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Measurement of urinary medium chain acyl glycines by gas chromatography–negative ion chemical ionization mass spectrometry*

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Abstract: Medium chain acyl-CoA dehydrogenase (MCAD) deficiency is an inborn error of fatty acid metabolism, which is difficult to diagnose, partly because of its unpredictable clinical presentation. A specific diagnostic marker is an increased excretion of certain medium chain acyl glycines. A sensitive and specific method has been developed for the extraction, derivatization, identification and quantitation of urinary medium chain acyl glycines by gas chromatography–negative ion chemical ionization mass spectrometry (GC–NICIMS). The following series of standard acyl glycines has been synthesized and characterized: hexanoyl, octanoyl, 3-phenylpropionyl and suberyl and their respective isotopomers (using $^{13}C_2$-glycine; for use as internal standards). The range of excretion of these compounds in normal subjects has been established using this method and increased excretion of acyl glycines, particularly hexanoyl, 3-phenylpropionyl and suberyl was successfully demonstrated in three MCAD deficient subjects from one family.

Keywords: *MCAD deficiency; acyl glycines; hexanoyl glycine; octanoyl glycine; 3-phenylpropionyl glycine; suberyl glycine; urine; GC–NICIMS.*

Introduction

There are at least eight inborn errors of mitochrondrial fatty acid β-oxidation and, of these, medium chain acyl-CoA dehydrogenase (MCAD) deficiency (McKusick 22274) is the most common [1]. It was first described by Gregerson and co-workers in 1976 [2] who reported an increased urinary excretion of suberyl glycine by a patient who subsequently died. Clinical presentation usually occurs in the first 2 years of life and varies from mild hypoglycaemia to a Reye-like syndrome, coma and sudden unexpected death [3–14]. An initial episode may occur in previously healthy children and there is a high mortality rate; however, some affected subjects may remain asymptomatic [6, 7]. There is good evidence that a significant percentage of sudden infant deaths is due to MCAD deficiency [4, 7, 10, 12, 15]. Diagnosis of MCAD deficiency is difficult, partly because of its unpredictable clinical presentation, but accurate diagnosis is very important because of the severity of the clinical symptoms. These can be prevented, in some cases, by administering a high carbohydrate–low fat diet, with L-carnitine supplements.

Methods for the identification of MCAD deficiency must be sensitive enough to detect the condition in asymptomatic subjects. The decrease in β-oxidation of medium chain fatty acids caused by this inborn error prevents them from being fully utilized as an energy source and also leads to a toxic excess of fatty acids in the mitochondria. The excess fatty acids, present as their reactive coenzyme A esters, acylate carnitine enabling them to be transported out of the mitochondria and eventually excreted; they react similarly with glycine to facilitate excretion. It has been shown that urinary excretion of two medium chain acyl glycines, hexanoyl glycine and 3-phenylpropionyl glycine (hydrocinnamoyl glycine), normally minor metabolites of fatty acid metabolism, was increased markedly in patients with MCAD deficiency [16]. The levels were increased not only during acute episodes, but also during remission and those increases were highly specific for MCAD deficiency. The concentration of suberyl glycine was also in-

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creased in some samples of urine, but it was not reliably diagnostic in isolation since the normal range was too wide.

These measurements were made using gas chromatography–mass spectrometry (GC–MS) in conjunction with a stable isotope dilution technique to achieve the degree of reproducibility and sensitivity required, and to compensate for losses of the extremely small amounts of endogenous acyl glycines. Other workers have shown that increased urinary excretion of acyl carnitines, particularly octanoyl carnitine, measured by fast atom bombardment-mass spectrometry, are diagnostic of MCAD deficiency [7, 17]. Indeed, there has been a debate as to which of the two series of metabolites is preferred for detecting MCAD deficiency [16–20]. A recent comparative study found that the measurement of acyl glycines was more reliable than acyl carnitine analysis for accurate diagnosis of MCAD deficiency; probably because tissue concentrations of glycine are higher than those of carnitine. Urinary acyl glycine excretion was significantly increased in all samples from MCAD deficient subjects, so that unambiguous diagnosis of MCAD deficiency was possible regardless of the condition of the patient [21].

