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Research Article

Enantioselective analysis of proteinogenic amino acids in cerebrospinal fluid by capillary electrophoresis–mass spectrometry

D-Amino acids (AAs) are increasingly being recognized as essential molecules in biological systems. Enantioselective analysis of proteinogenic AAs in biological samples was accomplished by CE–MS employing β-CD as chiral selector and ESI via sheath-liquid (SL) interfacing. Prior to analysis, AAs were fully derivatized with FMOC, improving AA-enantiomer separation and ESI efficiency. In order to optimize the separation and MS detection of FMOC-AAs, the effects of type and concentration of CD in the BGE, the composition of the SL, and MS-interfacing parameters were evaluated. Using a BGE of 10 mM β-CD in 50 mM ammonium bicarbonate (pH 8) containing 15% v/v isopropanol, a SL of isopropanol-water-1 M ammonium bicarbonate (50:50:1, v/v/v) at a flow rate of 3 μL/min, and a nebulizer gas pressure of 2 psi, 15 proteinogenic AAs could be detected with enantioresolutions up to 3.5 and detection limits down to 0.9 μM (equivalent to less than 3 pg AA injected). The selectivity of the method was demonstrated by the analysis of spiked cerebrospinal fluid, allowing specific detection of D-AAs. Repeatability and linearity obtained for cerebrospinal fluid were similar to standard solutions, with peak area and migration-time RSDs ($n = 5$) below 16.2 and 1.6%, respectively, and a linear response ($R^2 \geq 0.977$) in the 3–90 μM range.

Keywords:
Amino acids / Capillary electrophoresis–mass spectrometry / Cerebrospinal fluid / Chiral separation
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1 Introduction

Amino acids (AAs) play a major role in the physiology of organisms. They are the building units of proteins, but also essential in for example, metabolic processes, neurotransmission, and lipid transport. AAs are precursors for the synthesis of hormones and nitrogenous substances with significant biological importance [1, 2]. The α-carbon of AAs, except for glycine, is a chiral atom and, consequently, proteinogenic AAs in principle can occur as L- and D-enantiomers. Enantiomers have identical physical and chemical properties, but may have substantially different biological activities, reactivities, and metabolic rates or pathways. Therefore, specific analysis of D-AAs next to L-AAs, including determination of enantiomeric ratios, may be important in diverse areas such as pharmaceutical, environmental, and food science [3–6] where questions on quality, function, stability, toxicology, and safety are to be answered. For example, the chiral analysis of DL-AAs can be also used for the identification of food adulterations, the monitoring of fermentation and microbiological contamination, and the evaluation of aging, treatment, and storage effects [7]. Moreover, during synthesis of peptide drugs, unwanted racemization of AA residues is a concern [8]. In humans, AAs most commonly occur in their L- form, but there is increasing evidence that D-AAs also play essential roles in human biology. D-AAs are involved in the pathogenesis of psychiatric disease and abnormal levels were found in human disorders, such as schizophrenia and Alzheimer's disease [9–13]. In the aforementioned applications, the AA enantiomers often are minor components of complex mixtures, such as biological fluids and food, requiring the use of highly selective separation and detection techniques for their unambiguous assessment.

A number of analytical techniques are capable of probing molecular chirality, including optical spectroscopic [14, 15], MS, and NMR [16, 17] methods, frequently in combination with chiral chemistry. NMR is highly selective, but exhibits a relatively low sensitivity. Optical spectroscopy and MS can be very sensitive, but often lack the selectivity for direct analysis of biofluids. Specific and reliable detection of (low levels of) AA enantiomers in complex samples often requires use of separation techniques prior to detection. Employing chiral stationary phases, GC has been used for quantification of selected AA enantiomers in for example, wines [18],

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Abbreviations: AA, amino acid; **CSF**, cerebrospinal fluid; **SL**, sheath liquid

vinegars [19, 20], human urine, and blood [21, 22]. GC intrinsically can provide efficient separations, but its main drawback is the laborious sample preparation and derivatization to achieve analyte volatility [23]. LC is the most common separation technique used to achieve enantioresolution and has been used for the chiral analysis of AAs in biological samples, employing chiral derivatization [24–28], chiral stationary phases [29, 30], or chiral selectors in the mobile phase [31–33]. LC techniques require costly chiral columns or large amounts of chiral selector [34, 35]. Upon repeated analysis of biological samples, chiral LC columns may suffer from instability and loss of performance. Moreover, coupling of LC techniques to MS while employing a chiral selector in the mobile phase can be problematic as the selectors are often nonvolatile and their relatively high amounts can cause serious ionization suppression [36].

CE is a microscale technique that is particularly suited for the separation of ionic compounds, such as AAs, as its selectivity is based on molecular charge-to-size ratio. Chiral CE most commonly involves addition of chiral selectors, such as CDs, to the BGE [37]. The differential interaction of L- and D-enantiomers with the selector molecules induces differences in their overall electrophoretic mobilities, thus yielding their separation. Employing the intrinsically high separation efficiency (i.e. narrow peaks) of CE, the selectivity of chiral selectors can be fully utilized. As the required separation buffer volumes are small and the chiral selectors can be simply added to the separation buffer, consumption of (expensive) chiral selectors is very low, and various selectors can be evaluated easily and rapidly. Moreover, injection volumes in CE are minute, which is advantageous when sample volumes are limited and/or repeated analysis of the same sample is needed. On the other hand, the small injection volume may result in relatively poor detection limits (in concentration units), which means that sensitive detection is required when low levels of enantiomers have to be analyzed.

