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Method for on-line derivatization and separation of aspartic acid enantiomer in pharmaceuticals application by the coupling of flow injection with micellar electrokinetic chromatography

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Abstract

A novel, easy and accurate capillary electrophoresis (CE) coupled with flow injection (FI) method for the separation and determination of aspartic acid (Asp) enantiomers by on-line derivatization had been developed, and it had been applied to the real sample for the first time. The derivatization reagents were *o*-phthalaldehyde (OPA) and mercaptoethanol (ME), which were obtained easily, the chiral selector was β-cyclodextrin (β-CD), the micellar chemical was sodium dodecyl sulfate (SDS), and the modifier was methanol. By on-line derivatization, aspartic acid enantiomers were automatically and reproducibly converted to the ultraviolet (UV)-absorbing diastereoisomer derivates, which were separated by micellar electrokinetic chromatography (MEKC). According to the factors affecting the separation and sensitivity of aspartic acid enantiomer and other amino acids in the real sample, the pH value and concentration of the buffer, the concentration of β-CD and SDS, the volume percentage of the methanol (v/v) in the buffer, the applied voltage and the conversion time were selected as the investigating variates. Under the investigated separation conditions, D-aspartic acid (D-Asp), L-aspartic acid (L-Asp) and other four amino acids achieved the baseline separation in not only the standard mixture of amino acids but also the real sample (Compound Amino Acid Injection (6AA)). The repeatability (defined as relative standard deviation (RSD), $n = 5$) was 4.0% and 4.0% with peak area evaluation, and 4.2% and 3.7% with peak height evaluation for D-Asp and L-Asp in the real sample. Recovery at added standard levels of 1.0, 3.0 and 6.0 mM was 92%, 104% and 109%, respectively.

Keywords: Enantiomer separation; On-line derivatization; Capillary electrophoresis; Flow injection

1. Introduction

Amino acids were important biological compounds that were associated with peptides and proteins and were frequently found in food, feeds, body fluids and tissues. It was well known that in nature amino acids occur in L-forms and that they played an important role in the food and pharmaceutical industries. In the food industry, racemization of amino acids could occur during food processing, for example, in roasting or treatment of food protein under alkaline conditions [1]. The D-forms have been discovered in species of lower animals [2,3], mammalian organs and blood [4,5]. Some D-form amino acids were also detected in various vegetables and fruits [6]. However, recent research suggested important physiological roles for some D-amino acids including D-aspartic acid (D-Asp), D-glutamic acid and D-serine [7]. For example, D-Asp was an endogenous chemical species in human and various animals [8–10]. It might have the key effect in neurocrine and endocrine functions [11]. L-Aspartic acid (L-Asp) racemization ratios in human femur have been used for age estimation [12]. An age-related accumulation of D-Asp in the human brain has been observed [13].

In general, most amino acids lacked strong ultraviolet (UV) absorbance, except for the three aromatic amino acids, phenylalanine, tryptophan and tyrosine. Thus, it was necessary to derivate in many cases for sensitive detection of amino acids. Such a derivatization could lead to a

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considerable increase in sensitivity. In addition to sensitivity enhancement, the derivatization was expected to improve selectivity. As regard derivatization reagent, although a large number of different derivatization reagents had been applied for amino acid analysis in high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) [14–16], only some of them had so far been employed for enantiomeric separations in CE. As far as speed and easiness of derivatization, *o*-phthalaldehyde (OPA) was optimum; it reacted with amino acids in the presence of a reducing agent, such as mercaptoethanol (ME), to form a substituted isoindole ring. This reagent reacted with amino acids rapidly and the excess OPA reagent was non-fluorescent and had virtually no interference with the electrophoretic separation, which was favorable for on-line derivatization.

The chiral separation of amino acid derivatives by HPLC [17–20], gas chromatography (GC) [21] and supercritical fluid chromatography (SFC) [22] had been reported, while recently the use of CE dramatically increased [23,24]. CE was a powerful alternative technique due to its very high efficiency. In comparison with HPLC or GC, it had many advantages such as short analysis times and limited consumption of analytes and buffers, which make it ideal for the high sample throughput provided by combinatorial chemistry.

