Journal of Chromatography, 423 (1987) 297–303
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3910

Note

Analytical method for urinary glutarylcarnitine, acetylcarnitine and propionylcarnitine with a carboxylic acid analyser and a reversed-phase column

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(First received April 29th, 1987; revised manuscript received July 27th, 1987)

Carnitine is essential for the transport and metabolism of fatty acids. It also generates intramitochondrial free coenzyme A (CoASH) through transport of excess acyl groups out of the mitochondrial matrix. By this mechanism the specific acylcarnitine analysis provides the recognition of defects in mitochondrial oxidation and a biochemical marker for the detoxifying effect of carnitine therapy in metabolic disorders [1].

The current methods for the analysis of acylcarnitine in physiological samples are limited in number. They include high-performance liquid chromatography (HPLC) with radioisotope exchange [2], HPLC alone [3, 4], fast atom bombardment (FAB) with mass spectrometry (MS) [1, 5, 6], HPLC–MS [7], and chemical ionization MS [8]. The radioisotopic [2] and the HPLC methods with CoASH measurement [4] are sensitive. However, the former requires radioisotopes, and the latter requires a rather complicated procedure. Although FAB–MS analysis is excellent for acylcarnitine profiling without complicated preparations of samples [6], it requires an expensive mass spectrometer.

Our laboratory has developed a simple and reliable method for the identifica-

0378-4347/87/$03.50

tion of glutarylcarnitine in glutaric aciduria type 1 [9]. This method is characterized by the combination of on-line chromatographic separation and the specific colorimetric determination of the carboxyl group using a carboxylic acid analyser (CAA) and an ODS column. We here describe a CAA method for analysis of acetylcarnitine, glutarylcarnitine and propionylcarnitine in urine.

EXPERIMENTAL

Chemicals

Acylcarnitines were synthesized according to the method of Bohmer and Bremer [10]. They were characterized by FAB–MS and ^{1}H NMR. The purity of compounds was over 95%, as determined by a CAA with an ODS reversed-phase column as described below.

L-Carnitine was a generous gift from Sigma Tau (Rome, Italy). Acetyl chloride, propionyl chloride and glutaryl anhydride were purchased from Tokyo Chemical Industries (Tokyo, Japan). All other reagents used were of the highest purity available.

Samples

Urine samples were obtained from a patient with glutaric aciduria type 1 [9], a patient with propionic acidemia [11], a patient with methylmalonic aciduria [12] and five healthy infants.

Sample preparation

A 1-ml volume of urine was lyophilized. The residue, dissolved in 300 μl of deionized water, had a pH between 5.4 and 7.2. Acetylcarnitine, glutarylcarnitine and propionylcarnitine added to normal urine (pH 5.2–7.4) were eluted within 40 min (elution volume, 8 ml; flow-rate, 0.2 ml/min) from a 500 mm × 3 mm I.D. anion-exchange column (SA-08SA, chloride form, 11–15 μm, Tokyo Rika Kikai, Tokyo, Japan) using deionized water. Therefore, the residue was applied to the anion-exchange column for elimination of organic acids without pH adjustment. Acylcarnitines and some amino acids in urine samples were eluted with deionized water (10 ml). After lyophilization the residue was stored at -20°C prior to analysis. The residue was dissolved in deionized water (1 ml), and 100 μl of the sample were analysed using a CAA fitted with an ODS column. Fractions of acylcarnitines from patients' samples were hydrolysed in the previously described manner [9] and analysed again with a CAA and an ODS column. For FAB–MS analysis, urine was methylated according to the method of Roe et al. [6].

Analysis using a CAA and an ODS column

A Model S-14 CAA (Tokyo Rika Kikai) was used along with a CLC-ODS reversed-phase column (15 cm × 6 mm I.D., 5 μm nominal particle diameter) (Shimadzu, Kyoto, Japan).

