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Chiral separation of amino acids in biological fluids by micellar electrokinetic chromatography with laser-induced fluorescence detection

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Abstract

A method is presented for the chiral analysis of amino acids in biological fluids using micellar electrokinetic chromatography (MEKC) and laser-induced fluorescence (LIF). The amino acids are derivatized with the chiral reagent (+/−)-1-(9-anthryl)-2-propyl chloroformate (APOC) and separated using a mixed micellar separation system. No tedious pre-purification of samples is required. The excellent separation efficiency and good detection capabilities of the MEKC-LIF system are exemplified in the analysis of urine and cerebrospinal fluid. This is the first time MEKC has been reported for chiral analysis of amino acids in biological fluids. The amino acids D-alanine, D-glutamine, and D-aspartic acid have been observed in cerebrospinal fluid, and D-alanine and D-glutamic acid in urine. To the best of our knowledge no measurements of either D-alanine in cerebrospinal fluid or D-glutamic acid in urine have been presented in the literature before. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Amino acids

1. Introduction

It has long been presumed that amino acids occur solely as L-enantiomers in proteins and body fluids of mammals. However, increasing evidence for the occurrence of D-amino acids has been published in scientific journals during the last fifteen years. Free D-aspartic acid has been found in tissues of rat periphery organs [1,2], in rat brain [2–5] and plasma [4]. In humans, D-aspartic acid has been found in the prefrontal cortex [6], blood serum [2,7], and cerebrospinal fluid [8,9]. D-Serine has been detected in rat brain [3–5], in blood serum [7,10], in human cerebrospinal fluid [9], and in human prefrontal cortex [6]. A substantial part of the serine and alanine found in urine has been present as D-serine and D-alanine [7,11].

D-Serine, D-alanine and D-proline have been detected in mice lacking D-amino acid oxidase [12]. The regional distribution of D-serine has been studied in rats and has been shown to correlate to the *N*-methyl-D-aspartate (NMDA)-type excitatory amino acid receptor. While the concentration of free

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D-aspartic acid in brain tissue decreases shortly after birth in both rats and humans the concentration of D-serine is constant [3,6]. Other D-amino acids found include D-leucine in rat brain [12] as well as reports of the presence of D-phenylalanine, D-tyrosine, D-tryptophan, and D-leucine in human urine [13].

As of today few attempts have been made to correlate the abundance of D-amino acids to disease states in humans. However, correlations have been found between the total amount of D-amino acids [14,15], D-serine [15,16], and D-asx (a combined value calculated for D-aspartic acid and D-asparagine) [16] in blood serum and creatinine in patients with renal disorders. Increased amounts of D-aspartic acid, and increased total concentrations of D-amino acids have been found in the cerebrospinal fluid of patients suffering from Alzheimer's disease as compared to healthy control patients [8,9]. Methods for assaying D-amino acids in biological fluids and proteinaceous samples have been reviewed by Imai and co-workers [17,18].

Micellar electrokinetic chromatography (MEKC) has received a great deal of attention owing to its high separation efficiency [19]. The major drawback of the technique is the limited elution range of MEKC separations, due to the finite length of the separation window [19]. This can make a total separation of, for instance, all the biologically active amino compounds present in biological fluids difficult. The MEKC system can, however, easily be optimized for a limited number of analytes. Different aspects of the optimization of MEKC separations have been covered by Terabe et al. [20,21] and Vindevogel and Sandra [22]. A review article on the subject has been published by Corstjens et al. [23].

A number of separations using MEKC have been developed to date [24], including several MEKC separations of analytes in urine [25–33] and plasma [28–36]. The combination of capillary electrophoresis (CE) and laser-induced fluorescence (LIF) has been used for the analysis of substances such as LSD [38], anthracyclines [36,37], and moxifloxacin in biological fluids [39]. Amino acids have been analyzed as 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) [40] or fluorescein isothiocyanate (FITC) [41,42] derivates in cerebrospinal fluid using CE-LIF.

In this article a method is presented for the chiral analysis of amino acids in human cerebrospinal fluid and urine without the need for time consuming pre-purification. The amino acids were derivatized with either the (+)- or the (−)-enantiomer of the chiral reagent 1-(9-anthryl)-2-propyl chloroformate (APOC) and detected by an argon ion LIF system. The APOC reagent has been shown to provide attomole detection limits in combination with LIF detection, facilitating enantiomeric trace level determination of amino acids [43]. An internal standard for quantification of the amino acids, tranexamic acid, is also introduced.

