

# Evaluation of Urinary Acylglycines by Electrospray Tandem Mass Spectrometry in Mitochondrial Energy Metabolism Defects and Organic Acidurias

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**We analyzed the urinary acylglycine excretion in 26 patients with mitochondrial energy metabolism disorders and in 55 patients with organic acidurias by electrospray tandem mass spectrometry (ESI-MS/MS), monitoring precursor ions of  $m/z$  90. Urinary concentrations of the different acylglycines were quantified using deuterated internal standards. Normal values for the most important acylglycines were established. In MCAD and MAD (neonatal form) deficiencies, typical excretion patterns of urinary acylglycines were found in all the samples. In isovaleric aciduria, propionic aciduria, and 3-methylcrotonylglycinuria typical glycine conjugates were always found. Methylmalonic aciduria (mutase deficiency), multiple carboxylase deficiency, and 3-hydroxy-3-methylglutaric aciduria revealed pathological acylglycine profiles, even if not specific for the disease. In all these diseases acylglycine excretion seems to be less influenced by the clinical status than organic acid excretion. This method is a useful diagnostic tool for these metabolic disorders, complementary to organic acids and acylcarnitine profiles.** © 2000 Academic Press

**Key Words:** acylglycine; ESI-MS/MS; fatty acid oxidation; organic aciduria.

Glycine conjugation, an important detoxification system of the human body, takes place mainly in the liver (1). The mitochondrial enzyme glycine-*N*-acylase is responsible for conjugation of glycine with exogenous compounds to enhance their excretion in urine (1–3). Glycine conjugation is also an effective detoxification system for preventing accumulation of acyl-CoA esters in several inherited metabolic disorders. Acylglycines in urine are the direct expression of accumulation of the correspondent acyl-CoA esters in the mitochondrion (3). The study of acylglycines in urine can be diagnostic for inherited metabolic disorders, such as the defects in fatty acid oxidation (acyl-CoA dehydrogenases) and leucine catabolism (isovaleryl-CoA dehydrogenase, methylcrotonyl-CoA carboxylase). Furthermore, other enzyme defects involved in the catabolism of isoleucine, valine, and lysine (propionyl-CoA carboxylase, glutaryl-CoA dehydrogenase) are characterized by an accumulation of specific glycine conjugates. Common to all these disorders are life-threatening, “intoxication-like” metabolic crises, sometimes with the features of Reye-like syndromes. Specific metabolites, reflecting directly the enzymatic block and indirectly the intermediate metabolism impairment, are detectable in urine and blood during crisis. Urinary organic acid analysis and free and total carnitine in plasma are the main diagnostic tools in these situations, followed by the study of blood and urine acylcarnitine profiles. In the absence of metabolic decompensation, when the

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**TABLE 1**  
**Summary of Patients and Samples Analyzed in Different Disorders**

Disease	Number of patients	Number of samples
Mitochondrial energy metabolism disorders		
Multiple acyl-CoA dehydrogenase deficiency (MAD) or glutaric aciduria type II (ETF, ETF-dehydrogenase deficiency)		
Neonatal form	7	29
Late onset form	2	2
Fatty acid oxidation defects:		
Medium-chain acyl-CoA dehydrogenase deficiency (MCAD)	5	15
Long-/very-long-chain acyl-CoA dehydrogenase deficiency (LCAD/VLCAD)	7	10
Short-chain acyl-CoA dehydrogenase deficiency (SCAD)	1	9
Long-chain hydroxyacyl-CoA dehydrogenase deficiency (LCHAD)	1	1
Short-chain hydroxyacyl-CoA dehydrogenase deficiency (SCHAD)	1	1
Carnitine-palmitoyl-transferase II deficiency (CPT II)	2	5
Organic acidurias		
Isovaleric aciduria, isovaleryl-CoA dehydrogenase deficiency (IVA)	2	5
Propionic aciduria, propionyl-CoA carboxylase deficiency (PA)	16	47
Methylmalonic aciduria (MMA)		
Methylmalonyl-CoA mutase deficiency	15	48
Cobalamin C deficiency	6	13
3-Methylcrotonyl glycinuria, 3-methylcrotonyl-CoA carboxylase deficiency (MCG)	1	1
Multiple carboxylase deficiency (MCD)		
Holocarboxylase synthetase deficiency	2	7
Biotinidase deficiency	3	3
3-Hydroxy-3-methylglutaric aciduria (3-OH-3-methylglutaryl-CoA lyase deficiency)	3	10
Glutaric aciduria type I	7	29
Total	81	235

subject is doing well, these parameters are often not informative, and early diagnosis is essential to treat the disease and prevent a metabolic crisis.

