paper on methodology

Quantitative analysis of plasma acylcarnitines using gas chromatography chemical ionization mass fragmentography

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Abstract A stable isotope dilution gas chromatography chemical ionization mass spectrometry (GC–CI–MS) method was developed for the quantitative profiling of plasma acylcarnitines. The clean-up procedure was comprised of a solid-phase cation exchange extraction using PRS-columns from which the acylcarnitines were eluted with a barium chloride solution. Isolated acylcarnitines were transformed into acyloxylactones and analyzed by positive GC–CI–MS using isobutane as reactant gas. The selected monitoring of a common ion at m/z $[85]^+$ and the protonated molecular ion enabled a selective and sensitive detection of all C_2–C_{18} acylcarnitines. An accurate quantitation was achieved by the use of stable isotope-labeled internal standards (C_2–C_{18}) and acylcarnitines could be analyzed in the sub-nanomolar range. Control values for C_2–C_{18} acylcarnitines in plasma were established. Concentrations ranged from 0.02 μmol/L for C_{14}-acylcarnitine to 4.90 μmol/L for C_2-acylcarnitine. The diagnostic suitability of the method was demonstrated for patients with medium-chain acyl-CoA dehydrogenase deficiency and very long-chain acyl-CoA dehydrogenase deficiency.—**Costa, C. G., E. A. Struys, A. Bootsma, H. J. ten Brink, L. Dorland, I. Tavares de Almeida, M. Duran, and C. Jakobs.** Quantitative analysis of plasma acylcarnitines using gas chromatography chemical ionization mass fragmentography. *J. Lipid Res.* 1997. **38:** 173–182.

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Carnitine (β-OH-γ-trimethylaminobutyric acid) is a quaternary amine with a key role as a carrier of long-chain fatty acids into the mitochondrial matrix. Prior to β-oxidation, long-chain fatty acids are activated to acyl-coenzyme A (CoA) esters which are unable to cross the mitochondrial membrane. However, a transesterification to an acylcarnitine by carnitine palmitoyltransferase I (CPT I) allows them to readily transverse the mitochondrial membrane by the action of carnitine-acylcarnitine translocase. Once inside the mitochondrial matrix, acylcarnitines are converted back to the acyl-CoA by carnitine palmitoyltransferase II (CPT II) while carnitine is regenerated. It is the resulting acyl-CoA that enters now the β-oxidation cycle (1, 2).

Whereas the import of long-chain fatty acids into the mitochondria is the main function of carnitine, several other metabolic roles have been elucidated, including *a*) export of fatty acids from mitochondria and peroxisomes, *b*) restoration of the free CoA/esterified CoA ratio, and *c*) removal of potentially toxic acyl-CoA groups accumulating as a consequence of the metabolic block and thus allowing them to leave the cell as acylcarnitines (3). As a consequence, patients with a disturbed fatty acid oxidation or branched-chain amino acid catabolism will accumulate abnormal acyl-CoA species (at or near the metabolic block), eventually leading to the accumulation of related unusual acylcarnitines (4, 5). These processes make acylcarnitine profiling an important biochemical tool for the diagnosis of various inherited metabolic diseases. Especially good results were ob-

Abbreviations: GC, gas chromatography; MS, mass spectrometry; CI, chemical ionization; FAB/MS/MS, fast atom bombardment tandem mass spectrometry; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; CoA, coenzyme A; CPT I, carnitine palmitoyl transferase I; CPT II, carnitine palmitoyl transferase II; MCAD, medium-chain acyl-CoA dehydrogenase; vLCAD, very long-chain acyl-CoA dehydrogenase; MCT, medium-chain triglyceride.

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served in the β-oxidation defects where the other diagnostic approaches are often inconclusive. However, its diagnostic importance fully relies on well-defined methods for the isolation and separation of the acylcarnitines (6). Several methods using chromatographic separation of acylcarnitines have been published including thin-layer chromatography (7), high performance liquid chromatography (HPLC) (8–10), gas chromatography (GC) (11), or gas chromatography mass spectrometry (GC–MS) (12, 13). However, until now most of our knowledge of acylcarnitines was derived from the fast atom bombardment tandem mass spectrometry (FAB/MS/MS) which combines the advantages of speed, little sample preparation, and sensitivity, but on the other hand is very much compromised by the need for sophisticated and high cost instrumentation (6). All these methods have been carefully reviewed by Lowes and Rose (14) and Millington et al. (15). Virtually all chromatographic methods were applied only to urine which is probably not the ideal material for acylcarnitine profiling, because long-chain acylcarnitines tend to remain in the plasma or membrane-bound. Thus, in long-chain fatty acid oxidation disorders, urinary excretion of acylcarnitines is very low and theoretically should not occur (16). On the other hand, total plasma long-chain acylcarnitines in normal controls have been estimated to be approximately 2 μmol/L (17). Therefore, a very sensitive method for the quantitation of individual long-chain acylcarnitines in the sub-micromolar level is required.

