ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
21 (2000) 1215–1224

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Quantitative analysis of urinary acylglycines for the diagnosis of β-oxidation defects using GC-NCI-MS

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Received 28 May 1999; received in revised form 7 September 1999; accepted 17 September 1999

Abstract

The analysis of acylglycines is an important biochemical tool for the diagnosis of inherited disorders of mitochondrial fatty acid β-oxidation. A stable isotope dilution gas chromatography negative chemical ionisation mass spectrometry method for the quantitative analysis of short- and medium-chain acylglycines as their bis(trifluoromethyl)benzyl (BTFMB) ester derivatives is described. The diagnostic usefulness of the method was demonstrated in nine patients with medium-chain acyl-coenzyme A (CoA) dehydrogenase (MCAD) deficiency, and seven patients with multiple acyl-CoA dehydrogenation defect (MAD). The urinary acylglycine profiles in these patients were compared to those in controls ($n = 19$), children on a medium-chain triglyceride (MCT) supplemented diet ($n = 4$), and patients with various other diseases ($n = 5$). © 2000 Elsevier Science B.V. All rights reserved.

Keywords: BTFMB derivatives; Butyrylglycine; Hexanoylglycine; Isovalerylglycine; 2-Methylbutyrylglycine; Mitochondrial fatty acid oxidation; Phenylbutyrylglycine; Stable isotope dilution; Suberylglycine

1. Introduction

Intramitochondrially formed acyl-coenzyme A (CoA) esters derived from fatty acids and amino acids can follow alternative metabolic pathways, especially when their β-oxidation is hampered. Conjugation of acyl-CoAs with carnitine [1,2], glucuronic acid [3] or glycine leads to the forma-

Abbreviations: BG, butyrylglycine; BTFMB, bis(trifluoromethyl)benzyl; CoA, coenzyme A; GC-NCI-MS, gas chromatography negative chemical ionisation mass spectrometry; HG, hexanoylglycine; IBG, isobutyrylglycine; IVG, isovalerylglycine; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; MAD, multiple acyl-CoA dehydrogenation defect; 2MBG, 2-methylbutyrylglycine; MCAD, medium-chain acyl-CoA dehydrogenase; MCT, medium-chain triglycerides; PPG, phenylpropionylglycine; SCAD, short-chain acyl-CoA dehydrogenase; SG, suberylglycine; vLCAD, very long-chain acyl-CoA dehydrogenase.

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PII: S0731-7085(99)00235-6

tion of polar compounds that can be excreted in urine. These conjugation reactions are important in balancing the ratio between free and esterified CoA within the mitochondrion. In several inborn errors of mitochondrial amino acid and fatty acid metabolism, accumulation of acyl-CoA esters occurs, resulting in elevated concentrations of glycine and carnitine conjugates in plasma and urine. Analysis of glycine conjugates [4,5] and acylcarnitines [2,6] in urine and plasma, respectively, provides a tool for the biochemical diagnosis of these disorders. Acylglycines result from the transesterification of an acyl-CoA ester with glycine through the action of acyl-CoA:glycine-*N*-acyltransferase (glycine-*N*-acylase) [7]. This enzyme is involved in the formation of straight-chain, branched-chain and aromatic acylglycines [7–10]. Glycine-*N*-acylase is an exclusively mitochondrial enzyme. Increased acylglycine excretion is therefore directly related to the intramitochondrial accumulation of the corresponding acyl-CoA esters.