So far, urinary acylglycine excretion was studied by gas chromatography mass spectrometry (GC/MS) (4,5) and liquid secondary ion tandem mass spectrometry (LSI-MS/MS) (6) mainly in medium-chain acyl-CoA dehydrogenase deficiency. The aim of the present study was to analyze the urinary acylglycine excretion pattern in a wide spectrum of inborn errors of metabolism by means of electrospray tandem mass spectrometry (ESI-MS/MS).

## METHODS

**Subjects.** We analyzed 235 urine samples of 81 patients (aged a few days to 30 years) affected by 14 different metabolic diseases (Table 1). The samples were collected from patients with known diagnoses, in different clinical situations, and studied retrospectively. Fifty-four urine samples from children, aged a few days to 15 years, were analyzed as controls. No significant age-dependent difference in acylglycine excretion was observed. Organic acid profiles were normal in all the control samples.

**Internal standard solution.** Fifty microliters of internal standard solution contained 150 ng d<sub>3</sub>-acetyl glycine, 20 ng d<sub>3</sub>-propionyl glycine, 30 ng d<sub>7</sub>-butyryl glycine, 30 ng d<sub>9</sub>-valeryl glycine, and 20 ng d<sub>3</sub>-hexanoyl glycine (in methanol). All the internal standards were <sup>2</sup>H-labeled in the acyl group. The deuterated acylglycines were purchased from H. ten Brink, Academic Hospital, Amsterdam.

**Preparation of acylglycine-methylesters.** All the urine samples were derivatized to methylesters. Other derivatization procedures were used only for structure elucidation of unknown peaks. The amount of urine used for acylglycine analysis depended on the creatinine concentration (see Table 2). Fifty microliters of internal standard solution were added to the urine sample, which was subsequently dried in a SpeedVac concentrator (Savant, Farmingdale, NY) at 42°C and treated with 50 μl of 3 M methanolic HCl (Supelco, Bellefonte, PA) at 65°C for 15 min in a screw-capped vial. After evaporation of the solvent, the residue was reconstituted in 100 μl of methanol/water (50/50, vol/vol) containing 0.1% acetic acid.

**TABLE 2**  
**Creatinine-Dependent Amount of Urine Used**  
**for Acylglycine Analysis**

Creatinine concentration	Volume of urine
<1.0 mmol/L	200 $\mu$ l
1.0–2.0 mmol/L	100 $\mu$ l
2.1–3.4 mmol/L	60 $\mu$ l
3.5–4.9 mmol/L	40 $\mu$ l
5.0–6.9 mmol/L	30 $\mu$ l
7.0–9.0 mmol/L	20 $\mu$ l
>9.0 mmol/L	10 $\mu$ l

*Preparation of acylglycine-butylesters.* The same procedure as for acylglycine-methylesters was used for acylglycine-butylesters. Instead of methanolic HCl, 3 M butanolic HCl (Regis, Morton Grove, IL) was used as derivatization reagent.

*Preparation of acetylated acylglycine-butylesters.* The urine samples were derivatized to butylesters prior to acetylation of the hydroxy groups. The butylated acylglycine samples were dried in a Speed-Vac concentrator at 42°C. The residue was reconstituted in 100  $\mu$ l of acetic anhydride/pyridine (50/50, vol/vol). The sample was allowed to react at room temperature for 60 min. After evaporation of the solvent, the residue was redissolved in 100  $\mu$ l of methanol/water (50/50, vol/vol) containing 0.1% acetic acid.

*Mass spectrometry.* ESI-MS/MS was carried out on a Perkin-Elmer SCIEX API 365 LC/MS/MS system (Toronto, Canada). Samples were infused into the electrospray ion source via a 50  $\mu$ m i.d. fused silica transfer line using a Kontron Model 420 HPLC pump (Rotkreuz, Switzerland) at 20  $\mu$ l/min. The mobile phase used for all experiments was methanol/water (50/50, vol/vol) containing 0.1% acetic acid. The sample solution (20  $\mu$ l) was injected with a Rheodyne 8125 injector valve (Cotati, CA) equipped with a 20- $\mu$ l sample loop. Nitrogen was used as the collision gas at a pressure of 7 mTorr. Positive-ion electrospray MS/MS was performed at an orifice voltage of 0 V and the collision energy was set to 13 eV. For acylglycine-methylester profiles, the MS/MS method proposed by Millington *et al.* (6) was applied; precursor ions of  $m/z$  90 were monitored in the range  $m/z$  125–300 with step size 0.1 (4 s per scan). Additionally, precursor ions of  $m/z$  91 were monitored in the range  $m/z$  132–138, 164–170, and 180–186, with step size 0.1 (0.6 s per scan). Precursor ion scans of  $m/z$  91 are performed for analysis of the isotopically

labeled standards  $d_3$ -acetylglycine ( $m/z$  135),  $d_7$ -butyrylglycine ( $m/z$  167), and  $d_9$ -valeryl-glycine ( $m/z$  183), all of which have a deuterium atom at C-2 of the acyl group. The deuterium atom participates in the formation of the daughter ion at  $m/z$  91 (6). By alternatively switching the scan function from parents of  $m/z$  90 to parents of  $m/z$  91, the internal standards were acquired simultaneously from the urine sample. Fifteen to 20 scans were averaged to obtain the final spectrum.

