Journal of Chromatography, 577 (1992) 205–214
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6328

Identification of urinary acylcarnitines using gas chromatography–mass spectrometry: preliminary clinical applications

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(Received January 14th, 1992)

ABSTRACT

Many disorders of organic acid metabolism are associated with abnormalities in the levels of acylcarnitines excreted in urine. Profiling of urinary acylcarnitines allows diagnosis and characterisation of many acidurias and acidemias, monitoring dietary treatment of such patients, and elucidation of the metabolism of some exogenous acidic compounds. Urine (*ca.* 0.5 ml) was subjected to a simple work-up by ion-exchange chromatography, and the isolated acylcarnitines were derivatized by cyclization in 35 min to give volatile lactones that are compatible with gas chromatography–mass spectrometry using electron or chemical ionization. The feasibility of this new and affordable procedure has been confirmed by identifying urinary acylcarnitines in cases of medium-chain acyl-coenzyme A dehydrogenase deficiency, propionic acidemia and isovaleric acidemia.

INTRODUCTION

Acylcarnitines are important urinary metabolites for the diagnosis of a number of genetic disorders [1], including defects in the metabolism of fatty acids and of branched-chain amino acids. In some such disorders, acylcarnitines (**1**, Fig. 1) are formed in the mitochondria when potentially toxic excesses of acyl groups are scavenged by carnitine (3-hydroxybutyrobetaine). This detoxification role of carnitine is subsidiary to its primary function in the transport of long-chain fatty acids across the mitochondrial membrane for β-oxidation [1,2]. In some disease states, the formation and excretion of excessive amounts of carnitine esters may result in carnitine deficiency with serious consequences for its primary role. A

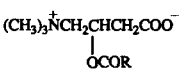

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Fig. 1. Structure of acylcarnitines (**1**).

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convenient method of analysing urine for acylcarnitines would aid the diagnosis and characterization of organic acidurias and acidemias, a number of which are poorly understood, and would assist the study of carnitine balance in such conditions.

A number of methods are available for detection of acylcarnitines [3]. The most successful approaches to date involve mass spectrometry (MS) and particularly the fast atom bombardment (FAB) procedures developed by Millington and co-workers [4–6]. The basic FAB approach, requiring methyl esterification of the acylcarnitines for optimal sensitivity, underpins (i) a continuous-flow FAB analysis that provides a rapid screen but complex spectra [4,5], (ii) continuous-flow FAB-MS–MS with a triple quadrupole instrument that gives greater structural selectivity [4–6], (iii) a continuous-flow microbore liquid chromatography (LC)–FAB-MS technique that attempts to overcome the limitations of MS–MS to characterize fully the acyl groups [5], and (iv) an isotope labelling method [5,7]. The MS–MS method is particularly fast and routine for blood from Guthrie cards [6]. The LC–MS approach (iii) needs further development [5] but may be more appropriate than the MS–MS method for elucidating the structures of unknown acylcarnitines because the former would provide complete conventional spectra and, if the chromatographic conditions were optimized, separation of mixtures of isomers.

To date, the combination of continuous-flow FAB and MS–MS offers the most successful screening for urinary acylcarnitines. However, it requires relatively expensive instrumention, still requires derivatization of the analytes, has limitations for unknown acylcarnitines that require LC–MS for full characterization [5], and may not be amenable to mixtures of isomers. To address these disadvantages, we are developing a complementary approach based on an ion-exchange work-up of urine followed by a simple and novel 35-min derivatization and capillary-column gas chromatography (GC)–MS [8]. The routine, low-cost GC–MS instrumentation provides full electron (EI) or chemical ionization (CI) spectra allowing unambiguous detection and structure elucidation of acylcarnitines. For example, the GC–MS method allowed the observation of urinary 3-phenylpropanoylcarnitine, a new metabolite of 3-phenylpropanoic acid [8] which was later also detected by the FAB approach [9], and a spectrum consistent with the derivative of 2-propyl-3-oxovalerylcarnitine, a new metabolite of the anti-epileptic drug, valproic acid (2-propylvaleric acid) [10]. This paper illustrates some initial clinical applications to cases of medium-chain acyl-coenzyme A dehydrogenase deficiency (MCADD), propionic acidemia and isovaleric acidemia (IVA).

EXPERIMENTAL

Materials

Acylcarnitines were synthesised according to the method of others [11], except for acetyl-, octanoyl- and palmitoyl-DL-carnitine hydrochloride which were obtained from Sigma (Poole, UK). N,N-Diisopropylethylamine was purchased from Aldrich (Gillingham, UK), ethyl acetate (Distol grade) and acetonitrile (Distol grade) from Fisons (Loughborough, UK), Analar water from BDH (Poole, UK), Reacti-Vials of 1-ml volume from Pierce (Chester, UK), and analytical-grade ion-exchange resins from Bio-Rad (Hemel Hempstead, UK).

