Talanta 88 (2012) 193–200

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Quantification of acylglycines in human urine by HPLC electrospray ionization-tandem mass spectrometry and the establishment of pediatric reference interval in local Chinese

Bonnie Mei-Wah Fong[a,b], Sidney Tam[b], Kelvin Sze-Yin Leung[a,*]

[a] Department of Chemistry, Hong Kong Baptist University, Kowloon, Hong Kong, China
[b] Department of Pathology and Clinical Biochemistry, Queen Mary Hospital, Hong Kong, China

ARTICLE INFO

Article history:
Received 29 August 2011
Received in revised form 24 October 2011
Accepted 26 October 2011
Available online 29 October 2011

Keywords:
Acylglycines
HPLC–ESI-MS/MS
Pediatric reference interval

ABSTRACT

Urinary organic acids, plasma amino acids and acylcarnitine profile analyses are the main tools used to diagnose inborn errors of metabolisms (IEMs). However, without metabolic decompensation, these parameters are often not helpful. On the other hand, in cases of IEM, acylglycines are consistently raised even when patients appear to be in remission. This study aims to set-up a simple liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) method for the determination of urine acylglycines, complementary to organic acid and acylcarnitine profiles, for the diagnosis of IEM. In addition, local reference intervals for various acylglycines are established by using this method. Acylglycines were isolated by solid-phase extraction, derivatized with n-butanol, separated by HPLC, and detected by ESI-MS/MS. Acylglycines were quantified with deuterated internal standards. Mean recoveries of acylglycines ranged from 90.2 to 109.3%. Within- and between-run imprecisions for all acylglycines have CVs less than 10%. Linear regression coefficients were greater than 0.99. Reference intervals were established according to CLSI guidelines by analyzing 204 samples from apparently healthy individuals less than 18 years of age. The distributions of AG in the "normal" urine were skewed towards the right. After log transformation, all the results were normally distributed. Partitioning into age group reference intervals was not indicated, according to the Harris and Boyd approach. In this context, a single reference interval for each acylglycine could be used. This method of urine acylglycines analysis is a powerful diagnostic tool, complementary to urine organic acids and plasma acylcarnitine profiling, for detecting certain inborn errors of metabolism.

1. Introduction

Inborn errors of metabolism (IEM) are genetic disorders that result in alterations of a specific chemical reaction in the metabolism. These alterations could be impaired activity of enzymes, transporters, or cofactors resulting in accumulation of abnormal metabolites (substrates) proximal to the metabolic block or in the absence of necessary products. The accumulated abnormal metabolites in turn may produce abnormal byproducts. Although individually rare, IEM collectively account for a significant proportion of illnesses. Today it is estimated that about 1 infant in every 1500 [1] is affected (i.e., about 0.067% of the population).

Originally, the diagnosis of IEM was generally made by observation or recognition of abnormality in people who become ill or had severe dysmorphic features or mental retardation. These disorders were understood to be inherited by observation of recurrence in family members. With the identification of specific enzymes and metabolic pathways, understanding the physical processes underlying these IEM has evolved. This knowledge has been accompanied by an awareness of the possibility of errors at various levels in different metabolic pathways.

Many IEM have a similar clinical presentation. Common to all these disorders are life-threatening metabolic crises. During these crises, specific metabolites, reflecting directly the enzymatic block involved and indirectly the intermediate metabolic impairment, are detectable in urine and blood. The detection and quantification of some of these metabolites or byproducts is the basis of biochemical diagnosis of IEM by tandem mass spectrometry.

Urinary organic acids, plasma amino acids and acylcarnitine profile analyses are the main diagnostic tools in these conditions. However, in the absence of metabolic decompensation—that is, when the patient is doing well, these parameters are often not informative. Acylglycines (AG) are formed by the action of the mitochondrial enzyme glycine-N-acylase (EC 2.3.1.13) when acyl-CoA

* Corresponding author. Tel.: +86 852 3411 5297; fax: +86 852 3411 7348.
E-mail address: s9362284@hkbu.edu.hk (K.S.-Y. Leung).

0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.10.031

esters accumulate in the body [2], and AG are consistently raised, even when patients appear to be in remission.