In short-chain acyl-CoA dehydrogenase (SCAD) deficiency, the excretion of butyrylglycine (BG) is increased [11]. A combined elevation of urinary hexanoylglycine (HG), phenylpropionylglycine (PPG) [12,13] and suberylglycine (SG) [14] is considered pathognomonic for medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. Patients with a multiple acyl-CoA dehydrogenation defect (MAD) display a considerable variation in acylglycine excretion, depending on the severity of the defect and the clinical condition of the patient [15,16]. In the more severely affected patients, elevations of both the branched-chain and the straight-chain acylglycines are found. The mildly affected patients only excrete increased amounts of BG, isobutyryl glycine (IBG) and isovalerylglycine (IVG). For differential diagnosis of fatty acid β-oxidation defects, a wide range of acylglycines in urine should be quantified. After the report by Gregersen et al. [17], in which the syntheses and analyses of eleven biochemically interesting acylglycines are described, several other methods have been published. The GC-MS methods reported by Rinaldo et al. [4,18] and Bonham Carter et al. [19] essentially focused on the diagnosis of MCAD deficiency by measurement of medium-chain acylglycines (HG, PPG and SG). Later, BG and IVG were included for the study of a limited group of patients with a MAD [15]. Here, we present a new method for the simultaneous measurement of BG, IBG, 2-methylbutyryl glycine (2MBG), IVG, PPG, SG and HG by stable isotope dilution GC-NCI-MS. We applied this method to (A) establish control and patient values, and (B) to investigate whether the acylglycine profile obtained after a medium-chain triglyceride (MCT)-enriched diet mimics that of a metabolic fatty acid oxidation defect.

2. Experimental

2.1. Biological samples

2.1.1. Controls

Control urine samples, which were used to establish control values, were collected from 19 children aged 2 days–12 years. All children were receiving a normal diet at the time of urine collection. To investigate the effect of an enriched dietary intake of MCT, urine samples from four children (aged 1–10 months) receiving an MCT-enriched diet were collected. None of the children was affected with an inborn error of metabolism.

2.1.2. Patients

Thirteen urine samples of ten proven patients with MCAD deficiency were analysed in this study. In addition, eight urine samples from seven proven MAD patients were analysed. Six patients from this group were classified as having the mild form while one patient suffered from the severe neonatal form. An extra set of eight urine samples from a group of five patients with proven mitochondrial inborn errors of metabolism were included in this study. Two patients from this group suffered from long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency, two patients suffered from very-long-chain acyl-CoA dehydrogenase (vLCAD) deficiency and one patient suffered from a respiratory chain defect.

2.2. Materials

3,5-bis(Trifluoromethyl)benzyl (BTFMB) bromide was purchased from Fluorochem (Old Glossop, UK). Both [$^2H_7$]butyric acid (98% isotopic purity) and [$^2H_7$]isobutyric acid (98% isotopic purity) came from C/D/N Isotopes (Pointe-Claire, Québec, Canada). [$^{13}C_2$]glycine (99% isotopic purity) was purchased from Isotec (Miamisburg, OH, USA). 2-Methylbutyryl chloride was from Acros Organics (Geel, Belgium). Suberyl-[2-^{13}C,^{15}N]glycine, [4,4,4-$^2H_3$]IVG, n-hexanoyl-[$^{13}C_2$]glycine and 3-phenylpropionyl-[2-^{13}C,^{15}N]glycine were generous gifts of Dr P. Rinaldo (Yale University Medical School, USA). IBG, BG and IVG were kind gifts from Dr G. Dacremont (Gent, Belgium). The CP-Sil 19 CB capillary analytical gas chromatography column came from Chrompack (Middelburg, The Netherlands). All other chemicals and solvents were of analytical grade.

2.3. Syntheses of labelled and non-labelled standards

The following compounds were synthesised: n-[$^2H_7$]BG, [$^2H_7$]IBG, 2-methylbutyryl-[$^{13}C_2$]glycine and 2MBG. All syntheses followed the same procedure; an ice-cooled aqueous glycine or [$^{13}C_2$]glycine-containing solution was treated with 1.2 equivalents of the appropriate acylchloride and stirred for 2 h. After warming to room temperature, the pH of the solution was adjusted to 1 with concentrated HCl, followed by an extraction with ethyl acetate. The extract was dried over magnesium sulphate, filtered and concentrated in vacuo. The synthesised acylglycines were obtained as crystals by treating the remaining concentrate, an oil, with petroleum ether (bp 40–60°C). The yields of the different syntheses were in the range of 70–80%. Both chemical and isotopic purities were assessed by GC-MS and found to be >95 and >98%, respectively. No significant degradation of both labelled and non-labelled acylglycines in analytical solutions has been observed.