Methods

The extraction of acylcarnitines from urine and derivatization to the corresponding volatile acyloxylactones have been reported previously [8]. Modifications to the method are presented below.

Aqueous standard sample

Acylcarnitines, about 2 mg of each, were dissolved in Analar water (100 ml). An aliquot (0.1 ml) was transferred to a 1-ml Reacti-Vial and lyophilized. The residue (*i.e.* 2 μg of each acylcarnitine) was treated with acetonitrile (0.5 ml) and N,N-diisopropylethylamine (1.25 μl). The Reacti-Vial was sealed with a PTFE-lined septum and heated to 125°C for 35 min to effect deriv-

atization. After cooling, the volume of solvent was reduced to a few drops by a stream of nitrogen and the residue taken up in ethyl acetate (0.2 ml). Any remaining solid material was filtered and the filtrate analysed for the resulting acyloxylactones by GC and GC–MS (typical injection volumes were 1–2 μl).

Clinical urine samples

An aliquot (1 ml) of each urine sample was spiked with the internal standard, 4-phenylbutanoylcarnitine, at a level of 200 nmol.

An ion-exchange method was employed for the extraction of acylcarnitines from urine [4,5]. A column of 1 cm diameter was packed with Bio-Rad AG1-X8, 100–200 mesh, formate form, anion-exchange resin (2 ml). The resin was converted to its chloride form by eluting with aqueous HCl (1 *M*; 10 ml) and then equilibrated with Analar water. The urine sample (0.5 ml) was applied to the head of the column and washed through with Analar water (2 ml). To acidify the eluent, aqueous HCl (1 *M*; 0.1 ml) was added.

Bio-Rad AG50W-X8, 100–200 mesh, hydrogen form, cation-exchange resin (2 ml) was used to pack a 1 cm diameter column. The acylcarnitine-containing eluent from above was applied to the column. Neutral and loosely bound cationic species were washed off the column with aqueous HCl (0.01 *M*; 5 ml) followed by Analar water (5 ml). Acylcarnitines were selectively eluted with 1 *M* NH_4OH in 20% aqueous ethanol, the first 1 ml being discarded and the following 6 ml collected and freeze-dried.

The resulting residue was dissolved in aqueous HCl (5 m*M*; 0.5 ml), transferred to a 1-ml Reacti-Vial and freeze-dried. The residue was then treated with acetonitrile and N,N-diisopropylethylamine and derivatized as for the aqueous standard samples.

Gas chromatography

A Carlo Erba 5300 Mega series gas chromatograph with flame ionization detection was used. An SGE BP5 fused-silica capillary column, 24.5 m × 0.33 mm I.D. and 0.5 μm film thickness, was used. Helium, 35 cm/s average linear velocity, was used as carrier gas. The hydrogen and air inlet pressures for the detector were 70 and 120 kPa, respectively. The temperature of the detector was maintained at 280°C. For each analysis, the temperature programme for the GC oven was 87°C (on injection) to 250°C at a rate of 10°C/min. On reaching 250°C the oven was maintained at this temperature for 15 min. Cold on-column injections (Grob-type injector) were performed with a 5-μl Hamilton syringe (7.5-cm needle).

Gas chromatography–mass spectrometry

The first instrument used was a VG 20-250 quadrupole system coupled to a Hewlett-Packard 5890 gas chromatograph equipped with an SGE BP5 fused-silica capillary column, 25 m × 0.22 mm I.D. and 0.25 μm film thickness, and an SGE OCI-3 on-column injector. Helium (35 cm/s average linear velocity) was used as carrier gas. On-column injections were made at an initial oven temperature of 50°C. The temperature was immediately ramped to 80°C at 30°C/min then increased to 250°C at 10°C/min and held for 15 min. The direct-line interface was maintained at 250°C and a source temperature of 200°C was used. EI was effected with an electron beam energy of 70 V and emission current of 100 μA. CI was effected with ammonia as reactant gas. Similar GC–MS conditions were also utilized with two other instruments: (i) Finnigan MAT 800 Series ion trap detector (but with a Varian GC, SE-54 stationary phase and, for CI, isobutane as reactant gas); and (ii) Hewlett Packard 5971A mass-selective detector (with methane as CI reactant gas). Cold on-column injections were preferred but, in the absence of such an injector, splitless injection was carried out at a temperature of 230°C.

