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Separation of D/L-carnitine enantiomers by capillary electrophoresis

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Abstract

Carnitine is an essential component of the tissue of animals, higher plants and many microorganisms. Whereas the L-carnitine enantiomer plays an important role during the metabolism of long chained fatty acids, D-carnitine has a considerably toxic influence on biochemical processes. The analytical separation of D/L-carnitine is possible only after derivatization with UV- or fluorescence active substances, e.g. FMOC (9-fluorenylmethyl chloroformate) and (+)/(−)-FLEC ((+)-1-(9-fluorenylethyl)chloroformate). The capillary electrophoretic separation of enantiomeric FMOC derivatives was performed using acidic buffers and different cyclodextrins as chiral selectors. Baseline separation as well as detection limits in the micromolar range could be achieved using UV detection. The migration order of both enantiomeric derivatives was reversed by the application of substituted cyclodextrins (CDs), thus making the analysis of low amounts of D- or L-carnitine in high concentrations of the other enantiomeric form possible. Different kinds of coated capillaries are compared with respect to separation time and resolution. Examples for the analysis of pharmaceuticals with high L- and low D-carnitine contents and of serum samples spiked with D/L-carnitine are shown.

Keywords: Enantiomer separation; Pharmaceutical analysis; Buffer composition; Capillary columns; Carnitine

1. Introduction

L-Carnitine is an essential component in various tissues of animals, higher plants and many microorganisms. It was found to play an important role in the metabolism of long chain fatty acids [1]. Also, some short-chain esters of L-carnitine possess interesting pharmacological properties [2]. In contrast, D-carnitine has been found to have a considerable toxic influence on biochemical processes [3] due to inhibition effects on the carnitine acetyltransferase, leading to a depletion of the body's L-carnitine stock. Therefore, in pharmaceutical formulations applied to overcome L-carnitine deficiencies, the use of the pure L-enantiomer of carnitine and its esters is recommended.

Today the industrial production of L-carnitine is performed by fractional crystallization or stereoselective synthesis, often coupled to microbiological processes. Purity testing for residual content of D-carnitine and byproducts of the synthesis is necessary.

Previously, this problem was solved by using NMR [4] or specific enzymatic reactions [5,6]. At present, chromatographic methods using chiral [7] and achiral [8,9] columns after derivatization of the D/L-carnitine and its esters with pure enantiomeric or racemic reagents are applied. The first separation of diastereomeric carnitine esters after derivatization with (+)-FLEC ((+)-1-(9-fluorenylethyl)chloroformate) using capillary electrophoresis was published recently [8].

In a paper [9] published last year the expensive purified enantiomeric derivatization reagent (+)- or (−)-FLEC was replaced by racemic FMOC (9-fluor-

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enylmethyl chloroformate). The separation of the enantiomeric FMOC–carnitine esters by capillary electrophoresis using γ-cyclodextrin as a chiral selector was demonstrated. Due to the high individual absorbance of buffer components, the detectable amounts of D/L-carnitine have been reported to be in the millimolar range.

We used various capillaries, buffers and chiral buffer additives for the electrophoretic separation to establish a practical method for the determination of derivatized enantiomeric carnitines with detection levels in the micromolar range. It was found that the determination of carnitine at lower micromolar levels was possible using coated capillaries with reduced, removed or reversed osmotic flow. The migration order of the D- and L-carnitine esters could be reversed by selecting the appropriate modified cyclcodextrin (CD) as buffer additive.

In this paper we describe a capillary electrophoretic method with pre-column derivatization leading to enantiomeric derivatives for the simultaneous determination of D- and L-enantiomers of carnitine. The optimization of the separation conditions as well as the validation of the method and its application to pharmaceutical products are described. The first results for the direct determination of carnitine enantiomers in plasma samples are discussed.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical grade. Only triple distilled water was added to standard solutions and buffers. Racemic D/L-carnitine, the enantiomers D-carnitine and L-carnitine, heptakis(2,6-di-O-methyl)-β-cyclodextrin and heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin were purchased from Sigma (Deisenhofen, Germany).