2.4. Sample preparation

The amount of urine taken into preparation depended on its creatinine concentration. For creatinine concentrations below 5 mmol/l, 1 ml of urine was used, otherwise 0.5 ml of urine was used and adjusted to 1 ml with water. The absolute amount of internal standard added to the urine sample was 20 nmol of [$^2H_7$]BG, [$^2H_7$]IBG, [$^{13}C_2$]2MBG, [$^2H_3$]IVG, [$^{13}C_2$]HG and [2-^{13}C,^{15}N]SG and 4 nmol of [2-^{13}C,^{15}N]PPG. After adjustment of the pH to 1–2 with 2 N HCl and saturation with sodium chloride, the acylglycines were extracted three times with 3 ml ethylacetate. The combined organic layers were dried over anhydrous sodium sulphate and evaporated to dryness with a stream of nitrogen at 40°C. 3,5-BTFMB derivatives were formed by treating the dry residue with 10 μl triethylamine and 100 μl 10% 3,5-BTFMB bromide in acetonitrile (v/v) for 15 min at room temperature. After addition of 150 μl 0.5 N HCl, the derivatives were extracted with 1.5 ml hexane. The hexane fraction was blown to dryness with nitrogen at 40°C and was reconstituted in 50 μl of ethyl acetate. From this ethyl acetate fraction 1 μl was injected into the GC-MS.

2.5. Gas chromatographic and mass spectrometric conditions

GC-MS analyses were performed on a 610 ATI-Unicam GC coupled to an Automass series I ATI-Unicam mass spectrometer (Cambridge, UK). Chromatographic separation was achieved on a CP-Sil 19 CB (25 m × 0.25 mm I.D., film thickness 0.4 μm) capillary column coated with a medium polar phase. Samples were injected using a split/splitless injector set at a temperature of 280°C, operating at a split ratio of 1:5. The initial oven temperature was maintained at 140°C for 1 min, followed by a ramp of 12°C/min to 320°C. Helium was used as carrier gas at a head pressure of 0.6 bar. The temperature of the transfer line to the mass spectrometer was kept at 270°C. The column was inserted directly into the ion source, which was set at 250°C. Compounds were detected in the electron capture negative chemical

ionisation mode using methane as moderating gas at an optimised source pressure. Ionisation was achieved with an electron energy of 150 eV. Detection of the [M-BTFMB]$^-$ carboxylate anions was performed in the selective ion monitoring mode using the target ions listed in Table 1.

Six-point calibration curves were established by carrying aqueous standard solutions, containing various amounts of acylglycines and constant amounts of the labelled analogues, through the entire procedure. The observed peak area ratios of standard towards internal standard were used for linear regression analysis. The concentration of an acylglycine in a urine sample was calculated by interpolation of the found peak area ratio into the regression curve.

3. Results

3.1. Mass spectrometric and gas chromatographic behaviour of BTFMB derivatives

The use of BTFMB derivatives of glycine conjugates results under negative chemical ionisation in a single [M-BTFMB]$^-$ fragment, which allows sensitive and accurate detection. The negative chemical ionisation mass spectra of the glycine conjugates involved in this study are shown in Fig. 1. SG having two derivatised carboxylic groups, loses only one BTFMB group in the ion source, yielding the $-m/z$ 456 fragment. Due to the excellent gas chromatographic separation of the different acylglycines, no co-elution of the straight and branched-chain acylglycines was observed, as can be seen in Fig. 2. The good stability of the BTFMB-derivatives permits that samples can be analysed well, weeks after sample preparation.

