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Short communication

Separation of carnitine and acylcarnitines by capillary electrophoresis

S. Kiessig, C. Vogt*

University of Leipzig, Faculty of Chemistry and Mineralogy, Institute of Analytical Chemistry, Linnéstrasse 3, 04103 Leipzig, Germany

Abstract

Free carnitine and specific acylcarnitines (carnitinyl esters) play an important role in the metabolism of long-chain fatty acids and many metabolic disorders. The optical properties of the carnitine molecule and the esters make a derivatization necessary to introduce ultraviolet or fluorescent chromophores into the molecule. The separation of carnitine and different acylcarnitines as derivatives of 9-anthryldiazomethane was performed with capillary electrophoresis successfully. An electrophoretic buffer system with very high methanol content (88%), phosphoric acid and sodium dodecyl sulfate has been optimized with respect to the best resolution of the derivatives. The separation of carnitine and its acyl esters up to the palmitoylcarnitine was achieved in aqueous standards as well as in standard plasma samples. For carnitine and most of the acylcarnitines detection limits below physiological concentrations were determined using laser induced fluorescence.

Keywords: Carnitine; Acylcarnitines

1. Introduction

Free carnitine and specific acylcarnitines (carnitinyl esters) play an important role in the metabolism of long-chain fatty acids and many metabolic disorders. Various methods for the analysis of carnitine and its acyl esters have been developed, among them radioisotope exchange, high-performance liquid chromatography (HPLC) and fast atom bombardment mass spectrometry (FAB-MS) [1,2]. To introduce ultraviolet or fluorescent chromophores into the carnitine or acylcarnitine molecules derivatization is required. For this purpose derivatization of the carboxyl group was performed with different agents, like 4-bromophenacyl trifluoromethansulfonate [3–5], 2-(2,3-naphthyl(imino)ethyl) trifluoromethansulfonate [6], 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone [7] and 9-anthryldiazomethane (ADAM) [8–10].

In capillary electrophoresis (CE) [11], separation is achieved by significant differences in the electrophoretic mobilities of charged molecules. Due to the characteristics of the separation mechanism and the physical properties of the separation technique good resolution even for very similar compounds could be obtained. Thus, the technique provides perfect preconditions for the separation of the permanently charged derivatives of carnitine and carnitine esters [12–14]. On the basis of recently published results for the separation of carnitine, acetylcarnitine and palmitoylcarnitine as ADAM derivatives by CE [15] we established a method for the separation of a wide variety of acylcarnitines, including isomeric forms, in electrophoretic buffers with up to 88% of organic solvent. Due to the robustness of the separation method, no further sample preparation despite the

*Corresponding author.

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derivatization procedure was necessary. The detection limit of all compounds could be decreased to or below real sample concentrations of plasma or serum. Successful separation was also performed with carnitine and acylcarnitine standards, diluted by a standard plasma.

2. Experimental

2.1. Chemicals

Methanol, 2-propanol and acetone of HPLC-grade were from Roth (Karlsruhe, Germany). 9-Anthryldiazomethane (ADAM) was obtained from Serva (Heidelberg, Germany); a stock solution of 5 mg/ml in acetone was used for the derivatization. D/L-Carnitine (1) was purchased from Sigma (Deisenhofen, Germany) as inner salt, the acylcarnitines L-acetylcarnitine (2) and L-palmitoylcarnitine (11) were from Aldrich (Steinheim, Germany), all other acylcarnitines [propionylcarnitine (3), *n*-butyrylcarnitine (n-4), isobutyrylcarnitine (i-4), valerylcarnitine (5), hexylcarnitine (6), octylcarnitine (7), decylcarnitine (8), dodecylcarnitine (9) and tetradecylcarnitine (10)] were from Stichting Klinische Genetica Amsterdam, (Amsterdam, Netherlands). Mixed stock solutions of carnitine and acylcarnitines with concentrations from 0.3 to 6.0 *M* were prepared by dissolving in triply distilled water. Sodium dodecyl sulfate (SDS) was obtained from Riedel-de Haën (Seelze, Germany), H_3PO_4 (85%) and glacial acetic acid from Merck (Darmstadt, Germany). For the preparation of all stock solutions only triply distilled water was used. Dilution procedures were performed with triply distilled water or a plasma standard (Precinorm S, Boehringer Mannheim, Germany).

Derivatization was performed adapting [12] a previously described procedure of Yoshida et al. [8]. The final solution was directly injected into the electrophoretic separation system.