2. Experimental

2.1. Chemicals

Boric acid, sodium tetraborate, and methanol were supplied by Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was from ICN (Aurora, OH, USA). All amino acids and the sodium salt of 7-deoxycholic acid were provided by Sigma (St. Louis, MO, USA). Sodium hydroxide pellets were from Eka Nobel AB (Bohus, Sweden). High purity hexane for residue analysis was obtained from Fisher Scientific (Loughborough, Leicestershire, UK). All buffer solutions were prepared using water from an Elgastat UHQII (Elga, High Wycombe, UK). The tranexamic acid used was a gift from Pharmacia Upjohn (Stockholm, Sweden).

2.2. Human cerebrospinal fluid samples

This investigation has been approved by the human Ethics Committee at the Faculty of Medicine, Göteborg University, Sweden. Human control cerebrospinal fluid (CSF) samples were obtained from the Institute of Neuroscience, Department of Neurochemistry at Sahlgrenska University Hospital. Control samples were chosen from patients without symptoms or signs of major neurological or psychiatric disorders undergoing lumbar puncture for diagnostic purposes. Routine CSF analyses gave normal values, without any sign of inflammation or damage to the blood–brain barrier function. Lumbar puncture was performed in the lateral decubitus position in the L4–L5 vertebral interspace. The first 12 ml of CSF

was collected on ice in a chilled plastic tube and gently mixed to avoid possible gradient effects. The CSF samples were centrifuged at 2000 *g* (4°C) for 10 min to eliminate cells and other insoluble material, aliquoted into 1 ml fractions and kept at −80°C until analysis. The samples were not pooled. The results presented here are from one individual.

2.3. Human urine samples

The human urine sample was collected and directly derivatized without any purification or pretreatment. The urine was obtained from a 29-year-old male.

2.4. Derivatization procedure

The amino acids present in the samples were derivatized by mixing sample solution with a 0.50 *M* boric acid buffer solution adjusted to pH 8.2 to a final buffer concentration of 0.20 *M*. For the cerebrospinal fluid sample this involved mixing 10 μl of cerebrospinal fluid with 20 μl of deionized water and 20 μl derivatization buffer in a 0.5-ml Eppendorf tube. To the buffered sample 30 μl of APOC solution (40 m*M* in acetonitrile) was added and the reaction was allowed to proceed for 15 min at room temperature. The derivatization reaction was terminated by extraction of excess reagent with 200 μl hexane. A brief centrifugation step (5 min at 14 000 *g*) was performed after the addition of hexane and mixing the aqueous and organic phases to remove proteinaceous material or other insoluble material. The aqueous phase was then transferred to a new Eppendorf tube.

The derivatization procedure used for the cerebrospinal fluid was also used for the urine sample. In the urine sample an internal standard, tranexamic acid, was also included. Tranexamic acid was added to the sample prior to the derivatisation by exchanging the 20-μl of deionized water for 10 μl of tranexamic acid solution (10 μ*M* in deionized water) and 10 μl of deionized water. The tranexamic acid can be used for the cerebrospinal fluid samples in analogous manner with the urine sample. The focus of this study was, however, the enantiomeric composition of the sample and the quantitative aspects, including the use of the internal standard is left for further evaluation in future work.

All derivatized samples were diluted 10-fold with deionized water prior to injection to ensure a lower conductivity in the sample plug as compared to the separation electrolyte.

2.5. Apparatus

The capillary electrophoresis (CE) equipment used was a system built in-house consisting of a high-voltage power supply (Brandenburg, Thornton Heath, UK) and an untreated fused-silica capillary with an internal diameter of 25 μm and an external diameter of 150 μm (Polymicro Technologies, Phoenix, AZ, USA).

The length of the capillary used for the analysis of the urine and CSF-samples was 92 cm, with the polyimide coating removed 84 cm from the injection end to enable LIF detection. The separation voltage applied was 30 kV giving a current of 9 μA. A shorter capillary from a different batch was used for the standard separation shown in Fig. 1. This capillary was 82 cm long with the detection point 74 cm from the injection end. This resulted in a slightly decreased resolution of the different amino acids and a current of 13 μA when 30 kV was applied.