Our goal was to develop a method for the analysis of plasma acylcarnitines that combines simplicity, reliability, and sensitivity, thus enabling moderately equipped laboratories to perform the analyses. In this paper we present a stable isotope dilution GC–CI–MS method for the accurate quantitation of acylcarnitines in plasma. Examples of its diagnostic suitability are illustrated by the analysis of plasma samples from known patients with proven MCAD and vLCAD deficiency. Moreover, normal control values were also established for all acylcarnitines studied ranging from C_2 to C_{18}.

MATERIAL AND METHODS

Chemicals

Acylcarnitine hydrochlorides with chain lengths of C_2, C_3, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}, and C_{18} were obtained from Sigma Chemical Company (Poole, UK) while C_4, isoC_4, and isoC_5 were a gift from Dr. P. Verhaege (Gent, Belgium).

Methanol and water were HPLC grade and were obtained from E. Merck AG (Darmstadt, Germany). Extra dry acetonitrile was DNA synthesis grade (Biosolve, Barneveld, The Netherlands). Ethylacetate was analytical grade and was obtained from J. T. Baker B. V. (Deventer, The Netherlands).

Methanolic HCl was obtained from Supelco (Bellefonte, PA) and N,N-diisopropylethylamine (98%) was from Janssen Chimica (Beerse, Belgium). Cation-exchange solid-phase extraction columns (PRS, Analytichem Bond Elut), containing 500 mg of silica matrix with covalently bonded propylsulfonic acid groups (sodium form) were obtained from Varian Analytical Equipment (Harbor City, CA).

Synthesis of labeled acylcarnitines

Deuterium-labeled acylcarnitines, with labeling at the terminal carbon atom, were synthesized essentially as described by Ziegler, Bruckner and Binon (18). In a typical preparation, L-carnitine·HCL was acylated by reaction with a slight excess of labeled acyl chloride, (prepared from the appropriate labeled acid and thionyl chloride by heating at 70°C for 4 h) by heating at 45°C for 18 h in trichloroacetic acid. After cooling, the product was precipitated by addition of diethyl ether, separated, and washed 3 times with diethyl ether, 2 times with acetone, and again with diethyl ether. Deuterium-labeled acyl-L-carnitine chlorides were obtained in yields of 80–90% on a 1 mmol scale. Their purities were checked by ^{1}H-NMR analysis (^2H_2O).

Biological samples

Controls. Eleven fresh plasma samples were taken from hospitalized children after an overnight fast. Their ages ranged from three months to twelve years. All were receiving their usual (MCT-free) diet at the time of sample collection. An inborn error of metabolism was excluded by our usual screening for disorders of amino acid, organic acid, oligosaccharides, mucopolysaccharides, and purine/pyrimidine metabolism.

Patients. We analyzed 4 plasma samples from 3 patients with a proven fatty acid oxidation disorder. One of the patients had medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, he was homozygous for the common A985G mutation (19). The other two patients had a very long-chain acyl-CoA dehydrogenase (vLCAD) deficiency (20) proven by measurement of the enzyme activity in fibroblasts (Dr. C. Vianey-Saban, Lyon). Two of the 3 vLCAD samples had been stored at −20°C for up to 7 years.

Sample preparation

The solid-phase, cation-exchange procedure used here was based on a method developed for urine acylcarnitines (6).

Plasma samples (500 µl) were deproteinized with 2 ml of methanol after the addition of 50 µl of labeled internal standard mixture (the concentration of each individual internal standard is given in Table 1). After centrifugation (3000 rpm for 5 min), the supernatant was diluted with 3 ml of water and acidified to pH 2 with 0.5 M HCl. This mixture was then applied on top of an activated PRS column (6) and the sample was allowed to run dropwise. The column was washed twice with 3 ml of water and twice with 3 ml of methanol. Acylcarnitines were finally eluted with 3 ml of a 0.04 M $BaCl_2$ solution (in methanol–water, 75:25, v/v). The eluate was then evaporated to dryness at 50°C under a gentle stream of nitrogen. To the residue 3 ml of acetonitrile was added and agitated for 5 min in the ultrasonic bath. After centrifugation (4000 rpm, 10 min), the supernatant was collected and dried at room temperature under a gentle stream of nitrogen. Acylcarnitines were finally transformed into acyloxylactones (12) by adding 750 µl of 0.75‰ diisopropylethylamine in acetonitrile and heating at 130°C for 45 min. After allowing the tubes to cool down, the derivatization solution was evaporated at room temperature with a stream of nitrogen and the residue was dissolved in 50 µl of ethylacetate. One or 2 µl of the ethylacetate solution was used for GC–MS analysis.

GC–CI–MS analysis

GC–CI–MS analysis was carried out with an Automass Serie I, ATIUnicam (Cambridge, England) quadrupole mass spectrometer interfaced with a 610 ATIUnicam gas chromatograph (GC). Samples were introduced using a cold on-column injector and GC separation was achieved on a WCOT fused silica capillary column (25 m × 0.32 mm ID) coated with CPsil8 CB-MS, film thickness 0.4 µm (Chrompack BV, Middelburg, The Netherlands). The column oven temperature was held at 100°C for 1 min and then programmed to 280°C at a rate of 10°C/min. The final temperature was maintained for 5 min. Helium (99.996% pure) was used as carrier gas at a pressure of 0.6 bar (flow rate 2 ml/min.). The column was inserted directly into the ion source of the mass spectrometer and selected ion monitoring was performed in the positive ion mode. The interface was kept at 280°C and the source temperature was 200°C. For chemical ionization mass spectrometry, isobutane was used as reactant gas and ionization was performed with 120 eV electrons with an emission current of 0.3 mA. Detection of target compounds was performed by single ion recording of the ion intensities from a common fragment ion of acyloxylactones at m/z $[85]^+$ and from a specific ion from each individual acylcarnitine at m/z $[M + H]^+$ with a dwell time of 50 ms.