Acetone (dried), glacial acetic acid, phosphoric acid (85%), NaOH, HCl, $NaHCO_3$, Na_2CO_3, NaH_2PO_4 and Na_2HPO_4 were obtained from Merck (Darmstadt, Germany), FMOC, α- and β-cyclodextrin were from Fluka (Buchs, Switzerland). γ-Cyclodextrin was supplied by Wacker-Chemie (Munich, Germany) and hydroxypropyl-β-cyclodextrin was supplied by Beckman Instruments (Fullerton, CA, USA). 2-Amino-4,6-dimethylpyrimidine was from Fluka (Buchs, Switzerland) and was used as the internal standard. The drug Biocarn (1 g L-carnitine/3.3 ml) was from Medice (Iserlohn, Germany).

Derivatization of D/L-carnitine enantiomers in aqueous solution was performed as described earlier [9] (Fig. 1). Using the same buffers and reagent solutions, plasma samples were prepared as follows: A 250-μl volume of plasma standard (Precinorm S, Boehringer Mannheim, Germany) was mixed with 100 μl of carbonate buffer. After the addition of 200 μl of FMOC solution, the sample was derivatized for 1 h at 45°C. To complete the reaction, 200 μl of acetic acid buffer and 250 μl of triple distilled water were added to a final volume of 1 ml. The solution was then filtered and injected. No further sample pretreatment was performed.

2.2. Apparatus

For capillary electrophoresis measurements P/ACE Systems 2100 and 5510 from Beckman Instruments (Palo Alto, USA) equipped with a UV-detection system either with fixed wavelengths or a diode array and controlled by System Gold software were used. The following separation parameters were used in all analyses; pressure injection; detection at 214 nm; applied voltage, 17 or 25 kV; cathode at the detection end of the capillary. Polyimide-clad coated fused-silica capillary material was purchased from Beckman Instruments (Fullerton, CA, USA) and Supelco (Bellefonte, PA, USA). An eCAP neutral capillary (Beckman) of 30 cm×50 μm I.D. to the detector as well as a CElect Hl50 (Supelco)-coated capillary of 60 cm×50 μm I.D. were used as separation capillaries.

3. Results and discussion

3.1. Determination of migration behavior and pK_a of D/L-carnitine–FMOC esters

To determine the optimum pH range for enantioseparation, phosphate buffers with pH values between 2.60 and 5.30 have been applied. A racemic mixture of D- and L-carnitine–FMOC esters was