Experimental

Chemicals

All solvents used for extraction were HPLC grade (Rathburn Chemicals, Peebleshire, UK). Chemicals were obtained from the following sources: hexanoyl chloride, octanoyl choride, 3-phenylpropionyl chloride (hydrocinnamoyl chloride) and 2,2,2-trifluoroethanol (TFE) — Aldrich Chemical Co. (Gillingham, Dorset, UK); suberoyl chloride and $^{13}C_2$-glycine — Sigma Chemical Co. (Poole, UK); glycine — BDH (Glasgow, UK) and pentafluoropropionic anhydride (PFPA) — Fluorochem Ltd (Old Glossop, Derbyshire, UK).

Preparation of external and internal standards

Hexanoyl glycine, octanoyl glycine and 3-phenylpropionyl glycine were prepared on a gram scale essentially according to the method of Bondi and Eissler [22] by reacting the appropriate acid chloride with an excess of glycine in 1 N sodium hydroxide on ice. The reaction mixture was acidified to pH 1 with 6 N hydrochloric acid, the product extracted into diethyl ether, the solvent removed from the combined dried organic extracts by rotary evaporation and the crystalline product washed with petroleum ether (60–80°C). The crude products were purified by recrystallization from appropriate mixtures of ethyl acetate and petroleum ether. Internal standards for these compounds were prepared by a similar method but on a milligram scale using $^{13}C_2$-glycine as the starting material.

Suberyl glycine was prepared using the method described by Gregersen *et al.* [2]. However, since it is dibasic, its synthesis proved to be much more difficult and the yield of pure suberyl-mono-glycine was very low (*vide infra*). The internal standard for suberyl glycine was prepared as required by reacting a small amount of suberoyl chloride with $^{13}C_2$-glycine in phosphate buffer (pH 7.4), and extracting the product into ethyl acetate after acidification of the reaction mixture. Calibration of the extract, in order to determine the concentration of suberyl $^{13}C_2$-glycine, was made against appropriate amounts of a standard solution of pure unlabelled suberyl glycine (see section on standard solutions).

The identities of the acyl glycines thus prepared were confirmed by elemental analysis, mass spectrometry, NMR spectrometry and melting point determination. Melting points and yields obtained were as follows: hexanoyl glycine, 92–96°C, 62%; hexanoyl $^{13}C_2$-glycine, 91–95°C, 70%; octanoyl glycine, 106–109°C, 82%; octanoyl $^{13}C_2$-glycine, 105–107°C, 85%; 3-phenylpropionyl glycine, 112–114°C, 90%; 3-phenylpropionyl $^{13}C_2$-glycine, 110–112°C, 81%; suberyl glycine, 125°C, 0.3%.

Standard solutions

For all the acyl glycines, except for suberyl $^{13}C_2$-glycine, stock solutions of 1 mg ml^{-1} in aqueous acetonitrile (1:9, v/v) were prepared. Equal amounts of the stock solutions of the $^{13}C_2$-glycines were taken and diluted together in acetonitrile, giving a final concentration of 10 μg ml^{-1} of each, for use as internal standards. Similarly, equal amounts of the stock solutions of all the unlabelled acyl glycines and acyl $^{13}C_2$-glycines were taken and diluted together in acetonitrile, to a final concentration of 10 μg ml^{-1} for use as a 1:1 (w/w) standard solution. These solutions were stored at −20°C when not in use.

Urine samples

Samples of urine were collected from normal subjects of both genders aged between 5 months and 56 years. Urine samples were also collected from all the members of a particular family where it was known that at least one of the children was MCAD deficient. All the samples were analysed for creatinine using a creatinine kit based on complex formation with picric acid (Boehringer, Lewes, UK) and they were stored at −20°C until analysis.