Injections were performed hydrodynamically by placing the capillary in the sample liquid and raising the injection end 11 cm. Injection volumes are presented in the figure legends. All electrolytes used were filtered through syringe filters having a poresize of 0.2 μm. The capillary was rinsed with at least three capillary volumes 0.1 *M* sodium hydroxide solution and at least five capillary volumes of deionized water when starting up the instrumentation for a new series of experiments. The capillary was then conditioned with at least five capillary volumes of separation electrolyte. Between runs the capillary was rinsed with approximately three capillary volumes of separation electrolyte.

The laser used for LIF detection was a full frame Innova argon 304 model (Coherent, Palo Alto, CA, USA), in an optical arrangement similar to that described by Yeung et al. [44]. The 351.0- and 351.5-nm emission lines from the laser were used for detecting APOC-derivatized amino acids. An UG11 shortpass filter was employed to filter away light

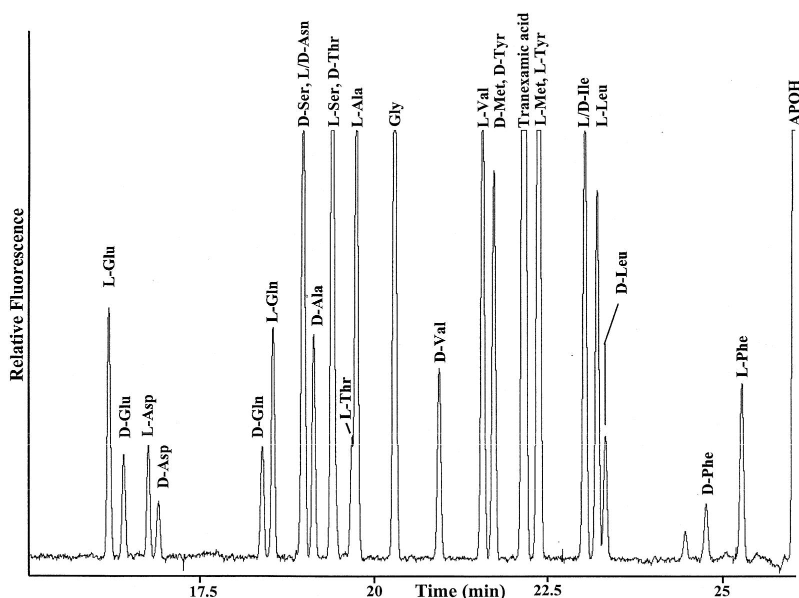

Fig. 1. MEKC-LIF separation of (+)-APOC-derivatized D- and L-amino acid standards. The injection volume is 1.4 nl and the injected amounts of all L-amino acids except glutamic acid, aspartic acid, and phenylalanine correspond to 0.14 pmol. The injected amounts of D-amino acids are 0.07 pmol. Glutamic acid, aspartic acid, and phenylalanine are present at half the concentration of the other amino acids. The length of the capillary used was 82 cm, with the polyimide coating removed 74 cm from the injection end to enable LIF detection. The separation voltage applied was 30 kV giving a current of 13 μA.

from other light sources in the room. Emitted light was collected at a 90° angle and focused onto a R212-UH photomultiplier tube (Hamamatsu, Hamamatsu City, Japan) using a microscope objective. Scattered light was filtered out using a KV389 longpass filter and Raman emission from the buffer was filtered using a 20-nm wide bandpass filter centered around 412 nm. All filters used were from Schott (Mainz, Germany). The high-voltage electrode was placed in a Plexiglas box at the injection end of the capillary. All instrumentation was arranged on a spring mounted optical bench.

3. Results and discussion

3.1. Derivatization and sample considerations

Primary and secondary amino compounds form stable carbamate reaction products with chloroformate reagents such as 9-fluoronyl-methyl chloroformate (FMOC), 1-(9-fluoronyl)ethyl chloroformate (FLEC), 9-anthryl-ethyl chloroformate (AEOC), or APOC. The reaction is base catalyzed and reaches completion in less than 20 s for most amino acids. The reaction with the acidic amino acids aspartic acid and glutamic acid is slower and requires a reaction time of at least 2 min.

