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Analysis of carnitine and acylcarnitines in urine by capillary electrophoresis

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Abstract

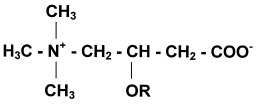
A capillary electrophoresis method is described for the simultaneous analysis of carnitine and short-chain acylcarnitines in aqueous standard solutions and urine samples. Samples were worked up using silica gel extraction and derivatization with 4′-bromophenacyl trifluoromethanesulfonate. Separation was performed in less than 8 min using a binary buffer system containing phosphate/phosphoric acid and sodium dodecyl sulfate. 3-(2,2,2-Trimethylhydrazinium)propionate (mildronate) was used as an internal standard. The method was developed with aqueous standard solutions and then applied successfully to spiked and unspiked human urine samples. The limit of detection for both carnitine and acetylcarnitine is 3 μ*M*.

Keywords: Derivatization, electrophoresis; Carnitine; Acylcarnitines; Bromophenacyl trifluoromethanesulfonate; Mildronate; Trimethylhydrazinium propionate

1. Introduction

Carnitine is an endogenous compound, which is either biosynthesized (from the amino acids L-lysine and L-methionine) or taken up in food (mainly in meat and milk) [1]. Carnitine is essential for the transport of fatty acids into the mitochondrial matrix, where they are metabolized (β-oxidation for energy production). Fatty acids bind carnitine via an ester binding on the hydroxyl group in position 3 of carnitine (see Fig. 1), thereby generating various acylcarnitines with different chain lengths. Carnitine is also important to maintain the cellular pool of free coenzyme A [2]. Free (non-esterified) carnitine and acetyl-carnitine represent the major constituents of the body fluids and tissue carnitine pools in normal subjects. Under pathological conditions, other acylcarnitines can accumulate, for instance in inherited or acquired organic acidurias such as propionic or isovaleric acidemia. Profiling of the acylcarnitines in body fluids is therefore useful for diagnostic purposes.

Existing analytical methods are problematic in that



R	Name	R	Name
H	Carnitine	$COC(CH_3)_3$	Isovalerylcarnitine
$COCH_3$	Acetylcarnitine	$CO(CH_2)_4CH_3$	Hexanoylcarnitine
$COCH_2CH_3$	Propionylcarnitine	$CO(CH_2)_6CH_3$	Octanoylcarnitine

Fig. 1. Chemical structures of carnitine and short-chain acylcarnitines analyzed in this study.

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they either do not allow differentiation between acylcarnitines (radioenzymatic method) or are time-consuming [high-performance liquid chromatography (HPLC) method]. The radioenzymatic assay is strictly enantioselective for L-carnitine, but it allows only the determination of the free carnitine, short-chain acylcarnitine (difference between total acid-soluble and free carnitine) and long-chain acylcarnitines [3]. HPLC methods have been developed to obtain a profile of acylcarnitines [4–8]. As carnitine has no specific UV absorption, it has to be derivatized for detection. Different chromophores have been described in the literature, reacting either with the carboxyl or the hydroxyl group of carnitine. Examples of such derivatizing agents include 1-aminoanthracene [4], (+)-1-(9-fluorenyl)ethyl chloroformate [(+)FLEC] [5] and 9-fluorenyl-methyl-chloroformate (FMOC) [6]. Minkler and Hoppel used 4′-bromophenacyl trifluoromethanesulfonate which reacts with the carboxyl group of carnitine and can be detected at 260 nm [7,8].

Capillary electrophoresis (CE) may have several advantages in comparison to HPLC for the separation of carnitine and acylcarnitines. The separation time is generally shorter and the amount of solvent used smaller, rendering the analysis faster and less expensive. Accordingly, CE procedures for the separation of carnitine and acylcarnitine standard solutions have been described [5,6,9–12]. Most recently, also a CE–mass spectrometry (MS) method for the separation of carnitine/acylcarnitines in biological fluids has been published, but MS is not widely available [13]. In the present paper we describe a CE method to separate acylcarnitines up to C_8 in standard solutions and in urine, after derivatization of the carboxyl group with 4′-bromophenacyl trifluoromethanesulfonate.