Table 1
Selected ion monitoring target ions of acylglycines and their corresponding labelled analogues

Standard	Internal standard	Target ions ($-m/z$)	
		Standard	Internal standard
IBG	[$^2H_7$]IBG	144	151
BG	[$^2H_7$]BG	144	151
2MBG	[$^{13}C_2$]2MBG	158	160
IVG	[$^2H_3$]IVG	158	161
HG	[$^{13}C_2$]HG	172	174
PPG	[2-^{13}C,^{15}N]PPG	206	208
SG	[2-^{13}C,^{15}N]SG	456	458

3.2. Standard curves of acylglycines

The linearity of the GC-MS response was determined over a range of 0.5–10 nmol of the acylglycines added to water in a total sample volume of 1 ml, with the exception of PPG (0.1–2 nmol). Using the observed peak area ratios of the standard versus the internal standard, in all cases a linear relationship between the absolute added amount of standard and the observed ratio was found with a correlation coefficient, $R > 0.999$.

3.3. Inter- and intra-assay variation

Table 2 shows the results of the inter- and intra-assay variability for the glycine conjugates involved in this procedure. Intra-assay variability was established by the analyses of five identical urine samples in one sample preparation. For the establishment of the inter-assay variation another urine sample was processed in five independent sample preparations on 5 different days, using five successive calibration curves. The intra-assay variability ($V = \mathrm{sd}\bar{x} * 100\%$) ranged from $V = 0.62\%$ for 2MBG to $V = 5.62\%$ for BG and the inter-assay variation ranged from $V = 4.67\%$ for 2MBG to $V = 13.8\%$ for BG.

3.4. Limit of detection and limit of quantification

The limit of detection (LOD) ($S/N = 5$) was estimated by verifying the observed baseline noise in a urine sample nearby the chromatographic region of the acylglycine of interest and found to range from 0.2 nmol/l for 2MBG to 3 nmol/l for SG in the described sample volume. The lower limit of quantification (LLOQ) ($S/N = 10$) ranged from 0.4 nmol/l for 2MBG to 6 nmol/l for SG.