RESULTS AND DISCUSSION

Being involatile and zwitterionic, acylcarnitines (**1**) are not directly amenable to GC–MS. However, in a simple cyclization reaction (Fig. 2), brought about by brief heating, they can be converted to acyloxylactones (**2**) that are readily examined by GC and GC–MS [8]. The reaction

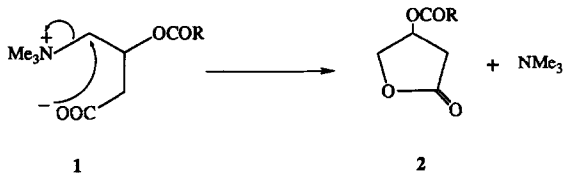

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Fig. 2. Cyclization reaction converting acylcarnitines (**1**) to acyloxylactones (**2**).

shown in Fig. 2 forms products (**2**) that are clearly derived from acylcarnitines. This should be contrasted with another GC approach [12,13] in which acylcarnitines are hydrolysed to fatty acids. GC analysis of the liberated fatty acids does not confirm their conjugation to carnitine because there may be other urinary sources of acyl groups. The lactones generated as shown in Fig. 2 are characteristic of the original acylcarnitines, thus providing an unambiguous analysis.

Several aqueous mixtures of standard acylcarnitines have been subjected to drying, derivatization and GC and/or GC–MS. A typical chromatogram is shown in Fig. 3 for a mixture of eleven standard acylcarnitines. Note from the figure that all eleven acyloxylactones are completely resolved, including two sets of isomers [valproyl (R = $(CH_3CH_2CH_2)_2CH$) and octanoyl (R = $CH_3(CH_2)_6$) derivatives; and 2-methylbutanoyl (R = $CH_3CH_2CH(CH_3)$), isovaleryl (R = $(CH_3)_2CHCH_2$) and valeryl (R = $CH_3(CH_2)_3$) derivatives]. For analysis of the lower homologues (R = CH_3 and CH_3CH_2), it has been found important that, after the cyclization reaction when the acetonitrile solvent is evaporated in a stream of gas, the sample is not subjected to prolonged blowing down. Excessive evaporation leads to losses of the more volatile lactones and the lactones from acetyl- and propanoylcarnitine in particular. As reported in the Experimental section, it is prudent to concentrate the solution to a few drops, and not to dryness, before dissolution in ethyl acetate. Thus far, carnitine esters of many straight-chain and branched-chain monocarboxylic acids from acetic (R = CH_3) to palmitic acid (R = $CH_3(CH_2)_{14}$) inclusively have been resolved readily by temperature-programmed GC on a BP-5 column following this

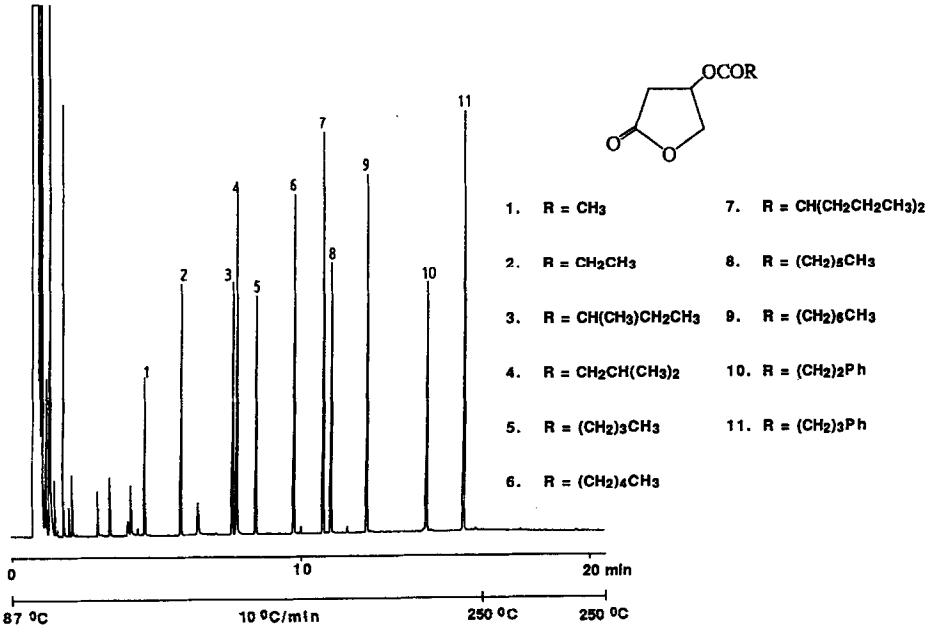

Fig. 3. Gas chromatogram for a standard mixture of eleven acylcarnitines following derivatization according to Fig. 2. The GC column contained BP-5, as described in the Experimental section.