2. Experimental

2.1. Chemicals

L-Carnitine was from Fluka (Buchs, Switzerland), and acetylcarnitine, hexanoylcarnitine, octanoylcarnitine and sodium dodecyl sulfate (SDS) from Sigma (St. Louis, MO, USA). Propionylcarnitine and isovalerylcarnitine were a gift of Sigma–Tau (Zofingen, Switzerland). Potassium hydroxide, potassium dihydrogensulfate, sodium dihydrogenphosphate dihydrate, silica gel and acetic acid were purchased from Merck (Darmstadt, Germany). Phosphoric acid was from Fluka. *N*,*N*-Diisopropylethylamine was from Aldrich (Buchs, Switzerland). Methanol and acetonitrile were HPLC grade from Biosolve (The Netherlands). 3-(2,2,2-Trimethylhydrazinium)-propionate (mildronate) was synthesized using the methods of Le Berre and Porte [14] and Kosower and Patton [15] with some modifications. 3-(2,2,2-Trimethylhydrazinium)propionate was used as an internal standard. The derivatizing agent 4′-bromophenacyl trifluoromethanesulfonate was synthesized as described elsewhere [16,17].

2.2. Standard solutions

A stock 100 m*M* L-carnitine solution was prepared in water, standardized by a spectrophotometric method [18], stored in aliquots at −20°C and diluted with water to the desired concentration just before use. Stock 20 m*M* solutions of all other acylcarnitines were prepared in water (not standardized), stored in aliquots at −20°C and diluted with water to the desired concentration as needed. A 50 μ*M* internal standard solution (mildronate) was prepared in water and stored at −20°C.

2.3. Sample preparation and derivatization

Carnitine, acylcarnitines and total carnitine were extracted and derivatized according to Minkler et al. [19] with minor modifications. Extraction was performed from 50 μl human urine (undiluted or after dilution up to 10 times) or aqueous standard mixtures. A 50-μl volume of aqueous mildronate solution (50 μ*M*) was used as internal standard. The samples were dried with an evaporator under reduced pressure at 35°C. The derivatized and dried samples were dissolved with 50 μl acetonitrile–water (80:20, v/v) and injected into the CE system. If necessary, samples were diluted further with the same solvent.

2.4. Capillary electrophoresis conditions

Electrophoresis was carried out on a BioFocus 3000 CE system (Bio-Rad, Hercules, CA, USA)

equipped with a multi-wavelength detector operating at 260 nm. The separation took place in a 40 cm (35.4 cm to detector)×50 μm I.D. fused-silica capillary thermostated at 20°C. A voltage of 18 kV was applied in a constant and cationic mode for a current of about 19 μA. Samples were hydrodynamically injected with pressure for 2 p.s.i. s (1 p.s.i.=6894.76 Pa).

The capillary was conditioned each morning during 5 min with 0.1 *M* NaOH, then during 5 min with water and finally for 30 min with the running buffer. Between runs, a 2-min wash with the running buffer was performed. At the end of the day, the capillary was rinsed for 5 min with 0.1 *M* NaOH and for another 15 min with water. The optimized running buffer (optimized for separation of carnitine and acylcarnitines) was 20 m*M* NaH_2PO_4, 20 m*M* H_3PO_4 and 20 m*M* SDS in acetonitrile–water (50:50, v/v). Fresh buffer was prepared each day from a 2 *M* salt aqueous solution and a 1 *M* SDS aqueous solution. Both inlet and outlet vials were refreshed every two analyses.

2.5. Radioenzymatic analysis of urine samples

Urine samples of healthy persons were assayed for free and for total carnitine with the radioenzymatic method as described by Hoppel [3].

3. Results and discussion

3.1. Choice of the internal standard

The internal standard has to have a similar behavior in the analytical system as the analytes but should not be present in biological samples. Mildronate (Fig. 2), an inhibitor of γ-butyrobetaine hydroxylase, the enzyme catalyzing the last step in carnitine biosynthesis, was chosen due to its structural similarity to carnitine [20,21]. Pilot studies showed that extraction and derivatization properties of mildronate did not differ from carnitine.