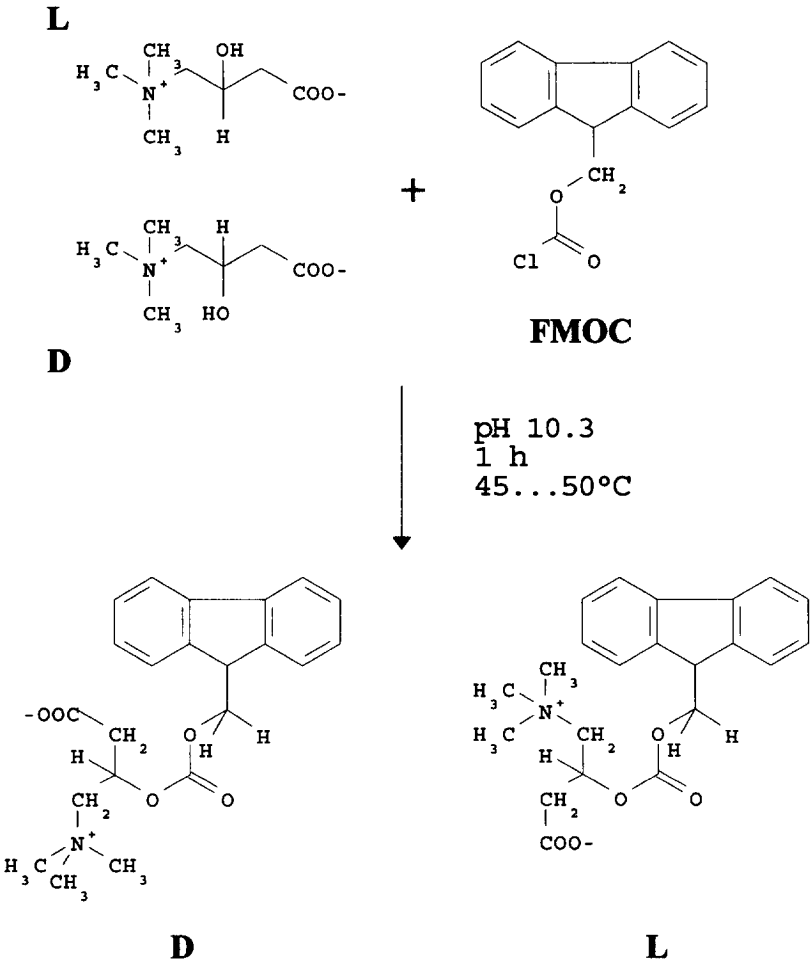

Fig. 1. Derivatization procedure.

separated in an achiral environment using both totally and partly coated capillaries. To achieve nearly the same enantioresolution in further experiments, the partly coated capillary used was twice the length (67 cm) of the totally coated (37 cm) capillary.

The dependence of the migration behavior on the pH of the buffer is shown in Fig. 2. The underivatized carnitine possesses a pK_a of 3.80. Because the derivatization with FMOC takes place at the hydroxyl group and the structural changes caused do not influence the acid–base properties of the carnitine derivative to a great extent, the pK_a of the FMOC derivatives of D- and L-carnitine should be similar to the pK_a of the underivatized species. Therefore, at pH 2.60 the carboxyl group is uncharged and a fast migration of the fully positively charged (+1) species to the cathode takes place. With increasing pH of the buffer dissociation of the carboxyl group takes place, thus reducing the net charge of the molecule and increasing the migration time. Using the neutral capillary, the separation at different pH values should provide the pK_a of the carnitine derivatives at the turning point of the curve, where half of the molecules are dissociated and the net charge should be +0.5. From the diagram in Fig. 2, a pK_a of 3.85 was calculated.

The application of only partly coated capillaries

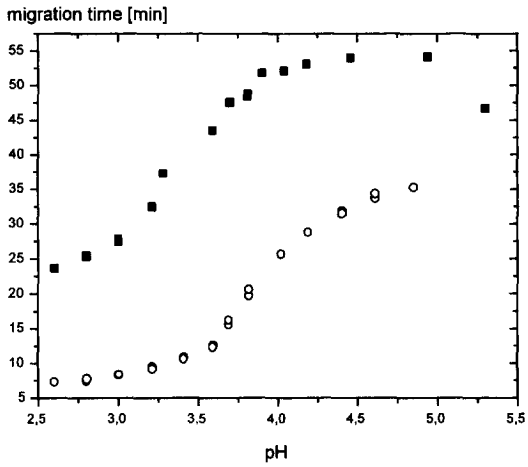

Fig. 2. Migration behaviour of D/L-carnitine–FMOC esters in 20 m*M* phosphate buffers as a function of the buffer pH. ○, eCAP neutral capillary, 30/37 cm×50 μm I.D., U=17.5 kV; ■, CElect H150, 60/67 cm×50 μm I.D., U=25 kV.

leads to slightly different results. When calculating the pK_a of FMOC derivatives from this diagram, increased errors for the determination must be taken into account. Even below pH 4, interaction of the analytes with the capillary wall could take place. A reduction of the migration velocity, which was not caused by a reduced charge of the molecule due to further dissociation, and an increased separation time will occur. As a result, the curve for pH dependence of migration time will rise more rapidly and a lower pK_a value should be obtained. The determination of a pK_a of 3.55 from the migration of D/L-carnitine–FMOC esters using the partly coated capillary confirms the discussed supposition. From the observed migration behavior, it could be concluded that the maximum influence of pH on enantioseparation should be expected for pH values between 3.5 and 4.5. Small changes of buffer pH will cause distinct modification of charge and mobility of the analytes. Therefore, optimization of chiral separation should be performed in buffers with pHs of between 3.5 and 4.5.

