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Gas chromatographic profiling and determination of urinary acylcarnitines

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Abstract

A method is described for the routine profiling and determination in urine of most of the acylcarnitines clinically relevant for the diagnosis of organic acidurias. The procedure, which does not require expensive apparatus, involves extraction of the acylcarnitines on strong cation-exchange disposable columns, mild alkaline hydrolysis and gas chromatography of the liberated monocarboxylic acids. The different steps were optimized in order to increase the analytical performance. No significant interferences were encountered, the limit of detection (signal-to-noise ratio = 3:1) ranged from 0.1 to 4 mg/l and the between-day coefficient of variation from 3.6 to 17.7%, depending on the acyl species. The rapidity of the method results from the application of a single solid-phase extraction on disposable columns. The acyl moieties are chromatographed underivatized in order to permit the identification of short-, medium- and long-chain acylcarnitines. The method was assessed by analysing fourteen urine specimens from patients presenting an organic aciduria.

1. Introduction

The detection of acylcarnitines in urine, especially after a carnitine load, may reveal the acyl species accumulated in the mitochondria above the enzymatic block responsible for organic acidaemia and aciduria. The analysis of the esterified fraction of urinary carnitine is a valuable and complementary aid for the biochemical diagnosis of several organic acidurias [1–6], particularly when the urinary organic acid profiles are non-specific, such as in medium-chain acylCoA dehydrogenase deficiency.

The determination of acylcarnitines is not yet widely used, even in specialized laboratories, owing to the lack of a method that is at the same time sufficiently sensitive, rapid and not requiring expensive apparatus. Methods described previously include thin-layer chromatography [7], high-performance liquid chromatography (HPLC) of precolumn [8–11] or postcolumn [12] derivatized acylcarnitines, HPLC of the acylCoA thioesters yielded by the carnitine acetyltransferase (EC 2.3.1.7) reaction [13], HPLC after radioisotopic exchange [14], LC–mass spectrometry (MS) [15,16], gas chromatography (GC) [17,18] or GC–MS [3] of the acyl moieties liberated by mild alkaline hydrolysis, GC–MS of derivatized acylcarnitines [19,20], desorption chemical ionization MS [21], fast atom bombard-

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ment (FAB) MS [6,15], FAB tandem MS [16] and nuclear magnetic resonance spectrometry [22]. Comprehensive reviews of these methods have been published by Lowes and Rose [23] and Millington [15]. Two recent GC–MS methods [19,20] provide the best criteria of reliability, sensitivity and unambiguous identification on the one hand, and of relative simplicity and practicability on the other. However, they involve double column extraction, the time and cost of which limit their use as routine procedures. Recently, Van Bocxlaer and De Leenheer (24) described a method involving a faster extraction and the derivatization procedure of Lowes and Rose.

Our aim was to develop a method allowing the profiling and determination of urinary short-, medium- and long-chain acylcarnitines while being simple, reliable and easily accessible for non-highly equipped laboratories.

2. Experimental

2.1. Equipment

The analyses were performed on an Intersmat IGC 120 DFL gas chromatograph (Delsi Instruments, Suresnes, France) equipped with an SGE OCI-3 manual on-column injector (Scientific Glass Engineering, Ringwood, Australia) and a flame ionization detector coupled to an Enica 21 integrator (Delsi Instruments). We used a Superox II FA bonded fused-silica capillary column (30 m × 0.32 mm; film thickness 0.25 μm) obtained from RSL (Eke, Belgium).

Other instruments consisted of a Reacti-Therm heating module (Pierce Chemical Europe, Oud-Beijerland, Netherlands), a vacuum manifold (Alltech, Deerfield, IL, USA) and a Bransonic 32 ultrasonic bath (Branson Europa, Soest, Netherlands).