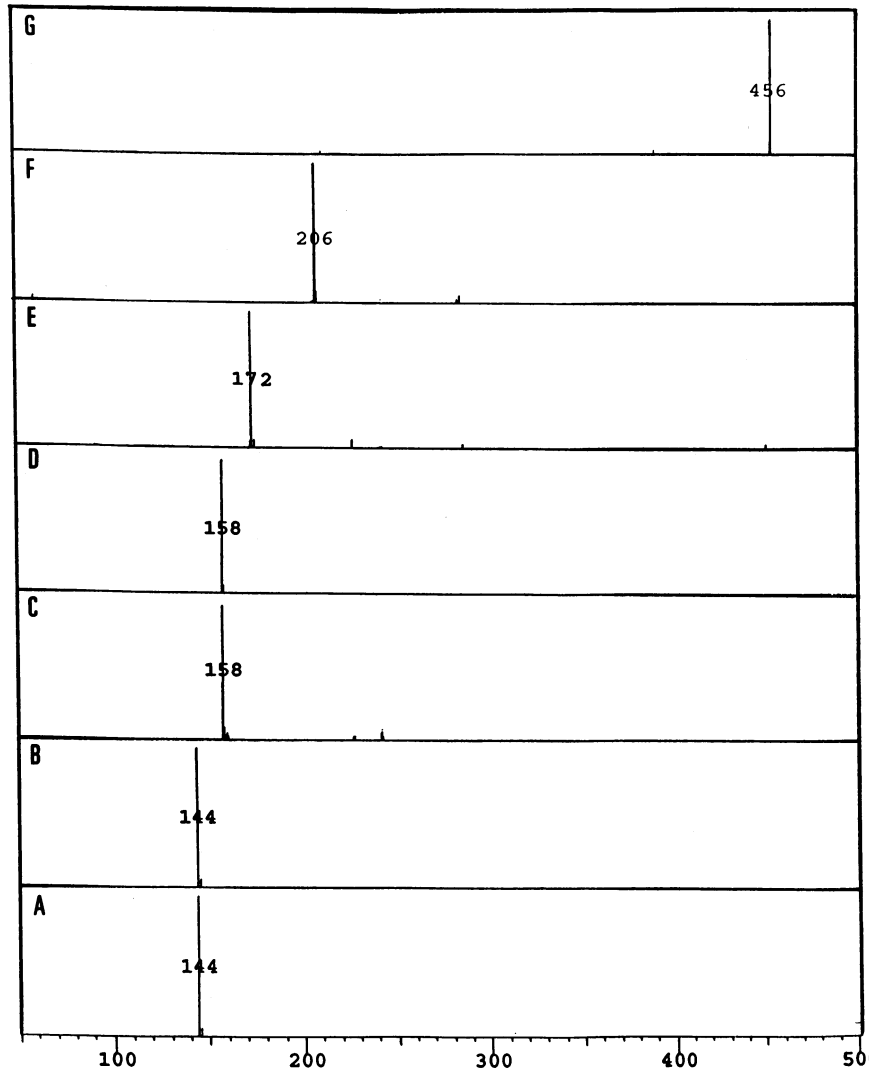

Fig. 1. Mass spectra of BTFMB ester derivatives of glycine conjugates obtained by NCI-MS using methane as reagent gas; (A) IBG, (B) BG, (C) 2MBG, (D) IVG, (E) HG, (F) PPG, and (G) SG.

3.5. Extraction efficiency

The efficiency of the ethyl acetate extraction was monitored by adding the labelled internal standard to a urine sample prior to the ethyl acetate extraction versus the addition of the labelled internal standards to the combined ethyl acetate extraction fractions. This experimental set-up was performed in fivefold using each time the same urine sample. The efficiency of the ethyl acetate extraction was calculated by the relation of the observed peak area ratios of the two different extracts (addition of internal standards prior to, and just after ethyl acetate extraction). The average recovery of the different acylglycines, expressed as percentage recovered analyte, ranged from 87% for SG to 103% for BG.

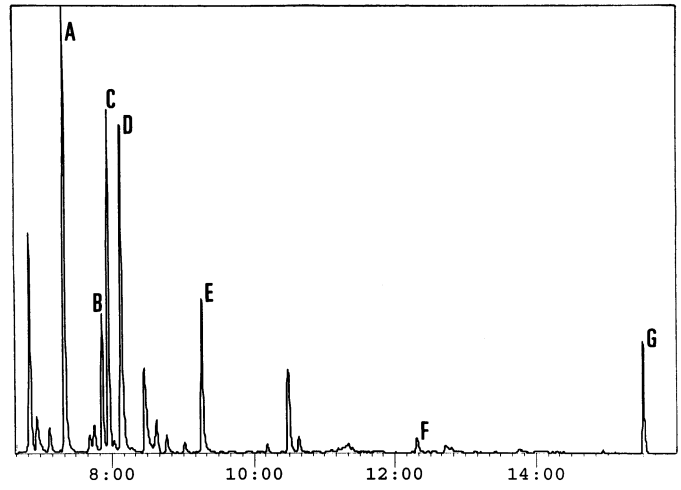

Fig. 2. Ion current trace for NCI-MS at m/z (M-BTFMB) from a pool urine spiked with a mixture of glycine conjugates. (A) IBG, (B) BG, (C) 2MBG, (D) IVG, (E) HG, (F) PPG, and (G) SG.

3.6. Reference values and pathological values

An overview of the urinary excretion of the glycine conjugates in controls and patients is shown in Table 3 and Fig. 3A and B. The urinary acylglycine profile in controls was characterised by a preponderance of the branched-chain glycine conjugates, especially IBG and IVG. HG was found to be the most abundant straight-chain glycine conjugate while PPG and BG were the glycine conjugates excreted in the lowest amounts in controls. In the four children fed a MCT-supplemented diet, no significant increase was found in the excretion of branched-chain acylglycines while the ones derived from fatty acid metabolism showed a slight increase. In the patients with vLCAD, LCHAD and a respiratory chain defect no significantly different profile was found compared to controls. Fig. 3A and B illustrate the excretion patterns of the control and patients groups. An impressive increase of the whole range of acylglycines, especially IBG and HG, characterised the urinary profile of the single neonatal MAD patient. The profile of the mild MAD patients, although qualitatively similar to the neonatal form, was characterised by much lower acylglycines concentrations. The acylglycine pattern of MCAD deficient patients was characterised by a marked increase of SG, HG and PPG while BG as well as the branched chain conjugates were sometimes slightly elevated.