Sample extraction and derivatization

A volume of urine equivalent to 0.5 mg of creatinine was diluted to 1 ml with water. Known amounts of the acyl $^{13}C_2$-glycines (100–500 ng) were added as internal standards. The urine was then acidified to pH 1–2 with hydrochloric acid (0.1 ml, 2 M), saturated with sodium chloride and extracted with ethyl acetate (1.5 ml). The ethyl acetate layer was carefully removed and acidic substances were back-extracted into dilute sodium hydroxide (1 ml, 0.5 M). After discarding the solvent, the aqueous layer was re-acidified (0.2 ml, 6 M HCl), saturated with sodium chloride and re-extracted with ethyl acetate (1.5 ml). The solvent layer was carefully removed and dried by passage through a column of anhydrous sodium sulphate. The ethyl acetate was removed from the eluant by evaporation under a stream of nitrogen. The dried extract was then derivatized by the addition of TFE (10 μl) and PFPA (40 μl) and heating the reaction mixture for 1 h at 70°C. Excess reagents were removed under a stream of nitrogen and the derivatized extract reconstituted in ethyl acetate (100–500 μl) prior to analysis by GC–NICIMS. Considerably smaller amounts of urine were taken for extraction when samples obtained from MCAD-deficient subjects were analysed (volumes equivalent to 0.01–0.05 mg creatinine).

A blank sample, comprising water containing the same amount of acyl $^{13}C_2$-glycines as the urine samples, was submitted to the same analytical procedure when every batch was analysed in order to check for contamination of reagents and solvents with unlabelled standards or endogenous acyl glycines. Similarly a 1:1 (w/w) standard, comprising water containing equal quantities of unlabelled acyl glycines and the corresponding acyl $^{13}C_2$-glycines, was included with each batch of samples in order to compare extraction, derivatization and instrument response of the endogenous compounds and their respective internal standards.

GC–NICIMS analysis

Analysis was carried out using a Hewlett–Packard capillary gas chromatograph (GC) interfaced to a Hewlett–Packard mass spectrometer (Model No. 5988A), operated in the NICI mode with methane as reagent gas. The GC was fitted with a BP1 aluminium or polyimide clad fused silica column (12 or 25 m × 0.22 mm i.d.); helium was used as carrier gas at a head pressure of 5 or 25 p.s.i. The GC was programmed as follows: 100°C for 1 min then 10°C min^{-1} to 300°C. Selected ion monitoring (SIM) was employed to give increased sensitivity and specificity and also to reduce the effects of background interference.

Results and Discussion

Concentrations of three of these four endogenous acyl glycines can be measured in most samples of urine using this method. However, often, the levels of octanoyl glycine in samples obtained from normal subjects were below the limits of detection (10 ng) of the method. The extraction and back-extraction procedures employed reduced the degree of interference from the biological matrix, but these manipulations also resulted in poor recovery of the acyl glycines. However, since the excretion of these substances was greatly increased in MCAD-deficient subjects, this did not cause any problems with diagnosis of disease.

The derivatization procedure effected the conversion of carboxyl groups to their trifluoroethyl esters and the replacement of the amide hydrogen with a pentafluoropropionyl group: this afforded highly electron-capturing, volatile derivatives (PFP–TFE derivatives), which are suitable for analysis by GC–NICIMS. In the NICI spectra of all the derivatized acyl glycines that have been prepared a large proportion of the ion current was carried by an ion resulting from the loss of hydrogen fluoride from the molecular ion. This is exemplified in Fig. 1, which shows the mass spectrum of the PFP–TFE derivative of hexanoyl glycine and hexanoyl $^{13}C_2$-glycine. The gas chromatographic and mass spectral characteristics are typical of all four acyl glycines and give a potential limit of detection of these derivatives below the picogram level but, in practice, the sensitivity was reduced by

