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Screening for fatty acid beta oxidation disorders Acylglycine analysis by electron impact ionization gas chromatography–mass spectrometry

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Abstract

Urinary acylglycine analysis by chemical ionization (CI) GC–MS has been utilized for more than a decade to screen of fatty acid oxidation disorders. We have developed an alternative GC–MS method involving *tert*.-butyldimethylsilyl derivatization and standard electron impact ionization. Using six stable isotope labeled internal standards, this method allows the biochemical diagnosis of glutaric aciduria type II and medium chain acyl-CoA dehydrogenase deficiency, and could contribute to the diagnosis of other FAO disorders when used in combination with other biochemical investigations on blood and urine. This method can be conveniently applied to GC–MS system routinely used for organic acid analysis.

Keywords: Fatty acids; Acylglycine

1. Introduction

The biochemical diagnosis of fatty acid oxidation disorders is a challenging process because patients may appear perfectly normal when not distressed by an episode of fasting intolerance. In addition to organic acid analysis, acylglycine analysis by gas chromatography–mass spectrometry [1–3] and more recently acylcarnitine analysis by tandem mass spectrometry [4] have been developed as more informative diagnostic tools. Acyglycine analysis was originally developed by Rinaldo et al [1,2]. They reported that hexanoylglycine and phenylpropionylglycine measurements would recognize patients with medium chain acyl-CoA dehydrogenase even when asymptomatic. In this study, we modified and simplified their method using *tert*.-butyldimethylsilyl (*t*-BDMS) derivatization and electron impact ionization (EI).

2. Materials and methods

2.1. Reagents

Urease was purchased from Sigma, St. Louis, MO; the hydrocarbon mixture (C10-C26, even numbers) and tetracosane (C24) from Seikagaku-Kogyo, Tokyo, Japan; and *N*-(*tert*.-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (BDMMTFA) from Aldrich, Milwaukee, WI; and dimethylformamide

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(DMF) from Nakarai Tesque, Tokyo, Japan. Ethylmalonate was purchased from Nakarai Tesque, Tokyo, Japan, and isovalerylglycine from Tokyo Kasei-Kogyo, Tokyo, Japan. Other acylglycine standards were made available by Dr. Piero Rinaldo (Yale University, New Haven, CT).

2.2. Stable-isotopes labeled internal standards

[D3]-Ethylmalonate, [D3]-isovalerylglycine, hexanoyl-[1,2-^{13}C] glycine, suberyl-[1,2-^{13}C] glycine, were synthesized according to the methods previously described [5,6]. [1,2-^{13}C]-Dodecanedioic acid and [D4]-glutarate were purchased from MSD Isotopes (Montreal, Canada), and C/D/N Isotopes (Quebec, Canada), respectively. The purity of the stable isotopes was >99% as judged by the lack of additional peaks on GC–MS. The isotopic enrichment of the stable isotopes was also more than 99%.

2.3. Sample preparation

Urine samples containing 0.4 mg of creatinine were analyzed. At first, 20 units of urease was added followed by incubation at 37°C for 30 min. As internal standards, 20 nmoles of [D3]-ethylmalonate, [D4]-glutarate, [D3]-isovalerylglycine, hexanoyl-[1,2-^{13}C] glycine and suberyl-[1,2-^{13}C] glycine, and 10 nmoles of [1,2-^{13}C] dodecanedioic acid were added, and the final volume was adjusted to 4.0 ml with distilled water. After acidification to pH 1.0 with 6 N HCl and the addition of 1 g of NaCl, the organic acid fractions were extracted three times with 6 ml of ethylacetate. After centrifugation, the organic phases were combined and dehydrated with 5 g of anhydrous sodium sulfate. The supernatant was evaporated under a gentle nitrogen stream at 60°C. The final dry residue was derivatized by the addition of 100 μl of a mixture of BDMMTFA and DMF (1:1, v/v) [7], allowed to react at 80°C for 30 min.