Quantitation and linear response

Standard curves were obtained in the concentration range of interest by spiking 500 µl of a plasma pool with 0, 5, 10, 20, 50, 100, and 500 µl of a standard mixture of acylcarnitines (C_2 to C_{18}) where concentrations varied between 2.28 and 13.45 µmol/L (see Table 1). Fifty µl of the stable isotope-labeled internal standard mixture was used. Sample preparation was the same as for controls and patients. The peak–area ratios of unlabeled over labeled were calculated and submitted to a linear regression analysis that was posteriorly used to calculate the concentrations of plasma acylcarnitines. Unsaturated acylcarnitines were quantitated on the basis of the calibration curve from the respective saturated acylcarnitine. Butyrylcarnitine was also quantified on the basis of the calibration curve from the respective iso form.

Reliability

Extraction recoveries from plasma for 11 acylcarnitines were determined by comparing the peak–area ratios (standard/internal standard) after extraction from a pool plasma with the ones obtained for a directly derivatized standard mixture. Four 500-µL aliquots of standard solution were freeze-dried after the addition of 50 µL of labeled internal standard solution. Two of them were redissolved in 500 µL of plasma pool and extracted as described above. Samples were finally analyzed in triplicate by GC–CI–MS.

Interassay reproducibility or day-to-day variability was assessed by quantifying the acylcarnitines in six pool plasmas extracted and analyzed on 6 different days. Concentrations were determined based on the calibration curve processed on the same day (average concentration, coefficients of variation, and standard deviation for each acylcarnitine were calculated).

Precision. A pool plasma spiked with 50 µL of standard mixture was extracted after the addition of 50 µL of internal standard mixture. This sample was then injected 5 times in the GC–MS system and the individual acylcarnitine concentration was determined on the basis of the calibration curves processed on the same day.

RESULTS

Chemical ionization mass spectrometry from acyloxylactones generates mass spectra that show prominent ions corresponding to $[M + H]^+$. However, fragmentation was also characterized by a common ion for all acylcarnitines at m/z $[85]^+$ which was minimal for short- and medium-chain acylcarnitines, while for long-chain acylcarnitines it gave rise to the base peak (21). This

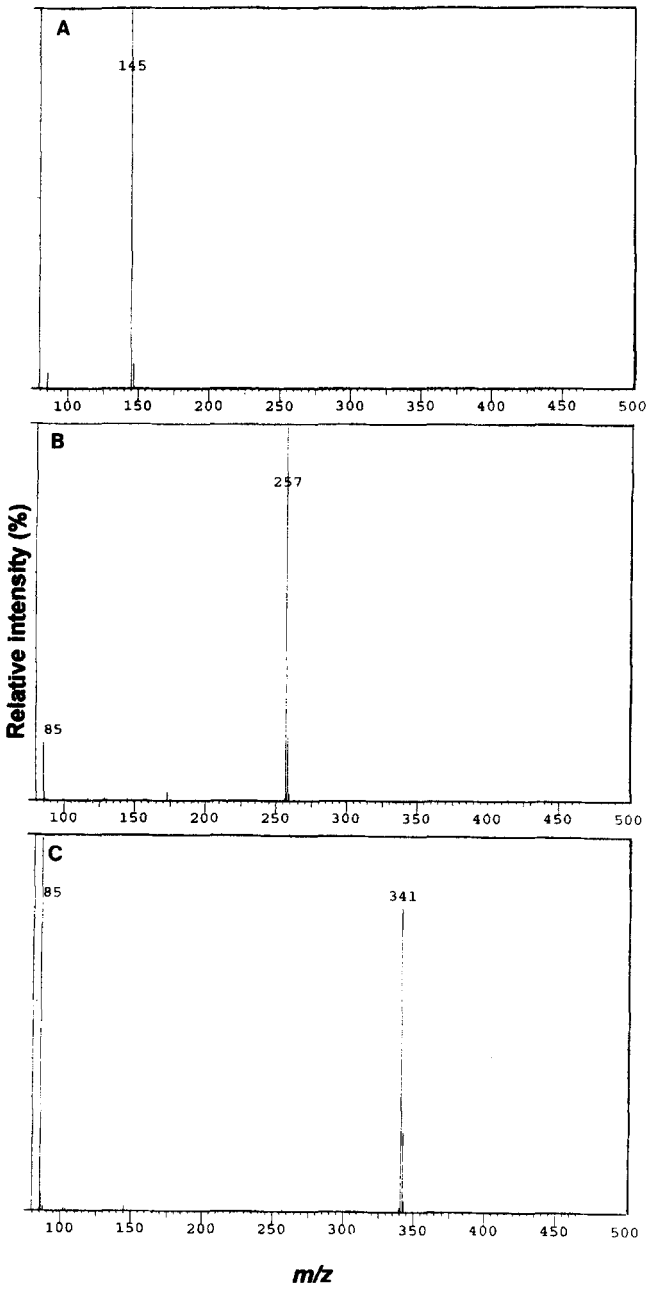

Fig. 1. Mass spectra of acyloxylactones obtained by CI–MS using isobutane as moderating gas; A: acetylcarnitine; B: decanoylcarnitine; C: hexadecanoylcarnitine.