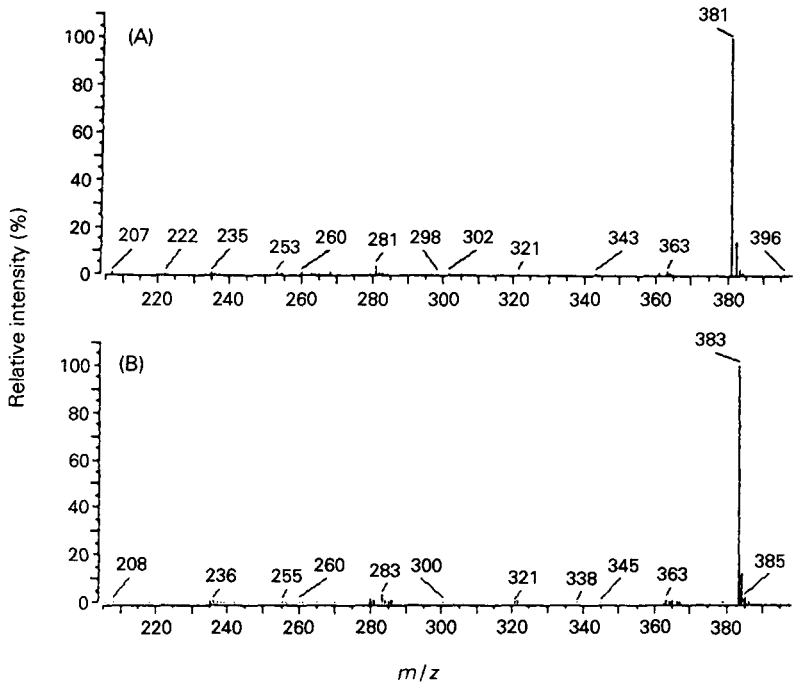

Figure 1
NICI mass spectrum of the PFP–TFE derivative of (A) hexanoyl glycine and (B) hexanoyl $^{13}C_2$-glycine.

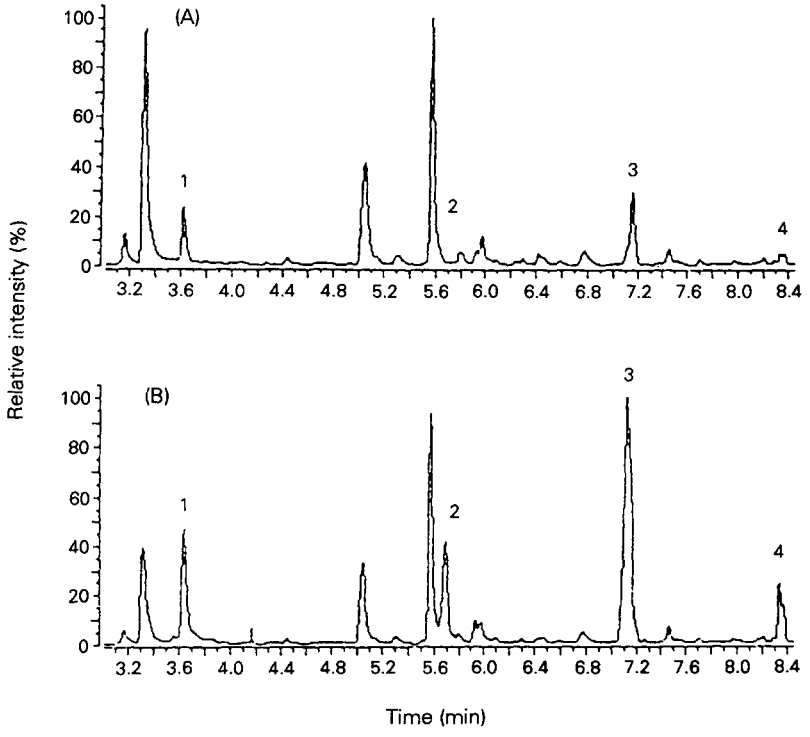

Figure 2
SIM trace of (A) PFP–TFE derivatives of endogenous medium chain acyl glycines extracted from human urine and (B) PFP–TFE derivatives of medium chain acyl $^{13}C_2$-glycines, as internal standards, extracted from the same urine sample. 1 = hexanoyl; 2 = octanoyl; 3 = 3-phenylpropionyl; 4 = suberyl.