2.2. Reagents and standards

Disposable strong cation-exchange (SCX) columns (Adsorbex, 100 mg) were manufactured by Merck (Darmstadt, Germany). Bond-Elut SCX columns from Varian (Harbor City, CA, USA) were also occasionally used with the same results. Even-carbon acylcarnitines (from acetyl to stearyl) and propionylcarnitine were purchased from Sigma (St. Louis, MO, USA). Other solvents and reagents, all of analytical-reagent grade, were obtained from Merck and used as received. Water was purified in a Milli-Q apparatus (Millipore, Bedford, MA, USA). Standard solutions of acetyl-, propionyl-, butyryl-, hexanoyl-, octanoyl-, lauryl- and myristoylcarnitine were prepared by dissolution in methanol in order to obtain 1 g/l solutions for each compound. Aliquots of these solutions were mixed and evaporated to dryness at 40°C under nitrogen and the residues were dissolved in water in order to obtain concentrations of 5–100 mg/l of each acylcarnitine. A 0.5 g/l aqueous solution of decanoylcarnitine was also prepared. All these solutions may be stored at −20°C for at least 3 months.

2.3. Extraction procedure

The SCX columns were conditioned with 2 ml of methanol, 2 ml of water and 2 ml of 0.01 mol/l hydrochloric acid. Aliquots of 1 ml of urine samples and standard solutions were acidified to pH 2 with 1 mol/l hydrochloric acid (checking with a pH 0.5–5 paper indicator) and supplemented with 50 μl of 0.5 g/l decanoylcarnitine solution (internal standard). The whole volume of these mixtures was applied to the extraction column and drained. The columns were washed with 3 ml of 0.01 mol/l hydrochloric acid solution in acetone and flushed with air for 1 min. The fixed carnitine esters were eluted with 3 ml of 0.1 mol/l ammonia solution in ethanol–water (65:35, v/v), the eluates being collected in a 10-ml glass-stoppered centrifuge tube and evaporated to dryness under nitrogen at 60°C. The dry residues were dissolved in 220 μl of water–ethanol–2 mol/l potassium hydroxide (10:10:2, v/v/v), heated at 40°C for 60 min (saponification) and evaporated to dryness as above. A 10-μl volume of phosphoric acid–water (50:50, v/v) was added to the residues and the freed acids were then extracted with 300 μl of

diisopropyl ether by sonication for 10 min, vortex mixing and centrifuging. The organic extracts were decanted into 1.5-ml PTFE screw-capped vials. The extracts may be stored at −20°C for at least 3 months.

2.4. Gas chromatography

Gas chromatography was performed as follows: oven temperature 100 s at 50°C; then increased from 80 to 245°C at 4°C/min with a final hold for 175 s; detector temperature, 260°C; injector, not heated; linear velocity of the carrier gas (nitrogen), 30 cm/s; electrometer, set at $10^{-12} \times 64$ A/mV; and injection volume, 2.5 μl.

3. Results

Fig. 1 shows chromatograms obtained after the extraction of (A) an aqueous standard solution, (B) a normal urine sample and (C) a urine sample from a patient with ethylmalonic/adipic aciduria. Retention times of the acids are acetic 8.1, propionic 10.0, isobutyric 10.6, butyric 12.1, 2-methylbutyric 13.0, isovaleric 13.1, valeric 14.8, hexanoic 17.6, tiglic 17.7, octanoic 23.1, decanoic 28.2, lauric 32.9, myristic 37.1 and palmitic acid 41.0 min. The chromatogram of the ethylmalonic/adipic aciduria (Fig. 1C) shows a high content of butyrylcarnitine, together with the presence of hexanoylcarnitine and octanoylcarnitine.

To evaluate the linearity, precision and recovery, we adapted the evaluation protocols of the NCCLS [25]. Four aliquots of a normal urine pool, each randomly spiked at a different concentrations with acetyl- (13.6, 33.9, 67.8 or 135.7 mg/l), hexanoyl- (3.5, 8.8, 17.5 or 35.1 mg/l), octanoyl- (7.1, 17.4, 35.5, or 71.0 mg/l) and laurylcarnitine (3.6, 9.0, 18.1 or 36.2 mg/l), were submitted to the whole extraction procedure eight times in the course of 6 weeks, each time in duplicate. Sixty-four values were thus obtained for each carnitine ester. Concentrations were determined on the basis of aqueous acylcarnitine standards also analysed in duplicate during the same runs.