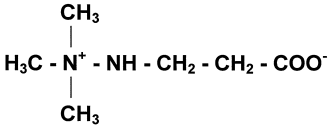

Fig. 2. Chemical structure of 3-(2,2,2-trimethylhydrazinium)-propionate (mildronate) which was used as an internal standard.

3.2. Separation of acylcarnitines

Carnitine and acylcarnitines are zwitterionic compounds, which are positively charged in acidic solutions (Fig. 1). The derivatization with 4′-bromophenacyl trifluoromethanesulfonate is performed on the carboxyl group of carnitine/acylcarnitines and introduces a permanent positive charge (the charge of the nitrogen in position 4 of carnitine, see Fig. 1). Therefore, a positive voltage polarity (anode at the inlet) was chosen. As all analyzed compounds have the same charge, they were expected to be separated mainly according to their molecular mass: the small internal standard shows the fastest migration, followed by carnitine and then by the acylcarnitines in the order of increasing chain length of the acyl group. The molecular masses of the analytes are quite close, however, requiring the use of SDS to widen the separation window. Due to decreased hydrophilicity after introduction of an apolar group with derivatization, an organic solvent had to be added to the running buffer to solubilize all compounds. Non-aqueous or binary running buffers do not only allow analysis of more hydrophobic compounds by solubilization, but also can introduce different selectivity [22,23]. Furthermore, the interaction between analytes and silanol groups appears to be favorably modified.

Acetonitrile was chosen as an organic solvent, as it had proved its efficiency in solubilizing and separating carnitine and acylcarnitines by HPLC [7]. Different concentrations of acetonitrile in the buffer (initially 10 m*M* NaH_2PO_4, 10 m*M* H_3PO_4 and 10 m*M* SDS) were tested. With 30% (v/v) acetonitrile in water, the solubility of the analytes was insufficient, resulting in very broad peaks. Fifty and 80% (v/v) acetonitrile in starting buffer showed similar separation profiles of carnitine and acylcarnitine standards. A concentration of 50% (v/v) acetonitrile in starting buffer was chosen as a compromise between solubility of carnitine/acylcarnitines and prevention of buffer evaporation during the analysis.

The SDS concentration was varied between 10 to 30 m*M*. In the presence of 10 m*M* SDS, peaks were

not well separated, whereas in the presence of 30 m*M* SDS, the migration time increased dramatically. A concentration of 20 m*M* SDS resulted in an acceptable resolution of the analytes with a short running time. Finally, the phosphate/phosphoric acid concentration of the starting buffer was adjusted to 20 m*M* in order to sharpen the peaks.

A typical profile of a standard solution of short-chain acylcarnitines is shown in Fig. 3. All peaks were attributed to the acylcarnitine indicated by injecting each standard alone. As expected, the migration order corresponds to the order of the molecular masses. The peak attribution was confirmed by the disappearance of the corresponding acylcarnitine peaks and the simultaneous increase in the carnitine peak after hydrolysis, by which time all acylcarnitines are converted into carnitine (Fig. 4). Fig. 3 demonstrates that the five acylcarnitines chosen can be separated from carnitine and internal standard in less than 8 min. The separation is acceptable for all analytes assayed except isovalerylcarnitine, which could be resolved hardly from an interfering peak of the derivatizing agent.

3.3. Method characterization

The optimized method was characterized for carnitine and acetylcarnitine standards.

The linearity was tested over a concentration range of 5 to 100 μ*M* for both carnitine and acetylcarnitine. Regression lines were calculated with the corrected area/migration time versus concentration. As shown in Table 1, a good linearity ($r>0.99$) was obtained for both compounds.

The limit of detection, defined as a signal-to-noise ratio of 3, was established by injecting serial dilutions of the corresponding 100 μ*M* standard solutions. For both carnitine and acetyl-carnitine the limit of detection was 3 μ*M*.