2.4. GC–MS analysis

A capillary GC–MS system, Shimadzu model QP 5000 (Shimadzu Co. Ltd., Kyoto, Japan), equipped with a Class 5000 data processing system, was used. The capillary column was a fused-silica DB-5 (30 m×0.25 mm I.D.) with a 0.25 μm film thickness of 5% phenylmethyl silicone (J&W, Folsom, CA). Mass spectra were obtained by electron impact ionization–selective ion monitoring (EI–SIM) at the scan rate of 0.5 s/cycle. The temperature program was started at 100°C with initial holding for 4 min, and was increased at the rate of 4°C/min to 290°C, with final holding for 10 min. The temperatures of the injection port and transfer line were both 280°C. The flow-rate of the helium carrier was 1.3 ml/min, and the linear velocity was 43.1 m/s. One microliter of the final derivatized aliquot was injected into the GC–MS in the splitless mode. Quantitation was performed by calculation of the relative peak areas (RPA,%) between the quantitative ions of the compound, $[M-57]^+$, and the corresponding ions of the stable isotope standards (Table 1). A standard methanol solution, which contained unlabeled ethylmalonate, glutarate, isovalerylglycine, hexanoylglycine and suberylglycine, each at the concentration of 200 nmol/ml, and dodecanedioic acid at 100 nmol/ml, was prepared.

Standard curves for the stable isotope dilution method were obtained in the following way. Fixed amount of the stable isotopes (20 nmol each, except dodecanedioic acid, 10 nmol) and a various amount of the standard methanol solution (5, 25, 50, 100, or 500 μl) were analyzed.

Table 1
Quantitative ions of the labelled and unlabelled marker compounds

	m/z [M-57]+		*m/z* [M-57]+
Ethylmalonate	303	Hexanoylglycine	230
[D3]-Ethylmalonate	306	Hexanoyl-[1,2-13C] glycine	232
Glutarate	303	Suberylglycine	402
[D4]-Glutarate	307	Suberyl-[1,2-13C] glycine	404
Isovalerylglycine	216	Dodecanedioic acid	401
[D3]-Isovalerylglycine	219	[1,2-13C]-Dodecanedioic acid	403

Reproducibility was determined by analyzing 100 μl of the standard methanol solution five times. Moreover, a urine sample from a patient with glutaric aciduria type 2 (GA2) in the acute condition, was also analyzed five times for the reproducibility of each metabolite.

2.5. Patient samples

Urine specimens from three patients with GA2, including one in a stable condition, three patients with medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, also including one in a stable condition, a patient with short-chain acyl-CoA dehydrogenase (SCAD) deficiency, and a patient with very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency with two samples in both stable and fasting conditions, were analyzed. The diagnoses of these patients were confirmed by immunoblotting and/or enzyme assays. Urine specimens from 3 patients with ketotic dicarboxylic aciduria and 10 healthy children were also examined.

3. Results

The EI mass spectrum of unlabeled and labeled hexanoylglycine are shown in Fig. 1. The $[M-57]^+$ ions at m/z 230 and 232, respectively, were selected for quantitative purpose. The $[M-57]^+$ ion peaks were also prominent for the other compounds.

The coefficients linear correlation for calibration points between 1 nmol and 100 nmol were as follows: ethylmalonate, 0.998; glutarate, 0.997; isovalerylglycine, 1.000; hexanoylglycine,1.000; suberylglycine, 0.990; and dodecanedioic acid, 0.996. The CV values for five assays of the standard solution were as follows: ethylmalonate, 1.5%; glutarate, 1.4%; isovalerylglycine, 4.3%; hexanoylglycine, 0.8%; suberylglycine, 6.0%; and dodecanedioic acid, 1.2%. Moreover, the reproducibility using urine samples of a patient with glutaric aciduria type 2 was shown in Table 2. The CV value of glutarate was high, possibly because the peak of glutarate on the chromatogram was huge and the ions were saturated.