highly characteristic fragment represents the lactone as such and corresponds to the loss of the acyl moiety of acylcarnitines (**Fig. 1**).

Figure 2 represents the GC–CI–MS chromatogram recorded in the single ion monitoring mode from a pool plasma spiked with a standard mixture of acylcarnitines. Despite the fact that the presented chromatogram is just the result from the single ion monitoring of the common ion at m/z $[85]^+$ and thus much less sensitive than $[M + H]^+$, it readily demonstrates that the chromatographic conditions permitted baseline resolution of all the carnitine components.

Standard curves for quantifying acylcarnitines were set up by the addition of known amounts of a standard mixture to a pool plasma (**Table 1**). Even using an extensive range of concentrations (e.g., 0.022–2.20 μmol/L for isobutyrylcarnitine or 0.135–13.45 μmol/L for octadecanoylcarnitine) all the calibration curves showed a linear relationship between concentration and peak area, while correlation coefficients were in all cases $\geq$ 0.998. Detection limits were determined in plasma samples and varied from 1 nmol/L for the short-chain (C_2–C_4) acylcarnitines to 25 nmol/L for the long-chain ($\geq C_{14}$) acylcarnitines, although with some variation even between the same chain-length depending on the specific acylcarnitine. Extraction recoveries for the different acylcarnitines ($C_2$to C_{18}) from plasma are presented in **Table 2.** In addition, this table shows the interassay reproducibility and the precision of the method for the quantitation of plasma acylcarnitines. As such, sensitivity and reliability of the method appeared suitable for the determination of physiological levels of acylcarnitines in clinical samples.

We determined control values for all the acylcarnitines studied. Eleven plasma samples were taken from control children with no evidence of a metabolic disease. As expected, the plasma acylcarnitine profile was characterized by the predominance of acetylcarnitine, followed by propionylcarnitine. The other acylcarnitines (C_4 to C_{18}), although present, had much lower concentrations. Among these, palmitoyl and stearoylcarnitine were the most abundant ones with concentrations 3-fold in excess of the acylcarnitines with a chain length from C_4 to C_{14} (**Table 3**).

To show the diagnostic power of our method, we analyzed four plasma samples from well-characterized patients with MCAD and vLCAD deficiency. In the MCAD-deficient patient, octanoylcarnitine was the predominant acylcarnitine followed by decenoylcarnitine and hexanoylcarnitine. Octenoylcarnitine was not detected in this patient. For the vLCAD patients, the acylcarnitine profile was characterized by the prominent accumulation of long-chain acylcarnitines, whose profile was more or less parallel to the one usually observed for the plasma free fatty acids in vLCAD deficiency. A high level of tetradecenoylcarnitine dominated the acylcarnitine profile while specimens with a chain-length of C_{16} and C_{18} were also elevated. Concentrations of each individual plasma acylcarnitine from these patients are given in Table 3.

Figure 3 shows the GC–CI–MS chromatogram recorded in the single ion monitoring mode of plasma