The inter-day reproducibility performed over 5

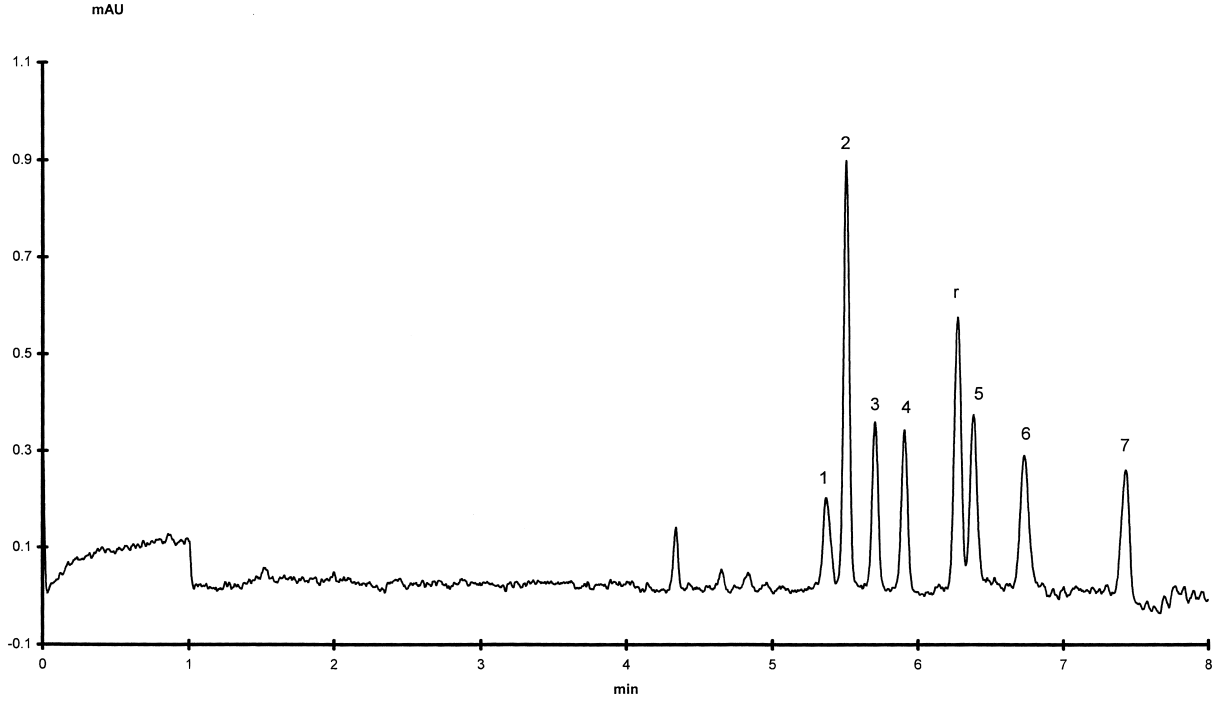

Fig. 3. Typical electropherogram of carnitine and short-chain acylcarnitines standards. The concentrations are 100 μ*M* for carnitine/acylcarnitines and 50 μ*M* for the internal standard. The final preparation was diluted 10-times before injection. Capillary electrophoresis conditions are given in the Experimental section. The peaks are: 1: internal standard, 2: carnitine, 3: acetylcarnitine, 4: propionylcarnitine, 5: isovalerylcarnitine, 6: hexanoylcarnitine, 7: octanoylcarnitine and r: peak from the derivatizing agent.