In this study, a novel, easy and accurate detection method for the analysis of D-Asp, L-Asp and other amino acids was proposed by on-line derivatization with OPA and ME in a flow injection (FI) system coupled to CE equipment. The microfluidic CE system with ultraviolet detection coupled with FI sample introduction was constructed from components readily available in the analytical laboratory. The main separation components were fixed on a 30 mm × 80 mm × 2 mm microscope slide. This microchip with an H-channel design was produced from plastic slides, PVC pump tubing and short several centimeter-long separation capillaries, and was readily coupled to FI sample introduction system. It presented an alternative for more basic studies on microfluidic system and could be inexpensively produced without resorting to microfabrication techniques [25–29]

The objective of this study was to explore rapidly, easily and inexpensively experimental domain and to find conditions at which the separation of D-Asp, L-Asp and other amino acids was sufficiently good for quantification with acceptable analysis time. The developed method was feasibility and applicability in separating and determining the real sample.

2. Experiments

2.1. Standards and reagents

o-Phthaldialdehyde and mercaptoethanol were purchased from Yatai Jingxi Huagong Chang (Beijing, China) and Shanghai Chemical Reagent Fourth Factory Hewei Chemical Engineer Limited Company (Shanghai, China), respectively. Amino acids (valine (Val), leucine (Leu), isoluecine (Ile), arginine (Arg), glutamic acid (Glu), L-Asp, D-Asp) were purchased from Kangda Amino Acids Factory (Shanghai, China). β-Cyclodextrin (β-CD) was purchased from Shanghai Chemical Reagent Factory (Shanghai, China). Sodium dodecyl sulfate (SDS) was bought from Xi'an Chemical Reagent Factory (Shanxi, China). Compound Amino Acid Injection (6AA) was bought from Lanzhou Medicine College and was filtered through a 0.45-μm syringe filter, and then was directly injected into the CE equipment by the FI system. All the chemicals used were of analytical reagent grade. Distilled water was used in all the experiments.

The carrier solution (functioning also as electrophoretic buffer) was composed of 15 mM borate, 12 mM β-CD, 8 mM SDS and 10% (v/v) methanol, and was prepared daily from stock solution of 0.1 M borate, 0.02 M β-CD and 0.1 M SDS, and then adjusted with 2 M NaOH or 2 M HCl to the required pH. The derivatization reagents, OPA and ME were prepared daily in methanol–water (1:1, v/v) and 15 mM borate (pH 9.5), respectively. Ten millimolar stock solutions of amino acids were prepared in distilled water and diluted to the required concentration with distilled water. All solutions were filtered through a 0.45-μm syringe filter before use and distilled water was used throughout the study.

2.2. Apparatus

A model HPE-100 CE system with 12 kV maximum voltage (Bio-Rad, Hercules, CA) was used for the separations, which was connected to a 486 PC with a chroma chromatography collection system (Bio-Rad) for integration and data treatment. Uncoated silica separation capillaries of 50 μm I.D., 350 μm O.D., and 30 cm length (27 cm effective length) (Yongnian Optical Fiber Factory, Hebei, China) were used throughout the study. UV detection was performed at 214 nm.

A FIAstar 5020 analyzer (Tecator, Sweden) equipped with two four-channel peristaltic pumps was used for automated FI operations. A 0.5 mm I.D. polytetrafluoroethylene (PTFE) tubing was used for connecting all components of the FI system, including 16 cm length transport line from the valve to the split-flow interface and 9 cm length mixing coil. A sample loop of 40 μl was made from 0.5 mm I.D. PTFE, and Tygon pump tubes were used for delivering all solutions. The time period for the injecting sample was defined through thumbwheel settings.

The manifold for the FI–CE system is shown schematically in Fig. 1. The H-channel chip constructed using a planar plastic slides B (30 mm × 80 mm × 2 mm) as the base plate integrated a Microsampler 100 Cartridge (Bio-Rad) encasing a separation capillary of 27 cm effective length and two reservoirs. The two reservoirs were produced from two Tygon tubing (T1) (3.0 mm I.D. × 4.0 mm O.D, 70 mm length for anodic reservoir and 110 mm length for cathode reservoir) which were first fixed sideways on the slide B with epoxy, and then connected with the separation capillary whose both