the extraction procedures employed to diminish interference from the biological matrix. At present it is possible to detect 10 ng of material above the background. The precision of the method is good since the physical properties of the ^{13}C-labelled compounds are identical for all practical purposes to those of the endogenous compounds which accurately compensates for any losses in the extraction and derivatization procedures. Calibration curves constructed were linear over a wide concentration range of all four acyl glycines and correlation coefficients were as follows: hexanoyl glycine, 0.9990, $n = 10$; octanoyl glycine, 0.9990, $n = 10$; 3-phenylpropionyl glycine, 0.9981, $n = 10$, over the range 5 ng–1 μg and 0.9987, $n = 8$, for suberyl glycine over the range 20 ng–1 μg. Relative standard deviation between assays were as follows: hexanoyl glycine, 3.6%, $n = 18$; octanoyl glycine, 7.0%, $n = 18$; 3-phenylpropionyl glycine, 7.3%, $n = 18$; suberyl glycine, 7.9%, $n = 10$.

Figure 2 shows a SIM trace of derivatized endogenous acyl glycines extracted from a sample of urine from a normal subject compared with a corresponding trace of the derivatized ^{13}C-labelled acyl glycines (as internal standards) extracted from the same sample of urine. It is clear from Fig. 2 that there was very little, if any, endogenous octanoyl glycine in this particular sample and this was observed frequently.

Table 1 gives the normal range of values obtained for excretion of these four acyl glycines and these are somewhat lower than those reported by Rinaldo *et al.* [16]. Their highest values for 3-phenylpropionyl glycine and suberyl glycine were 1.1 and 95 μg mg^{-1} creatinine respectively; however it is pertinent to note that all of their control subjects were infants. The method which we have developed using highly electron-capturing derivatives and NICIMS will be more sensitive but, as previously mentioned, sensitivity is not normally a major problem for the diagnosis of MCAD deficiency using acyl glycines since the excretion of these metabolites is greatly increased. However, it may be of paramount importance when only extremely small amounts of biological samples are available (e.g. in cases of sudden infant death).

Table 2 gives the excretion of acyl glycines by all the members of a family which has three MCAD-deficient subjects. It can be seen clearly that excretion of all four acyl glycines exceeded the upper limit of the range, which was obtained for normal subjects: this was particularly pronounced for hexanoyl glycine, 3-phenylpropionyl glycine and suberyl glycine. The excretion of acyl glycines by the unaffected family members was within the 'normal range' except that the excretion of 3-phenylpropionyl glycine by two subjects (F,37 and M,11) was higher. One of these is the mother of the family who, as a parent of three MCAD-deficient children, is an obligate heterozygote for MCAD deficiency. It is interesting to note that the excretion of all four acyl glycines by the father (M,46) was within the 'normal ranges'. Only one (F,7) of the three affected children has suffered any symptoms of MCAD

Table 1
Normal range of urinary medium chain acyl glycine excretion (μg/mg creatinine) for 29 subjects, aged between 5 months and 56 years

Hexanoyl glycine	Octanoyl glycine	3-Phenylpropionyl glycine	Suberyl glycine
0.03–1.15	N.D.–0.17	0.01–0.09	0.02–1.53

N.D. = below limit of detection.

Table 2
Urinary medium chain acyl glycine excretion (μg/mg creatine) by a family with three MCAD-deficient subjects

Subject (age, y)	Hexanoyl glycine	Octanoyl glycine	3-Phenylpropionyl glycine	Suberyl glycine
M (46)	0.38	0.01	0.03	0.19
F (37)	1.13	0.02	0.11	0.37
M (12)	0.58	0.01	0.06	0.20
M (11)	0.77	N.D.	0.28	0.23
M (10)	0.53	N.D.	0.06	0.13
M (2)	0.62	N.D.	0.08	0.32
M* (14)	25.71	0.81	50.34	14.06
F* (7)	14.09	0.28	87.74	8.23
M* (0.5)	56.56	1.24	6.07	130.79

N.D. = below the limit of detection.
*MCAD-deficient subjects.