In order to achieve selective detection of AAs in biological samples, hyphenation of CE with MS is indicated [38–40]. As reviewed by Shamsi [41], several procedures have been developed for chiral CE–MS. With partial-filling techniques [42–47] or use of reverse-migrating phases [48–55], chiral selectors are prevented from entering the MS ion source. Unfortunately, chiral separation often is compromised using these adapted CE schemes and AA-specific optimization is needed. Direct coupling of chiral CE to MS would be very attractive as optimal CE methods can be transformed without major adjustment ensuring optimum chiral resolution. Chiral molecular micelles have shown to be compatible with MS [56, 57]. Interestingly, as the absolute amounts of common chiral selectors, such as CDs, present in the BGE are relatively small, adequate sensitivity can also be obtained with direct chiral CE–MS [53, 58–63]. The use of chiral CE–MS for the separation of D- and L-AAs has been relatively limited so far. Few studies report on the chiral analysis of multiple AAs in complex samples, such as soy, vinegar, orange juice, and fertilizers [60, 62, 64], but chiral CE–MS of AAs in human biofluids has not been described yet.

Enantioselective analysis of AAs by CE–MS may be facilitated by chemical derivatization. Derivatized AAs often show improved ESI efficiencies and allow detection in a mass range (>280 m/z) where the MS background noise usually is much lower [65]. Moreover, derivatization may enhance AA chiral separation. FITC and dansyl chloride have been successfully used as derivatization agent in CE–MS of AAs yielding LODs in the 0.05–1 μM range [60–62, 64]. However, the main disadvantage of FITC and dansyl chloride is that AA derivatization normally takes at least several hours [60–62, 64, 66]. Derivatization of AAs with FMOC is much faster (up to 10 min) and, therefore, could be an attractive alternative for chiral CE–MS of AAs. FMOC has been used for chiral CE–MS of carnitine [67–69] and, more recently, of proteinogenic AAs [62], achieving LODs down to 0.06 and 0.1 μM, respectively. Notably, in the latter method, a partial-filling approach had to be used to prevent interference of the chiral selector vancomycin with the MS detection of the AAs [70].

In the present study, we aimed to develop a direct chiral CE–MS method for proteinogenic AAs using the advantage of fast FMOC derivatization. The derivatization efficiency and recovery were studied, and due attention was given to the effect of the BGE composition (including chiral selector), the sheath-liquid (SL) composition, and several ESI–MS parameters on AA separation and detection. The applicability of the optimized chiral CE–MS method was evaluated by the enantioselective analysis of AAs in cerebrospinal fluid (CSF). LODs, concentration linearity, and repeatability of migration time and peak area were assessed.

2 Materials and methods

2.1 Chemicals

All reagents were of analytical grade. Ammonium hydroxide (28%, w/v) was from VWR (Amsterdam, The Netherlands). (2-Hydroxypropyl)-β-CD (2-HP-β-CD), (2-hydroxypropyl)-γ-CD (2-HP-γ-CD), isopropanol, ammonium bicarbonate, DL-methionine, DL-tyrosine, formic acid, and γ-CD were supplied by Fluka (Steinheim, Germany). Methanol and ACN (both HPLC grade) were from Biosolve (Valkenswaard, The Netherlands). L-phenylalanine was from Acros Organics (Geel, Belgium). FMOC-Cl, α-CD, β-CD, pentane, sodium hydroxide, sodium tetraborate, glycine, D-glutamic acid, D-histidine, D-threonine, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, DL-alanine, DL-arginine, DL-asparagine, DL-aspartic acid, DL-cysteine, DL-glutamic acid, DL-histidine, DL-isoleucine, DL-leucine, DL-lysine, DL-phenylalanine, DL-proline, DL-serine, DL-tryptophan and DL-valine were from Sigma Aldrich (Steinheim, Germany). Methyl-β-CD was from Wacker-Chemie GMBH (München, Germany). 6-Monodeoxy-6-mono-(3-hydroxy)-propylamino-β-CD (6-3HP-β-CD) and sulfated-γ-CD were from Cyclolab (Budapest, Hungary). Heptaxis

(2,3-di-O-methyl-6-O-sulfo)-β-CD (2,3-OM-6-OS-β-CD) was from ElphoTech (College station, TX, US). Octakis(2,3-dihydroxy-6-Sulfato)-γ-CD (6-OS-γ-CD) was from TM Chemicals (Deer Park, TX, US). Water was deionized and purified with a Milli-Q purification system (Millipore, Bedford, MA, USA) prior to use. Human CSF was provided by the Leiden University Medical Centre (Leiden, The Netherlands) and stored at −80°C until analysis.

The optimized BGE was 0.05 M ammonium bicarbonate (pH 8) isopropanol (85:15, v/v). The pH of the ammonium bicarbonate solution was adjusted with 1 M ammonium hydroxide. The BGE was filtered prior use through 0.45 μm pore size disposable nylon filters from VWR (Amsterdam). Stock solutions (10 mM) of AAs were prepared in 0.2 M sodium tetraborate (pH 9).

2.2 Sample preparation and AA derivatization

CSF samples were thawed and 250 μL were mixed with 1500 μL ice cold (−20°C) ACN and vortexed vigorously. The samples were kept at −20°C for 5 min and then were centrifuged for 15 min at 15 000 rcf at 4°C. The supernatants were dried and then reconstituted in 250 μL of 0.2 M sodium tetraborate (pH 9).

The FMOC derivatization was carried out based on a procedure reported by Wan et al. [71]. Briefly, 250 μL of an AA solution or the CSF reconstituted sample were mixed with 250 μL of 20 mM FMOC in ACN and kept at room temperature for 10 min achieving near 100% derivatization yield. In order to remove excess of FMOC, the solution was extracted with 0.75 mL pentane. The aqueous phase was diluted 1:1 with water prior to CE–UV or CE–MS analysis. Derivatized samples were stored at 4°C showing good stability for at least one month. For analysis, samples were brought to room temperature showing no degradation within one week.