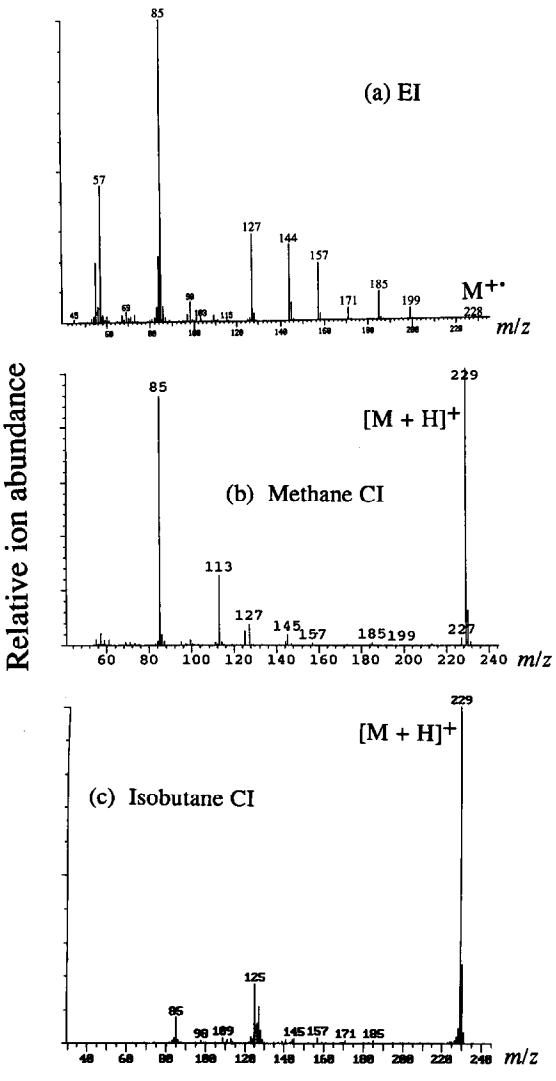

Fig. 4. Mass spectra of the octanoyloxylactone (**2**, R = $CH_3(CH_2)_6$) recorded during GC–MS in (a) the EI mode with a quadrupole mass spectrometer, (b) the CI mode with methane as reactant gas and a mass-selective detector, and (c) the CI mode with isobutane as reactant gas and an ion trap detector.

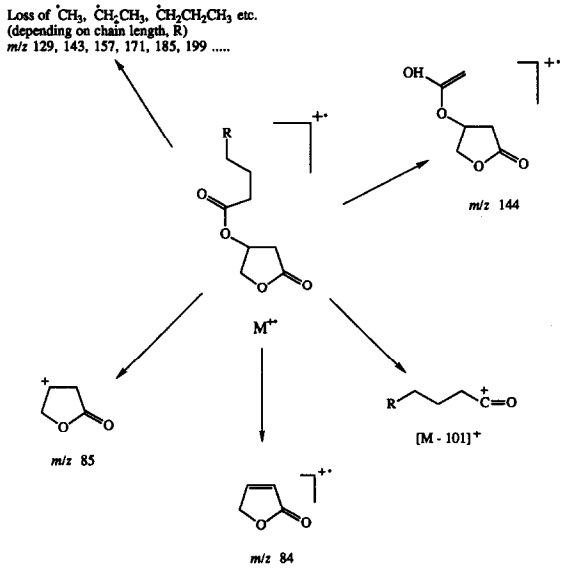

Fig. 5. Generalized fragmentation behaviour of acyloxylactones (**2**).

work-up and derivatization by cyclization (see also ref. 8).

The EI mass spectra of the acyloxylactones, **2**, act as fingerprints for their identification in urine. The considerable fragmentation observed [8] also allows structure elucidation by interpretation of mass spectra when the authentic acylcarnitine or corresponding acyloxylactone is not available and has not been synthesised. However, the molecular ions of aliphatic lactones **2** are not abundant. Molecular ion peaks are usually small in the spectra of standards and often absent when the amount of sample is limited, as with urine extracts. A typical EI mass spectrum is shown in Fig. 4a for the lactone from octanoylcarnitine and generalized fragmentation behaviour is given in Fig. 5.

The paucity of information on relative molecular mass can be addressed by CI. Methane and isobutane CI mass spectra of acyloxylactones have been recorded and typically $[M + H]^+$ ions give rise to the base peak. Fig. 4 compares the EI, methane CI and isobutane CI spectrum of **2** (R = $CH_3(CH_2)_6$). Clearly, the recording of both CI and EI mass spectra of unknown acyloxylactones aids identification by providing clear information on relative molecular mass and on structure through fragmentation, respectively.

The ammonia CI spectrum of the acyloxylactone from acetylcarnitine (**2**, R = CH_3) shows $[M + H]^+$ ions at *m/z* 145 and $[M + NH_4]^+$ ions at *m/z* 162, and a major fragment ion at *m/z* 102 due to loss of acetic acid from the $[M + NH_4]^+$ ion. It is interesting that the thermospray mass spectrum of acetylcarnitine, recorded in the pres-

ence of ammonium acetate, also has a peak at *m/z* 162 (the base peak) and a predominant fragment ion at *m/z* 102 [14,15]. The ready cyclization of acetylcarnitine, at 125°C as reported here, may constitute an alternative explanation for the origin of these two peaks in the thermospray mode (in which the LC–MS transfer line and vaporizer were held at 300 and 225°C respectively [14]). The reported variation of ion abundances in the thermospray spectra with the temperature of the transfer line may also support a thermal origin for peaks at *m/z* 102 and 162; that is, the reaction in Fig. 2 may occur to some extent in the hot LC–MS interface prior to thermospray ionization. An exactly analogous situation exists for the homologue, propanoylcarnitine (**1**, R = CH_3-CH_2).