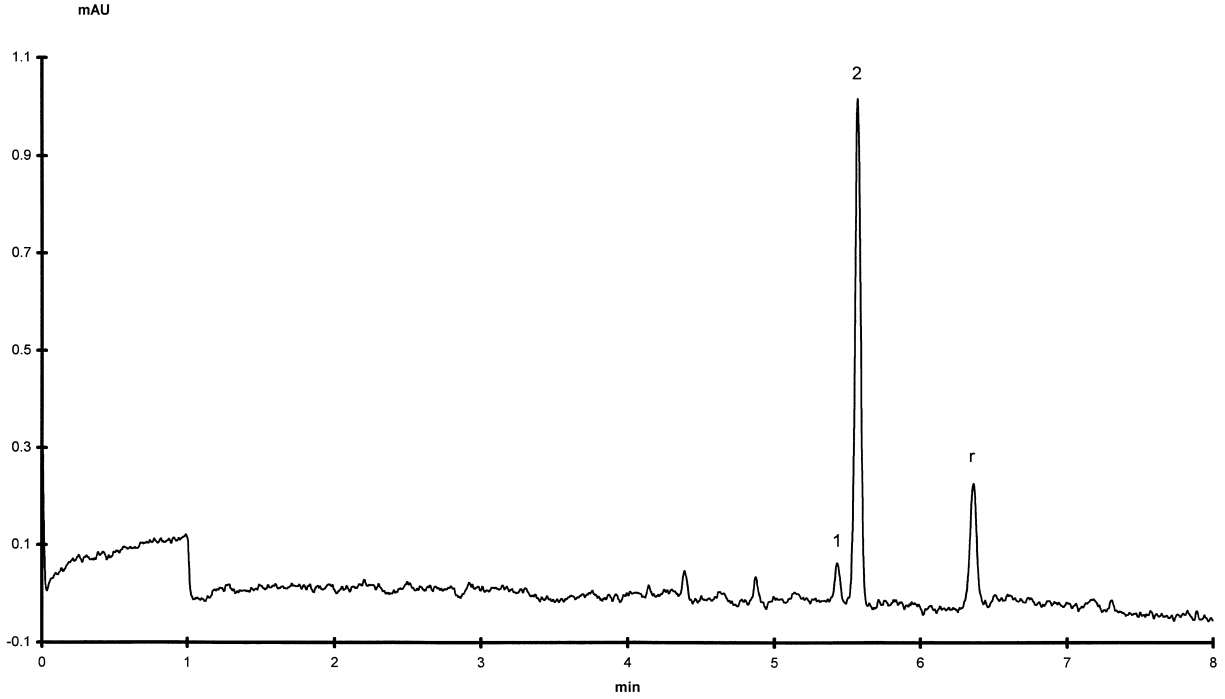

Fig. 4. Electropherogram of hydrolyzed carnitine and short-chain acylcarnitine standards. The concentrations are 100 μ*M* for carnitine/acylcarnitines and 50 μ*M* for the internal standard. The final preparation was diluted 10-times before injection. Conditions and peaks as in Fig. 3.

days (complete work-up of four samples at four different concentrations) is shown in Table 2. For both compounds the relative standard deviation (RSD) is below 7% for the higher concentrations (25–100 μ*M*) but increases to approximately 20% with the lowest concentration (5 μ*M*). The intra-day reproducibility, also shown in Table 2, was performed by analyzing three individual samples on the same day. The results obtained are similar to the ones obtained for the inter-day assays.

Table 1
Regression data for carnitine and acetylcarnitine standard solutions[a]

	$y=ax+b$		Correlation
	a	*b*	*r*
Carnitine	1.03±0.05	−0.002±0.024	0.999±0.001
Acetylcarnitine	1.10±0.06	0.014±0.026	0.999±0.001

[a] The concentration range was 5–100 μ*M*. $n=5$ individual determinations for every curve.

3.4. Application to urine samples

The next step was to analyze urine spiked with a mixture of the same acylcarnitines (Fig. 5). The separation of the acylcarnitines added proved to be as efficient as for the standard solutions in Fig. 3. Peak attribution was performed by spiking with individual acylcarnitines and by hydrolyzing the spiked urine samples (not shown). An additional peak can be observed in urine which elutes just before the internal standard, is not hydrolyzed and does not interfere with the analytes of interest.