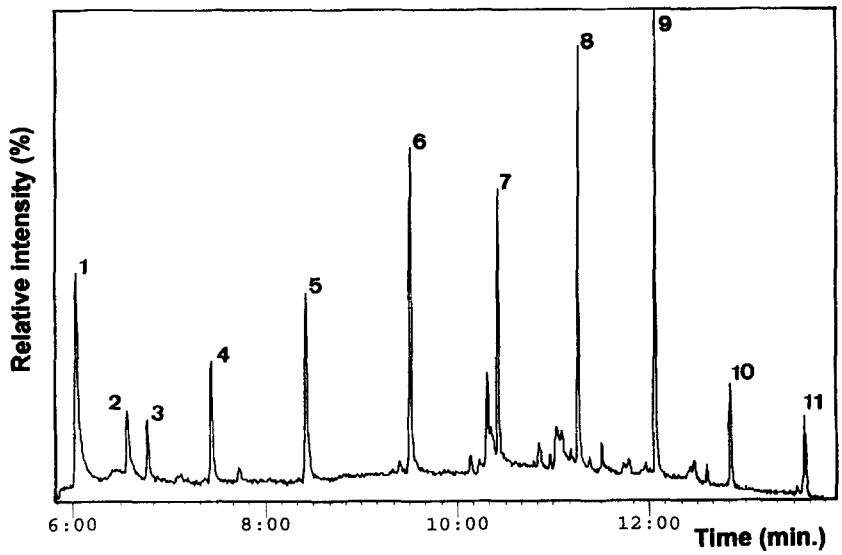

Fig. 2. Ion current trace for CI–MS at m/z $[85]^+$ from a pool plasma spiked with a mixture of acylcarnitines containing: 1, acetylcarnitine (0.56 nmol); 2, propionylcarnitine (0.61 nmol); 3, isobutyrylcarnitine (0.23 nmol); 4, isovalerylcarnitine (0.50 nmol); 5, hexanoylcarnitine (0.70 nmol); 6, octanoylcarnitine (0.70 nmol); 7, decanoylcarnitine (0.38 nmol); 8, dodecanoylcarnitine (0.68 nmol); 9, tetradecanoylcarnitine (0.77 nmol); 10, hexadecanoylcarnitine (0.83 nmol); and 11, octadecanoylcarnitine (1.35 nmol).

TABLE 1. Concentration of each individual acylcarnitine in both standard or internal standard mixture

Standard Mixture		Internal Standard Mixture	
Acylcarnitine	Conc (μM)	Acylcarnitine	Conc (μM)
Acetylcarnitine	5.58	[$^2H_3$]acetylcarnitine	2.23
Propionylcarnitine	6.10	[$^2H_3$]propionylcarnitine	2.44
Isobutyrylcarnitine	2.28	[$^2H_7$]butyrylcarnitine	0.91
Isovalerylcarnitine	5.00	[$^2H_9$]valerylcarnitine	2.00
Hexanoylcarnitine	6.95	[$^2H_3$]hexanoylcarnitine	2.78
Octanoylcarnitine	7.00	[$^2H_3$]octanoylcarnitine	2.80
Decanoylcarnitine	3.75	[$^2H_3$]decanoylcarnitine	1.50
Dodecanoylcarnitine	6.80	[$^2H_3$]dodecanoylcarnitine	2.78
Tetradecanoylcarnitine	7.70	[$^2H_3$]tetradecanoylcarnitine	3.08
Hexadecanoylcarnitine	8.30	[$^2H_3$]hexadecanoylcarnitine	3.32
Octadecanoylcarnitine	13.45	[$^2H_3$]octadecanoylcarnitine	5.38

TABLE 2. Extraction recoveries from plasma, reproducibility, and precision for 11 acylcarnitines

Acylcarnitine	Recovery (n = 3)	Precision (n = 5)			Day-to-Day Reproducibility (n = 6)		
		$\bar{X}$	CV%	SD	$\bar{X}$	CV%	SD
	%	μmol/L			μmol/L		
Acetylcarnitine	42	8.861	1.02	0.090	6.818	5.31	0.362
Propionylcarnitine	47	1.214	0.62	0.008	0.518	8.91	0.046
Isobutyrylcarnitine	54	0.396	1.16	0.005	0.159	6.70	0.011
Isovalerylcarnitine	60	0.600	1.99	0.012	0.053	9.70	0.005
Hexanoylcarnitine	62	0.959	1.45	0.014	0.147	7.84	0.012
Octanoylcarnitine	62	0.832	0.47	0.004	0.174	8.03	0.014
Decanoylcarnitine	67	0.560	1.75	0.009	0.197	7.82	0.020
Dodecanoylcarnitine	66	0.712	1.21	0.009	0.064	7.02	0.005
Tetradecanoylcarnitine	62	0.774	1.36	0.011	0.027	15.44	0.004
Hexadecanoylcarnitine	56	1.021	0.84	0.009	0.265	9.95	0.026
Octadecanoylcarnitine	52	1.322	0.80	0.011	0.059	10.96	0.007

TABLE 3. Concentration (μmol/L) of plasma acylcarnitines from control children, MCAD patient, and vLCAD patients, along with the detection limits for some of the acylcarnitines

Acylcarnitine	MCAD (n = 1)	vLCAD (n = 3)[a]		Controls (n = 11)	
		Mean	Range	Mean	Range
C2	0.512	0.316	0.091–0.760	4.90	2.48–8.62
C3	0.003	0.058	0.022–0.085	0.564	0.209–1.159
C4	0.011		<0.005–0.009	0.079	0.023–0.174
IsoC4	0.009	0.034	0.009–0.084	0.069	0.015–0.203
IsoC5	0.020	0.052	0.022–0.068	0.049	0.032–0.082
C6	0.279		<0.001–0.004	0.031	0.015–0.053
C8	1.484	0.025	0.014–0.034	0.061	0.006–0.127
C10	0.084		<0.005–0.012		<0.005–0.124
C10:1	0.212		<0.005		<0.005–0.057
C12	0.018	0.124	0.090–0.162	0.038	0.005–0.069
C12:1	0.165	0.353	0.276–0.461		<0.005
C14	0.024	0.719	0.478–0.983	0.016	0.002–0.043
C14:1(1)	0.035	5.223	2.658–7.891	0.032	0.005–0.097
C14:1(2)	0.004	0.278	0.105–0.595	0.008	0.002–0.023
C14:2	0.016	1.088	0.655–1.469	0.015	0.002–0.046
C16	0.224	1.909	1.012–2.233	0.153	0.073–0.227
C16:1(1)	0.021	1.554	0.710–2.911		<0.005–0.044
C16:1(2)	0.026	0.412	0.105–0.883		<0.005–0.042
C16:2	0.010	0.907	0.277–1.484		<0.010
C18	0.087	0.408	0.293–0.525	0.053	0.027–0.086
C18:1	0.326	1.591	0.764–2.981	0.165	0.084–0.316
C18:2	0.121	0.710	0.201–1.155	0.090	0.039–0.165

[a]Three plasma samples from two different vLCAD patients.

acylcarnitines from a vLCAD patient. Special attention is given to the power of the chromatographic system that allowed us to readily quantify 3 different acylcarnitines with the same chain length and whose respective retention times differ by less than 10 sec.