2.2. Capillary electrophoresis apparatus

Electrophoretic separations were performed on P/ACE 2050 and 5510 systems from Beckman (Palo Alto, CA, USA) monitored by an IBM personal computer using GOLD 8.1 and P/ACE 3.0 software (Beckman). For laser induced fluorescence (LIF) an He–Cd laser Omnichrom Series 74 from Omnichrom (Chino, USA) with an excitation wavelength of 320 nm and 26 mW laser power was used. Detection was performed at 405 nm.

Fused-silica capillary material (CS Chromatographie Service, Langerwehe, Germany) of 57 or 77 cm length (50 or 70 cm to the detection window)× 50 μm I.D. were used. Operation voltages were 20 and 28 kV, respectively. Injection was performed in the electrokinetic mode. Injection time was set between 10 and 120 s using a voltage of 10 kV.

3. Results and discussion

Carnitine and acylcarnitines derivatized with ADAM are compounds with permanent positive charges. This property represents an ideal starting point for electrophoretic separations. However, the poor solubility in aqueous or partly aqueous solutions make a separation in common electrophoretic buffers impossible. In this case organic buffers, introduced in 1994 for CE separations [16] are suited to achieve sufficient separation of charged analytes.

First results for electrophoretic separation of acylcarnitines [15] gave cause for optimism that the separation of most of the clinical important acylcarnitines will be possible. For this purpose the buffer composition was slightly changed. Separation of carnitine, acylcarnitine and palmitoylcarnitine was achieved in a buffer with 92% methanol, 4% of concentrated (85%) phosphoric acid and 4% of a 5% SDS solution. To extend the migration window between the derivatized carnitine and palmitoylcarnitine the content of the 5% SDS solution was increased from 4 to 8% vol in the buffer. The percentage of the buffering phosphoric acid was kept constant, therefore the content of methanol was decreased to 88%. The increase of the SDS content leads to reduced migration velocities of the analytes due to better compensation of the permanent positive charge of the derivatized acylcarnitines by ion pairing effects. Therefore, the resolution for sample components with similar migration velocities should be improved. For the derivatization of carnitine and the eleven acylcarnitines under investigation in this work a procedure described earlier [15] was used.

Standard mixtures containing carnitine and different acylcarnitines were prepared as stock solutions.

Prior to the derivatization procedure these mixtures were diluted either by water or by the standard plasma Precinorm. After the derivatization step only filtration of the sample is necessary before the sample is injected into the capillary. Due to the derivatization conditions the original sample concentration is decreased 50-fold by the derivatization agents added. For every separation the injection was performed electrokinetically. By this way only charged sample compounds are introduced into the capillary, because no osmotic flow is present under the separation conditions applied. Along with the positive selective effect of this injection mode discrimination of the different acylcarnitines have to be taken into account. Faster migrating short-chain acylcarnitines and the carnitine itself are injected to a greater extend and detection limits for these compounds should be lower than for the long-chain acylcarnitines.

Separation of a standard mixture of carnitine (1) and five acylcarnitines (2, 5, 7, 9 and 11) of different concentrations is demonstrated in Fig. 1. In Fig. 1A the separation of a standard diluted 10-fold with water is shown. All sample components are marked according to increasing number of carbon atoms in the acyl chain of the molecules (except for carnitine with no carbon atom in the side chain, but marked with number 1). Baseline resolution of all five acylcarnitines as well as the carnitine itself could be observed. The space between different acylcarnitines in the electropherogram should also make the separation of a more complex mixture possible. In comparison to previous work an improvement of the reproducibility of the migration times was observed for the buffer with higher SDS content. A slight increase in migration times for the first and second run on new capillaries is followed by stable runs with reproducibilities below 2% for corrected migration times, if each run is followed by a cleaning step with 1 *M* NaOH mixed with methanol (50:50). A deterioration of the reproducibility was observed using only 1 *M* NaOH or methanol or both in succession, but in separate vessels. Fig. 1B shows the separation of the same standard as in Fig. 1A, but diluted with standard plasma prior to derivatization. A reproducible shift in migration times of all components was observed, which was probably caused by differences in the sample viscosity. The resolution for palmitoylcarnitine (11), comigrating with one of the plasma peaks, could be improved by changing the SDS content in the separation buffer. Despite the standard plasma contains less components than a real plasma, the maintenance of the good resolution is a prerequisite for real sample resolution. The sample concentrations applied for both separations in Fig. 1 are far away from real sample values. To prove the ability of the method to separate acylcarnitines at or below real sample concentrations, the standard stock solution was diluted 1000-fold with the standard plasma. Fig. 2 shows the separation of carnitine and five acylcarnitines near the detection limit. In this case sample components of the plasma and hydrolysis products of the derivatization agent (in front of the carnitine 1 peak) have significant influence on the separation and resolution of the low concentrated acylcarnitines. A detection limit of $6 \cdot 10^{-8}$ *M* was calculated for dodecylcarnitine under these separation conditions. In this buffer no separation of the palmitoylcarnitine from the plasma peak was possible. An increased SDS content or the application of