Acylcarnitine analysis was carried out by using the CAA techniques described previously [9], with a 5% acetonitrile–phosphate buffer as the eluent [0.005 *M* phosphoric acid, 0.005 *M* sodium dihydrogenphosphate dihydrate, 0.12 *M* sodium

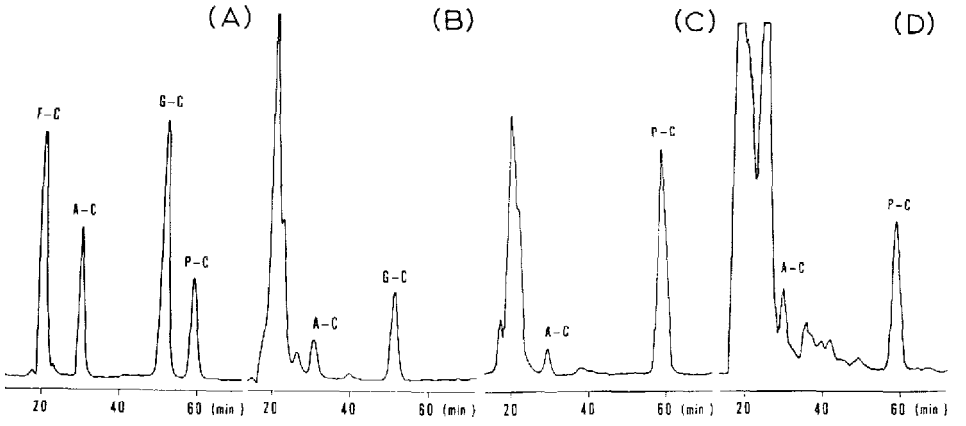

Fig. 1. (A) Chromatogram of a strandard acylcarnitine mixture obtained by a CAA and an ODS column. Peaks and quantities: free L-carnitine (F-C), 74 nmol; acetylcarnitine (A-C), 52 nmol; glutarylcarnitine (G-C), 43 nmol; propionylcarnitine (P-C), 34 nmol. The analytical conditions were as described in the text. Column, 150 mm×6 mm I.D. CLC-ODS (5 μm nominal particle diameter) at room temperature; eluent, 0.005 *M* phosphoric acid, 0.005 *M* sodium dihydrogenphosphate dihydrate, 0.12 *M* sodium perchlorate monohydrate in water–acetonitrile (95:5, v/v); injection volume, 100 μl; flow-rate, 0.2 ml/min. (B–D) Chromatograms of the urinary short-chain acylcarnitines from (B) the patient with glutaric aciduria type 1, (C) the patient with propionic acidemia and (D) the patient with methylmalonic aciduria. The chromatographic conditions were the same as for Fig. 1A.

perchlorate monohydrate in water–acetonitrile (95:5, v/v)] at room temperature. The injection volume was 100 μl, and the flow-rate was 0.2 ml/min.

Quantification

The calibration curves of acetylcarnitine, glutarylcarnitine and propionylcarnitine were obtained by adding a known amount of standard acylcarnitines to normal urine. All reference samples were determined in duplicate for calibration curve generation. The linearity of the detector response over a sample concentration range was established by algebraic least-squares fit of determined chromatographic peak-height ratios and their respective sample concentrations to a linear equation.

Acylcarnitines recovery

The recovery of the acylcarnitines through the sample preparation was obtained by adding acetylcarnitine (517 nmol), glutarylcarnitine (428 nmol) and propionylcarnitine (339 nmol) to 1 ml of five normal urine samples.

FAB–MS analysis

FAB mass spectra of samples were recorded oscillographically on a JMS DX-300 double-focusing mass spectrometer (JEOL, Tokyo, Japan) at low resolution equipped with the manufacturer's FAB ion source and sample introduction system. The data were stored and processed by a JMA 3500 data system of JEOL. Typically, 2–3 μl of methylated sample solution was applied to the stainless-steel

TABLE I

ELUTION TIMES OF CARNITINE, ACYLCARNITINES, ORGANIC ACIDS AND AMINO ACIDS

Determined with a CAA and a CLC-ODS reversed-phase column (150 mm × 6 mm I.D.) as described in Experimental.

