The method precision was evaluated with repeatability and intermediate precision, which were expressed as RSD. A microdialysate sample spiked with 0.005 mmol/L of each standard was employed to test the precision. The repeatability and intermediate precision were measured on the same day ($n=3$) and on the sequential three days ($n=9$), respectively. The results for repeatability ranged from 1.4% to 3.2% (Table 2). The intermediate precision yielded values ranging from 2.0% to 4.3% (Table 2). To carry out the recovery study, a microdialysate sample was spiked each standards at a closed concentration to the original values. The recoveries were calculated based on the formula of (measured value − original value)/added value × 100%. All analyses were carried out in triplicate. A good degree of accuracy was achieved for most of the compounds with recovery percentages ranging from 92.5% to 105.6% (Table 2). These results indicate that quantification of AAs in rat brain microdialysate can be carried

Table 3
Content of free amino acids in rats brain microdialysates from a salicylate-induced tinnitus animal model ($X \pm S$, $n = 3$, pmol/μL microdialysate).

Amino acids	Values before drug application	Values in 90–180 min after drug injection	Amino acids	Values before drug application	Values in 90–180 min after drug injection
Arg	0.34 ± 0.02	0.18 ± 0.01	Val	1.19 ± 0.04	1.31 ± 0.05
Asp	0.49 ± 0.02	1.10 ± 0.03	Trp	–	–
Ser	3.24 ± 0.11	3.46 ± 0.13	Phe	0.85 ± 0.03	0.36 ± 0.02
Glu	1.67 ± 0.05	1.34 ± 0.05	ILe	0.67 ± 0.04	0.26 ± 0.02
Thr	1.54 ± 0.04	0.85 ± 0.04	Leu	0.81 ± 0.03	0.58 ± 0.02
Gly	5.62 ± 0.12	3.08 ± 0.09	$(Cys)_2$	3.75 ± 0.18	4.19 ± 0.22
Ala	1.08 ± 0.05	2.06 ± 0.06	His	1.12 ± 0.05	0.43 ± 0.03
GABA	0.28 ± 0.01	0.11 ± 0.01	Orn	1.45 ± 0.05	1.15 ± 0.07
Pro	0.64 ± 0.03	0.67 ± 0.03	Lys	1.03 ± 0.04	0.85 ± 0.03
Met	–	–	Tyr	0.38 ± 0.02	0.26 ± 0.01

out with acceptable accuracy and precision using the proposed method.

3.6. Determination of amino acids in rat brain microdialysate from a salicylate-induced tinnitus animal model

Sodium salicylate is often used to produce an animal model of tinnitus, however, the mechanism underlying the development of salicylate-induced tinnitus is unknown [29]. The inferior colliculus is an important auditory structure that plays many roles in processing and relaying acoustic information. The characterization of AA changes induced by salicylate in inferior colliculus might provide a better understanding of neurological mechanism of salicylate-induced tinnitus. Results in electrophysiology and behavior studies indicated that the strongest responses occurred at about 2 h after drug treatment [30,31]. Therefore, the microdialysate collected in 1.5–3 h after salicylate injection were analyzed using the proposed methods, and microdialysate before drug treatment were also determined as the original values for self-control method. The representative chromatogram is shown in Fig. 5, and the content of AAs in these microdialysate samples are displayed in Table 3. The released aspartic acid (excitatory neurotransmitter) increased significantly after salicylate injection; the release of 4-aminobutanoic acid and glycine (inhibitory AA neurotransmitter) decreased significantly; glutamic acid and most neutral AA were not significantly changed. These results indicate that the DBCEOC–Cl derivatization coupled with HPLC–FLD provided a novel and efficient method to determining trace levels of AAs in microdialysate samples.

4. Conclusions

In the present study, DBCEOC–Cl has been reported as a new reagent for labeling AAs. The reagent DBCEOC–Cl exhibits superior spectral properties to previous reagents, such as CEOC–Cl and BCEC–Cl. With DBCEOC–Cl as pre-column labeling reagent, a HPLC–FLD method has been developed for the analysis of AAs in rat brain microdialysate. The method has high sensitivity, excellent accuracy and precision. The proposed method provides a good technique for the quantitative analysis of trace levels of AAs from microdialysate or other biochemical samples.