As a summary, **Fig. 4** illustrates the differences in the plasma acylcarnitines between control subjects, patients with MCAD deficiency, and patients with vLCAD deficiency.

DISCUSSION

Acylcarnitine profiling either in plasma or urine has been claimed to be one of the most valuable diagnostic tools for determining inborn errors of fatty acid oxidation (22). Until now, several methods for their analysis have been published. However, the most reliable ones require sophisticated and costly instrumentation such as the FAB-MS/MS procedure, while the more accessible GC–MS methods have been used successfully only in a qualitative way. Our goal was to achieve a GC–MS procedure for the quantitative acylcarnitine analysis in plasma. However, due to the wide range of polarities from short- to long-chain acylcarnitines, their simultaneous extraction is difficult. Most of the extraction procedures such as the one developed by Norwood, Kodo, and Millington (23) and Millington et al. (24) and later on used by Lowes and Rose (11) or the one used by Huang et al. (12) involve a double ion exchange extraction which limited its suitability for a routine analytical procedure.

Based on a faster clean-up procedure by solid-phase cation exchange extraction (6) and using acyloxylactone formation (11), we developed a GC–MS method for the quantitative plasma acylcarnitine analysis. For the first time, quantitative data from short- to long-chain acylcarnitines could be achieved by a GC–MS procedure. After deproteinization with methanol, we simultaneously extracted all acylcarnitines from plasma including the protein-bound long-chain acylcarnitines. The subsequent clean-up by solid-phase cation exchange extraction resulted in very clean acylcarnitine extracts that contributed enormously to the selectivity of the method. Moreover, detection of acyloxylactones by chemical ionization mass fragmentography with isobutane as the reactant gas proved to be of crucial importance to achieve the necessary sensitivity, especially for long-chain acylcarnitines that could finally be detected in concentrations down to 25 nmol/L.

Methane and ammonia were also tried but fragmentation of acylcarnitines was much higher than with isobutane. A low source temperature of 200°C was also used in order to decrease fragmentation. However, the problem of sensitivity for long-chain acylcarnitines does not deal only with their high fragmentation in the ion source but also with their breakdown during lactoniza-