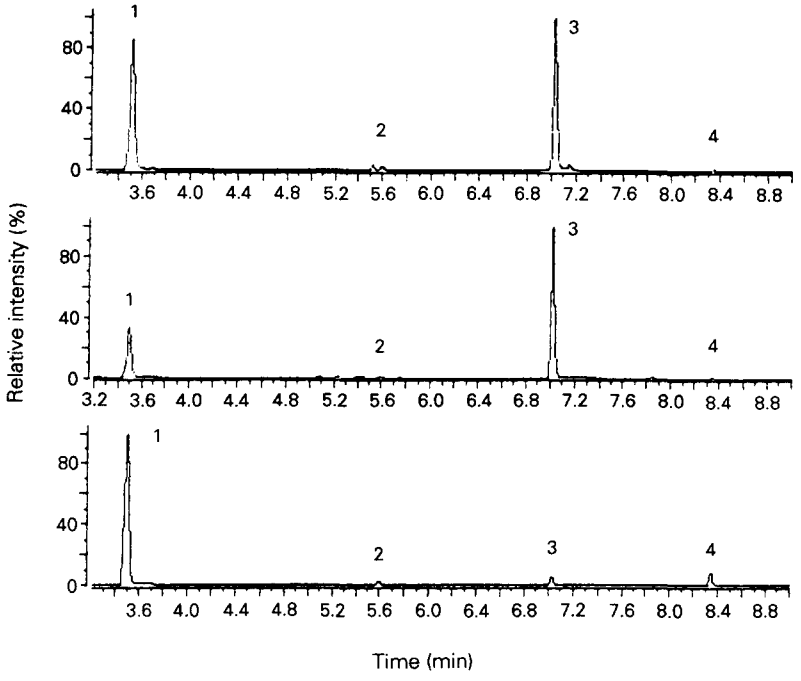

Figure 3
SIM trace of PFP–TFE derivatives of endogenous medium chain acyl glycines extracted from urine samples of three MCAD-deficient subjects: (A) M aged 14 y; (B) F aged 7 y; (C) M aged 0.5 y. 1 = hexanoyl; 2 = octanoyl; 3 = 3-phenylpropionyl; 4 = suberyl.

deficiency and all were asymptomatic at the time these urine samples were collected.

Figure 3 compares the SIM traces for derivatized endogenous acyl glycines extracted from the urine samples of the three MCAD-deficient children in order to illustrate the different patterns of excretion. The poor extraction efficiency and a relatively lower instrumental response for suberyl glycine accounts for the relatively small peak observed for this substance. This was especially noticeable in the case of the youngest family member (M, 0.5 y), where the excretion of suberyl glycine was 131 $\mu g\ mg^{-1}$ creatinine. The excretion of 3-phenylpropionyl glycine by this subject, the only infant in the family, was the lowest of the three affected children. 3-Phenylpropionyl glycine is derived from phenylpropionate, a gastrointestinal bacterial metabolite which is normally metabolized to benzoate. This reacts with glycine and is excreted as hippurate and it has been shown that the excretion of 3-phenylpropionyl glycine in MCAD deficiency requires adequate colonization of the gastrointestinal tract, which is certainly insufficient in the neonatal period [20]. Although this infant was by no means a neonate and the excretion of 3-phenylpropionyl glycine was well above the normal range, this, together with an infant diet, may partly explain the different excretion patterns observed. It has been proposed that analysis of urinary 3-phenylpropionyl glycine by high-performance liquid chromatography, following an oral dose of phenylpropionate, could be used as a diagnostic loading test for MCAD deficiency [23] and it could be a useful adjunct to the analysis of urinary acyl glycines by GC–MS, especially in the neonatal period [20].

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