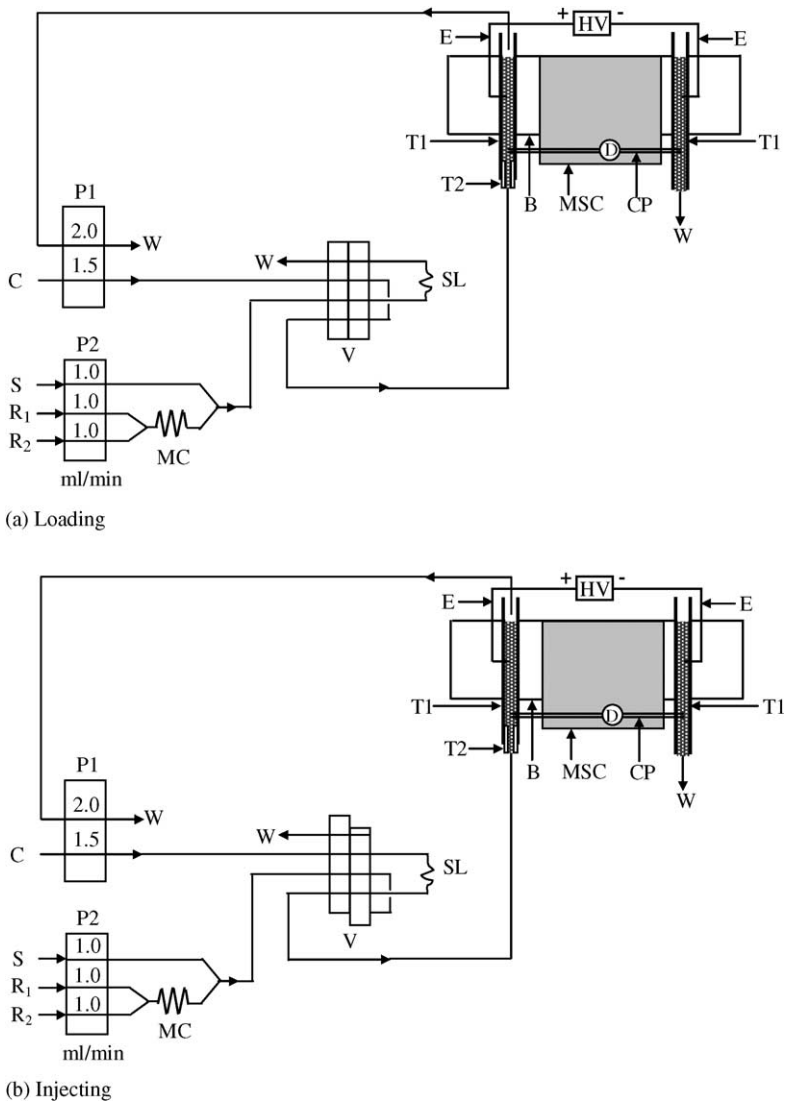

Fig. 1. The manifold of the automated on-line derivatization device combined with the CE system (not to scale): (a) loading; (b) injecting. C, carrier solution; S, sample; R1, OPA; R2, ME; MC, mixing coil; P1 and P2, peristaltic pumps; SL, sampling loop; V, injector valve; B, planar plastic base; MSC, Microsampler 100 Cartridge (Bio-Rad); T1, Tygon tubing; T2, PVC tubing; CP, separation capillary column; E, platinum electrode; W, waste; C/S, carrier/sample; HV, high voltage; D, detector.

ends were inserted in the center of the Tygon tubing (T1) through holes punctured with a hypodermic stainless steel needle. Two platinum electrodes that were used as the anode and cathode were, respectively, inserted into the two reservoirs 30 mm above the capillary tip to avoid entrainment of electrolytically generated oxygen bubbles into the capillary. A 30 mm length PVC tubing (T2) was push-fitted into the left Tygon tubing until 12 mm below the capillary tip, and functioned as the carrier/sample (C/S) inlet. The lower section of the right Tygon tubing (T1) was controlled by a screw clamp except for filling or changing of electrolyte solution, or for capillary cleaning. The entire setup was fixed on the plastic slide by applying epoxy glue at the connecting points. The H-channel chip was inserted into the standard capillary cassette of the commercial HPE-100 CE instrument.