2.3 CE with UV absorbance detection

CE–UV analysis was carried out using a P/ACE MDQ CE instrument from Beckman Coulter (Brea, CA, USA) equipped with an UV detector operating at 257 nm (band width, 10 nm). Separations were performed on a bare fused-silica capillary of 50 μm id with a length of 70 cm to the detector and 80 cm of total length (Polymicro Technologies, Phoenix). The capillary was thermostated at 20°C. Hydrodynamic injections were made at the anodic end for 20 s at 0.5 psi (approximate injection volume of 9.8 nL) and the applied separation voltage was +25 kV. The P/ACE MDQ instrument was controlled by a PC running 32 Karat software version 8 (Beckman Coulter). New capillaries were preconditioned by rinsing, respectively, with 1 M NaOH and water for 15 min each using a pressure of 30 psi. Between runs, the capillary was rinsed at 30 psi with 0.2 M NaOH for 2 min, followed by 2 min with milli-Q water, and then conditioned for 6 min with running buffer. At the end of the day, the capillary was rinsed with milli-Q water for 10 min at 30 psi.

2.4 CE with MS detection

The P/ACE MDQ CE instrument described above was coupled to an Agilent 6300 Series LC/MSD XCT IT mass spectrometer (Agilent Technologies, Waldbronn, Germany) through a coaxial CE–MS sprayer (Agilent Technologies). LC/MSD Trap software 6.1 was used for MS control and data analysis. The SL was provided by a 2.5 mL syringe (Hamilton, Bonaduz, Switzerland) and a syringe pump of KD Scientific (Holliston, MA, US) at 3 μL/min. After optimization, the following ESI-MS interface conditions were used: dry gas temperature, 325°C; dry gas flow, 4 L/min; nebulizer gas pressure, 2 psi; ESI voltage, 4.5 kV. Data were acquired in positive ionization mode in the scan range 100–650 *m/z* with a repetition rate of 3 Hz. The trap parameters were programmed in expert mode with 50 V of capillary exit offset and 45 for trap drive. The ion charge control mode operated to accumulate 50 000 ions for a maximum accumulation time of 300 ms. Extracted ion electropherograms (EIEs) were obtained using the smooth option of the software (Gauss at 1 point). A bare fused-silica capillary of 50 μm id and 80 cm length was used and preconditioned at the start of the day by rinsing with, respectively, 1 M NaOH and water for 15 min each at 30 psi. During this rinsing the outlet of the capillary was disconnected from the ion source. Between runs, the capillary was rinsed at 30 psi with 0.2 M NaOH for 2 min, followed by 2 min with milli-Q water, and then conditioned for 6 min with running buffer. During this flushing the solvent emerging from the capillary outlet was sprayed into the ion source using a nebulizer gas pressure of 25 psi and applying no ESI voltage. At the end of the day, the capillary was rinsed with milli-Q water for 10 min at 30 psi. The capillary was thermostated at 20°C and samples were injected for 20 s at 0.5 psi. The applied voltage was +25 kV. The SL was isopropanol-water-1 M ammonium bicarbonate (50:50:1, v/v/v) and was delivered at a flow rate of 3 μL/min. For MS infusion experiments, a solution of 50 μM FMOC-L-phenylalanine was pushed through the capillary at 1.5 psi (103 mbar), inducing a flow rate of about 80 nL/min into the CE–MS interface.

3 Results and discussion

Ammonium bicarbonate was selected as BGE for its compatibility with ESI–MS and its buffering capacity in the pH 6.8–11.3 range. After derivatization with FMOC, most of the AAs will be overall negatively charged at pH 6 or higher, and migrate slower than the EOF when applying normal voltage polarity. Isopropanol was added to the BGE to decrease the EOF and potentially improve resolution. For starting experiments, the chiral selector β-CD was selected.

3.1 Derivatization of AAs with FMOC

Derivatization of AAs with FMOC appeared to favor chiral separation. This is illustrated for the CE–UV analysis of DL-tryptophan and DL-phenylalanine. Using a BGE of 50 mM

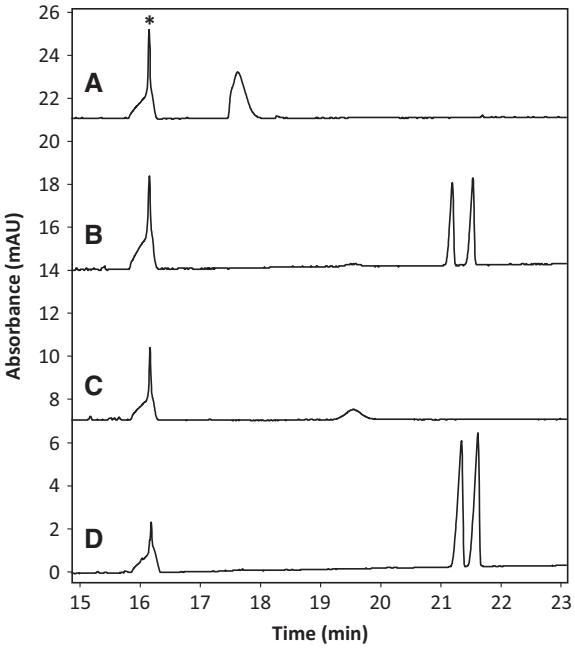

Figure 1. CE–UV of (A) DL-phenylalanine, (B) FMOC-DL-phenylalanine, (C) DL-tryptophan, and (D) FMOC-DL-tryptophan. The asterisk indicates unreacted FMOC that migrates at the EOF time. Conditions: BGE, 50 mM ammonium bicarbonate (pH 8) containing 15% v/v isopropanol and 10 mM β-CD. Further conditions, see Section 2.

ammonium bicarbonate (pH 8) containing 15% v/v isopropanol and 10 mM β-CD, no enantioseparation for tryptophan and phenylalanine was observed. However, the enantiomers of FMOC tryptophan and FMOC phenylalanine could be baseline separated (Fig. 1). Apparently, the changes in the analyte structure result in more enantiodiscriminative interactions with the CD, leading to significant improvement in chiral separation [60, 66]. As noted above, FMOC reacts with the amino group of the AA leaving the derivatized AA negatively charged at pH 6 and higher. This explains why the underivatized AAs—which are zwitterions at pH 8—migrate faster than the FMOC-AAs.