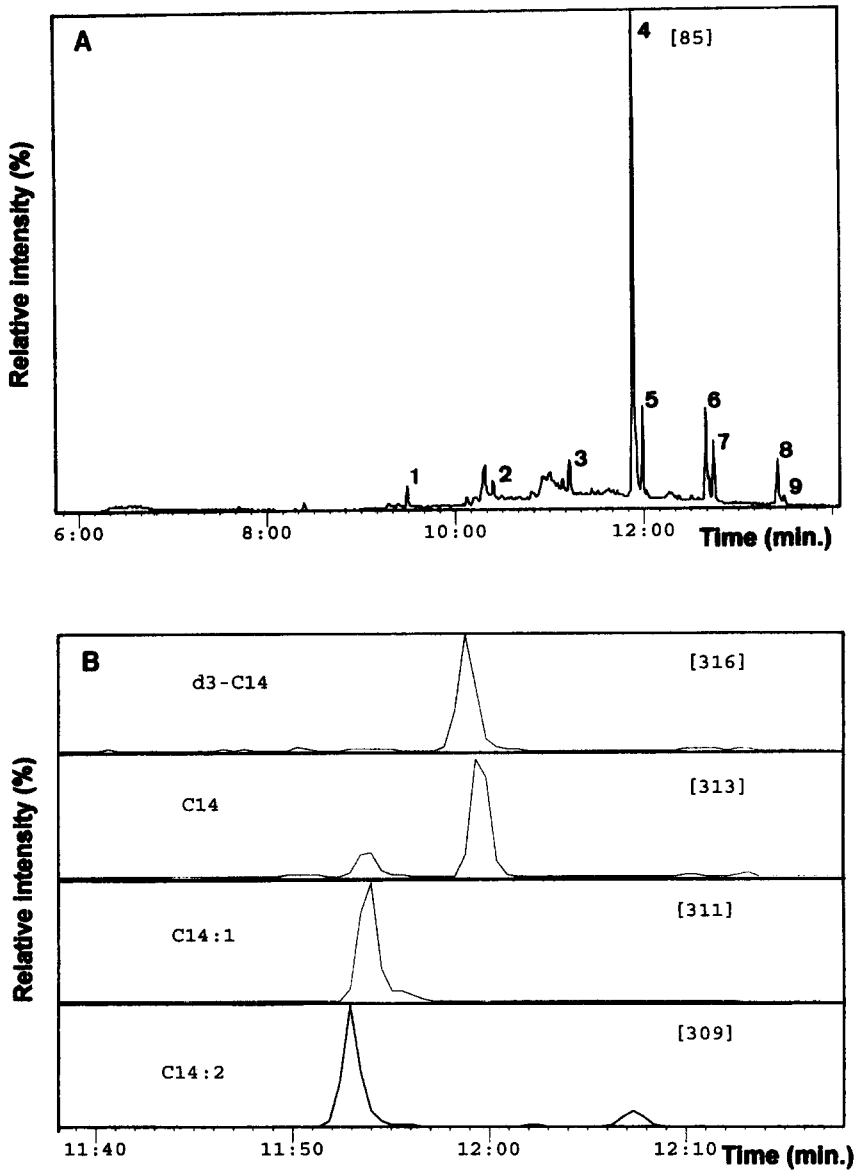

Fig. 3. Ion current trace for GC–CI–MS from a vLCAD patient. A: Represents the single ion monitoring at m/z $[85]^+$: 1, octanoylcarnitine; 2, decanoylcarnitine; 3, dodecanoylcarnitine; 4, tetradecenoylcarnitine + tetradecadienoylcarnitine; 5, tetradecanoylcarnitine + $[^2H_3]$-tetradecanoylcarnitine; 6, hexadecenoylcarnitine + hexadeca-dienoylcarnitine; 7, hexadecanoylcarnitine + $[^2H_3]$-hexadecanoyl-carnitine; 8, octadecenoylcarnitine + octadeca-dienoylcarnitine; 9, octadecanoylcarnitine + $[^2H_3]$-octadecanoylcarnitine. B: Single ion monitoring of $[M + H]^+$ from d3-tetradecanoylcarnitine at m/z $[316]^+$, tetradecanoylcarnitine at m/z $[313]^+$, tetradecenoylcarnitine at m/z $[311]^+$ and tetradeca-dienoylcarnitine at m/z $[309]^+$.

tion and in the GC injection port. This was overcome by a careful control of time and temperature during lactonization and by the use of a cold on-column injector. A split/splitless injector was also tried, again with acceptable results for short- and medium-chain but not for long-chain acylcarnitines. Moreover, stable isotope-labeled internal standards were shown to have an important role not only as quantitative standards but also as a carrier, whereby they protect against the loss of small amounts of endogenous acylcarnitines during the sample preparation. As such, labeled internal standards were essential for the sensitivity and reproducibility demonstrated for the overall analytical procedure.

The reliability of this quantitative plasma acylcarnitine profiling for the diagnosis of some of the inborn errors of fatty acid oxidation has been illustrated by the data obtained for MCAD and vLCAD patients as well as for normal children. The end product of an undis-