Linearity was assessed by testing for the lack of fit of the linear model [25]. Linear relationships between analytical response and concentration were accepted for the four investigated acylcarnitines: the G values are 0.179 (acetylcarnitine), 0.776 (hexanoylcarnitine), 0.115 (octanoylcarnitine) and 1.717 (laurylcarnitine), compared with an $F_{(2,60)}$ of 3.15 for $P = 0.05$. Within-day and between-day precision estimates for two concentration levels were calculated by analysis of variance (two random effects). The recoveries were calculated from concentrations measured in urines supplemented with acylcarnitines, relative to aqueous standard solutions and taking into account levels of endogenous carnitine esters present in the unspiked pooled urine. All these data are presented in Table 1.

The specificity of the method was evaluated by extraction of aqueous solutions of potentially interfering substances at concentrations of 50–500 mg/l. The results, expressed as the fraction of the response given by an equal amount of acyl species originating from carnitine esters, were 0.03 for acetylcholine, 0.00 for propionic and valeric acid, 0.01 for palmitic acid, 0.04 for propionylglycine, 0.02 for isovalerylglycine and 0.01 for hexanoylglycine.

The qualitative performance of the extraction procedure was assessed by analysing fourteen urine specimens representing seven different organic acidurias. The results are given in Table 2 and show the clinical relevance of the method, as the predominant acylcarnitines we found are those previously described for the same disorders [1–6,14,18]. Urines from eleven normal subjects were also analysed and never yielded abnormal acyl species in significant amounts. The anticonvulsant drug valproic acid was detected in one urine specimen.

4. Discussion

Our method is a modification of the extraction procedure briefly described by Roe et al. [3] and which is derived from that of Bieber and Kerner