Compound	Elution time (min)	
	Range	Mean ± S.D. ($n=6$)
Amino acids*	16.2–24.0	
Carnitine	21.0–21.1	21.1 ± 0.03
Acetic acid	24.6–24.7	24.6 ± 0.03
Acetylcarnitine	29.7–29.7	29.7 ± 0.03
Glutaric acid	33.6–33.6	33.6 ± 0.03
Glutarylcarnitine	51.0–51.0	51.0 ± 0.03
Propionic acid	37.2–37.2	37.2 ± 0.02
Propionylcarnitine	59.4–59.4	59.4 ± 0.02

*Mixture of 17 amino acids: lysine, histamine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine.

target coated with glycerol (2–3 μl) and exposed to argon atoms (6 kV translational energy) in the FAB source.

RESULTS

Separation of carnitine, acetylcarnitine, glutarylcarnitine and propionylcarnitine was achieved isocratically within 65 min, as shown by a typical chromatogram in Fig. 1A. Elution times of the acylcarnitines, the corresponding acids and some amino acids in urine are shown in Table I. The coefficients of variation for the elution times of the acylcarnitines were as follows: acetylcarnitine, 0.09%; glutarylcarnitine, 0.06%; propionylcarnitine, 0.03%.

Calibration curves for free L-carnitine, acetylcarnitine, glutarylcarnitine and propionylcarnitine in urine were linear over a sample concentration range from 30 to 1000 nmol/ml.

The recoveries (mean ± S.D., $n=5$) of the three acylcarnitines in urine (pH 5.2–7.4) were as follows: acetylcarnitine, 92.6 ± 3.0%; glutarylcarnitine, 70.2 ± 4.6%; propionylcarnitine, 93.2 ± 3.0%.

Chromatograms of the patients' samples revealed an acetylcarnitine peak and a specific acylcarnitine peak: the latter was glutarylcarnitine in glutaric aciduria type 1 (Fig. 1B), and propionylcarnitine both in propionic acidemia (Fig. 1C) and in methylmalonic aciduria (Fig. 1D). The specific acylcarnitine fraction of patients' samples had carnitine and corresponding acid peaks (glutaric acid to glutarylcarnitine and propionic acid to propionylcarnitine) following hydrolysis. The glutarylcarnitine and the propionylcarnitine peaks were not observed in healthy infants.

Table II shows the concentrations of urinary short-chain acylcarnitines in pa-

TABLE II

URINARY ACYLCARNITINE VALUES IN GLUTARIC ACIDURIA TYPE 1, PROPIONIC ACIDEMIA AND METHYLMALONIC ACIDURIA

GA1 = glutaric aciduria type 1; PA = propionic acidemia; MMA = methylmalonic aciduria; N.D. = not detected. Chromatograms of samples numbered 1 are shown in Fig. 1B–D.

Case	Age (years)	Sex	Sample No.	Acylcarnitine (nmol/mg of creatinine)		
				Acetyl	Glutaryl	Propionyl
GA1*	2	Male	1	409	602	N.D.
			2	49	372	N.D.
PA*	3	Male	1	459	N.D.	3997
			2	99	N.D.	5245
MMA	13	Female	1	351	N.D.	1510
Control ($n=5$)				N.D. to 64	N.D.	N.D.

*During carnitine therapy (100 mg/kg per day).