The FMOC derivatization yield was assessed by derivatizing phenylalanine in the concentration range of 0.01–20 mM with 20 mM FMOC (see Section 2). Subsequently, the derivatized sample was analyzed by achiral CE–UV using a BGE of 50 mM ammonium bicarbonate (pH 8) containing 15% v/v isopropanol. Results were compared with the analysis of the same concentrations of a commercial FMOC-phenylalanine standard. In the concentration range of 0.01-6 mM, FMOC-derivatization yields appeared to be above 98% when taking a reaction time of 10 min, indicating highly efficient derivatization and good extraction recovery. When derivatizing AA concentrations above 6 mM, the yield was below 90%. As overall AA concentrations in real samples normally will not exceed 2 mM, 20 mM FMOC was concluded to provide sufficient excess to allow quantitative derivatization of target AAs. In order to assess derivatization efficiency for other AAs, a mixture of DL-lysine, DL-tryptophan, DL-proline, DL-glutamic acid and DL-aspartic acid was derivatized in the concentration range of 0.01-6 mM. For all tested AAs, good concentration linearity (coefficients of determination (R^2) ≥ 0.99) was obtained during CE–UV analysis, indicating stable derivatization yields and extraction recoveries.

3.2 Chiral separation of FMOC-AAs

In order to select an appropriate chiral selector allowing chiral separation of multiple FMOC-derivatized AAs simultaneously, various CDs were tested in a BGE of 50 mM ammonium bicarbonate (pH 8) containing 15% v/v isopropanol. For this purpose, a test solution comprising DL-lysine, DL-tryptophan, DL-proline, DL-glutamic acid, and DL-aspartic acid was used. These AAs represent divers chemical properties (polarity, basic, acidic) and exhibit different charge after derivatization. Moreover, D-aspartic acid is found in human brains and neurons, and is reported to be related with Alzheimer's disease. Ten CDs (10 mM each) were tested: three native CDs (α-CD, β-CD, and γ-CD) and seven modified CDs of which four neutral (2-HP-β-CD, 2-HP-γ-CD, methyl-β-CD, and 6-3HP-β-CD) and three negatively charged CDs (2,3-OM-6-OS-β-CD, 6-OS-γ-CD, and sulfated-γ-CD). For achieving chiral resolution of analytes, the CD concentration in the BGE most commonly has to be at least 5–10 mM and often much higher [72, 73]. However, when coupling chiral CE directly to MS, the CD concentration should be kept as low as possible in order to avoid excessive ionization suppression of the analytes by the CD. Therefore, we selected 10 mM as CD concentration for the screening the potential of the different CDs. A BGE with a slightly basic pH was selected in order to ensure the FMOC-AAs were charged. After FMOC derivatization, a large part of the AAs will have no basic functional groups and, therefore, will be neutral at low pH, exhibiting no electrophoretic mobility.

Using the neutral α, γ, and modified CDs, the AAs could be detected, but their enantioseparation was poor. In presence of the negatively-charged CDs, no chiral separation was obtained for lysine and tryptophan. Moreover, glutamic acid and aspartic acid could not be detected, most probably because their overall electrophoretic mobility was toward the anode with the applied BGE. Detection as well as chiral separation of all tested AAs was obtained only with β-CD, which was selected for further studies. When using β-CD in 50–200 mM ammonium acetate (pH 6.7) or 0.1 M formic acid (pH 1.7), some AAs were detected, but no chiral separation was obtained. In ammonium bicarbonate (pH 8), β-CD provided enantioresolutions of 0.9, 2.3, 2.0, 3.8, and 2.4, respectively, for lysine, tryptophan, proline, glutamic acid, and aspartic acid.

The effect of the concentration of β-CD (0–20 mM) on the enantioseparation of the test AAs was studied. When no β-CD was in the ammonium bicarbonate BGE, most AAs were detected in the migration time range of 28–35 min, whereas glutamic acid and aspartic acid migrated

after 60 min. Using a BGE containing 5 mM β-CD, all tested AAs could be detected and chirally separated in the time frame of 24–52 min. Increasing the β-CD concentration to 10 mM, resulted in shorter migration times (range 23–43 min) and improved enantioseparations. Further increasing the β-CD concentration up to 15 mM resulted in migration times of 21–35 min, but a decrease of chiral resolution. As optimum concentration, 10 mM β-CD was chosen.

The effect of the percentage isopropanol (0–50%) of the BGE was evaluated. At low isopropanol content (0–5%) the AAs migrated relatively fast without exhibiting enantioseparation. Increase of the isopropanol concentration induced chiral separation of the FMOC-AAs. At high isopropanol content (30–50%), the migration of the AAs became very slow leading to analysis times over more than 1 h and also loss of enantioseparation. In order to achieve enantioresolution at reasonable analysis time, a concentration of 15% isopropanol in the BGE was selected.

The effect of the pH of the ammonium bicarbonate BGE on the enantioseparation of the tested AAs was studied in the range of pH 7–10. There was no significant effect of the pH on the chiral resolution, most probably because the tested pH range was well above the p*I* of the derivatized AAs. Surprisingly, the EOF time—which was 18–19 min—and the migration times of the AA did not significantly change when the pH was varied. We assume that an increase of the EOF by raising the pH was counteracted by a decrease of the EOF by the raise of the ionic strength related to the adjustment of the BGE to the appropriate pH. A BGE of 50 mM ammonium bicarbonate (pH 8) was selected as it required no further pH adjustment upon preparation.