In the procedure for identifying urinary acylcarnitines by GC–MS, synthetic 4-phenylbutanoylcarnitine (**1**, R = $C_6H_5(CH_2)_3$) as its HCl salt is added at the level of 100 nmol to each 0.5-ml urine sample as an internal standard. In the gas chromatogram of Fig. 3, its lactone derivative is seen to elute at 15.7 min (peak 11).

The ion-exchange procedure reported here (see Experimental) has increased selectivity, resulting in simpler chromatograms for urinary acylcarnitines than that reported previously [8]. For example, the gas chromatogram of Fig. 6 was obtained from the urine of an infant with MCADD, after work-up and derivatization. Most of the principal peaks are due to cyclized acylcarnitines and diketopiperazines (cyclic dipeptides). The latter are assumed to be formed artefactually from urinary dipeptides during the hot derivatization stage. As before [8], the presence of both hexanoyl- and octanoylcarnitine is proven by comparing EI mass spectra of the urinary components with the appropriate authentic lactones, **2**. A comparison of solvent extraction (using a range of solvents) with the ion-exchange method for isolating acylcarnitines from urine will be published elsewhere [16].

At this stage in the development of the method, only full-scan mass spectral data have been acquired. This has sufficient sensitivity to identify many of the acylcarnitines present. Greater sensitivity would be needed to address trace level acylcarnitines, selected ion monitoring [17] being the method of choice. It is notable that peaks at *m/z* 85 and 144 (see Fig. 5) occur in the EI mass spectra of all of the oxyacylactones examined to date, except acetyl and propanoyl derivatives, so these ions are suitable candidates for selected ion monitoring in order to achieve lower detection limits. The same methodology, coupled with the current practice of adding 4-phenylbutanoylcarnitine as internal standard, will be used to quantify urinary acylcarnitines in the future. In addition, specific acylcarnitines could be detected in the CI mode by monitoring the $[M + H]^+$ ions of each acyloxylactone of interest.

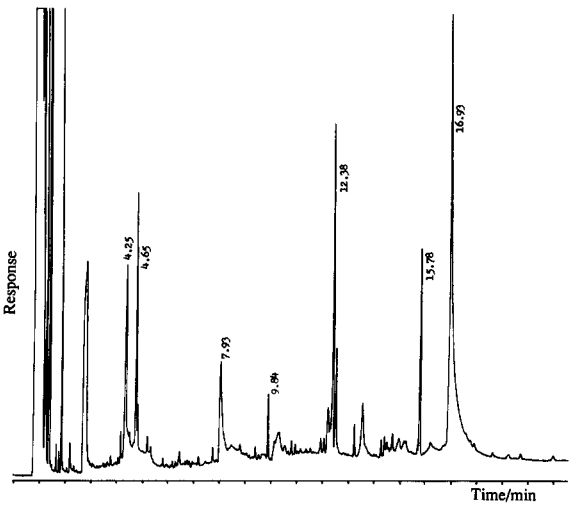

Fig. 6. Gas chromatogram for a urine sample taken from an infant diagnosed as an MCADD case, following ion-exchange work-up and derivatization according to Fig. 2. The GC column contained BP-5, as described in the Experimental section. The lactones derived from acetylcarnitine (4.65 min), hexanoylcarnitine (9.84 min), octanoylcarnitine (12.38 min) and 4-phenylbutanoylcarnitine (internal standard, 15.78 min) are very clear. Several of the smaller peaks are also derived from acylcarnitines. The mass spectrum of the substance eluting at 16.93 min suggests that it is a cyclic dipeptide.

The results obtained with eleven urine samples from nine MCADD cases are summarised as follows. The major acylcarnitines detected were acetyl-, hexanoyl-, octenoyl-, octanoyl- and decanoyl-carnitine. Two MCADD patients also excreted 3-phenylpropanoylcarnitine. This com-

pound is a consequence of administering 3-phenylpropanoic acid to suspected MCADD patients in order to test β-oxidation and, specifically, medium-chain acyl-CoA dehydrogenase activity. Some of the observed carnitine ester of 3-phenylpropanoic acid may be derived from the parent acid produced in the gut. The mass spectra of the 3-phenylpropanoylcarnitine derivative (**2**, R = $C_6H_5CH_2CH_2$) in both patients' urine samples matched the spectrum of authentic 3-phenylpropanoylcarnitine after treatment according to Fig. 2. An example of the match is given in Fig. 7. As stated above, the analyses described here are qualitative but the GC peak due to the 3-phenylpropanoylcarnitine derivative was similar in size to that of the lactone of the endogenous metabolite, octanoylcarnitine. Both peaks were roughly the same size as the peak due to the internal standard which is present at the concentration of 200 nmol ml^{-1} of urine. In many MCADD (and multiple acyl-CoA dehydrogenation deficiency) patients, more than one octenoylcarnitine was found in their urine. Current work aims to synthesise and identify these, and other unsaturated, isomers precisely. Illustrating typical results, the EI and isobutane CI mass spectra of one of the octenoylcarnitine derivatives are shown in Fig. 8.