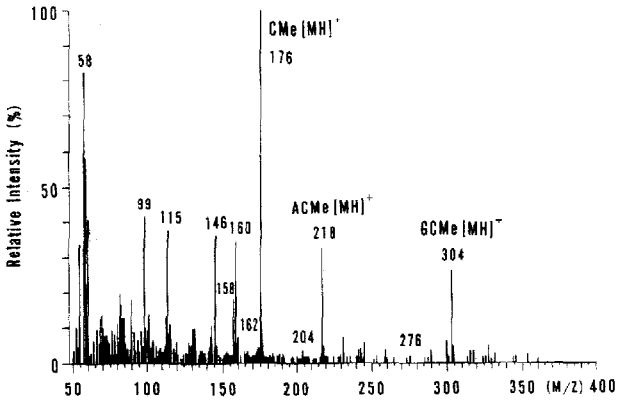

Fig. 2. Mass spectrum obtained by fast atom bombardment for methylated urine from glutaric aciduria type 1. CMe = carnitine methyl ester; ACMe = acetylcarnitine methyl ester; GCMe = glutarylcarnitine methyl ester.

tients. The acylcarnitine value was corrected for losses during the purification procedure.

The FAB mass spectrum of the methylated urine sample from the patient with glutaric aciduria type 1 is shown in Fig. 2. It had major MH^+ ions (m/z 176, carnitine methyl ester; 218, acetylcarnitine methyl ester; 304, glutarylcarnitine methyl ester). The FAB mass spectra of the samples from patients with propionic acidemia and methylmalonic aciduria had an MH^+ ion of propionylcarnitine methyl ester as well.

DISCUSSION

Although acylcarnitine analysis is important for the investigation of carnitine metabolism and for the evaluation of carnitine therapy in metabolic diseases,

studies in this area have been limited owing to the lack of a simple and quantitative method.

FAB–MS analysis is useful for acylcarnitine profiling when a strong MH^+ ion count is observed (as shown Fig. 2). No specific acylcarnitine signals were observed in the FAB–MS analysis for eluent fractions of acylcarnitines where sodium chloride and phosphoric acid were present in the eluent [9]. The relative abundances of the major FAB ions in the spectra of acylcarnitines vary considerably between different loadings of the same sample [5]. It would require the corresponding isotopic acylcarnitines to quantify acylcarnitines by FAB–MS analysis.

In our previous study, the eluent used for the identification of urinary glutarylcarnitine in glutaric aciduria type 1 was 0.1 *M* sodium perchlorate in a 0.01 *M* phosphate buffer without acetonitrile at 60°C [9]. Under those analytical conditions, the acetylcarnitine peak did not completely separate from the first urine peaks, and propionylcarnitine could not be quantified because of its long elution time (264 min). With the new analytical conditions presented here, the acetylcarnitine peak was separated from the first urine peaks, and propionylcarnitine was quantified.

The sensitivity (30 nmol/ml) appears to be well within the range of acylcarnitine concentations encountered in organic acidurias during carnitine therapy. In carnitine-deficient states, it may be necessary to concentrate the sample by lyophilization.

The concentrated urine sample (pH 5.4–7.2) was applied to the anion-exchange column without pH adjustment, and eluted with deionized water. With an 8-ml elution volume, glutarylcarnitine and five medium-chain acylcarnitines (isovaleryl-, hexanoyl-, heptanoyl-, valproyl- and octanoylcarnitine) added to normal urine (pH 5.2–7.4) were eluted from the column following acetylcarnitine and propionylcarnitine. However, recovery of glutarylcarnitine was relatively low compared with other short-chain and the five medium-chain acylcarnitines. Glutarylcarnitine should have a net negative charge at neutral pH. It is also more hydrophobic than monocarboxylic acid acylcarnitines (which can be partly retained owing to hydrophobic interaction with the polystyrene matrix of the Dowex resins [13]). Therefore, glutarylcarnitine may show a strong tendency to be retained in the anion exchanger.

For the specific detection of the carboxyl group, the degree of purification of samples is simple compared with other techniques. Our application requires only lyophilization and the removal of some organic acids from the urine sample by an anion-exchange resin. When an appropriate internal standard is used, the method described here appears to establish a quantitative analytical method for some short-chain acylcarnitines.

The acylcarnitine excretion and the required amount of L-carnitine may vary according to the patient's metabolic state. Therefore, repeated acylcarnitine analyses are essential. The simple CAA method will contribute to a better understanding of the organic acid metabolism and potential therapeutic applications of L-carnitine in metabolic diseases.

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