3.3 MS detection of FMOC–AAs

The effect of several system parameters on the MS response of FMOC-L-phenylalanine (50 μM) was studied by direct infusion at a flow rate of 80 nL/min through the CE capillary into the CE–MS sprayer. The MS signal intensity of FMOC-L-phenylalanine appeared to be hardly affected by the ammonium bicarbonate concentration (0–100 mM). A concentration of 50 mM was considered to provide sufficient buffer capacity. Subsequently, the effect of the β-CD concentration on the MS signal was studied. Solutions of FMOC-L-phenylalanine (50 μM) in 50 mM ammonium bicarbonate (pH 8) containing 15% v/v isopropanol and 0–20 mM β-CD were evaluated (Fig. 2). Compared to a BGE without CD, addition of 5 mM β-CD caused a threefold reduction of the MS signal for FMOC-L-phenylalanine, but a substantial intensity could still be obtained. Increasing of the β-CD concentration to 10 mM and higher, caused further decrease of the MS signal, but the change was less significant as compared to the first 5 mM added. Taking into account the effect of β-CD on both ionization efficiency and enantioseparation, a concentration of 10 mM β-CD was selected.

CE–MS was accomplished by SL interfacing. The effect of the SL composition and flow rate was studied by

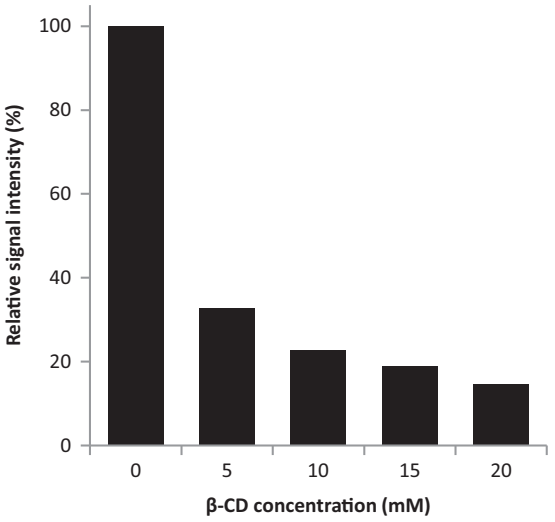

Figure 2. Effect of the β-CD concentration in the BGE on the MS signal intensity of 50 μM FMOC-L-phenylalanine. MS signal intensity obtained without β-CD was set at 100%. Experimental conditions, see Section 2.

infusion, monitoring the signal for FMOC-L-phenylalanine in BGE containing 10 mM β-CD. SLs composed of organic solvent–water 1 M ammonium bicarbonate (50:50:1, v/v/v) were tested employing isopropanol, methanol and ACN as respective organic solvents (Fig. 3A). Using the SL containing isopropanol, the MS signal intensity was, respectively, two and ten times higher than with the SLs containing methanol and ACN. The isopropanol–water ratio (1:3–3:1) in the SL was also investigated (Fig. 3B). Comparing to a SL with 50% isopropanol, the MS signal was, respectively, about 1.5 and five times lower when SLs with 25 and 75% isopropanol were used. A SL of isopropanol–water 1 M ammonium bicarbonate (50:50:1, v/v/v) was selected. The effect of the SL flow rate (1.5–5 μL/min) on the MS signal of FMOC-L-phenylalanine was also studied. Whereas a SL flow rate of 1.5 μL/min was too low to maintain a stable CE current, at SL flow rates of 2 μL/min or higher the MS signal intensity was stable and constant. A flow rate of 3 μL/min was selected ensuring spray stability, while minimizing sensitivity reduction by dilution.

The effect of the nebulizer gas pressure (2–20 psi) on the system performance was evaluated using DL-phenylalanine as test compound (Fig. 4). An increase of the nebulizer gas pressure causes a clear reduction of the enantiomer resolution while at the same time migration times decreased. Moreover, at higher nebulizer gas pressures, the MS signal intensities obtained for DL-phenylalanine were lower. Most probably, the nebulizing gas causes a suction effect inducing a hydrodynamic flow in the capillary that yields extra band broadening and, thus, loss of enantioresolution. The use of a relatively low nebulizer gas pressure evidently is mandatory for chiral CE–MS of FMOC-AAs. A nebulizer gas pressure of 2 psi—the minimum value for obtaining stable ESI—was selected.

The drying gas temperature (tested range, 250–365°C) and flow rate (tested range, 3–12 L/min) was also optimized

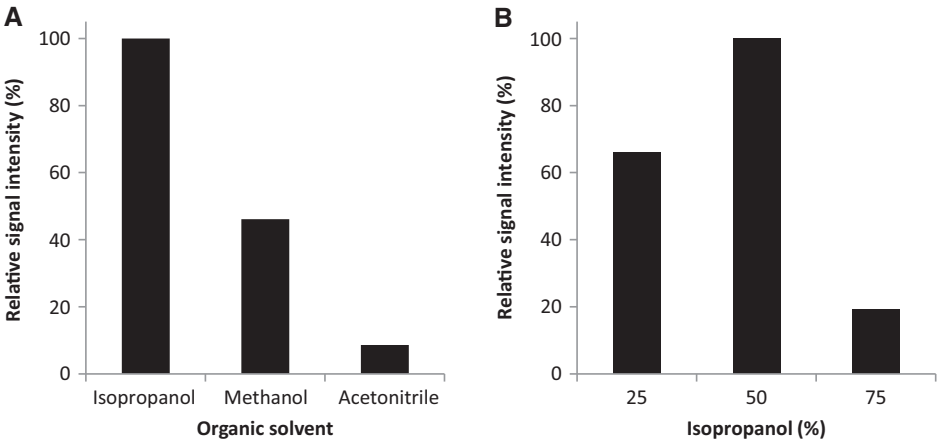

Figure 3. Influence of (A) the type of organic solvent in the SL, and (B) the isopropanol percentage in the SL on the MS signal of 50 μM FMOC-L-phenylalanine in BGE. MS signal intensity obtained with isopropanol-water-1 M ammonium bicarbonate (50:50:1, v/v/v) was set at 100%. Conditions: SL, (A) organic solvent-water-1 M ammonium bicarbonate (50:50:1; v/v/v); (B) isopropanol-water in different proportion containing 1% v/v ammonium bicarbonate. Further conditions, see Section 2.