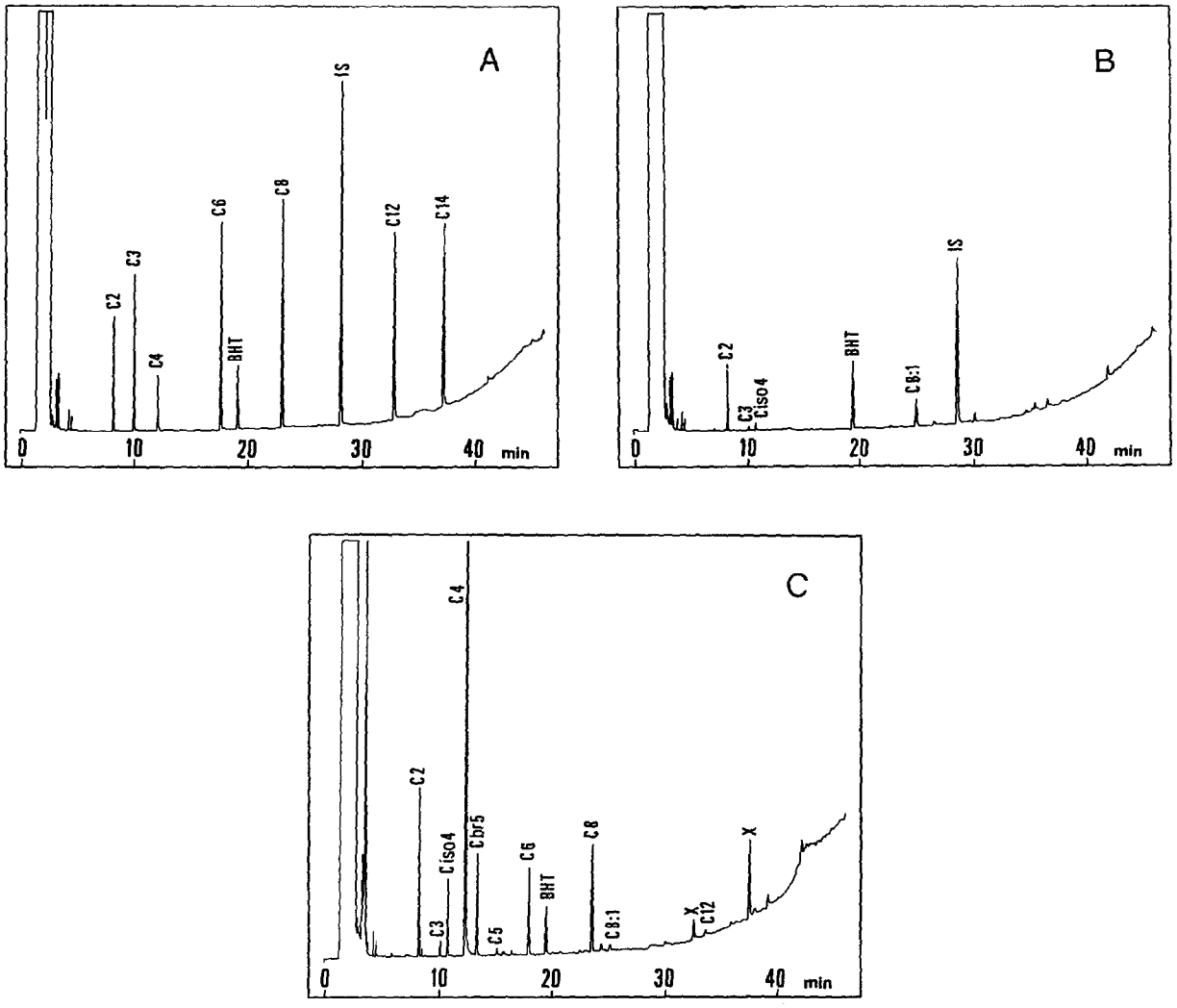

Fig. 1. Chromatograms obtained after extraction of (A) an aqueous standard solution containing 33.9 mg of acetylcarnitine (C2), 25.7 mg of propionylcarnitine (C3), 8.1 mg of butyrylcarnitine (C4), 17.5 mg of hexanoylcarnitine (C6), 17.7 mg of octanoylcarnitine (C8), 13.6 mg of laurylcarnitine (C12) and 12.7 mg of myristoylcarnitine (C14) per litre, (B) a normal urine sample and (C) a urine sample from a patient affected with ethylmalonic/adipic aciduria. The internal standard (decanoylcarnitine, IS) was not added to the pathological samples to avoid contamination of these rare samples with an acylcarnitine susceptible of being present. However, this was not the case for any of the samples studied. This validates the choice of the internal standard. Other peaks: isobutyrylcarnitine (Ciso4), 2-methylbutyryl and/or isovalerylcarnitine (Cbr5), valerylcarnitine (C5), butylated hydroxytoluene (BHT) (an antioxidant contained in diisopropyl ether), presumably 6-octenoylcarnitine (C8:1), unidentified peaks (X). For operating conditions, see text.

[18]. In order to obtain a simple and relatively rapid procedure applicable to all laboratories equipped with capillary GC, the different steps of the method were optimized.

To isolate the acylcarnitine fraction, we used strong cation-exchange columns [3,8]. They were selected after comparison with strong anion-exchange (SAX) resins [4,12], which retain acidic interfering compounds, and with cyano-bonded phases (partition chromatography). We observed that purification by SAX columns was not efficient: when fatty acids are added to a sample,

Table 1
Analytical performance of the assay method

	Acetylcarnitine		Hexanoylcarnitine		Octanoylcarnitine		Laurylcarnitine	
Precision in aqueous standards								
n	16		16		16		16	
Mean (mg/l)	67.8		17.5		35.5		18.1	
Within-day C.V. (%)	2.4		1.6		0.8		2.1	
Precision in spiked urines[a]								
n	32	32	32	32	32	32	32	32
Mean concentration found (mg/l)	28.8	110.8	7.0	28.7	13.1	54.5	5.9	25.9
Within-day C.V. (%)	9.6	17.1	8.9	7.2	3.6	3.9	4.2	4.1
Between-day C.V. (%)	10.3	17.7	9.0	7.7	3.6	4.3	8.1	5.2
Relative recovery[a]								
Mean concentration added (mg/l)	23.7	101.8	6.1	26.3	12.4	53.2	6.3	27.1
Recovery (mean ± S.D.) (%)	105 ± 19	104 ± 17	115 ± 10	108 ± 9	105 ± 6	103 ± 5	88 ± 7	94 ± 5
Detection limit[b] (mg/l)	4.0		0.2		0.1		0.5	
Upper limit of linearity[c] (mg/l)	135		35		70		36	
Calibration graph[d]								
Slope ± standard error	0.00714 ± 0.00023		0.03242 ± 0.00079		0.03702 ± 0.00036		0.04266 ± 0.00055	
Intercept ± standard error	0.0366 ± 0.0181		0.0048 ± 0.0159		0.0245 ± 0.0147		−0.0151 ± 0.0115	
Correlation coefficient	0.969		0.982		0.997		0.995	