2.3. Precolumn derivatization and operating procedure and conditions

According to Chen et al.'s study in our laboratory [30], the 1.5 ml/min flow rate was chosen for the carrier solution, 2.0 ml/min for the waste, 40 μl for the sample volume, respectively. The detailed operating procedure was as follows. Firstly, P2 was operated, the excess content of OPA (R1) in 50% ethanol, the excess content of ME (R2) and a certain amount of sample (amino acid solution, S) were simultaneously pumped into the FI system (see Fig. 1). While the sample loop (the sample volume: 40 μl) was full of the mixture, P2 was stopped and the mixture in the sample loop was vortexed adequately. Secondly, the derivatization of amino acid was carried out under the condition of ambient temperature and reaction time 1 min. Then, a little of the derivates of

amino acid were introduced into the capillary electrophoresis through the flow injection, and moved and separated in the running buffer (15 mM borate, 12 mM β-CD, 8 mM SDS and 10% (v/v) methanol at pH 9.5) till reached the detection window, the determination was carried out under the condition of the 9.0 kV applied voltage and 214 nm detection wavelength.

In order to maintain the capillary under good working conditions, its surface was regenerated once a day by consecutive rinsing with distilled water (5 min), 0.1 M NaOH (5 min), and distilled water (2 min) followed by the running buffer (10 min).

3. Results and discussion

3.1. The derivatization of amino acid

According to the review by Wan and Blomberg [31], OPA reacts with amino acids in the presence of the reducing agent, such as ME, to form a substituted isoindole ring. The OPA–amino acids have a strong absorbance at 260 and 340 nm. Most importantly, this reagent reacts with amino acids rapidly and the excess OPA reagent has virtually no interference with electrophoretic separation. Moreover, this reaction takes place easily at the ambient temperature.

Comparing with other reducing agents (for example, the chiral thiol), ME is very cheap and easily obtained. In addition, although the OPA derivative has the maximum wavelength of absorption at 260 or 340 nm, the separation of Asp with other components in Compound Amino Acid Injection is not satisfactory. As a compromise between selectivity and sensitivity, the wavelength of 214 nm was chosen.

So in the experiment, 0.1 M OPA, 0.1 M ME, 214 nm detection wavelength and ambient temperature were chosen for considering the total concentration of the amino acids.

3.2. Influence of the buffer pH on separation

In the investigating of the buffer pH, the four diastereoisomers (Val, Leu, Ile and Arg) and two enantiomers (D-Asp and L-Asp) were able to achieve the good baseline separation in the range of total investigating pH (i.e. from 8.0 to 10.5) in the real sample (Compound Amino Acid Injection (6AA)), however, D-Asp and L-Asp could not be separated adequately under the investigated pH. Under the conditions of 10 mM borate, 15 mM SDS, 10 mM β-CD, 10 kV applied voltage and 1 min reaction time, the six points pH were chosen to investigate, and the resolution between D-Asp and L-Asp (Rs) changed from 0.5 to 1.0. From the result, with the increasing of the pH, the Rs became larger and larger initially, moreover, the peak area also increased. At the pH 9.5, the Rs almost reached 1.0. However, as the pH >9.5, the peak area became smaller and smaller, and the analysis time became longer and longer. So, the pH 9.5 was chosen for the shorter analysis time, the better separation and sensitivity (expressed by the peak area of the analytes).

3.3. Influence of the buffer concentration on separation

Owing to influence the electroosmotic flow (EOF) and the viscosity of the electrolyte obviously, the buffer concentration had played an important role in the capillary electrophoresis separation. In order to achieve the best resolution of the analytes, the influence of the concentration of buffer at pH 9.5 was studied (from 5 to 30 mM every 5 mM), with 10 mM β-CD, 15 mM SDS, 10 kV applied voltage and 1 min reaction time. With the increasing of the concentration of the buffer (C_b), the analysis time, the Rs and the peak area of the OPA derivates of the enantiomer (D-Asp and L-Asp) increased till C_b was at 15 mM. When $C_b > 15$ mM, the Rs and the sensitivity decreased simultaneously, and the analysis time became longer. This may be explained as the higher Joule heating due to the higher conductivity of the increasing buffer. In order to obtain higher separation and sensitivity, while avoiding the generation of excessive Joule heating, a buffer concentration of 15 mM was believed to be a good comprise of analysis time, resolution and sensitivity.