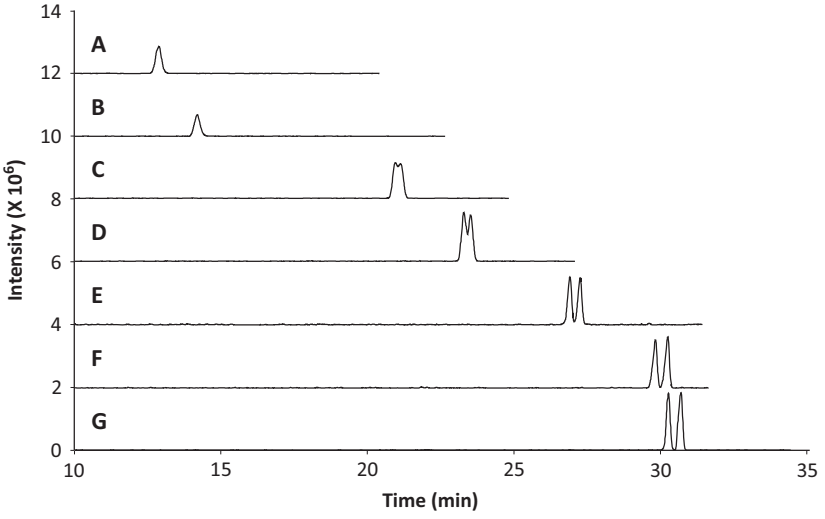

Figure 4. Chiral CE–MS of 50 μM FMOC-DL-phenylalanine using different nebulizer gas pressures. Conditions: nebulizer gas pressure, (A) 20 psi, (B) 15 psi, (C) 10 psi, (D) 8 psi, (E) 5 psi, (F) 3 psi, (G) 2 psi. Further conditions, see Section 2.

by infusion of FMOC-L-phenylalanine in BGE containing 10 mM β-CD. Highest intensities were obtained with a dry gas temperature of 325°C and 4 L/min, respectively.

3.4 Chiral CE–MS of FMOC-AAs

A BGE of 50 mM ammonium bicarbonate (pH 8) and 15% v/v isopropanol containing 10 mM β-CD and a SL of isopropanol-water-1 M ammonium bicarbonate (50:50:1, v/v/v) were selected for chiral CE–MS. A solution of 19 proteinogenic DL-AAs and glycine were derivatized and analyzed with the optimized CE–MS method (Fig. 5). The migration times of the FMOC-AAs were in the 30–37 min range except for DL-arginine (24 min), DL-glutamic acid (56/58 min), and DL-aspartic acid (61/63 min). All 20 AAs were detected, whereas 15 showed enantioseparation with a resolution above 0.5; nine AAs exhibited chiral resolution of at least 1.2 (Table 1). For DL-alanine, DL-arginine, DL-lysine, and DL-tyrosine no chiral resolution was obtained. The FMOC-DL-arginine migrated with the EOF-most probably because it is a zwitterion with an overall charge of zero-and exhibited no chiral resolution. DL-Cysteine, DL-lysine, DL-histidine, and DL-tyrosine were detected as derivatives carrying two FMOC groups due to the presence of a reactive functional group next to the α-amine. The LODs for the D-enantiomers of the enantioseparated AAs were in the low μM range (0.9–12.5 μM).

The migration order of the enantiomers of each AA was assessed by spiking DL-AAs with the L-enantiomer. Except for proline, the D-form of the AAs appeared to migrate faster than the L-form. This can be considered an advantage for the analysis of real samples where normally the minor D-enantiomer has to be assessed in the presence of the abundant L-enantiomer. The opposite order for the proline enantiomers is probably related to the secondary amine functionality of proline. Therefore, the FMOC is spatially differently bound and causing a different chiral interaction of the proline AA enantiomers with the β-CD.

The optimized CE–MS method was evaluated in terms of linearity and migration time and peak area repeatability (Table 2). Calibration curves were established using a mixture of DL-tryptophan, DL-proline, DL-glutamic acid, and DL-aspartic acid at enantiomer concentrations ranging from 1 to 40 μM. Each dilution was individually derivatized prior the analysis