Table 3 shows the urinary levels of the marker

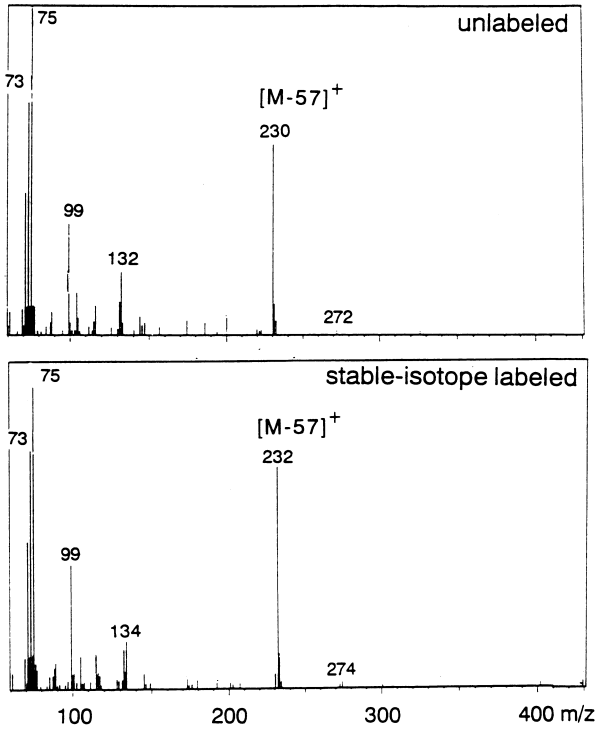

Fig. 1. Mass spectrum of *t*-BDMS derivatives of hexanoylglycine (upper) and stable isotope-labeled hexanoyl-[1,2-^{13}C] glycine.

Table 2
Reproducibility analyzed five times of a urine sample from a patient with glutaric aciduria type 2 in the acute condition[a]

	Mmol/mol Cr				
	Mean	Min	Max	SD	CV (%)
EMA	61.3	59.6	64.4	1.9	3.0
GA	244.2	225.6	263.8	18.0	7.4
IVA	179.0	175.2	181.5	2.9	1.6
HG	13.0	13.3	12.7	0.2	1.7
SG	9.8	9.7	10.1	0.2	1.8
C12	11.7	11.1	11.8	0.6	2.8

[a] Abbreviations: EMA, ethylmalonate; GLU, glutarate; IVG, isovalerylglycine; HG, hexanoylglycine; SG, suberylglycine; C12, dodecanedioic acid.

metabolites obtained with this method in various fatty acid oxidation disorders. In GA2, isovalerylglycine and ethylmalonate were elevated even in a sample obtained in a stable condition. In SCAD deficiency, ethylmalonate and glutarate were highly elevated (Fig. 2). In MCAD deficiency, hexanoylglycine and suberylglycine were characteristically increased. The profile of a patient with VLCAD deficiency was non-specific in a stable condition, but a marked elevation of dodecanedioic acid in response to fasting. In children with physiological ketosis, suberylglycine was mildly increased.

4. Discussion

We developed a method for the screening of fatty acid oxidation disorders involving stable isotope-labeled internal standards of selected acylglycines and organic acid. Originally, Rinaldo et al. reported the importance of the analysis of hexanoylglycine and phenylpropionylglycine in diagnosis of MCAD deficiency [2]. They used methylation and ammonia chemical ionization (CI) to generate intense peaks of $[M+H]^+$ ions, which were conveniently used for quantitation. In this study, we tested the use of EI and *t*-BDMS derivatization. *t*-BDMS derivatization yields an intense peak of the $[M\text{-}57]^+$ ion, which is suitable as quantitation [8]. Compared to CI, EI/*t*-BDMS derivatization does not require a reactive gas. This allows us to utilize a GC–MS in the same configuration used for routine urine organic acid analysis.