A prerequisite for enantiomeric analysis by the formation of diastereoisomers is that the enantiomeric purity of the reagent is known or that the reagent is optically pure [45–47]. The optical purity of the APOC reagent has previously been determined and can thus be corrected for [43]. The elution order of a specific D/L-amino acid pair will be interchanged depending on which enantiomer of the reagent that is used for the derivatization of the amino acids [7]. This is due to the fact that, for example, the diastereoisomer (+)-APO-L-serine is an enantiomer to (−)-APO-D-serine.

In the early developmental stages of the analysis a series of spikes in the chromatogram prevented the determination of early eluting amino acids. The spikes were presumably caused by aggregated proteins or particulate material with a diameter less than 2 μm since filtering the samples through a 2-μm

filter did not affect them. The spikes disappeared when the sample was subjected to ultrafiltration or passed through a solid-phase extraction cartridge (Varian bondelute C_{18}). A centrifugation step was included in the derivatization procedure to precipitate proteins remaining in the cerebrospinal fluid.

3.2. Optimization of the separation buffer

In our work we have used a mixed micellar buffer consisting of two surfactants: sodium dodecyl sulfate (SDS) and sodium deoxycholate (SDC). Khaledi et al. [48] have used linear solvation energy relationships to describe the influence of mixed micellar systems on retention and selectivity in MEKC. Wiedmer et al. [49] have presented a statistical approach to the optimization of a mixed micellar system consisting of SDS and the bile salt surfactant sodium cholate

In the early phases of this work the separation electrolyte used consisted of a 20 m*M* sodium tetraborate buffer solution at pH 9.8 with an SDS content of 17.5 m*M*. However, when tranexamic acid was introduced as an internal standard, it coeluted with two of the amino acids: (+)-APO-L-methionine and (+)-APO-L-tyrosine or (−)-APO-D-methionine and (−)-APO-D-tyrosine.

In order to change the selectivity of the separation electrolyte the addition of an organic modifier or a mixed micellar solution was suggested. A statistical approach (two-level factorial design) [50] was used to evaluate which parameters affected the selectivity in the appropriate k' region without diminishing the separation of analytes with other k' values. The parameters evaluated were pH, addition of urea to the separation electrolyte, and the addition of SDC as a second micelle-forming agent. The addition of SDC was determined to be the most significant factor for the resolution of the specific compounds of interest. The resolution of early eluting analytes increased slightly with the addition of small amounts of SDC, whereas addition of urea decreased the resolution of these, less hydrophobic, analytes.

The incorporation of SDC into micelles consisting of SDS changes the selectivity of the micelles by reducing the interactions between strong hydrogen bond acceptor analytes and the micelles [48]. Mixed micellar systems often have relatively large separation windows, which facilitates the separation of a wide range of analytes [19]. The structural difference between APOC-derivatized tranexamic acid, lacking the α-amino group common in the amino acids in this study, may explain the change in selectivity observed.

A second factorial design with the concentration of SDS and concentration of SDC as variable parameters was evaluated and the following separation system chosen for the actual experiments; 20 m*M* borax buffer at pH 9.8 with 20 m*M* SDS and 7.5 m*M* SDC as micelle-forming surfactants. The relative standard deviation for the retention time of the (+)-APO-glycine/GABA peak was 0.5% calculated as an average over 3 days consecutive analysis.

The influence of deoxycholate on the retention times of the different enantiomers of the amino acids is something that needs to be considered. By changing the optical form of the reagent, a reversal of elution order for analyte enantiomers is achieved. This gives valuable information concerning the identity of peaks in the chromatogram [2,5,7,11]. The (+)- and (−)-derivate of both chiral and achiral substances may, however, have different retention times in the mixed micellar system as deoxycholate is a chiral surfactant that has been used for chiral separations [19]. The addition of deoxycholate in this separation system was found to be sufficient to change the selectivity for the different amino acids but not substantial enough to achieve direct chiral separation of enantiomers. The retention times of (+)-APO-L-amino acids correlated well with the retention times of (−)-APO-D-amino acids. The same was true for (−)-APO-L-amino acids and (+)-APO-D-amino acids.