For acylglycine-butylester profiles and for acetylated acylglycine-butylester profiles, precursor ions of  $m/z$  132 were monitored in the range  $m/z$  170–450 with step size 0.1 (5 s per scan).

For analyzing daughter ions of the corresponding masses, the same conditions as those for precursor ion scans were applied.

A solution of 7.6  $\mu$ M acetylglycine, 6.9  $\mu$ M propionylglycine, 1.7  $\mu$ M butyrylglycine, 9.2  $\mu$ M valeryl-glycine, and 6.4  $\mu$ M hexanoylglycine in methanol was derivatized to acylglycine-methylesters according to the procedure described above; this solution was used for mass spectrometer tuning.

*Creatinine concentration.* Creatinine concentrations were measured by the Jaffé-modified kinetic method (7) by a Beckman Synchron CX5 analyzer (Brea, CA).

*Quantification of acylglycine-methylesters.* The acylglycine-methylester profiles are quantified by comparing peak heights of isotopically labeled acylglycines with natural acylglycines (stable isotope dilution method). All concentrations are expressed as millimole per mole of creatinine. Acylglycines for which no deuterated internal standards were available were quantified by using the standard with the closest molecular mass.

## RESULTS

*Structure elucidation.* In all the samples acylglycines were analyzed after derivatization to methylesters. Most of the peaks detected in urine correspond to acylglycines. Some of them, however, correspond to other compounds which yield also  $m/z$  90 without belonging to acylglycines. In order to distinguish between these compounds and acylglycines, various tandem mass spectrometric experiments were performed. (i) In addition to acylglycine-methylesters, acylglycine-butylesters were prepared. Butylester samples were analyzed by monitoring the precursor ions of  $m/z$  132. Candidates for acylgly-

**TABLE 3**  
**Masses and Abbreviations of Protonated Acylglycines**

Mass (amu)	Abbreviation	Acylglycine-methylester
132	ACG	Acetylglycine
135	d <sub>3</sub> -ACG	d <sub>3</sub> -Acetylglycine (internal standard)
146	PG	Propionylglycine
149	d <sub>3</sub> -PG	d <sub>3</sub> -Propionylglycine (internal standard)
150 <sup>a</sup>		
160	BG, IBG, C <sub>4</sub> -G	Butyrylglycine, isobutyrylglycine
167	d <sub>7</sub> -BG	d <sub>7</sub> -Butyrylglycine (internal standard)
172	TG	2-Methylcrotonylglycine (tiglylglycine)
172	MCG	3-Methylcrotonylglycine (3,3-dimethylacryloylglycine)
174	IVG, MBG, C <sub>5</sub> -G	Isovalerylglycine, 2-methylbutyrylglycine
176	HBG	Hydroxybutyrylglycine
183	d <sub>9</sub> -VG	d <sub>9</sub> -Valerylglycine (internal standard)
188	HG, MVG, C <sub>6</sub> -G	Hexanoylglycine, 4-methylvalerylglycine
190	HVG	Hydroxyvalerylglycine
191	d <sub>3</sub> -HG	d <sub>3</sub> -Hexanoylglycine (internal standard)
200	C <sub>7:1</sub> -G	Heptenoylglycine
202	MHG, C <sub>7</sub> -G	4-Methylhexanoylglycine, heptanoylglycine
208	PAG	Phenylacetylglycine
218	GG	Glutarylglycine
222	PPG	Phenylpropionylglycine
232	7-HOG, 8-HOG	7-Hydroxyoctanoylglycine, 8-hydroxyoctanoylglycine
260	SG	Suberylglycine

<sup>a</sup> Hcy, homocysteine.