3.4. Influence of the SDS on separation

The SDS was selected in the study, because it was an anionic surfactant and formed micelles lagging behind EOF, and therefore, functioned as a “pseudostationary phase”. To date, the effect of SDS concentration on chiral separations had usually been neglected in all previous studies. Usually, 20 mM was the typical value used in most studies [32]. In MEKC, the concentration of SDS (C_{SDS}) was always considered as one of the important factors, so it was necessary to investigate the influence of SDS. In the study, the six concentration points were selected from 5 to 30 mM every 5 mM and the other factors were kept constant. From the experimental result, at the concentration of 20 mM SDS, the sensitivity was the highest; however, the D-Asp could not be separated from L-Asp and other amino acids apparently. At the concentration of 15 mM, the sensitivity and Rs were both acceptable. Furthermore, it was found that the operating current increased from 28 to 45 mA, when C_{SDS} increased from 20 to 50 mM. Higher current is not desirable since it leads to lower resolution. So, 15 mM for SDS was chosen.

3.5. Influence of β-CD on separation

According to the review by Wan and Blomberg [31], β-CD and γ-CD are better chiral selectors than α-CD. Comparing with the γ-CD, the β-CD leads to the better selectivity for the smaller chromophore groups, indicating the importance of size-fit between CD cavity and derivative moieties for chiral recognition.

It is evident that OPA derivate and β-CD cavity are a perfect match in size, so β-CD was chosen in the study.

To investigate the effect of β-CD on the separations, the chiral separations were performed under two different conditions: (i) without β-CD; and (ii) with 10 mM β-CD, while all other reagents were kept the same for two systems, i.e. pH 9.5, 15 mM borate, 15 mM SDS, 10 kV applied voltage and 1 min reaction time. The result indicated that D-Asp and L-Asp overlapped totally in the system without β-CD; and Rs varied to some extent in the system with varying the concentration of β-CD. So, β-CD was a very important factor to be investigated in the experiment. The six concentration points (0, 6, 9, 12, 15 and 18 mM) were selected according to the experimental requirements. With increasing the concentration of β-CD, the sensitivity and Rs firstly became larger and larger, and then smaller and smaller when the concentration of β-CD exceeded 12 mM. On the other hand, the migrating time of the analytes became longer with the increasing of the concentration of β-CD, owing to the increasing of the viscosity of the bulk solution; moreover, the sensitivity and Rs were both the largest value at the 12 mM concentration point, respectively. Therefore, 12 mM for β-CD was considered as the optimum.

In the experiment, the separation principle was based on the synergetic effect of uncharged β-CD in combination with an achiral sodium dodecyl sulfate (SDS). Uncharged β-CD migrated with an identical velocity to that of the bulk solution. Partition of the hydrophobic analytes took place between the bulk solution, the β-CD and the negatively charged SDS micelle, which migrated in the opposite direction to the EOF, thus retaining the analytes.

3.6. Influence of organic modifier on separation

Although there were a serial of factors mentioned above to optimize, D-Asp and L-Asp could not achieve the best separation without adding the organic modifier, the largest Rs value was 1.0 or so, in other words, the enantiomer did not separate completely. So, finding other factors was apparently important. In general, adding the organic modifier was a very effect measure. In the study, various organic modifiers were studied to enhance the enantiomeric separation. These included acetonitrile (15%, v/v), acetone (15%, v/v), isopropanol (15%, v/v), ethanol (15%, v/v), and methanol (10–30%, v/v). Most of them showed little effect. However, the addition of methanol to the running buffer remarkably improved the enantiomeric separation. The best Rs (=1.67) was achieved with 10% (v/v) methanol. Further increasing of methanol concentration deteriorated the detection sensitivity. Moreover, binary system of methanol (15%, v/v)/acetonitrile (15%, v/v), methanol (15%, v/v)/acetone (15%, v/v), methanol (15%, v/v)/ethanol (15%, v/v) and ethanol (15%, v/v)/acetonitrile (15%, v/v) were evaluated. The effect of the binary system on separation was not all desired, its largest Rs value was 1.1, which was smaller than that obtained with methanol alone. So, according to the analysis mentioned above, 10% (v/v) for methanol was selected.