The estimated concentrations of urinary hexanoyl- and octanoylcarnitine from any single patient varied significantly, depending on the time of collection. Poor detector responses for these major acylcarnitines were especially noticeable for samples collected during or shortly after an episode of acute clinical symptoms (crisis condition). Generally, a pattern emerged whereby urine collected during a crisis stage of the disorder presented lower concentrations of carnitine esters compared with samples collected during the basal stage of the disease (with respect to urinary creatinine levels). The same observation was made with other disorders of mitochondrial β-oxidation, *e.g.* isovaleric acidemia (see below). Such observations may be due to carnitine insufficiency at crisis or administration of glucose. The latter treatment is often given at crisis and will "switch" the metabolism away from mitochondrial β-oxidation.

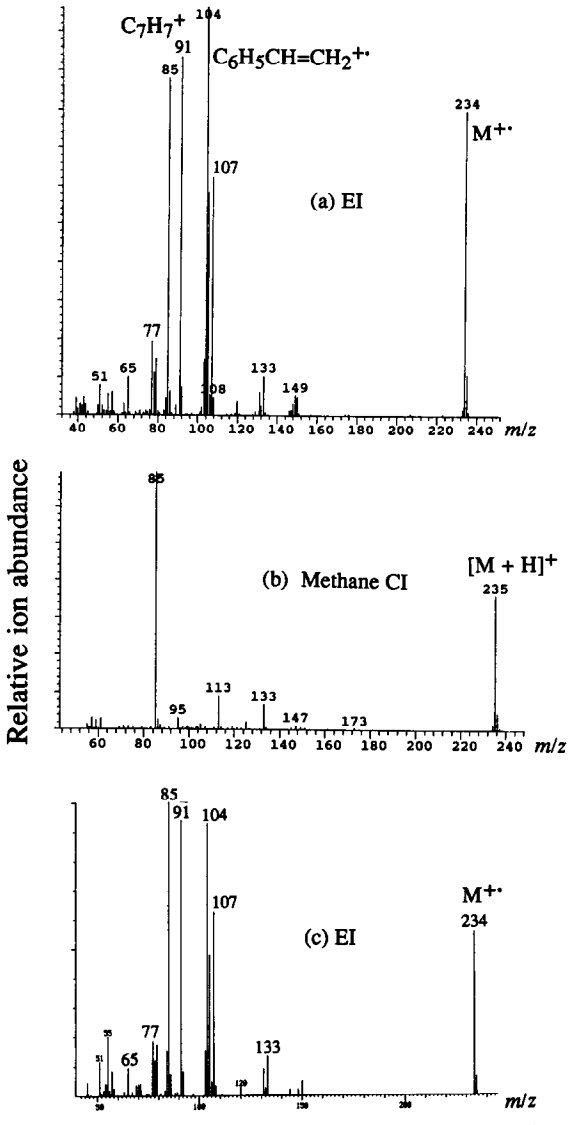

Fig. 7. Mass spectra of the acyloxylactone **2** (R = $C_6H_5(CH_2)_2$) from 3-phenylpropanoylcarnitine recorded during GC–MS. (a) EI mass spectrum of the derivative from authentic 3-phenylpropanoylcarnitine; (b) methane CI mass spectrum of the same standard; and (c) EI mass spectrum of a compound eluting at the same retention time in a clinical sample from an MCADD patient given a 3-phenylpropanoic acid load test. Spectra (a) and (c) were recorded at different times on different quadrupole mas spectrometers, but they still match well.

In contrast to the observations with MCADD patients, urine samples from five apparently healthy babies were shown to contain acetylcarnitine as the only endogenous acylcarnitine that