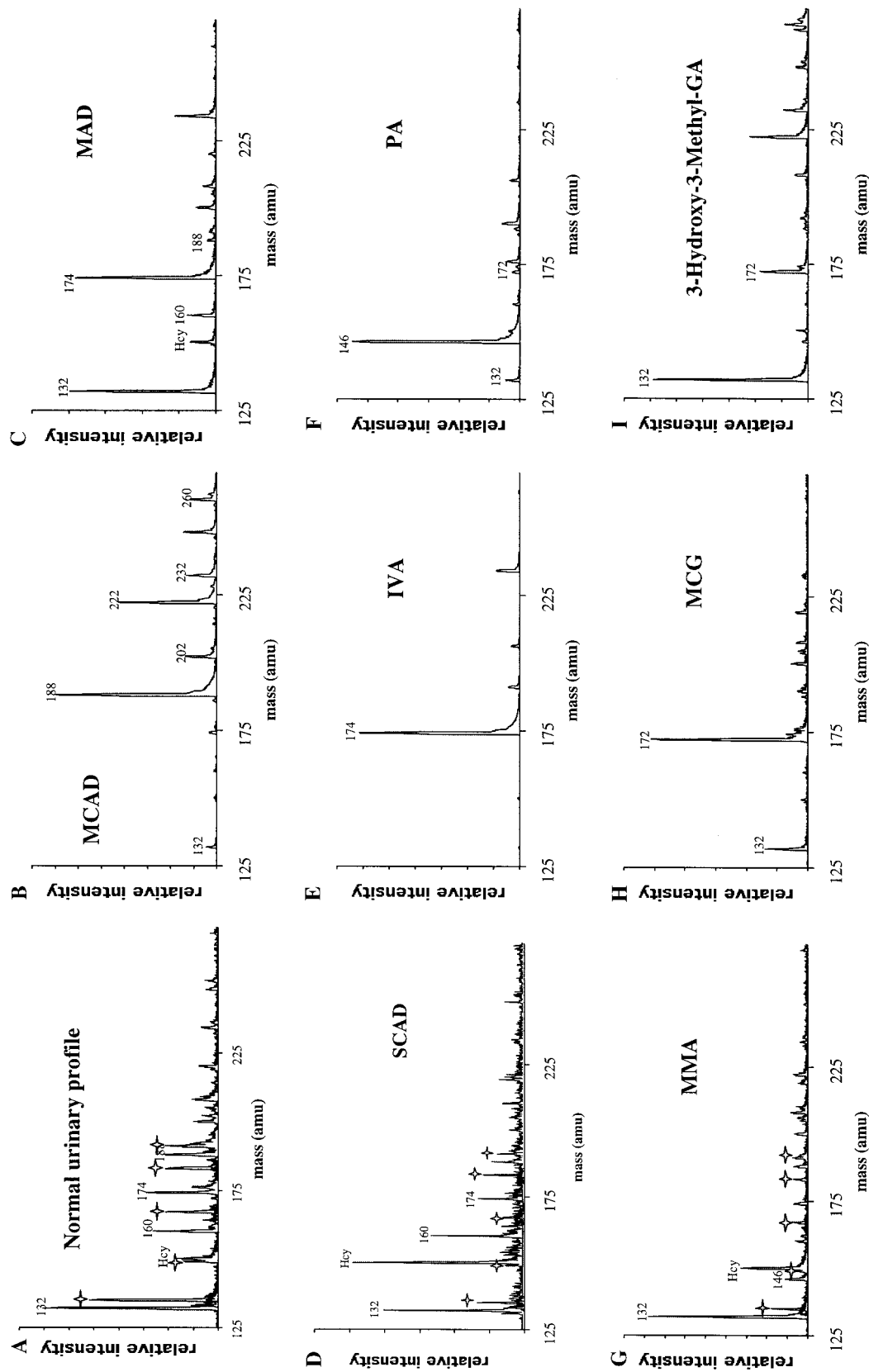
cines should exhibit a molecular weight shift of +42 amu compared to the corresponding methylester (dicarboxylic acylglycines: +84 amu). Comparison of the acylglycine-methylester and the acylglycine-butyrylester profiles of the same sample allows identification of the peaks that do not correspond to glycine conjugates. (ii) Hydroxy-acylglycines were distinguished from other acylglycines by acetylation of the esterified sample. After acetylation, hydroxy-acylglycines exhibit a molecular weight shift of +42 amu compared to the corresponding esterified acylglycines. (iii) Acquisition of daughter ion scan spectra of acylglycines was performed to obtain information about the structure of the compounds. Daughter ion spectra of the acylglycine-methylester ions [M+H]<sup>+</sup> usually exhibit pronounced fragment peaks at *m/z* 90, corresponding to the loss of the acyl group as ketene (6). In addition, the loss of methanol (−32 amu) and of 89 amu from [M+H]<sup>+</sup> yields fragments with high intensity.

Table 3 lists the corresponding protonated molecular weights of the acylglycine-methylesters. The different structures were assigned with the help of literature data and by executing the experiments described above. It must be mentioned, however, that isomeric structures (for example valeryl- and

isovalerylglycine) cannot be distinguished; neither could the position of the hydroxy group in the acylglycines be determined by our method.