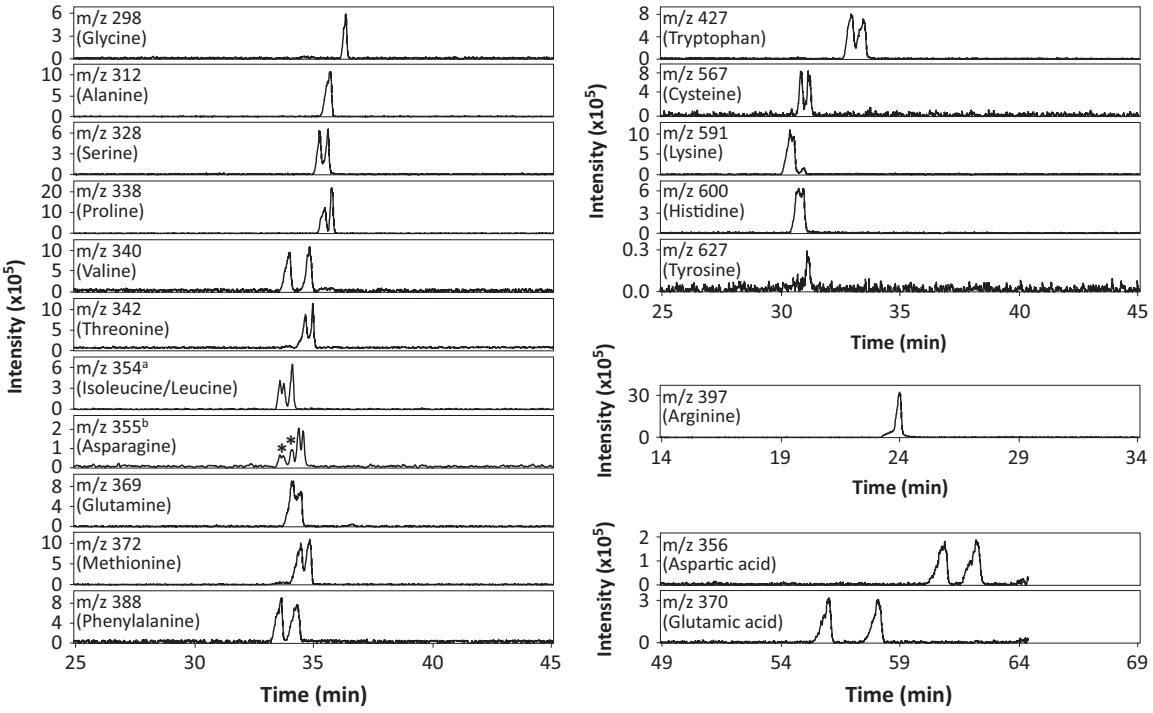

Figure 5. Extracted-ion electropherograms obtained during chiral CE–MS of glycine (*m/z* 298), and the DL-AAs alanine (*m/z* 312), serine (*m/z* 328), proline (*m/z* 338), valine (*m/z* 340), threonine (*m/z* 342), isoleucine and leucine (*m/z* 354), asparagine (*m/z* 355), glutamine (*m/z* 369), methionine (*m/z* 372), phenylalanine (*m/z* 388), tryptophan (*m/z* 427), cysteine (*m/z* 567), lysine (*m/z* 591), histidine (*m/z* 600), tyrosine (*m/z* 627), arginine (*m/z* 397), aspartic acid (*m/z* 356), and glutamic acid (*m/z* 370). Injected AA concentration, 45 μM of each enantiomer. Experimental conditions, see Section 2. The superscript letter "a" indicates migration order: D-isoleucine, D-leucine, L-isoleucine/L-leucine; asterisks indicate C13-isotopic peaks of isoleucine and leucine.

Table 1. Enantiomer resolution and LODs (μM) obtained during chiral CE–MS of a test mixture of 20 proteinogenic AAs

AA[a)]	Enantioresolution	LOD (μM)[b)]
Alanine	< 0.2	3.9
Arginine	< 0.2	0.5
Asparagine	0.8	3.8
Aspartic acid	2.3	8.3
Cysteine	1.2	12.5
Glutamic acid	3.5	4.7
Glutamine	0.6	2.6
Glycine	–	5.9
Histidine	0.5	5.7
Isoleucine	1.6	0.9
Leucine	1.7	1.3
Lysine	< 0.2	4.6
Methionine	0.9	3.6
Phenylalanine	1.2	7.9
Proline	0.9	1.4
Serine	1.3	3.1
Threonine	1.3	5.9
Tryptophan	0.9	1.8
Tyrosine	< 0.2	84.3
Valine	2.0	8.1

a) Injected concentration, 90 μM for each AA (45 μM per enantiomer).
b) Concentration yielding an S/N ratio of 3; calculated for d-enantiomer or dl-peak in case of enantioresolution <0.2.

Table 2. Analytical performance of the chiral CE–MS method for AAs in water

Analyte	Concentration linearity (R^2)[a)]	Migration time RSD (%)[b)]	Peak area RSD (%)[b)]
D-Tryptophan	0.993	0.97	11.8
L-Tryptophan	0.989	1.00	11.5
D-Proline	0.990	1.07	8.9
L-Proline	0.993	1.06	10.4
D-Glutamic acid	0.991	1.70	12.2
L-Glutamic acid	0.993	1.76	16.9
D-Aspartic acid	0.995	1.88	16.2
L-Aspartic acid	0.989	1.85	15.9

a) Five-point calibration curve (peak area vs. concentration (1-40 μM)) with two repeats per concentration.
b) $n = 5$; injected concentration, 24–36 μM.

and subsequently analyzed twice. Good linearities for peak area were observed with coefficients of determination (R^2) above 0.988 for all enantiomers. Migration time RSDs for five consecutive measurements were in the range of 0.9–1.9%. Peak area precision was satisfactory with RSDs between 8.9 and 16.9%.

The suitability of the developed chiral CE–MS method for the specific detection of D-AAs next to their L-enantiomers in biofluids was investigated by the analysis of CSF. D-AAs have