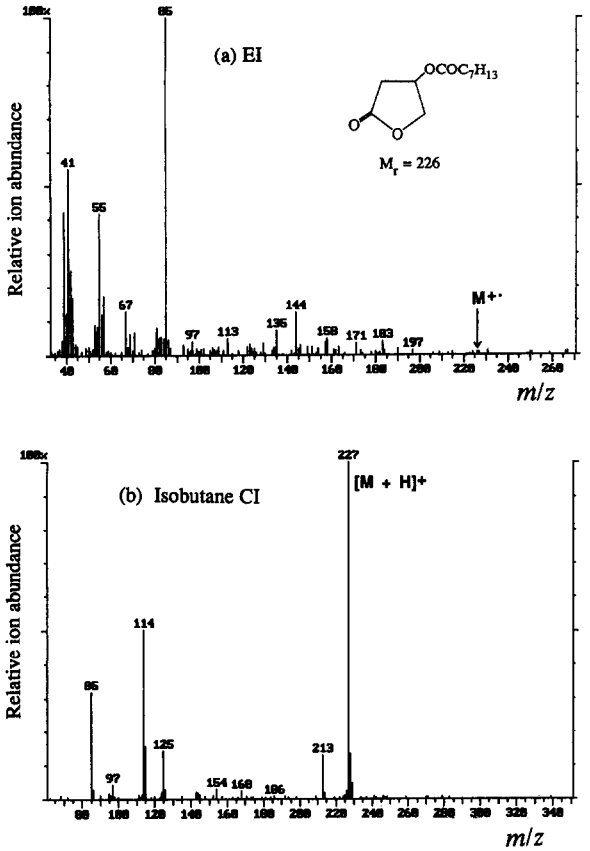

Fig. 8. (a) EI and (b) isobutane CI mass spectra of one of the octenoylcarnitine derivatives found in cases of MCADD and multiple acyl-CoA dehydrogenation deficiency. In (b), the peak at *m/z* 213 is thought to be due to a co-eluting substance. The GC–MS data were acquired with an ion trap detector (ITD 800 Series).

was present at the current detection limit of this full-scan GC–MS method. Even after oral administration of L-carnitine (50 mg kg^{-1} of body weight), a two-month-old infant with normal fatty acid metabolism excreted acetylcarnitine as the only carnitine ester at levels above the detection limit. Indeed, acetylcarnitine is known to be the only urinary acylcarnitine to increase in the normal neonate upon carnitine supplementation. The same carnitine administration to an MCADD patient of similar age resulted in increased excretion of hexanoyl- and octanoylcarnitine. These results agree with previous analyses by the FAB-MS–MS method. An examination of the variation in concentration of acylcarnitines excreted over the first four weeks of life of an MCADD patient, by both FAB-MS–MS and GC–MS, will be described elsewhere.

Fig. 9 shows typical GC profiles for children with (a) isovaleric acidemia, (b) propionic acidemia and (c) multiple acyl-CoA dehydrogenation deficiency (MADD). The MADD case will be described in detail separately [18]. In each example, the figure legend assigns the acylcarnitines that have been identified through comparison of retention times and mass spectra with authentic acyloxylactones. With both isovaleric acidemia and propionic acidemia cases, there is one prevalent carnitine ester present in each profile: isovaleryl- and propanoylcarnitine, respectively. This finding correlates very well with that for the FAB-MS–MS method [5].

In the urine of the three IVA cases examined, high levels of isovalerylcarnitine were found. The mass spectrum obtained for the corresponding acyloxylactone is illustrated in Fig. 10. Samples taken at stable stages of the disease were estimated to contain up to 4 μmol ml^{-1} isovalerylcarnitine. This large concentration made the metabolic disease particularly simple to assign. Excretion of isovalerylcarnitine at crisis stage was usually reduced when compared with excretion in the basal condition. In cases of IVA at the stable stage, it is appropriate to consider using smaller analytical samples of urine (*i.e.* less than 0.5 ml) because large concentrations of acylcarnitines can inhibit the intramolecular cyclization, presumably due to unwanted intermolecular interactions. Two urine samples from each of two propionic acidemia cases were availabe. Propanoylcarnitine was readily detected in all four of the samples. The estimated excretion of propanoylcarnitine was found to vary markedly from low nmol ml^{-1} to μmol ml^{-1} levels within each patient.

CONCLUSIONS

A procedure involving a simple chemical derivatization and GC–MS analysis can identify many urinary acylcarnitines. Even using full-scanning GC–MS, which is much less sensitive than select-