*Urine analysis.* The analysis of control urine samples showed that many acylglycines were detectable in small amounts (Fig. 1A). The most intense signal is ascribed to ACG. C<sub>4</sub>-G, C<sub>5</sub>-G, and HG are also present but PG, MCG, PPG, and SG were never detected. A significant peak with high intensity at *m/z* 150, corresponding to homocysteine (Hcy), is always detected in the profiles. Previous work demonstrated the presence of reduced homocysteine in plasma in low concentrations (8). A fragment ion at *m/z* 90 is produced by collision-induced dissociation (CID) from protonated molecular ions of homocysteine (9). In urine samples this amino acid is therefore analyzed simultaneously with the acylglycines. Low-intensity signals corresponding to HBG (*m/z* 176) and PAG (*m/z* 208) can be detected; other signals (*m/z* 220, 234, 248) are not glycine-conjugated compounds. Table 4 shows the acylglycine patterns found in the pathological samples by ESI-MS/MS compared to the literature reports about other methods.

It must be noted that the most prominent acylglycines in urine are hippuric acid and 4-hydroxyhip-



**FIG. 1.** ESI-MS/MS urinary acylglycine profiles in controls and in different disorders. (Peaks marked with asterisk correspond to internal standards. Unmarked peaks are not acylglycines).

**TABLE 4**  
**Acylglycines Detected in Different Disorders**

Disease	Detected by our method (ESI-MS/MS)	Detected by other methods <sup>c</sup>	References
MCAD deficiency	C <sub>4</sub> -G	BG	4,5,6,10,11,12,15,22
	C <sub>5</sub> -G	C <sub>5</sub> -G (MBG or IVG)	
	C <sub>6</sub> -G	C <sub>6</sub> -G (HG and MVG)	
	C <sub>7</sub> -G (MHG)	C <sub>7</sub> -G	
	HOG	7-HOG and 8-HOG	
	PPG	PPG	
	SG	SG	
	MAD deficiency	C <sub>4</sub> -G	
	C <sub>5</sub> -G	C <sub>5</sub> -G (MBG and IVG)	
	C <sub>6</sub> -G	C <sub>6</sub> -G (HG)	
	SG	SG	
LCAD/VLCAD deficiency	<sup>a</sup>	—	18,19,22
SCAD deficiency	C <sub>4</sub> -G (BG)	BG	16,17,18,19,22
LCHAD deficiency	<sup>a</sup>	—	18,19
SCHAD deficiency	<sup>a</sup>	—	18,19,20
CPT-II deficiency	<sup>a</sup>	—	18,19
Isovaleric aciduria	IVG	IVG	2,12,24
Propionic aciduria	PG	PG	2,12
	TG	TG	
3-Methylcrotonylglycinuria	MCG	MCG	2,13,24
Methylmalonic aciduria	PG	—	2
Multiple carboxylase deficiency	PG	PG	12
	TG, MCG	TG, MCG	
3-OH-3-methylglutaric aciduria	MCG	MCG	12,24
Glutaric aciduria I	GG <sup>b</sup>	GG	3

Note. For abbreviations see Table 3.

<sup>a</sup> Normal acylglycine pattern comparable to healthy controls.

<sup>b</sup> Found in 8/29 samples from 4/7 patients.

<sup>c</sup> GC/MS, LSI-MS/MS.

puric acid (1,2). Because of the lack of protons at position 2 of the acyl group, these compounds cannot be detected by the applied method (6).

Quantification of acylglycine concentrations in control urine samples and in different diseases is shown in Table 5.

*Mitochondrial energy metabolism disorders.* Urine from patients with MCAD and MAD deficiencies showed typical patterns of acylglycine excretion (Figs. 1B–1C). In MCAD patients (15 samples), HG, PPG, and SG are the main metabolites excreted in urine (Fig. 1B); C<sub>4</sub>-G, C<sub>5</sub>-G, C<sub>7</sub>-G, and HOG are also detectable. We observed that the acylglycine profiles are always pathological in MCAD, independent of the clinical status. HG is always excreted in large amounts and both HG and SG increase in the course of the illness; PPG and HOG are often detectable in the compensated state, and they are clearly elevated during acute crises.

In urine samples from the SCAD-deficient patient BG was not always elevated (Fig. 1D), being in the normal range in 6 out of 9 samples.

LCAD/VLCAD (10 samples) as well as LCHAD (1 sample), SCHAD (1 sample), and CPT-II (5 samples)-deficient patients showed acylglycine excretion comparable to that of normal subjects.

In MAD deficiency (neonatal form, 29 samples), C<sub>4</sub>-G (BG and IBG) and C<sub>5</sub>-G (MBG and IVG) are the most prominent acylglycines detected in urine (Fig. 1C), both in compensated and in decompensated states. HG is often excreted in large amounts and its excretion increases during illness along with C<sub>5</sub>-G. Sometimes, especially when dicarboxylic aciduria is elevated in the organic acid profiles, it is possible to detect SG. Normal profiles were found in the late onset form (2 samples).