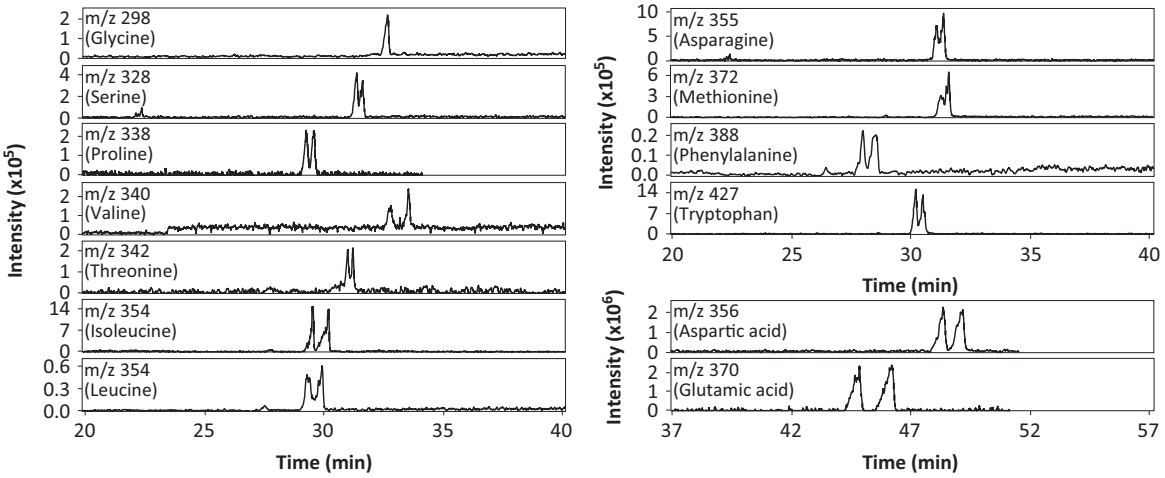

Figure 6. Extracted-ion electropherograms obtained during chiral CE–MS of CSF spiked with glycine (*m/z* 298) and the DL-AAs serine (*m/z* 328), proline (*m/z* 338), valine (*m/z* 340), threonine (*m/z* 342), isoleucine and leucine (*m/z* 354), asparagine (*m/z* 355), methionine (*m/z* 372), phenylalanine (*m/z* 388), tryptophan (*m/z* 427), aspartic acid (*m/z* 356) and glutamic acid (*m/z* 370). Injected D-AA concentration, 45 μM each; Experimental conditions, see Section 2.

Table 3. Analytical performance of the chiral CE–MS method for AAs in CSF

Analyte	Concentration linearity (R^2)[a)]	Migration time RSD (%)[b)]	Peak area RSD (%)[b)]	Enantioresolution
D-Tryptophan	0.984	0.94	13.1	1.4
L-Tryptophan	0.987	1.03	4.9	
D-Proline	0.995	1.09	11.1	1.2
L-Proline	0.978	1.04	13.6	
D-Glutamic acid	0.989	1.60	11.5	2.3
L-Glutamic acid	0.980	1.43	7.2	
D-Aspartic acid	0.977	1.49	16.2	1.6
L-Aspartic acid	0.988	1.51	15.4	

a) Five-point calibration curve (peak area vs. concentration (3-90 μM per enantiomer)).
b) n = 5; injected concentration, 28–36 μM per d-enantiomer.

been recognized as signaling molecules in the central nervous system [7–11], and therefore chiral analysis of AAs in CSF can be important. As proteins in the CSF may interfere with the CE–MS analysis [23], CSF samples were deproteinized using cold ACN prior to FMOC derivatization (see Section 2). The CSF sample preparation recovery was assessed by analysis of CSF spiked with DL-proline and DL-aspartic acid and compared with results for the respective standard solution. The obtained recoveries were above 80% for each enantiomer. Chiral CE–MS of blank CSF showed the presence of the L-enantiomers of alanine, serine, valine, leucine/isoleucine, glutamine, glutamic acid, methionine, histidine, phenylalanine, arginine, tryptophan, and lysine. In order to test the chiral performance of the CE–MS method, CSF was spiked with glycine and 13 DL-AAs that previously showed enantioresolutions of 0.8 or higher for the aqueous standard solutions. CE–MS analysis of the spiked CSF sample showed chiral resolution (0.9–4.0) for 12 of the spiked AAs (Fig. 6). Cysteine could not be detected due to poor S/N ratio at its *m/z*. LODs for glycine and the D-enantiomers of the other 12 AAs were in the range of 1.3–21 μM. Linear response in CSF analysis was assessed for DL-tryptophan, DL-proline, DL-glutamic acid, and DL-aspartic acid by spiking with the respective AAs at different concentrations. Linearity was observed in the 3–90 μM range with R^2 above 0.976 for all tested enantiomers (Table 3). RSDs for migration time were 0.9–1.6% for five consecutive measurements in the same day. Peak area repeatability was satisfactory with RSDs ranging between 4.9 and 16.2%. The CSF results are similar to the results obtained for aqueous solution of the same AAs enantiomers, indicating matrix effects were limited.

4 Concluding remarks

A chiral CE–MS method for the analysis of proteinogenic AAs was developed. Fast and efficient derivatization of the AAs with FMOC provided enhanced chiral separation and ESI–MS detection. The chiral selector (β-CD) in the BGE appeared to cause only moderate ionization suppression, allowing direct coupling to MS. The optimized method was found suitable for the chiral analysis of AAs in CSF with

calibration curves, detection limits, and migration time and peak area repeatabilities similar to those for standard AA solutions, indicating that matrix effects were marginal. The method employs a common bare-fused silica capillary and a simple easy-to-prepare BGE requiring very small absolute amounts of chiral selector. In-between-runs rinsing of the capillary with fresh BGE assures reproducible enantioresolution of AAs from CSF with LODs in the low μM range, which is quite favorable for a direct chiral CE–MS method.

Previously reported works on direct chiral CE–MS of AAs [60–62, 64] showed somewhat better sensitivity when using FITC as derivatizing agent. However, with these methods overall analysis times were quite long (above 15 hours) due to the slow derivatization. FMOC derivatization takes about 10 min, leading to total analysis times of less than 1.5 hours. Notably, compared to previous works, the presented method allowed enantioresolution of a larger number of proteinogenic DL-AAs in one run.

For the detection of endogenous levels of D-AAs in CSF and other biofluids further improvement of the sensitivity is needed. We plan to achieve this by applying on-line preconcentration (e.g. pH-mediated stacking [74]) and/or by using sheathless CE–MS interfacing. The latter option will circumvent the dilution of analyte by SL, which can result in a sensitivity enhancement of up to 50-fold, bringing the LODs in the low nanomolar range.

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