3.3. The determination of D-amino acid in biological fluids

A chromatogram of a (+)-APOC-derivatized standard mixture of 14 D- and L-amino acids and the proposed internal standard tranexamic acid is presented in Fig. 1. Aspartic acid, glutamic acid, glutamine, alanine, valine, leucine and phenylalanine were well separated with the separation buffer used. A few amino acids coeluted with other amino acids; D-serine coeluted with D- and L-asparagine, L-serine coeluted with D-threonine, and methionine coeluted

with tyrosine. Asparagine was not chirally separated under the conditions used. The different enantiomeric forms of isoleucine have not been assessed due to scarcity of pure standards containing only one enantiomer. Arginine, cysteine and tryptophan eluted close to or together with 1-(9-anthryl)-2-propanol (APOH), a hydrolysis product from unreacted reagent, and were not included in the study.

Chromatograms of (+)-APOC-derivatized human cerebrospinal fluid are shown in Fig. 2. Two injections are presented: a 1.2-nl injection of sample diluted 100-fold after the derivatization and a 5-nl injection of sample diluted 10-fold after derivatization. The amino acids identified in the chromatogram correspond to compounds that coeluted with synthetic standards when these were added to the sample. Possible evidence for the presence of D-aspartic acid, D-glutamine and D-alanine is shown. To verify that the compounds identified in the chromatogram in fact are the compounds of interest the sample was derivatized with the (−)-enantiomer of APOC. The assessment of peak identity by the reversal of elution order of the amino acid enantiomers is shown in Fig. 3.

Fig. 4 shows a separation of urine derivatized with (−)-APOC. The upper chromatogram has been obtained for a sample diluted 100-fold after derivatization. The lower chromatogram shows the same sample with added synthetic standards of (−)-APOC-derivatized amino acids. In Fig. 5 an enlarged

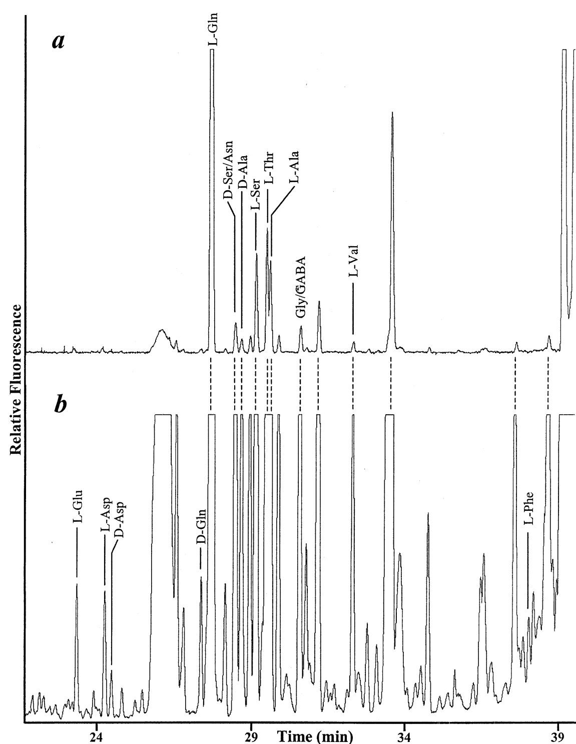

Fig. 2. MEKC-LIF separation of (+)-APOC-derivatized cerebrospinal fluid: (a) 1.2-nl injection of sample diluted 100-fold after the derivatization; and (b) 5-nl injection of sample diluted 10-fold after derivatization. The length of the capillary used was 92 cm, with the polyimide coating removed 84 cm from the injection end to enable LIF detection. The separation voltage applied was 30 kV giving a current of 9 μA.