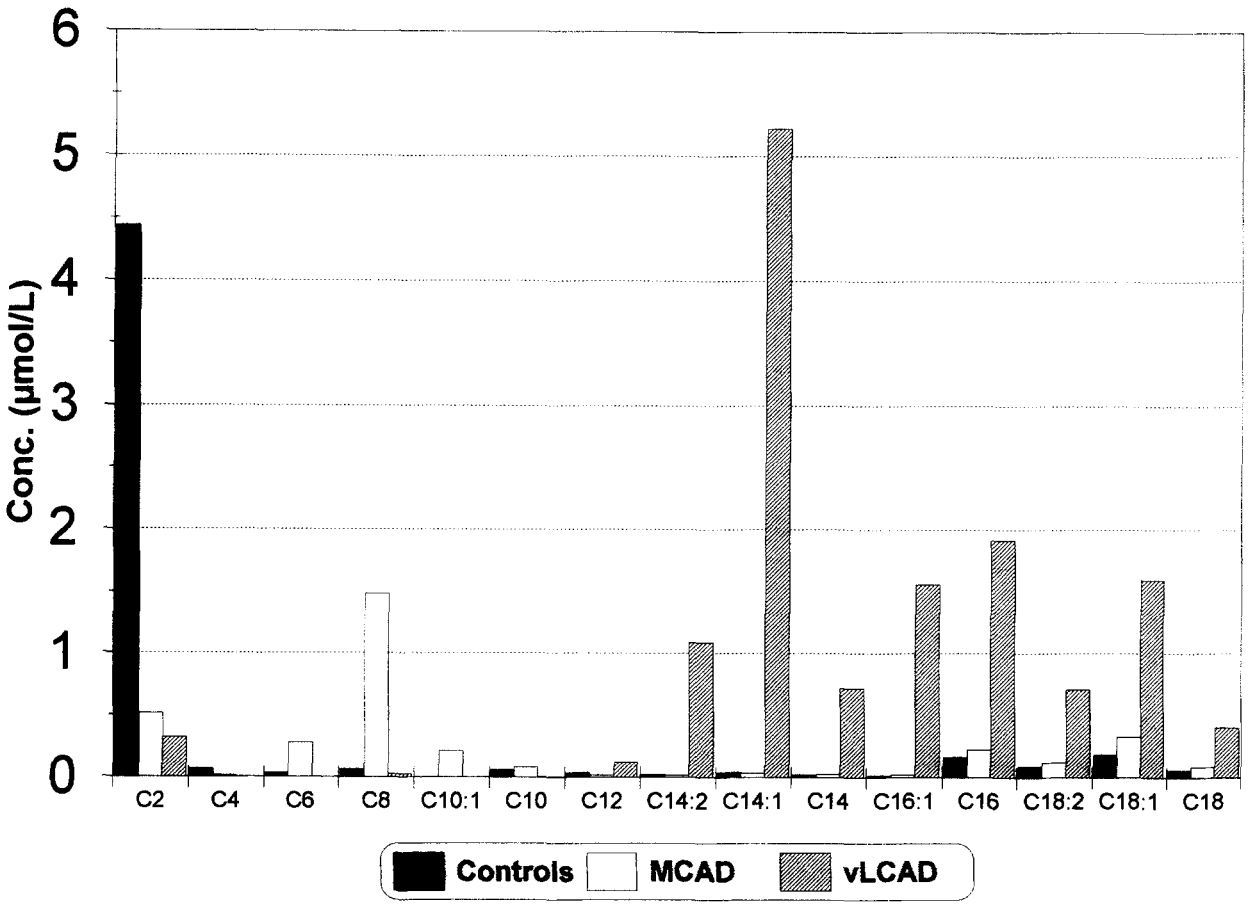

Fig. 4. Concentration (average) of plasma acylcarnitines from normal controls, as well as MCAD and vLCAD patients.

turbed β-oxidation is acetyl-CoA. Therefore, under the conditions of an undisturbed intermediary metabolism, virtually all esterified carnitine in plasma is represented by acetylcarnitine (4). However, all the other acylcarnitines from C_3 to C_{18} could also be quantified. Until now very few details about the levels of medium- and long-chain acylcarnitines in plasma, as well as about the physiological significance of their presence in normal subjects, have been reported.

Our data suggest that in controls straight-chain acylcarnitines (C_4 to C_{14}) originating from the β-oxidation of long-chain fatty acids have approximately the same low basal level, whereas long-chain acylcarnitines such as C_{16} and C_{18} appear in controls as the most abundant acylcarnitines after acetyl and propionylcarnitine. This more or less reflects the high concentration of the analogous fatty acids in plasma, i.e., palmitic, oleic, linoleic, and stearic acids.

In contrast with normal plasma, where acetylcarnitine is the predominant carnitine ester, patients with a fatty acid oxidation defect have a characteristically low acetylcarnitine due to the impaired β-oxidation and thus to the reduced production of acetyl-CoA. In the MCAD-deficient patient, the plasma acylcarnitine pattern was dominated by octanoylcarnitine, which can be explained by the low affinity of mitochondrial glycine-N-acylase to octanoyl-CoA while it is active with hexanoyl-CoA and suberyl-CoA (25). On the other hand, the acylcarnitine profiles from vLCAD patients were characterized by the accumulation of long-chain acylcarnitines (C_{14}–C_{18}). Tetradecenoylcarnitine was the predominant peak, whereas acylcarnitines with a chain-length of C_{16} and C_{18} were also increased. Their concentrations were in the same order of magnitude as that of octanoylcarnitine for MCAD deficiency.

Comparing both acylcarnitine patterns from MCAD and vLCAD patients (Fig. 4) it is evident that the profile from vLCAD patients is much more impressive. One of the reasons may be the easy urinary excretion of medium-chain fatty acids as well as their respective acylcarnitines in contrast with the hydrophobic long-chain acylcarnitines whose excretion in urine is negligible.

Moreover, octanoyl-CoA is also a substrate for glucuronyltransferase (26). Hence octanoylglucuronide formation is another detoxification mechanism for the accumulated octanoyl-CoA, while for long-chain acyl-CoA esters no glycine or glucuronic acid conjugate has been identified so far. This suggests that formation of acylcarnitines is the only conjugation mechanism that the accumulated long-chain acyl-CoA esters are able to undergo, although it does not seem to increase their urinary excretion. Recently, Rashed et al. (27) reported the long-chain acylcarnitine excretion in bile, suggesting that it is a major route of disposal of long-chain acylcarnitines. However, despite the unquestionable diagnostic importance, this fluid is more suited for a postmortem diagnosis than for a routine investigation due to the inherent difficulties of sample collection.

We conclude that this GC–CI–MS method for the analysis of acylcarnitines in plasma is highly sensitive and allows an accurate quantitation for all acylcarnitines studied. The power of this technique is illustrated by the reported control values for acylcarnitines with chain lengths from C_2 to C_{18} which makes it ideal for metabolic studies either in patients or in normal children. The diagnostic suitability of the overall analytical procedure was proven by the presented acylcarnitine profiles from either MCAD and vLCAD patients, two of the most frequent mitochondrial fatty acid oxidation disorders. It was shown to be highly sensitive for the detection of vLCAD deficiency even in very old samples, which makes it perfectly suited for retrospective studies. For the first time, quantitative profiling of plasma acylcarnitines using only routine instrumentation like bench-top GC–MS systems has been achieved.

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