In this study, we examined four kinds of fatty

Table 3
Urinary excretion of acylglycines and organic acids in fatty acid oxidation disorders determined by the EI/*t*-BDMS method[a]

	Patient no.	Mmol/mol Cr					
		EMA	GLU	IVG	HG	SG	C12
GA-2	1	37.1	9.4	5.2	3.2	1.0	1.5
	2	43.3	30.0	109.2	7.4	5.4	18.2
	3 (stable)	14.9	1.7	6.6	1.3	0.0	0.0
MCAD	1	2.6	1.5	2.7	5.9	4.5	0.0
	2	4.4	1.2	5.3	2.3	12.7	0.0
	3 (stable)	0.6	0.5	0.1	1.0	0.6	0.0
VLCAD	1 (stable)	1.8	0.4	0.2	0.2	0.1	0.0
	1 (fasting)	4.0	5.4	0.4	0.6	0.5	5.6
SCAD	1	61.8	17.1	0.0	0.5	1.9	3.8
Ketosis	1	4.1	2.2	0.2	0.3	0.3	0.0
	2	15.2	5.9	0.6	0.7	0.4	0.5
	3	5.9	30.4	0.1	1.1	0.3	3.7
Controls	10	1.4–6.9	1.5–10.5	0.2–1.9	0.0–1.4	0.0–0.1	0.0–0.2

[a] Abbreviations: GA-2, glutaric aciduria type 2; VLCAD, very long chain acyl-CoA dehydrogenase deficiency; MCAD, medium chain acyl-CoA dehydrogenase deficiency; SCAD, short chain acyl-CoA dehydrogenase deficiency. Stable: sampled in a stable condition; controls: 3-year-old children.