*Organic acidurias.* All the samples from isovaleric aciduria (5 samples), propionic aciduria (47

**TABLE 5**  
**Quantification of the Most Important Pathological Urinary Acylglycines in Different Disorders**

Disease ( <i>n</i> = number of samples)	Acylglycines	Median	Range
		(mmol/mol creatinine)	
MAD deficiency ( <i>n</i> = 29)	C <sub>4</sub> -G (BG, IBG)	6.4	2.3–38.5
	C <sub>5</sub> -G (MBG)	28.9	4.6–83.7
	C <sub>6</sub> -G (HG)	3.6	0.8–44.4
MCAD deficiency ( <i>n</i> = 15)	C <sub>6</sub> -G (HG)	31.4	11.8–103.2
	C <sub>7</sub> -G	3.8	1.3–17.8
	7-HOG or 8-HOG	1.1	0–16.7
	PPG	2.6	0.8–306.6
	SG	1.7	0–11.7
	BG	4.3	0.9–5.2
SCAD deficiency ( <i>n</i> = 9)	BG	4.3	0.9–5.2
Isovaleric aciduria ( <i>n</i> = 5)	IVG	3173.1	713.1–4760.4
Propionic aciduria ( <i>n</i> = 47)	PG	46.0	2.8–1183.3
	TG	2.0	0.1–81.9
3-Methylcrotonylglycinuria ( <i>n</i> = 1)	MCG	40.8 <sup>a</sup>	
Methylmalonic aciduria ( <i>n</i> = 48)	PG	1.8	0.1–80.6
	PG	1.0	0.5–311.1
Holocarboxylase synthetase deficiency ( <i>n</i> = 7)	TG, MCG	0.5	0.2–7.4
	MCG	4.6	0.6–21.2
3-OH-3-methylglutaric aciduria ( <i>n</i> = 10)	ACG	11.4	2.5–47.6
	PG	n.d.	n.d.
	C <sub>4</sub> -G	0.9	0–3.0
	TG/MCG	n.d.	n.d.
	C <sub>5</sub> -G	0.9	0.2–3.0
	HBG	0.5	0–1.8
	HG	0.7	0.2–2.4
	C <sub>7</sub> -G	n.d.	n.d.
	PAG	0.4	0–1.8
	GG	n.d.	n.d.
	PPG	n.d.	n.d.
	7-HOG or 8-HOG	n.d.	n.d.
	SG	n.d.	n.d.
	Controls ( <i>n</i> = 54)	ACG	11.4
PG	n.d.	n.d.	
C <sub>4</sub> -G	0.9	0–3.0	
TG/MCG	n.d.	n.d.	
C <sub>5</sub> -G	0.9	0.2–3.0	
HBG	0.5	0–1.8	
HG	0.7	0.2–2.4	
C <sub>7</sub> -G	n.d.	n.d.	
PAG	0.4	0–1.8	
GG	n.d.	n.d.	
PPG	n.d.	n.d.	
7-HOG or 8-HOG	n.d.	n.d.	
SG	n.d.	n.d.	

Note. For abbreviations see Table 3. n.d., not detectable.

<sup>a</sup> Single value.

samples), and 3-methylcrotonylglycinuria (1 sample) showed the typical glycine conjugates in urine in high concentrations (Fig. 1). IVG is excreted at a very high level in isovaleric aciduria (Fig. 1E) compared to unaffected children. In propionic aciduria, PG is the main metabolite, followed by TG (Fig. 1F). In methylmalonic aciduria (methylmalonyl-CoA mutase deficiency) no specific markers were found but all the 48 samples showed significant excretion of PG (Fig. 1G), which increases in metabolic decompensation. In some samples TG is also detected. In methylcrotonylglycinuria (1 sample) MCG is detected at high level (Fig. 1H). In 3-hydroxy-3-methylglutaric aciduria (10 samples), MCG is always excreted in urine (Fig. 1I). MCG is also detectable when organic acids are only mildly altered. In glu-

taric aciduria type I we observed normal acylglycine profiles. Traces of GG are detectable in only 8 out of 29 examined samples (in 4 out of 7 patients), and only in the decompensated state. In multiple carboxylase deficiency (holocarboxylase synthetase deficiency, 7 samples) the presence of PG is always relevant, even when organic acid levels are not significantly altered, and TG and/or MCG are usually detectable, in highest amounts during acidotic crises. Biotinidase-deficient patients showed normal acylglycine profiles (3 samples).

## DISCUSSION

All the studies on acylglycines so far focused mainly on MCAD deficiency, a relatively frequent

disease that shows a typical pattern of glycine conjugates in blood and urine (4,10,11). They demonstrate the sensitivity of this test also in patients without acute illness (5). Organic acidurias were also considered in some studies (2,12,13). The methods used until now were either GC/MS (4,5,10) or LSI-MS/MS (6). In this study we present the analysis of urinary acylglycine profiles in a wide spectrum of disorders, including the main fatty acid oxidation deficiencies and organic acidurias, by ESI-MS/MS.