4. Discussion

We developed a sensitive and accurate stable isotope dilution GC-NCI-MS method for quan-

Table 2
Intra- and inter-assay variability of the described NCI GC-MS method[a]

Acylglycine	Intra-assay variability ($n=5$)	Inter-assay variability ($n=5$)
IBG	1.91 ± 0.066 ($V=3.43\%$)	0.65 ± 0.062 ($V=9.57\%$)
BG	0.87 ± 0.048 ($V=5.62\%$)	0.057 ± 0.008 ($V=13.8\%$)
2MBG	0.77 ± 0.005 ($V=0.62\%$)	0.18 ± 0.008 ($V=4.67\%$)
IVG	1.91 ± 0.014 ($V=0.75\%$)	0.59 ± 0.051 ($V=8.72\%$)
HG	0.84 ± 0.007 ($V=0.82\%$)	0.15 ± 0.017 ($V=11.5\%$)
PPG	0.17 ± 0.009 ($V=5.15\%$)	0.030 ± 0.003 ($V=11.6\%$)
SG	0.65 ± 0.012 ($V=1.91\%$)	0.024 ± 0.003 ($V=12.5\%$)

[a] Two different pooled urine samples were used. Concentration of glycine conjugates in mmol/mol creatinine.

Table 3
Urinary acylglycine excretion in controls and the different patient groups[a]

	BG	IBG	2MBG	IVG	HG	PPG	SG
MAD-n[b]	144	299	10	84	182	6.8	50
MAD-m[c]	2.2 (0.17–1.0)	8.9 (2.0–22)	1.5 (0.33–3.3)	13 (1.3–6.1)	18 (0.77–38)	0.35 (0.037–0.83)	9.9 (0.30–38)
MCAD[d]	0.40 (0.14–0.91)	1.3 (0.078–3.6)	0.81 (0.20–2.0)	2.4 (0.28–6.7)	41 (5.5–122)	8.9 (0.10–28)	87 (5.2–359)
Others[e]	0.11 (0.056–0.20)	0.28 (0.048–0.68)	0.12 (0.032–0.32)	0.34 (0.059–0.90)	0.31 (0.055–0.60)	0.024 (0.008 –0.054)	0.33 (0.082–1.3)
MCT-diet[f]	0.12 (0.015–0.28)	0.30 (0.12–0.57)	0.15 (0.077–0.22)	0.17 (0.087–0.12)	0.65 (0.17–1.11)	0.11 (0.012–0.20)	1.1 (0.40–1.1)
Controls	0.041 (0.007–0.12)	0.36 (0.003–1.5)	0.20 (0.002–0.58)	0.37 (0.027–0.92)	0.30 (0.014–0.83)	0.033 (<0.002–0.15)	0.14 (0.024–0.52)

[a] Urine concentrations in mmol/mol creatinine expressed as mean and range.
[b] MAD-n, neonatal MAD ($n = 1$).
[c] MAD-m, mild MAD ($n = 6$).
[d] MCAD, deficiency ($n = 10$).
[e] Others, patients with other inborn errors of metabolism in whom an increased acylglycine excretion is not expected (two vLCAD patients, two LCHAD patients, and one patient with a respiratory chain defect).
[f] MCT-diet, MCTs-enriched diet ($n = 4$).