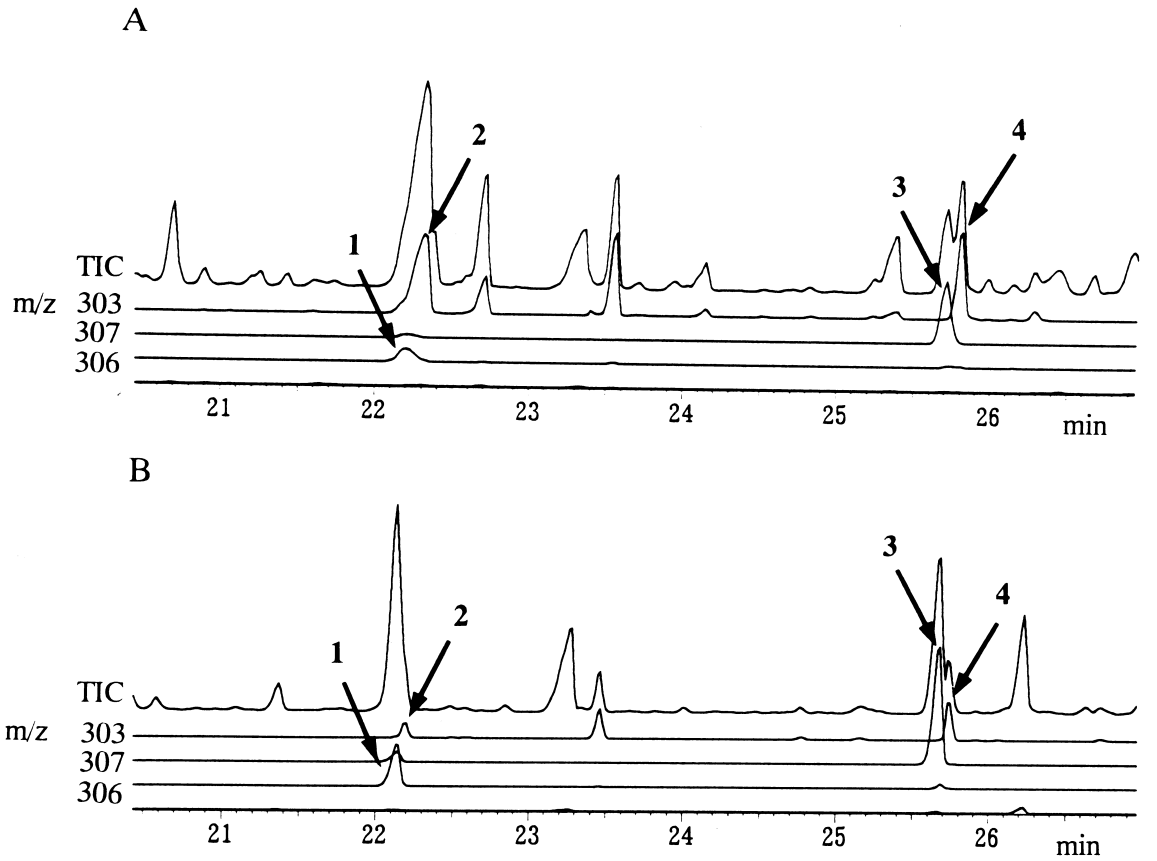

Fig. 2. Chromatograms (partly shown) from analysis of a urine sample with SCAD deficiency (A) and a control urine (B). Peak 1: [D3]-ethylmalonate, Peak 2: ethylmalonate, Peak 3: [D4]-glutarate, Peak 4: glutarate, TIC: total ion chromatogram (here, summation of ions in SIM). Ethylmalonate and glutarate were elevated in a patient with SCAD deficiency compared with those of a normal control.

oxidation disorders. Even in a stable condition, it was possible to correctly diagnose GA2 and MCAD deficiency. Isovalerylglycine was highly elevated in GA2, but also mildly elevated in MCAD deficiency. Ethylmalonate could differentiate GA2 from MCAD deficiency. The marked elevation of short chain fatty acids, ethylmalonate and glutarate, was compatible with SCAD deficiency. In MCAD deficiency, suberylglycine was elevated even in a stable condition. But suberylglycine was also mildly increased in ketotic dicarboxylic aciduria. In ketotic dicarboxylic aciduria, suberate is also elevated. Suberylglycine is formed from suberate through glycine-*N*-acylase-catalyzed conjugation [9]. In any conditions with an elevation of suberate, suberylglycine is possibly increased. Rinaldo et al. also reported that suberylglycine increased non-specifically, in ketosis and with a MCT formula [1,2]. As one sample of a patient with MCAD deficiency had a normal level of hexanoylglycine, it is recommended to rely on the determination of phenylpropionylglycine in addition to hexanoylglycine. In our experience, the clinical application of this method is found to be complementary to standard organic acid analysis especially when patients are asymptomatic.

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References

[1] P. Rinaldo, J.J. O'Shea, K. Tanaka, Lancet 2 (1987) 1158.
[2] P. Rinaldo, J.J. O'Shea, P.M. Coates, D.E. Hale, C.A. Stanley, K. Tanaka, New. Engl. J. Med. 319 (1988) 1308.
[3] S.M. Bonham Carter, J.M. Midgley, D.G. Watson, R.W. Logan, J, Pharmaceut. Biomed. Anal. 9 (1991) 969.
[4] D.S. Millington, N. Kodo, D.L. Norwood, C.R. Roe, J. Inherit. Metab. Dis. 13 (1990) 321.
[5] H.S. Ramsdell, K. Tanaka, Clin. Chem. Acta 74 (1977) 109.
[6] D.G. Hine, A.M. Hack, S.I. Goodman, K. Tanaka, Pediatr. Res. 20 (1986) 222.
[7] J. Straczek, F. Felden, B. Dousset, J. Chromatogr. 620 (1993) 1.
[8] A.P.J.M. De Jong, J. Elema, B.J.T. van de Berg, Biomed. Mass. Spectr. 7 (1980) 359.
[9] N. Gregersen, R. Lauritzen, K. Rasmussen, Clin. Chim. Acta 70 (1976) 417.