MCAD deficiency is the most frequent fatty acid oxidation disorder. MCAD catalyzes the oxidation of C<sub>6</sub>-C<sub>14</sub> fatty acids. Laboratory findings are as follows: low levels of free and total carnitine, as in all the mitochondrial fatty acid metabolism defects; massive C<sub>6</sub>-C<sub>10</sub> dicarboxylic aciduria; elevated plasma and urinary hexanoylcarnitine, octanoylcarnitine, and decanoylcarnitine, mainly during acute episodes. Diagnosis is usually difficult during remission because the characteristic metabolites may not be detectable. It is well known that urinary acylglycine determination is an accurate test for the diagnosis of MCAD deficiency (4,5,10). HG and PPG are characteristic, together with SG. HG and SG excretion increases during the acute phase, PPG excretion usually increases to a lesser degree (5,14). Other acylglycines detected in urine of MCAD-deficient patients are 4-MHG, 4-MVG, 7-HOG, and 8-HOG (15). After carnitine administration excretion of acylcarnitines increases and that of acylglycines decreases. In any case, glycine conjugation is considered to be the major pathway of removal of C<sub>6</sub> to C<sub>8</sub> acyl-CoAs (5,14). For MCAD deficiency, we confirmed the typical excretion of HG, PPG, and SG, even when organic acid levels are only mildly or not at all altered. With our method, HOG and MHG were also detected in MCAD deficiency, especially during illness. HOG is likely to be a product of microsomal  $\omega$ - $\omega_1$  oxidation and 4-MHG probably originates from branched-chain fatty acid catabolism. Most of the octanoyl-CoA is transformed to octanoylcarnitine but the high sensitivity of the method allows detection of even small amounts of HOG.

SCAD deficiency is a very rare disorder of C<sub>4</sub> fatty acid oxidation, of which two forms are known, a neonatal and an infantile form. The fasting state usually shows normal glycemia and ketosis. Laboratory findings are increased urinary excretion of 2-methylsuccinic acid, butyrylcarnitine, and BG (16,17). Dicarboxylic acids are usually only slightly increased. All these metabolites are intermittently

detectable and the diagnosis can often be missed. In SCAD deficiency, we found significantly elevated BG only in a few samples.

LCAD/VLCAD deficiencies are characterized by excretion of medium- and long-chain saturated dicarboxylic acids and long-chain acylcarnitines. No pathological acylglycine excretion has been described up to now (18). LCHAD deficiency is characterized by low levels of free and total carnitine, excretion of 3-hydroxydicarboxylic acids in nearly equimolar amounts of the saturated dicarboxylic acids, and long-chain acylcarnitines. SCHAD deficiency is a rare disorder of short-chain hydroxy fatty acid oxidation, and no pathological acylglycine excretion has so far been described (18-20). The defects in the transport of fatty acids into the mitochondria are carnitine uptake defect (CUD), carnitine palmitoyl transferase type I (CPT-I), carnitine translocase (TRANS), and carnitine palmitoyl transferase type II (CPT-II) deficiencies. Apart from CPT-II, these diseases are all associated with fasting hypoketotic hypoglycemia; no dicarboxylic aciduria nor pathological acylglycine excretion is expected as no accumulation of acyl-CoA esters occurs in the mitochondria (19). We confirmed, even if in a limited number of patients, that in LCAD/VLCAD, LCHAD, SCHAD, and in fatty acid transport defects (CPT-II) no pathological acylglycine excretion was detectable.

Multiple acyl-CoA dehydrogenase (MAD) deficiency, or glutaric aciduria type II, is a defect of electron transfer flavoprotein (ETF) or of electron transfer flavoprotein dehydrogenase (ETF-DH). All the mitochondrial flavin-containing acyl-CoA dehydrogenases are affected: long-, medium-, short-chain acyl-CoA dehydrogenases, isovaleryl-CoA dehydrogenase, 2-methylbutyryl-CoA dehydrogenase, glutaryl-CoA dehydrogenase, dimethylglycine dehydrogenase, and sarcosine dehydrogenase. Fatty acid and branched-chain amino acid metabolisms are both affected. Typical metabolites excreted in this disorder are medium-chain dicarboxylic acid, ethylmalonic acid, methylsuccinic acid, glutarate, 2-hydroxyglutarate, octanoylcarnitine, glutarylcarnitine, butyrylcarnitine, isovalerylcarnitine, C<sub>4</sub>-G (BG, IBG), IVG or MBG, HG, and SG (18,21). Diagnosis is difficult, especially in late-onset forms, because the organic acid profiles are intermittently altered. All the MAD deficiency samples from the neonatal form were pathological, no matter what the clinical status of the patient was at the time of urine collection. The same acylglycine excretion pattern

was found with and without riboflavin administration. The two samples from the late-onset form showed normal acylglycine profiles.