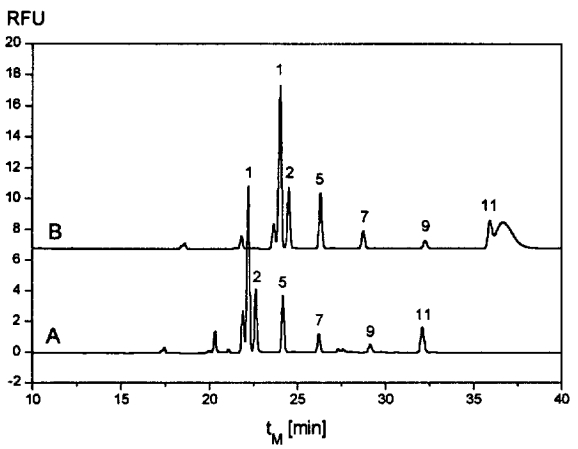

Fig. 1. Electrophoretic separation of carnitine and five acylcarnitines. (A) Aqueous standard 1:10 diluted with water, (B) aqueous standard 1:10 diluted with standard plasma. 20 kV; 23°C; 30 s electrokinetic injection at 10 kV; capillary: 50/57 cm length× 50 μm I.D.; buffer: 88% methanol, 8% of a 5% SDS solution, 4% of a 85% phosphoric acid; concentration of the separated compounds (after derivatization) in μ*M*: (1) carnitine 218.6; (2) acetylcarnitine 73.9; (5) valerylcarnitine 68.7; (7) octylcarnitine 19.2; (9) dodecylcarnitine 12.3 and (11) palmitoylcarnitine 41.8.

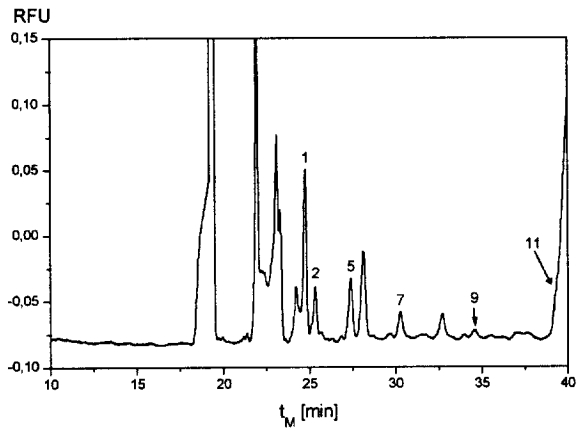

Fig. 2. Electrophoretic separation of carnitine and acylcarnitines with concentrations comparable with real samples. Aqueous carnitine/acylcarnitine standard 1:1000 diluted by standard plasma. 20 kV; 23°C; 120 s electrokinetic injection at 10 kV; capillary: 50/57 cm length×50 μm I.D.; buffer: 88% methanol, 8% of a 5% SDS solution, 4% of a 85% phosphoric acid; concentration of the separated compounds (after derivatization) in μ*M*: (1) carnitine 2.19; (2) acetylcarnitine 0.74; (5) valerylcarnitine 0.69; (7) octylcarnitine 0.19; (9) dodecylcarnitine 0.12 and (11) palmitoylcarnitine 0.42.