tification of short- and medium-chain acylglycines in urine. The applicability of the method was illustrated by the demonstration of abnormal levels of these acylglycines in urine samples of MCAD- and MAD-deficient patients and normal levels in urine samples of patients affected with long-chain fatty acid oxidation defects. Furthermore, the method was used to study the effect of an MCT enriched diet on the excretion of acylglycines. The method is based on formation of BTFMB derivatives providing a very good GC resolution and a favourable NCI mass spectrum showing one intense signal at m/z $[M\text{-}BTFMB]^-$. In literature, several derivatisation reagents for the GC-MS analysis of acylglycines have been described. Most of these reagents had serious drawbacks. Derivatisation as TMS-esters has been reported to yield multiple and unstable derivatives which give poor GC resolution [20]. Methyl esters [21], although more stable and giving better GC results, unfavourably fragment in the mass spectrometer (both in EI and CI), which negatively influences the sensitivity of the method [18]. Recently, Bonham Carter et al. [22] reported the formation of pentafluoropropionyl-trifluoroethyl derivatives of acylglycines. Their method has the disadvantage of producing multiple GC peaks for 2MBG, while SG was incompletely derivatised. In our method derivatisation with BTFMB-bromide proceeds under mild conditions, yielding stable derivatives which can be analysed well, weeks after sample preparation. Furthermore, the hexane extraction of the derivatives provides an additional clean-up procedure without loss of sensitivity. The acylglycine profile observed in controls was dominated by IBG and IVG, derived from branched-chain amino acid metabolism and HG from fatty acid metabolism. The concentrations of 2MBG and SG were lower, whereas BG and PPG were present in the lowest concentrations. This profile may reflect the various concentrations of the correspondent acyl-CoA esters within the mitochondrion. In addition, the substrate specificity of glycine-*N*-acylase [7] and the occurrence of other conjugation mechanisms [1,2] forms the basis of this profile.

The acylglycine profiles in the urine from children on a MCT enriched diet showed increased concentrations of glycine conjugates derived from medium-chain fatty acid metabolism. SG showed

the most prominent increase, reflecting the contribution of ω-oxidation as an alternative to the overloaded β-oxidation during MCT supplementation. In accordance with the findings of Rinaldo et al. [4], we observed a wide range of values of urinary SG in MCT fed children. We did not detect an overlap between the concentrations of SG in MCT fed children and in patients affected with MCAD deficiency, the latter being always higher, so no diagnostical errors can be expected in case a child receives a MCT enriched diet. The acylglycine profiles in urine from patients affected with long-chain fatty acid oxidation defects or with a respiratory chain defect were normal, as expected. Only one patient excreted elevated amounts of SG, which probably reflects an increased ω-oxidation as a consequence of a secondary defect in β-oxidation. The urinary acylglycine profiles in all patients with MCAD deficiency were clearly abnormal. In all cases, HG and SG were substantially increased. PPG was elevated in most of the patients, however normal