n = Number of assays; S.D. = standard deviation.
[a] Precisions and recoveries were calculated for two concentration levels, cumulating analytical data obtained from the two lowest or the two highest supplemented samples.
[b] Defined as the level corresponding to three times the peak (for acetylcarnitine) or the background noise (for the other acylcarnitines) observed in blanks.
[c] Higher concentrations were not tested.
[d] Regression line of acid peak area/internal standard peak area as a function of concentration in mg/l.

some of them are recovered in the extract, the most volatile being in fact eliminated during the freeze-drying step included in this extraction procedure. In contrast, SCX columns are able to yield a pure extract, long-chain fatty acids being efficiently removed on washing the column with an acetonic acidic solution but not if an aqueous acidic solution is used. This optimized sample preparation avoids the two- or three-step extraction procedures described previously [16–20]. We also tested several eluents, comparing sodium hydroxide and ammonium solution, 0.1 and 1 mol/l concentrations, different aqueous–ethanolic media and different elution volumes. With 0.1 mol/l ammonia solution in ethanol–water (65:35, v/v), 90–100% of each acylcarnitine (depending on the acyl moiety) added to the column was recovered in 3 ml of eluate.

The extraction of the acids liberated by the mild alkaline hydrolysis is the most crucial step. We compared two solid-phase extractions (on octadecanoyl- and aminopropyl-bonded phase columns) and several liquid–liquid extractions (*n*-hexane, chloroform, ethyl acetate, methyl isobutyl ketone, acetonitrile, acetone, ethanol, diethyl ether and diisopropyl ether). With the last solvent, several acidifying agents (hydrochloric, sulfuric, phosphoric, trichloroacetic and formic acid) and the addition of sodium or calcium chloride were also tested. The most efficient extraction was achieved by evaporating the hydrolysed mixture to dryness and taking up the

Table 2
Urine specimens from fourteen patients with organic aciduria: proportion of the identified acylcarnitines (as a percentage of the sum of their concentrations in mg/l)

Patient No.	Diagnosis	C2[a]	C3	Ciso4[b]	C4	Cbr5[c]	C5	Tiglic[c]	C6	C8:1[d,e]	C8
1	Propionic acidaemia	7	93								
2	Isovaleric acidaemia	45	7	2	4	42					
3		37	4			59					
4	Methylmalonic acidaemia	35	59	1	2				3		
5		89	9			2					
6		55	41		3				1		
7	Ethylmalonic/adipic aciduria	19	1	4	63	5	1		3	1	3
8	Medium-chain acylCoA										
9	dehydrogenase deficiency	47	2			2			8	2	39
10		38	2			1			6	3	50
11		76	7		3				3		11
		42	5	4	1	1			7	1	39
12	3-Hydroxydicarboxylic aciduria	87	6	1	2				1	2	1
13	2-Methylacetoacetyl-CoA										
14	thiolase deficiency	46	8	5	3	3		35			
		52	8	4				36			
15	Typical normal urine	90	2	5						3	

[a] C2 = Acetyl-; C3 = propionyl-; Ciso4 = isobutyryl-; C4 = butyryl-; Cbr5 = isovaleryl- and/or 2-methylbutyryl-; C5 = valeryl-; C6 = hexanoyl-; C8:1 = 6-octenoyl-; C8 = octanoylcarnitine.
[b] Expressed in equivalents of butyrylcarnitine.
[c] Expressed in equivalents of valerylcarnitine.
[d] The identity of this peak is not yet certain. As suggested by mass spectra and literature data [6,14,16], it is presumed to be 6-octenoic acid.
[e] Expressed in equivalents of octanoylcarnitine.

residue without the used of a salting-out reagent. Hydrochloric acid was used in the beginning of our study but was replaced with phosphoric acid because of rapid degradation of the chromatographic resolution. Finally, the use of an ultrasonic bath is of decisive importance, as some dried hydrolysed urine extracts are otherwise poorly taken up.