3.7. Influence of the applied voltage on separation

The applied voltage on separation was another important factor. Attempt was made to optimize the separation by using different applied voltages ranging from 8.0 to 11.0 kV under the optimum factors mentioned above. Varying the applied voltage did not give any changes in the migration order of the amino acid derivatives. The separation voltage determined the migration time directly and influenced the resolution. As it may be expected, the migration time and resolution decreased significantly with increasing separation voltage. Based on experiments, 9.0 kV was selected as the optimum voltage to accomplish a good compromise between the migration time and the separation efficiency.

3.8. Influence of the derivatization time on sensitivity

The derivatization time has the direct effect on the content of the derivates of the OPA and amino acids; furthermore, the sensitivity (expressed by the peak area of the derivates). Therefore, optimization of the derivatization time was a key step. The six time points (0, 0.5, 1, 1.5, 2, 2.5 and 3 min) were selected. The peak area became gradually large at the begin, then, it kept constant when the derivatization time exceeded 1 min, under the optimum conditions mentioned above (i.e. pH 9.5, 15 mM borate, 15 mM SDS, 12 mM β-CD, 10% (v/v) methanol 9.0 kV applied voltage and the excessive for OPA and ME). So, 1 min derivatization time was adequate.

3.9. Performance of the combined FI–CE system

Calibration graphs (peak area (y) versus concentration (x, mM)) for L-Asp and D-Asp which were commonly used as target compounds to quantitate Compound Amino Acid Injection (6AA) in the application section were obtained by injecting standard solutions in the range 0.3–10.0 mM. Each point on the calibration graph corresponded to the mean value obtained from five independent peak area measurements. The regression equation of the curve and the correlation coefficient are shown in Table 1.

The optimized conditions were as follows: pH 9.5, 15 mM borate, 15 mM SDS, 12 mM β-CD, 10% (v/v) methanol, 9.0 kV applied voltage, the excessive OPA and ME, 1 min conversion time and ambient temperature. Under the optimum conditions, a typical standard (concentration: 6 mM for D-Asp and L-Asp, respectively) and sample electropherograms for L-Asp, D-Asp and other amino acids was obtained and shown in Figs. 2 and 3, respectively. According to Figs. 2 and 3, the relative standard deviations (RSD, $n = 5$) of the peak area and the peak height of D-Asp and L-Asp were calculated and shown in Table 1.

Table 1
Analytical performance of the microfluidic FI–CE on separation testing system of the L-Asp and D-Asp acids ($n = 5$)

Item	D-Asp	L-Asp
LOD (S/N = 3) (μg/ml)	0.11	0.12
Peak height RSD (%)	4.0[a]; 4.2[b]	3.2[a]; 3.7[b]
Peak area RSD (%)	3.8[a]; 4.0[b]	4.2[a]; 4.0[b]
Linear range (mM)	0.3–10.0	0.3–10.0
Regression equation[c]		
a	6.6×10^2	2.2×10^2
b	1.0×10^3	1.1×10^3
Correlation coefficient	0.9962	0.9963
Sampling frequency (h^{-1})	10	

[a] RSD in Fig. 2.
[b] RSD in Fig. 3.
[c] The regression equation is $y = a + bx$, where y is peak area and x is the concentration (mM).

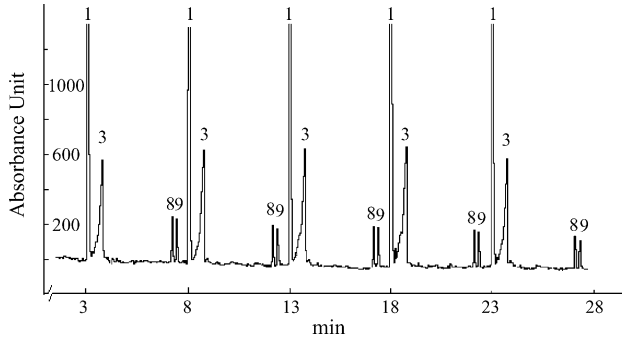

Fig. 2. Electropherogram of the OPA derivates of L-Asp and D-Asp standard solution (five times injected). CE conditions: 15 mM borate buffer solution, 15 mM SDS, 12 mM β-CD, pH 9.5; voltage, 9.0 kV; detection wavelength, 214 nm; capillary column, 50 μm I.D. × 375 μm O.D. × 30 cm length (27 cm effective length). FI conditions: buffer flow rate, 1.5 ml/min; R1 (OPA), R2 (ME) and S (sample) flow rate, 1.0 ml/min; sample volume, 40 μl. On-line conversion conditions: the excessive OPA and ME; reaction temperature, ambient; reaction time, 1 min; sample, 6 mM L-Asp and D-Asp in the distilled water, respectively. Peak 1: OPA; peak 2: ME; peak 8: D-Asp; peak 9: L-Asp.