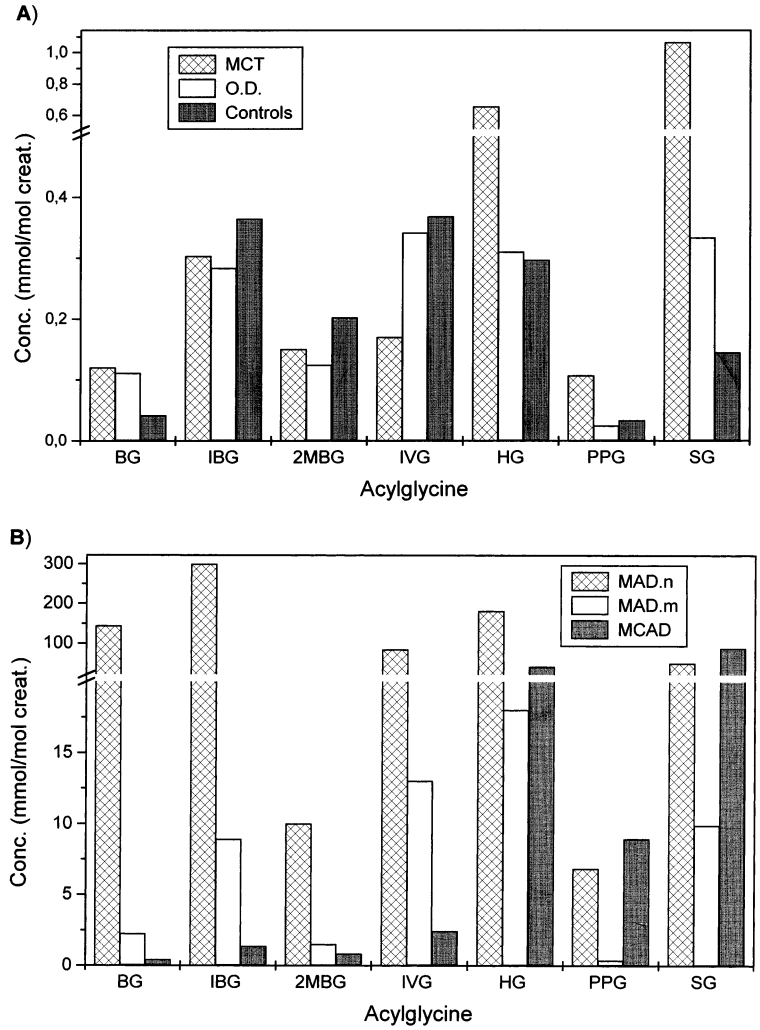

Fig. 3. Urinary acylglycine profile from: (A) Healthy children, patients with inborn errors of metabolism in whom an increased urinary excretion of glycine conjugates is not expected (O.D), and children under MCT diet. (B) Neonatal MAD (MAD-n), mild form of MAD (MAD-m), and MCAD deficiency.

in two samples from one MCAD deficient patient during periods of remission. These results are in accordance with those of Bennett et al. [5], who reported absence of PPG in blood from three proven MCAD deficient patients. Phenylpropionic acid (PPA), the precursor of PPG, is produced by anaerobic gut bacteria [23]. The amount of PPA produced depends on the ontogeny of colonisation and on antibiotic therapy [24]. Thus, absence of elevated levels of PPG does not rule out MCAD deficiency. The excretion of short-chain acylglycines in MCAD deficiency varies greatly. BG levels were in all cases higher than in controls, which may reflect the intramitochondrial accumulation of butyryl-CoA due to overlapping substrate specificity of MCAD and SCAD. The increased concentrations of IBG, 2MBG and IVG in some MCAD deficient patients may reflect the acceleration of amino acid catabolism in bad periods. MAD, both the severe and the mild form, is accompanied by variably increased excretion of acylglycines. The acylglycine profiles in urine show increased excretions of short- and medium-chain acylglycines. In the severe neonatal form of the disease, the extremely high concentrations of short- and medium-chain acylglycines directly point to the diagnosis and rule out any possible misdiagnosis. In the mild form however, a great heterogeneity in the concentrations of acylglycines was found. In all cases, concentrations of BG, IBG and IVG were elevated, whereas in all but one case (patient during remission) HG was elevated too. In accordance with previous reports, PPG and SG were normal in some urine specimens. The diagnosis of late onset MAD should thus be considered in cases of increased excretions of branched-chain acylglycines, accompanied or not by elevated levels of HG and SG. Furthermore, one should realise that the profile as a whole is more important than the individual acylglycines. Our results show that in MCAD deficiency, short-chain acylglycines may be elevated. A considerable overlap between the excretions in MCAD deficiency and in MAD was found. This overlap, however, will not cause diagnostic problems, as in MCAD deficiency the excretion of HG and SG was always preponderant, whereas in mild MAD several acylglycines are mildly increased, especially IBG, IVG, HG and SG. We conclude that this new method allows accurate quantification of the studied acylglycines. The sensitivity of the method allows differentiation between MCT fed children and patients affected with MCAD deficiency and allows differentiation between the severe and mild forms of MAD.

Acknowledgements

Part of this work was made possible by a grant (BD 2589/93-ID) of 'Junta Nacional de Investigacao Cientifia e Tecnologia-Programa Praxis XXI' (Lisbon, Portugal) to C.G. Costa.

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