3.2. Optimization of chiral resolution and change of migration order

Native and derivatized CDs have been tested for the separation of carnitine enantiomers. No chiral resolution could be obtained using α-, β- and 2,3,6-trimethyl-β-CDs, unlike with γ-, 2,6-dimethyl-β- and hydroxypropyl-β-CDs, where different resolutions could be achieved. As described earlier [9], the separation of FMOC derivatives was possible in fused-silica capillaries with phosphate buffers containing γ-CD and high quantities of cationic surface modifiers. Consequently, well separated peaks for D- and L-carnitine have been achieved on neutral and partly coated capillaries with γ-CD-containing buffers but with no cationic surfactant. Due to the disadvantage of the relatively high concentrations of γ-CD (≥20 m*M*) necessary to achieve a separation with baseline resolution, derivatized CDs have also been examined. The concentration of hydroxypropyl-β-CD has to be above 10 m*M* to attain baseline resolution. Only with 2,6-dimethyl-β-CD was excellent separation achieved using a concentration of CD that was below 10 m*M*. In Fig. 3a and b, the dependence of the enantiomeric resolution of γ- and 2,6-dimethyl-β-CD on the concentration of CD and on the applied capillary material is shown.

Using a neutral capillary (Fig. 3a) and γ-CD as the chiral selector, the optimum separation with regard to resolution and analysis time was observed in phosphate buffers with a pH of 3.40. At a higher pH, the separation time was dramatically increased but no significant improvement of the resolution could be observed. Baseline separation (R_s=1) was achieved with more than 13 m*M* γ-CD. The separation with 2,6-dimethyl-β-CD was optimal in buffers with a pH of 4.55. Although the analysis time was prolonged, baseline separation was possible with only 1 m*M* 2,6-dimethyl-β-CD in the buffer. Even with a 0.3 or 0.5 m*M* concentration of this CD, partial separation could be obtained, which illustrates the existence of big differences in the stability constants of both enantiomers with this type of CD. Maximum resolution (R_s=1.63) with 2,6-dimethyl-β-CD as the chiral selector was obtained at a concentration of 5 m*M* and for γ-CD (R_S=1.58) it was found to be 30 m*M*. At concentrations above the maximum resolution for both cyclodextrins, a loss in resolution was observed. This could be attributed to an oversupply of CD in the complex, forming an equilibrium with the enantiomers, which causes reduced differences in the interaction of both enantiomers with the chiral selector and therefore