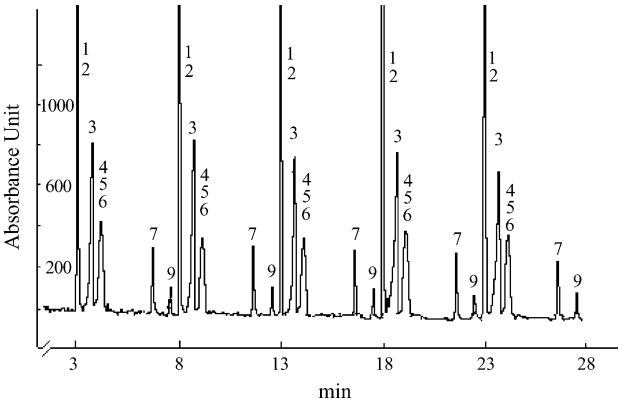

Fig. 3. Electropherogram of Asp in Compound Amino Acid Injection (6AA) (five times injected). The conditions as in Fig. 2. Peaks 1 and 2: OPA and Arg; peak 3: ME; peaks 4–6: Val, Leu and Ile; peak 7: Glu; peak 9: L-Asp.

Based on the principle that the ratio of signal and noise (S/N) was equal to 3, the baseline noise was evaluated by recording the detector response every 3 s within 2 min, and the signal of the sample response that caused a peak with a height was considered as three times the baseline noise level. So, the limits of detection (LOD) of D-Asp and L-Asp were calculated and shown in Table 1.

3.10. Application

The aim of developing the system was to service the practical applicability, which demonstrated the feasibility and precision of performing the determination of D-Asp and L-Asp in Compound Amino Acid Injection (6AA) with such system in pharmaceutical processing. The sample solution was obtained from the Lanzhou Medicine College. The solution was diluted 10 times with distilled water and filtered through a 0.45-μm syringe filter, and then was directly injected into the FI–CE after on-line conversion. The typical electropherogram of Compound Amino Acid Injection (6AA) is shown in Fig. 3. Peaks 1–7 were OPA, Arg, ME, Val, Leu, Ile and Glu, respectively; peak 9 was L-Asp that we tried our best to separate and determine, there was not D-Asp in the Compound Amino Acid Injection (6AA). All peaks were identified by the addition of the corresponding standard solution. The result for the determination of L-Asp in real sample showed agreement between the claimed (4.60 g/l) and found values (4.73 g/l). Recovery at added standard levels of 1.0, 3.0 and 6.0 mM was 92%, 104% and 109%, respectively.

Obviously, the FI–CE system was very effective and suitable for on-line conversion and determination of the chiral Asp in Compound Amino Acid Injection (6AA) in the pharmaceutical processing.

4. Conclusion

In this paper, the derivatization was fully automated in the FI system, and it was sensitive and quick. This greatly facilitated the analysis of amino acid, which was highly desirable in view of the increasing demand of analysis. On the other hand, MEKC has powerful separation function, which usually was applied to the chrial amino acids separation that other CE mode could not reach, and all chemicals that were used in MEKC system could expediently obtain in the lab on the study. Taking the advantage of synergistic effects of the easy, sensitive and rapid derivatization of OPA, ME with amino acids and better separation in MEKC, a novel, accurate and simple FI–CE method had been developed for the determination of the chrial Asp by on-line conversion, which had been successfully used to separate and identify D-Asp from L-Asp and other four amino acids. Moreover, it was the first time to utilize FI–CE to determine the content in the real sample (Compound Amino Acid Injection (6AA)). So, this method may be better alternative for the analysis of chrial amino acids.

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