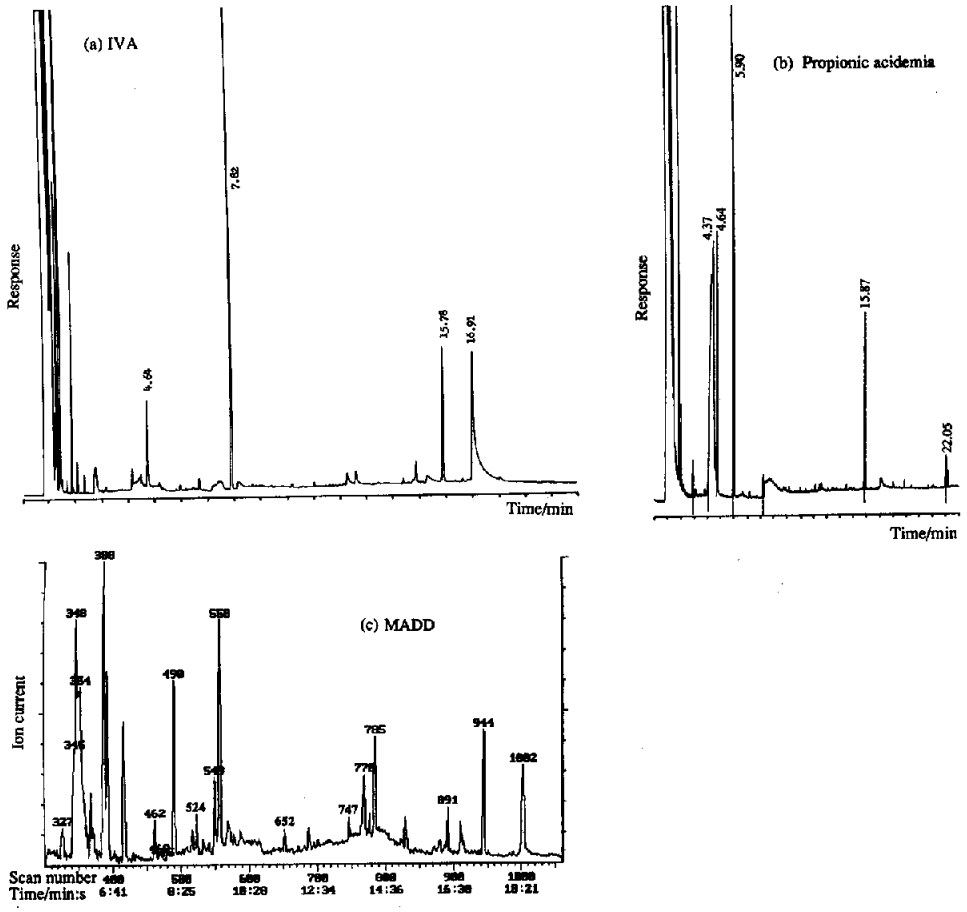

Fig. 9. Gas chromatograms for urine samples taken from infants with various metabolic diseases, following ion-exchange work-up and derivatization according to Fig. 2. (a) Isovaleric acidemia (BP-5 column): lactones derived from acetylcarnitine (4.64 min), isovalerylcarnitine (7.82 min) and 4-phenylbutanoylcarnitine (internal standard, 15.78 min) are prominent. The mass spectrum of the substance eluting at 16.91 min suggests that it is a cyclic dipeptide. (b) Propionic acidemia (BP-5 column): lactones derived from acetylcarnitine (4.64 min), propanoylcarnitine (5.90 min) and 4-phenylbutanoylcarnitine (internal standard, 15.87 min) are prominent. The broad peak at 4.37 min is interfered with by co-eluting urea. (c) MADD (ion current trace for GC–CI-MS on SE-54 column with ITD): lactones derived from isobutanoylcarnitine (scan number 490, assigned by interpretation only), butanoylcarnitine (scan number 524, assigned by interpretation only), 2-methylbutanoylcarnitine (549, assigned by interpretation only), isovalerylcarnitine (558), hexanoylcarnitine (652), an octenoylcarnitine (747, assigned by interpretation only), octanoylcarnitine (770) and 4-phenylbutanoylcarnitine (internal standard, 944) were found.

ed ion monitoring, the acylcarnitines are readily detected in the urine of patients with several inherited metabolic diseases. The method is less well established than that using FAB-MS–MS [4–6], is slower to execute and, thus far, has only been applied to urine. However, it does have several advantages. The procedure requires only routine and inexpensive instrumentation; in particular, benchtop GC–MS systems are ideal for the analysis. Conventional, whole mass spectra are recorded in the EI or CI modes and these

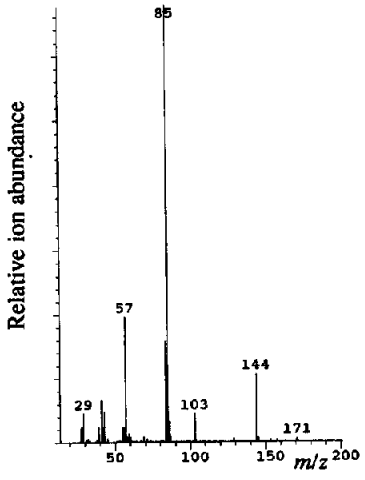

Fig. 10. EI mass spectrum of the major acyloxylactone **2** eluting in the case of a patient with isovaleric acidemia. The retention time and mass spectrum match those for the lactone derived from authentic isovalerylcarnitine ($M^{+\cdot}$ very small at *m/z* 186).

allow ready structure elucidation by interpretation. Mixtures of isomers are resolved prior to MS, thus allowing the spectra of each isomer to be examined separately.

ACKNOWLEDGEMENTS

We thank the Trustees of the Analytical Chemistry Trust Fund of The Royal Society of Chemistry for the award of an SAC Research Studentship (to S.L.). We gratefully acknowledge the Foundation for the Study of Infant Deaths and the British Mass Spectrometry Society for equipment grants. We thank Dr. P. Divry (Lyon Hospital) for useful discussions and the urine sample from a MADD patient, to be discussed fully in a separate publication.

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