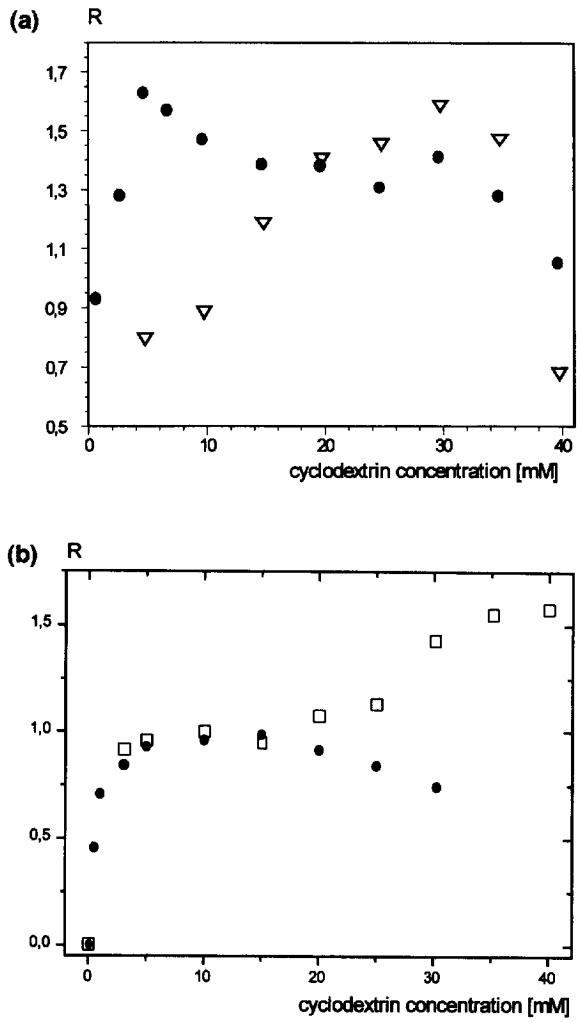

Fig. 3. Resolution of enantiomeric carnitine–FMOC esters as a function of the concentration of cyclodextrin and of the type of coated capillary. (a) eCAP neutral capillary, 30/37×50 μm I.D., U =17.5 kV; ▽, γ-cyclodextrin in 20 m*M* phosphate buffer, pH 3.40; ●, 2,6-dimethyl-β-cyclodextrin in 20 m*M* phosphate buffer, pH 4.55. (b) CElect 150, 60/67 cm×50 μm I.D., U =25 kV; □, γ-cyclodextrin in 20 m*M* phosphate buffer, pH 3.85; ●, 2,6-dimethyl-β-cyclodextrin in 20 m*M* phosphate buffer, pH 3.85.

reduced differences in migration velocities of the separated species.

For a partially coated capillary (Fig. 3b), a slightly lower γ-CD concentration of 10 m*M* was necessary to perform the separation with baseline resolution. However, the same resolution as obtained with the neutral capillary and γ-CD as the chiral selector was achieved only with pH≥3.85. At lower pHs, even at very high γ-CD concentrations, the resolution was $R_s \leq 1$. With 2,6-dimethyl-β-CD as the chiral selector in a buffer at pH 3.85, no resolution better than $R_s = 0.98$ was achieved. At increasing buffer pH, resolution increases slightly to up to 1.1. At pH>4.5 the increasing electroosmotic flow is responsible for a deterioration of the resolution and shorter migration times of the enantiomers. Neither the high resolution nor the baseline separation at very low concentrations of 2,6-dimethyl-β-CD, as obtained for the neutral capillary, have been achieved with the partially coated capillary.

In practice, the opportunity to change the migration order of both enantiomers plays an important role. To control a biochemical conversion of D- to L-carnitine [10], low amounts of L-carnitine should be detected in front of the main D-carnitine component. By contrast, in pharmaceutical products low D-carnitine contents have to be detected in the presence of high concentrations of L-carnitine. In Fig. 4 the reversal of the migration order of D- and L-carnitine–FMOC esters is shown. With γ-CD as the chiral selector, the migration of L-carnitine in front of D-carnitine was observed. If 2,6-dimethyl-β-CD was dissolved in the buffer, the migration order was reversed due to modified interactions of both enantiomers with the CD and at the same time

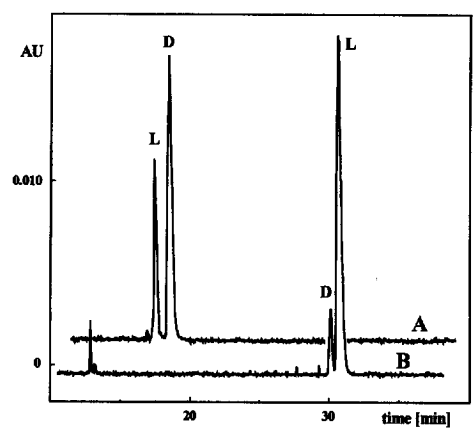

Fig. 4. Reversal of migration order of D- and L-carnitine–FMOC esters. eCAP neutral capillary, 30/37 cm×50 μm I.D., U =17.5 kV; A, 20 m*M* phosphate buffer, pH 3.40, 20 m*M* γ-cyclodextrin; L–D, 1:2; B, 20 m*M* phosphate buffer, pH 3.85, 20 m*M* 2,6-dimethyl-β-cyclodextrin, L–D, 10:1.

modified stability constants for the complexation equilibrium between the carnitine enantiomers and the CD.