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References

[1] H. Kaspar, K. Dettmer, W. Gronwald, P.J. Oefner, Anal. Bioanal. Chem. 393 (2009) 445.
[2] P. Uutela, R.A. Ketola, P. Piepponen, R. Kostiainen, Anal. Chim. Acta 633 (2009) 223.
[3] M. Dauner, U. Sauer, Biotechnol. Prog. 16 (2008) 642.
[4] C. Deng, X. Yin, L. Zhang, X. Zhang, Rapid Commun. Mass Spectrom. 19 (2005) 2227.
[5] K. Shimbo, T. Oonuki, A. Yahashi, K. Hirayama, H. Miyano, Rapid Commun. Mass Spectrom. 23 (2009) 1483.
[6] G. Noctor, G. Bergot, C. Mauve, D. Thominet, C. Lelarge-Trouverie, J.L. Prioul, Metabolomics 32 (2007) 161.
[7] H. Zhang, I.L. Potier, C. Smadja, J. Zhang, M. Taverna, Anal. Bioanal. Chem. 386 (2006) 1387.
[8] Q. Qu, X. Tang, C. Wang, G. Yang, X. Hu, X. Lu, Y. Liu, S. Li, C. Yan, Anal. Chim. Acta 572 (2006) 212.
[9] J. López-Cervantes, D.I. Sánchez-Machado, J.A. Rosas-Rodríguez, J. Chromatogr. A 1105 (2006) 106.
[10] V. Lozanov, B. Benkova, L. Mateva, S. Petrov, E. Popov, C. Slavov, V. Mitev, J. Chromatogr. B 860 (2007) 92.
[11] L. Bosch, A. Alegría, R. Farré, J. Chromatogr. B 831 (2006) 176.
[12] S. Hou, H. He, W. Zhang, H. Xie, X. Zhang, Talanta 80 (2009) 440.
[13] V. Lozanov, S. Petrov, V. Mitev, J. Chromatogr. A 1025 (2004) 201.
[14] M. Rigobello-Masini, J.C.P. Penteado, C.W. Liria, M.T.M. Miranda, J.C. Masini, Anal. Chim. Acta 628 (2008) 123.
[15] Y.V. Tcherkas, L.A. Kartsova, I.N. Krasnova, J. Chromatogr. A 913 (2001) 303.
[16] I. Fernández-Fígares, L.C. Rodríguez, A. González-Casado, J. Chromatogr. B 799 (2004) 73.
[17] M. Aito-Inoue, K. Ohtsuki, Y. Nakamura, E.Y. Park, K. Iwai, F. Morimatsu, K. Sato, J. Agric. Food Chem. 54 (2006) 5261.
[18] I. Krause, A. Bockhardt, H. Neckermann, T. Henle, H. Klostermeyer, J. Chromatogr. A 715 (1995) 67.
[19] E.H.J.M. Jansen, R.H. Van den Berg, R. Both-Miedema, L. Doorn, J. Chromatogr. 553 (1991) 123.
[20] R. Badoud, G. Pratz, Chromatographia 19 (1984) 155.
[21] R. Minocha, S. Long, J. Chromatogr. A 1035 (2004) 63.
[22] I. Molnár-Perl, J. Chromatogr. A 987 (2003) 291.
[23] T. Fukushima, N. Usui, T. Santa, K. Imai, J. Pharm. Biomed. Anal. 30 (2003) 1655.
[24] J.M. Rosenfeld, Trend. Anal. Chem. 22 (2003) 785.
[25] J. You, Y. Zhang, Chromatographia 56 (2002) 43.
[26] J. You, L. Liu, W. Zha, X. Zhao, Y. Suo, H. Wang, Y. Li, Anal. Bioanal. Chem. 387 (2007) 2705.
[27] J. You, T. Yan, H. Zhao, Z. Sun, L. Xia, Y. Suo, Y. Li, Anal. Chim. Acta 636 (2009) 95.
[28] A. Jámbor, I. Molnár-Perl, J. Chromatogr. A 1216 (2009) 3064.
[29] J. Liu, X. Li, L. Wang, Y. Dong, H. Han, G. Liu, Hear. Res. 175 (2003) 45.
[30] G.D. Chen, P.J. Jastreboff, Hear. Res. 82 (1995) 158.
[31] M. Yasuhiro, Y. Shigeru, S. Hitoshi, Hear. Res. 103 (1997) 192.