Free and total carnitine were quantified in five urines of healthy persons (Fig. 6). The urines were appropriately diluted so that their content in free and total carnitine were in the calibration range. The results were compared with those of the

Table 2
Reproducibility of the quantification of carnitine and acetylcarnitine standard solutions[a]

	Concentration (μM)	Corrected area/migration time		
		Average	RSD (%)	*n*
Inter-day				
Carnitine	100	2.092	5.4	5
	50	0.968	2.6	5
	25	0.530	6.6	4
	5	0.110	21.0	4
Acetylcarnitine	100	2.211	5.0	5
	50	1.137	6.5	5
	25	0.577	5.9	5
	5	0.117	16.1	3
Intra-day				
Carnitine	100	2.216	1.5	3
	25	0.635	4.6	3
Acetylcarnitine	100	2.514	6.4	3
	25	0.722	10.2	3

[a] Individual samples of standard solutions were worked up and analyzed on the same or on different days as indicated in the Table.

radioenzymatic method and are presented in Table 3. The correlation between the two methods was in an acceptable range ($r=0.998$ for free and 0.959 for total carnitine). On the other hand, the slopes were different from one (0.77 for free carnitine and 1.55 for total carnitine), indicating that in urine, the free carnitine concentration tends to be over- and the total carnitine concentration underestimated by the current CE method.

Additional urine samples contained variable amounts of propionylcarnitine but no other acylcarnitines (results not shown). Unfortunately, we did not have access to urine samples of patients with organic acidurias.

4. Conclusions

A new CE method was developed to analyze carnitine and acylcarnitines in standard aqueous solutions and in human urine samples. Seven compounds (carnitine, different acylcarnitines and inter-

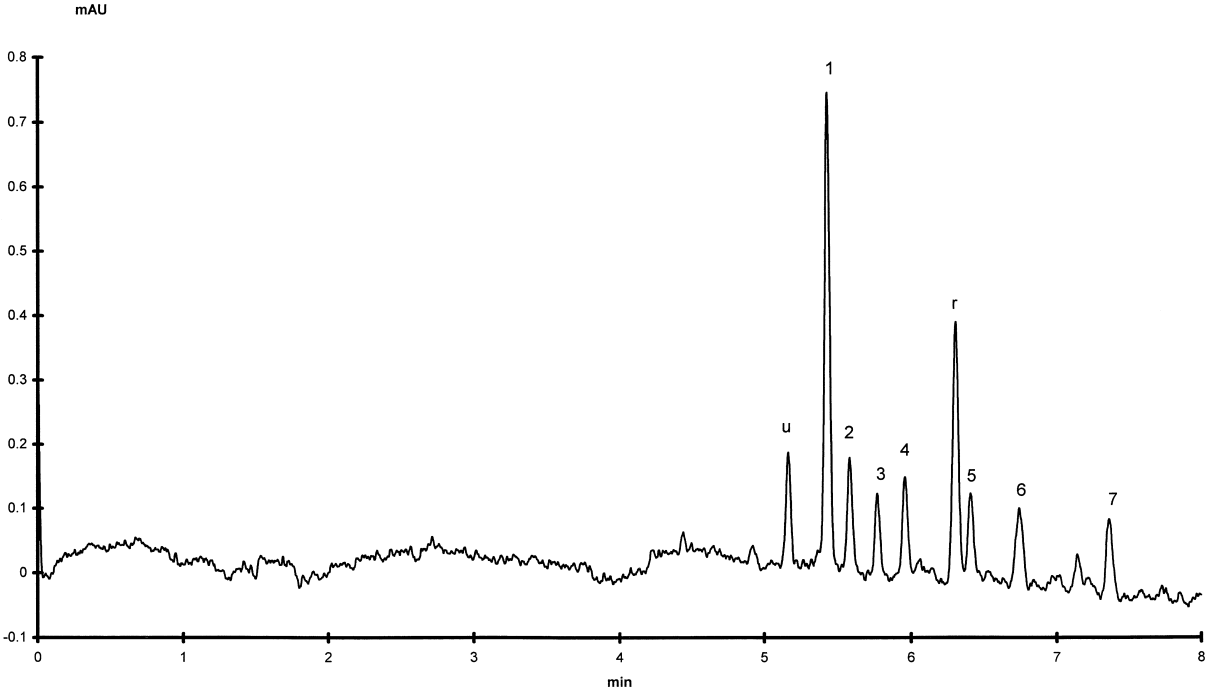

Fig. 5. Electropherogram of human urine spiked with 100 μM of each acylcarnitine and 50 μM of internal standard. Conditions and peaks are as in Fig. 3. u Represents a peak which is observed with urine samples but not with standard solutions.