3.3. Quantification and determination of the limit of detection (LOD)

Calibration was performed using both capillary materials and aqueous standards. Correlation coefficients of k_D=0.9991 (y=−0.0047+0.18754x) for D-carnitine and k_L=0.9998 (y=−0.001+0.160x) for L-carnitine have been achieved. Using a neutral capillary, the obtained values (k_D=0.9983, k_L=0.9962) were slightly worse. For both capillaries the determined limits of detection for L- and D-carnitine were 10 μM and 5 fmol, respectively. Only normalized peak areas have been used for quantification. For this purpose, peak areas have been divided by the migration time of the peak. The higher slope for the calibration of the D-enantiomer was observed independently of the capillary used, the time and the conditions of storage of the standard solutions and even of the applied derivatization procedure and analytical separation technique. For derivatization with (+)-FLEC or separation with HPLC, the same behavior was observed. Although a biochemical degradation of only the L-enantiomeric form could not be excluded, an incorrect declaration of the percentage by the manufacturer is likely. Different optical properties of the enantiomeric FMOC derivatives should not occur, but varying yields of derivatization reaction are within the bounds of possibility.

In Fig. 5 the separation of different ratios of D- and L-carnitine is shown. In Table 1, uncorrected and normalized peak areas are compared for these separations. The application of normalized peak areas reduces the error due to the different migration velocities of the separated enantiomers and the different dwelling times inside the detector [11]. Even near the detection limit can results be obtained with good accuracy (≤0.5%), as demonstrated for the 1:100 mixture of D- and L-carnitine.

3.4. Separation of D/L-carnitine in pharmaceuticals and plasma samples

To separate small amounts of D-carnitine from the main component, L-carnitine, in pharmaceuticals,

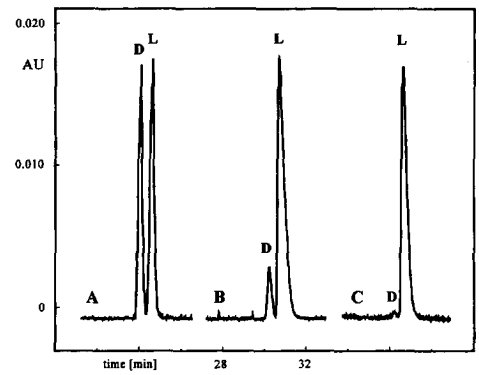

Fig. 5. Separation of D- and L-carnitine–FMOC esters at different ratios. A, L–D, 1:1; B, L–D, 1:10; C, L–D, 1:100. eCAP neutral capillary, 30/37 cm×50 μm I.D.; U=17.5 kV; 3 s pressure injection; buffer, 20 mM phosphate buffer, pH 3.85, 20 mM 2,6-dimethyl-β-cyclodextrin, L-carnitine concentration, 2 mM.

Biocarn was selected. Using (+)-FLEC for the derivatization of the carnitine enantiomers and the formation of diastereomeric products a D-carnitine content of ≤0.46% in the pharmaceutical formulation was determined by HPLC [9] under achiral separation conditions. The electrophoretic separation using 2,6-dimethyl-β-CD as the chiral selector and a coated neutral capillary is shown in Fig. 6. The buffer pH was adjusted to 4.55 and the CD concentration was set to 20 mM to guarantee a good resolution of both enantiomers. Problems arose from the selection of proper dilution conditions of the sample prior to the injection. Samples that were diluted to a lesser extent (1:10, 1:20) could not be baseline resolved, due to the extreme overloading of the separation unit. Smaller peaks and better resolution resulted for more dilute samples (1:100, 1:200), but quantification of the D-carnitine content

Table 1
Comparison of uncorrected (A_D:A_L) and normalized ($A_D t_D$:A_L/t_L) peak areas

D–L	A_D:A_L uncorrected area	A_D/t_D:A_L/t_L normalized area
1:1	1.013:1	0.998:1
	1.3%	0.2%
1:10	0.980:10	0.998:10
	2.0%	0.2%
1:100	0.981:100	0.995:100
	1.9%	0.5%