Organic acidurias lead to accumulation of organic acids in body fluids, resulting in disturbance of the acid-base balance and the intracellular biochemical pathways. The acute toxicity of organic acids during attacks leads to cerebral suffering and damage but chronic toxicity also leads to progressive neurologic impairment. The diagnosis is usually based on urinary organic acid analysis during decompensation.

It is known that some metabolites accumulating in these diseases are conjugated efficiently with glycine: isovaleryl-CoA, propionyl-CoA, tiglyl-CoA, and 3-methylcrotonyl-CoA are all substrates of the enzyme glycine-*N*-acylase (2). Isovaleryl-CoA has a very high affinity for the enzyme, which is always overwhelmed. Thus, isovalerylglycine is always present in the urine of isovaleryl-CoA dehydrogenase-deficient patients, reaching levels thousands of times those in normal controls (Table 5). Glycine conjugation is the main detoxification system in this disease, followed by isovalerylcarnitine production. In propionic aciduria, propionyl-CoA is excreted in high amounts as free propionic acid and in small amounts as propionylglycine. Tiglyl-CoA has the highest affinity for the enzyme and tiglylglycine is found under three conditions: 3-methylcrotonylglycinuria, propionic aciduria, and  $\beta$ -ketothiolase deficiency. In 3-methylcrotonylglycinuria, the main metabolite derived from methylcrotonyl-CoA is 3-hydroxyisovaleric acid which is excreted as 3-hydroxyisovaleryl-carnitine while a significant amount is assumed to be transformed to MCG; the excretion of 3-methylcrotonylglycine is enhanced by glycine supplementation (13). Methylmalonyl-CoA is not a substrate of the conjugating enzyme, therefore no specific acylglycine excretion is expected in methylmalonic aciduria (2). However, PG is always detectable in urine from mutase-deficient patients, being a possible marker for this disease.

In 3-hydroxy-3-methylglutaric aciduria the leucine catabolism and ketone body synthesis are impaired. The following metabolites are derived from 3-hydroxy-3-methylglutaryl-CoA: 3-hydroxy-3-methylglutaric acid (formed by hydrolysis), which is always present in urine; 3-methylglutaconic acid (formed by reverse reaction of 3-methylglutaconyl-CoA hydratase); 3-methylglutaric acid (from hydrogenation of 3-methylglutaconic acid). Another metabolite is 3-methylcrotonyl-CoA, which is hydrated to 3-hydroxyisovaleric acid or conjugated with gly-

cine to MCG, which is always present in the urine samples studied.

Glutaric aciduria type I is characterized by accumulation of glutaryl-CoA due to the lack of the enzyme glutaryl-CoA dehydrogenase. Glycine conjugation of glutaric acid occurs, even if this is not the main metabolic pathway. In our study traces of GG were detected in 8 out of 29 urine samples from 4 out of 7 patients with glutaric aciduria type I, and only when organic acid profiles were also altered.

Multiple carboxylase deficiency is caused by holocarboxylase synthetase deficiency or by biotinidase deficiency. In this disease four biotin-dependent enzymes are affected: propionyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase, pyruvate carboxylase, and acetyl-CoA carboxylase. The metabolic pathways of gluconeogenesis, fatty acid synthesis, and amino acid catabolism are disturbed. As a consequence, the expected glycine conjugates are PG, TG, and MCG, which were detected in our samples from holocarboxylase synthetase-deficient patients. Biotinidase-deficient patients showed normal acylglycine profiles.

In conclusion, urinary acylglycine analysis by ESI-MS/MS is a powerful diagnostic tool for some mitochondrial energy metabolism defects and organic acidurias. For MAD and MCAD deficiencies, acylglycines are always informative because their excretion is less influenced by the clinical status than the excretion of organic acids. For other diseases the excretion pattern of acylglycine is not specific but there is always a pathological profile to indicate a metabolic disorder. In fact, PG is always present in methylmalonic aciduria and in holocarboxylase synthetase deficiency, and MCG is constantly present in 3-hydroxy-3-methylglutaric aciduria. Further investigations should be done in order to define the acylglycine excretion pattern in SCAD deficiency.

The applied method is noninvasive, fast, and sensitive for very low concentrations of acylglycines in random urine samples. Therefore, it can be suitable for the selective screening of diseases such as MAD and MCAD deficiencies, isovaleric and propionic acidurias, and 3-methylcrotonylglycinuria. 3-Hydroxy-3-methylglutaric aciduria, multiple carboxylase deficiency, and methylmalonic aciduria can also be suspected from the presence of abnormal glycine conjugates. Urinary acylglycine measurement by ESI-MS/MS should be considered as a fast and useful analysis complementary to urinary organic acid and acylcarnitine profiles. Early diagnosis of meta-

bolic disorders, in fact, is the first goal for the clinician, as proper therapeutical intervention often prevents acute life-threatening events and later disability.

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