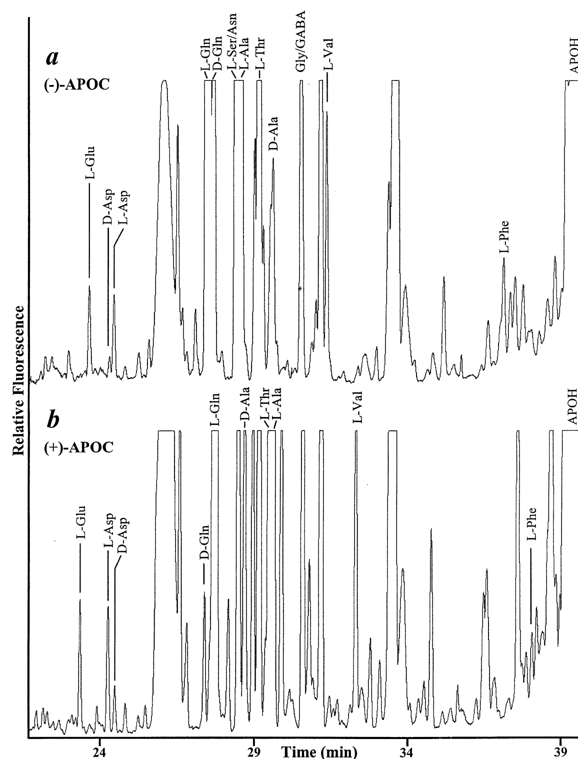

Fig. 3. MEKC-LIF separation of cerebrospinal fluid derivatized with (−)-APOC and (+)-APOC showing reversal of elution order of the derivatized amino acids depending on the choice of reagent enantiomer: (a) 5-nl injection of cerebrospinal fluid derivatized with (−)-APOC; and (b) 5-nl injection of cerebrospinal fluid derivatized with (+)-APOC. The length of the capillary used was 92 cm, with the polyimide coating removed 84 cm from the injection end to enable LIF detection. The separation voltage applied was 30 kV giving a current of 9 μA.

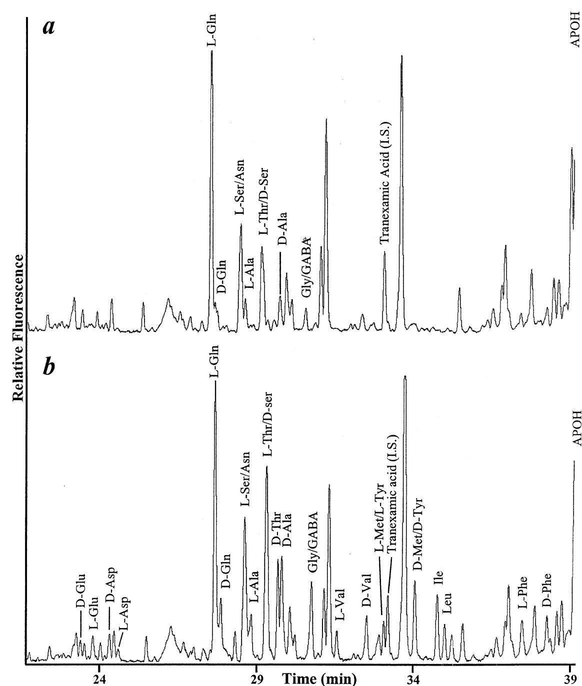

Fig. 4. MEKC-LIF separation of urine derivatized with (−)-APOC. (a) Urine sample diluted 100-fold after derivatization, 1.2-nl injection. (b) (−)-APOC-derivatized urine sample with added synthetic (−)-APOC-derivatized amino acid standards. The length of the capillary used was 92 cm, with the polyimide coating removed 84 cm from the injection end to enable LIF detection. The separation voltage applied was 30 kV giving a current of 9 μA.

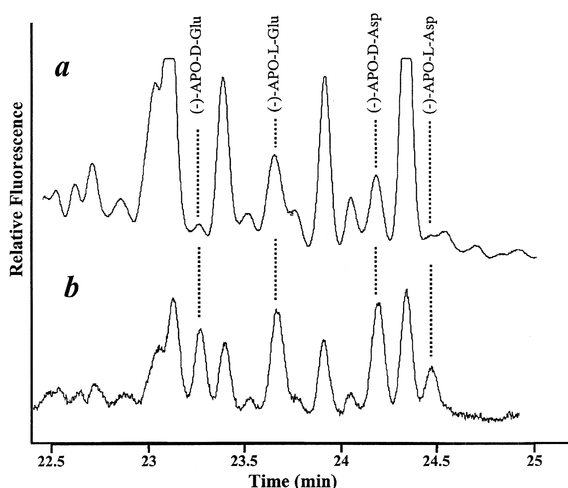

Fig. 5. MEKC-LIF separation of urine derivatized with (−)-APOC. (a) A 3.1-nl injection of sample diluted 10-fold after derivatization; and (b) a 1.2-nl injection of a 100-fold diluted sample spiked with (−)-APOC spiked with (−)-APOC-derivatized standards. The length of the capillary used was 92 cm, with the polyimide coating removed 84 cm from the injection end to enable LIF detection. The separation voltage applied was 30 kV giving a current of 9 μA.

portion of a 3.1-nl injection of a (−)-APOC-derivatized urine sample is shown along with a 100-fold diluted sample spiked with (−)-APOC-derivatized standards. The presence of D-glutamic acid and L-glutamic acid can be seen in the upper chromatogram. The lower chromatogram in Fig. 5 shows the same sample diluted 100-fold and spiked with (−)-APOC-derivatized amino acid standards. These substances, containing two acidic groups, are preconcentrated by sample stacking from the sample plug to a greater extent than the other amino acids in the chromatogram.