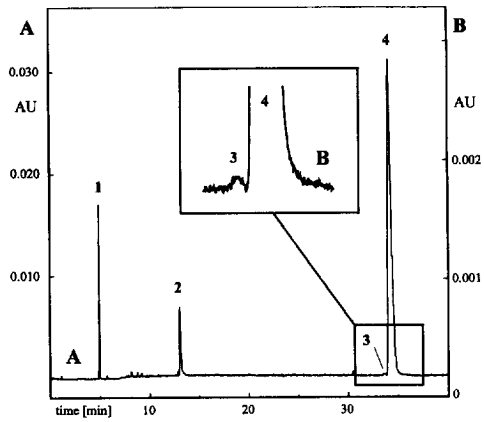

Fig. 6. Separation of D/L-carnitine in pharmaceuticals. eCAP neutral capillary, 30/37 cm×50 μm I.D.; U =17.5 kV; 2 s pressure injection. The Biocarn sample was diluted 1:50 (v/v) with water prior to derivatization. Buffer, 20 m*M* phosphate buffer, pH 4.55, 20 m*M* 2,6-dimethyl-β-cyclodextrin.

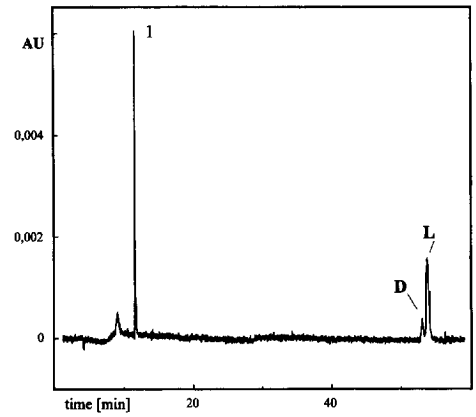

Fig. 7. Separation of D/L-carnitine–FMOC esters in spiked plasma. CElect H-150 (Supelco), 60/67 cm×50 μm I.D.; buffer, 20 m*M* phosphate buffer, pH 4.35, 20 m*M* 2,6-dimethyl-β-cyclodextrin; (1) internal standard. Sample preparation: 250 μl of standard serum Precinorm S was spiked with D/L-carnitine prior to derivatization. The carnitine concentration in the spiked serum was 0.1 m*M* D/0.5 m*M* L.

was impossible. By using normalized peak areas and a sample dilution of 1:50 prior to the derivatization, a D-carnitine content of 0.42% was determined, which shows good correlation with the value obtained from the HPLC determination.

To control carnitine deficiencies or therapeutic carnitine dosages, the determination of both enantiomers in biological liquids plays an important role. Therefore, the determination of carnitine in spiked plasma standards was tested using separation conditions optimized for aqueous samples. A very simple sample preparation was applied. The original plasma spiked with D- and L-carnitine was derivatized under the described conditions. After a simple filtration using filter paper, the sample was injected into the capillary. The removal of all interfering proteins and peptides takes place during the derivatization procedure, therefore no interfering components have been observed during the separation (Fig. 7). Under the described conditions, the calibration for D- and L-carnitine in plasma was successful. Correlation coefficients of $k_{L/D}$=0.998 for both enantiomers and a detection limit of 20 μ*M* (10 fmol of each enantiomer) have been achieved. Viscosity, the type of serum and the sample temperature at injection time have been identified as being the most important parameters that influence the sensitivity of detection and the reproducibility of results.

4. Conclusions

D/L-Carnitine could be well separated under chiral conditions using capillary electrophoresis. Unfortunately, the limit of detection of 10 μ*M* is still too high to perform the analysis of both enantiomers in any sample matrix at real concentrations. In the near future efforts will be made to apply fluorescence detection to the separation. This will allow us to reduce the LOD by one or two orders of magnitude, thus making the analysis of real samples possible, without preconcentration.

The robustness of the capillary electrophoresis separation unit enables the analyst in many cases to reduce the sample preparation of biological matrices, e.g. plasma and urine, to a minimum. As demonstrated for plasma samples, capillary electrophoresis can be used for the direct analysis of carnitine after derivatization. Lower detection limits and shorter analysis times will make this analytical procedure attractive for routine analysis in clinical laboratories.

Acknowledgments

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