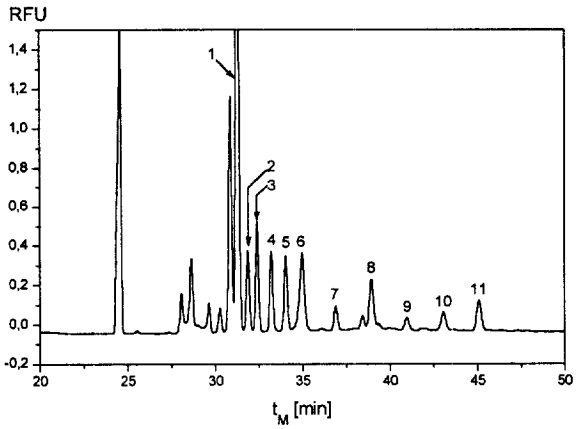

Fig. 3. Electrophoretic separation of carnitine and ten acylcarnitines with concentrations in the lower μ*M* range. Aqueous standard 1:50 diluted by water. 28 kV; 23°C; 30 s electrokinetic injection at 10 kV; capillary: 70/77 cm length×50 μm I.D.; buffer: 88% methanol, 8% of a 5% SDS solution, 4% of a 85% phosphoric acid; concentration of the separated compounds (after derivatization) in μ*M*: (1) carnitine 73.3; (2) acetylcarnitine 14.7; (3) propionylcarnitine 14.5; (n-4) butyrylcarnitine 8.72; (5) valerylcarnitine 13.7; (6) hexylcarnitine 7.7, (7) octylcarnitine 3.84; (8) decylcarnitine 6.2; (9) dodecylcarnitine 2.5; (10) tetradecylcarnitine 2.8 and (11) palmitoylcarnitine 4.2.

longer capillaries could help to overcome this problem. To obtain sufficiently high peaks and detection limits below real sample concentrations, a relatively long injection time of 120 s was used. Nevertheless, no negative influence on peak width or the reproducibility of the migration times was observed.

Fig. 3 shows the separation of carnitine and ten acylcarnitines, most of them important substances in the metabolism of fatty acids or the characterization of metabolic disorders. To obtain a baseline resolution of all compounds a longer capillary and higher voltage during the separation were applied. In comparison to the electropherogram in Fig. 2 peak intensities are reduced despite the same injection time. The longer capillary leads to a lower electric field, generated during electrokinetic injection across the capillary. Therefore, the resulting migration velocity of the sample components during the injection step is decreased and a lower amount of sample is injected. The higher voltage applied for the separation step allows a complete separation of all carnitine compounds as well as of the hydrolysis products of the derivatization agent.

The incapacity to separate isomeric forms, like the *n*- and *i*-isomer of distinct acylcarnitines, is a main disadvantage of all separation methods for acylcarnitines published until now. In most cases this effect is caused by insufficient separation capacity of the separation method. Therefore, the introduced buffer system was tested for the separation of *n*- and isobutyrylcarnitine. Depending on the metabolism of the patient both isomers could be components of real plasma samples. Fig. 4 shows the separation of both isomers under the same conditions as in Fig. 3. Although no baseline separation was achieved the method is able to distinguish between both forms. With slight changes of the buffer composition improvements of the resolution should be possible. Corresponding with the theory the iso-form migrates faster due to the more compact structure. Therefore this isomer possess the lower migration time under the separation conditions applied. Further investigations have to be confirm the applicability of the separation of isomers in complex mixtures and real plasma samples, where peaks from the plasma may interfere with one or both signals of the isomers.

The described separations of carnitine and eleven

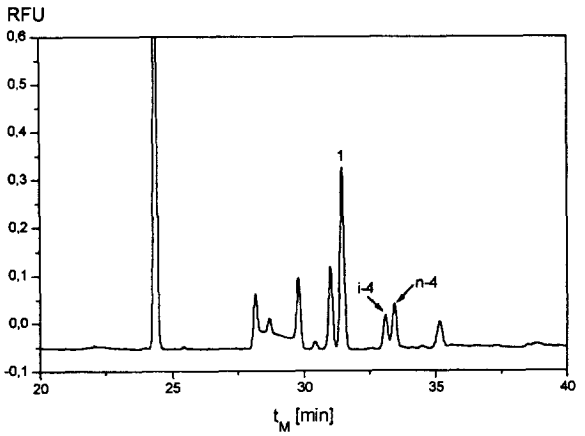

Fig. 4. Electrophoretic separation of carnitine and isomeric butyrylcarnitines. Aqueous standard 1:50 diluted by water. 28 kV; 23°C; 30 s electrokinetic injection at 10 kV; capillary: 70/77 cm length×50 μm I.D.; buffer: 88% methanol, 8% of a 5% SDS solution, 4% of a 85% phosphoric acid; concentration of the separated compounds (after derivatization) in μ*M*: (1) carnitine 26.1; (i-4) isobutyrylcarnitine 7.0 and (n-4) *n*-butyrylcarnitine 12.0.

acylcarnitines, among them isomers, have clearly shown that CE is a well suited method for the separation of these permanently charged biomolecules. High-resolution power and a very sensitive detection system make the determination of low acylcarnitine concentrations in real samples possible concurrently with carnitine. No preconcentration was necessary to detect acylcarnitine concentrations in a standard plasma at or below known concentrations in real plasma samples. Further investigations have to be performed to adapt the measurements to quite different samples, like real plasma, urine or muscle.

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