As trimethylsilylation or methylation of the acids yields a reagent peak that obscures short-chain fatty acids, capillary chromatography of the underivatized fatty acids on a polar phase was chosen in order to permit the separation of most of the acyl species of clinical interest. In fact, all even- and odd-carbon acids, from acetic to stearic, and also isobutyric, isovaleric, valproic, pivalic and levulinic acid, and the acid presumed to be 6-octenoic acid are resolved. Glutaric and other dicarboxylic acids cannot be chromatographed without derivatization.

From a qualitative point of view, our method is applicable to all acylcarnitines, including acetyl and propionyl, with the exception of dicarboxyl esters. Although no interfering substances have been found, the possibility that metabolites other than acylcarnitines could yield fatty acid peaks should remain in mind. However, this possibility is unlikely as these metabolites at the same time should be cationic and include a hydrolysable acyl moiety. Analysis of several pathological urines has shown the diagnostic value of our procedure, which is relatively rapid and simple,

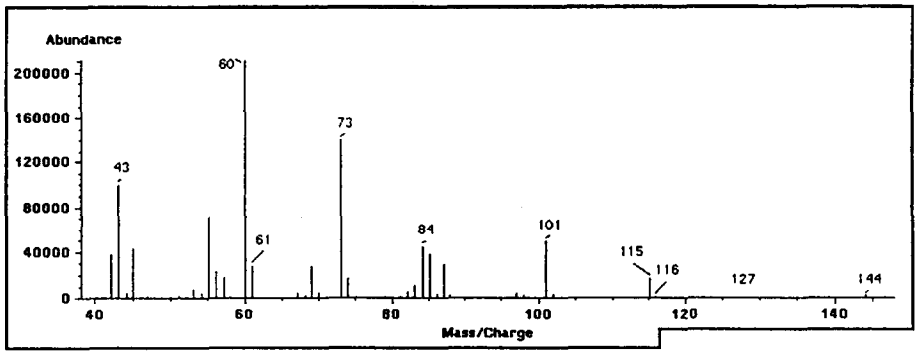

Fig. 2. Electron impact mass spectrum of the peak C8 found in a case of medium-chain acylCoA dehydrogenase deficiency (patient 11 in Table 2). The fragmentation pattern is identical with that given by authentic octanoic acid.

owing to the use of one solid-phase extraction on a disposable column.

A possible confirmation of the identity of the peaks can be provided by GC–MS: concentration at room temperature of the organic extract to about 30 μl and splitless injection of a 3-μl aliquot is sufficient to operate in the scanning mode and to yield interpretable mass spectra on a Hewlett-Packard Model 5970 mass-selective detector. An example of a mass spectrum is given in Fig. 2.

From a quantitative point of view, our method amply achieves medically acceptable detectability, linearity and precision. The recoveries relative to an aqueous standard range from 88 to 115% (Table 1). Standards should then be prepared in urine or, if aqueous standards are used, a correction in proportion to the recoveries should be introduced. As far as the acetylcarnitine is concerned, a higher limit of detection (near the lower limit of the physiological levels) is encountered. Our method is thus fully indicated for the analysis of pathological specimens where the acetylcarnitine excretion is elevated or where other carnitine esters are to be determined.

In conclusion, the described method is suitable for qualitative and quantitative analyses for abnormal acylcarnitines. Although it cannot compete with FAB tandem MS in terms of specificity and rapidity [14], it offers the advantage of being accessible to all non-specialized laboratories, as no expensive apparatus is needed.

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