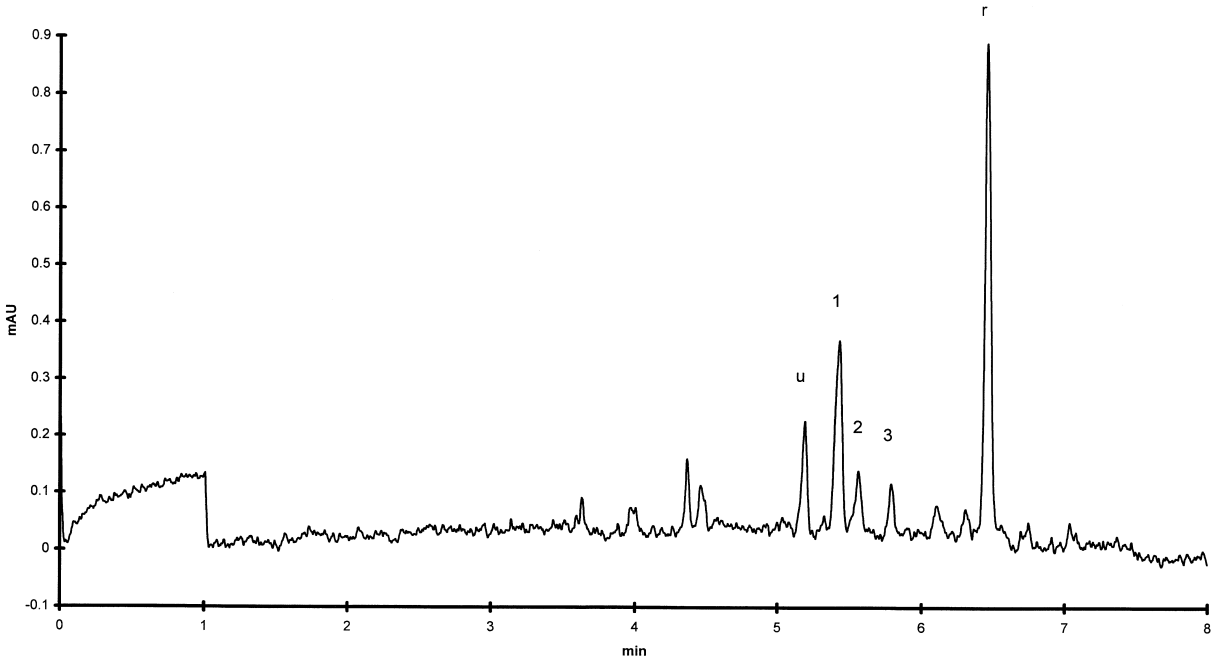

Fig. 6. Electropherogram of human urine spiked with internal standard (50 μ*M*). The final preparation was diluted five times before injection. Conditions and peaks are as in Fig. 3.

nal standard) could be separated and identified in less than 8 min. In comparison to similar HPLC methods, analysis of carnitine/acylcarnitines by CE is faster and consumes less solvents, rendering CE less expensive. While the current method is usable for separation of carnitine and acylcarnitines, the quantification of carnitine and acylcarnitines should still be improved.

Table 3
Quantification of free and total carnitine in samples of human urines[a]

Urine sample	Free carnitine (μ*M*)		Total carnitine (μ*M*)	
	CE	REA	CE	REA
1	8.8	10.0	30.4	22.8
2	27.9	40.0	119	112
3	7.6	10.5	14.6	16.5
4	11.0	7.1	137	143
5	113	144	379	233

[a] Free and total carnitine was determined by capillary electrophoresis (CE) and by the radioenzymatic assay (REA) in five human urines.

Acknowledgements

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