Also present in the sample is the added internal standard tranexamic acid. Tranexamic acid is a water-soluble substance clinically used as an antifibrinolytic agent. Given that the clinical history of a subject or patient is known it is unlikely that this substance is present in humans or other test subjects. Tranexamic acid is achiral yielding only one peak in the chromatogram.

The relative abundance of D-amino acids, in percent of total amino acid present, found in cerebrospinal fluid and urine samples are presented in Table 1. All values presented have been corrected for the optical impurity in the reagent. Evidence for the presence of D-alanine is found in both cerebrospinal fluid and urine. Elevated levels of D-alanine has earlier been reported in urine [7,16] but not in cerebrospinal fluid. D-Glutamine has not been reported earlier in cerebrospinal fluid. The difference between the relative amount determined by derivatization with (−)-APOC as compared to that found when derivatizing with (+)-APOC could imply that (−)-APO-D-glutamine (and possibly (+)-APO-L-glutamine) coelutes with some unknown substance. This lessens the ability to assess the peak identity of (+)-APO-D-glutamine. D-Aspartic acid has earlier been reported to be present in cerebrospinal fluid by Fisher and co-workers [8,9] (0.6% in healthy control cerebrospinal fluid and 1.68% in cerebrospinal fluid

Table 1
Relative abundance (%) of D-amino acid as compared with the total amino acid present[a]

	Cerebrospinal fluid		Urine	
	(+)-APOC	(−)-APOC	(+)-APOC	(−)-APOC
Alanine	13	6	56	59
Glutamine[b]	0.1	1	nd	nd
Aspartic acid	25	18	nd	nd
Glutamic acid	nd	nd	12	6

[a] Calculation of percent D-amino acids performed as percent D-amino acid$=100\times n_{D\text{-}AA}/(n_{D\text{-}AA}+n_{L\text{-}AA})$.
[b] Discrepancy in the values presented for glutamine are probably due to coelution of (−)-APO-glutamine with some unknown substance.

from subjects suffering from Alzheimer's disease) but not at the high amounts found in the samples analyzed here. The presence of D-glutamic acid in urine has not been presented earlier. Brückner and Hausch [16] have found possible evidence for glutamine or glutamic acid (presented as glx, a combined value for both glutamine and glutamic acid, as determined by chiral phase gas chromatography). As seen in Fig. 4, the D- and L-glutamic acid elute very close to other, unidentified amino compounds. This makes integration of peak area difficult which can account for the large difference in the values obtained when using (+)- and (−)-APOC. The same reasoning can apply to alanine in cerebrospinal fluid.

The limit of detection ($S/N=3$) for D-glutamic acid in the urine analysis was determined to be 20 amol. The injection volume was 3.1 nl resulting in a concentration of 7 n*M*. The injected volume represents 0.7% of the total volume of the capillary. Larger injections can be made but will result in a significantly increased baseline signal due to the multitude of compounds present in the sample. A less complex sample would facilitate further optimization of the detection limits.

4. Conclusions

The presented data clearly show the high separation potential and the excellent detection capabilities associated with the combination of MEKC using mixed micellar systems and LIF-detection. The capacity of the MEKC-LIF system as an analytical tool in the separation and detection of D- and L-amino acids has been demonstrated. The ability to study the chiral amino acid ratio in complex biological fluids, exemplified with human cerebrospinal fluid and urine, indicate the possibility to use the method for clinical screening purposes. We are for the first time able to present data suggesting the presence of D-alanine in human cerebrospinal fluid and D-glutamic acid in human urine. These findings together with the other D-amino acids presented may well reflect disturbances in metabolic pathways that could be part of mechanisms resulting in disorders. Further studies have to be performed on clinically well characterized subgroups of patient in order to elucidate this.

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