Glycine conjugation is an important detoxification system of the human body, taking place mainly in the liver [3]. It is also an effective detoxification system for preventing accumulation of acyl-CoA esters in several inborn metabolic diseases. The study of AG in urine can be diagnostic for several IEM [4–7], such as the defects in fatty acid oxidation (acyl-CoA dehydrogenases, (EC 1.3.99.3)) [8] and leucine catabolism (isovaleryl-CoA dehydrogenase (EC 1.3.99.10), methyl-crotonyl-CoA carboxylase (EC 6.4.1.4)). In addition, accumulation of specific glycine conjugates is diagnostic for other defects in enzymes involved in the catabolism of isoleucine [9], valine, and lysine (propionyl-CoA carboxylase (EC 6.4.1.3), glutaryl-CoA dehydrogenase (EC 1.3.99.7)).

Reference intervals of urine AG reported in normal subjects ranged from undetectable for propionylglycine (PG), tiglylglycine (TG), phenylpropionylglycine (PPG), suberylglycine (SG) to a few μmol mmol^{-1} creatinine for isobutyrylglycine (IBG) (0–3.0), butyrylglycine (BG) (0–3.0), 2-methylbutyrylglycine (2-MBG) (0.2–3.0), isovalerylglycine (IVG) (0.2–3.0) and heaxanoylglycine (HG) (0.2–2.4) [10]. For such low level determination, mass spectrometry based methods should be used. Urinary AG excretion has been mainly studied by gas chromatography–mass spectrometry (GC/MS) [11–15] and fast atom bombardment tandem mass spectrometry (FAB-MS/MS) [16], and more recently by electrospray tandem mass spectrometry (ESI-MS/MS) [10]. The GC/MS methods are labor-intensive and time-consuming; importantly, however, neither FAB-MS/MS nor ESI-MS/MS can distinguish glycine isomers which are in turn required to distinguish between certain disorders [17]. The aim of this work was to set up a simple liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) method for the determination of AG in urine, including the isomers (butyryl- and isobutyryl-glycine; 2-methylbutyryl- and isovaleryl-glycine), complementary to organic acids and acylcarnitine profiles, for the diagnosis of certain IEM. In addition, we intended to establish local pediatric reference intervals for various AG by using this method.

2. Experimental

2.1. Materials and reagents

Acylglycine standards (propionylglycine, butyrylglycine, isobutyrylglycine, 2-methylbutyrylglycine, isovalerylglycine, tiglylglycine, hexanoylglycine, phenylpropionylglycine and suberylglycine) were purchased from Dr. H.J. ten Brink (VU Medical Center, Amsterdam, The Netherlands). For deuterated acylglycines: $^{2}H_3$-propionylglycine, $^{2}H_3$-butyrylglycine, $^{2}H_7$-isobutyrylglycine, $^{2}H_9$-2-methylbutyrylglycine, $^{2}H_9$-isovalerylglycine, and $^{2}H_3$-hexanoylglycine were purchased from Dr. H.J. ten Brink (VU Medical Center, Amsterdam, The Netherlands). $^{2}H_2$-phenylpropionylglycine, $^{2}H_2$-tiglylglycine and $^{2}H_2$-suberylglycine were obtained from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Ammonium formate and formic acid were purchased from Sigma–Aldrich (Fluka, Switzerland). HPLC-grade methanol and acetonitrile were purchased from J.T. Baker (Philipsburg, NJ, USA). Oasis MCX, HLB, MAX solid phase extraction (SPE) cartridges were purchased from Waters (Milford, USA). Agilent SAX SPE cartridges were purchased from Agilent Technologies (Palo Alto, CA, USA). 3 N HCl in n-butanol solution was purchased from Regis Technologies Inc. (Morton Grove, IL, USA). All other chemicals were of analytical grade. All solutions were prepared using highly purified water produced by a Millipore Milli-RO/Milli-Q Gradient system (Millipore, Bedford, MA, USA).

2.2. Liquid chromatography

An HP 1200 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of a binary gradient pump, a vacuum degasser and autosampler was used. An Atlantis dC18 (Waters, Milford, USA) column (150 mm × 2.1 mm, 5 μm) was used at ambient temperature. A 10 μL mixture of butylated acylglycine was injected onto the column and eluted at a flow rate of 200 μL min^{-1} using a step gradient alternating between 20 mmol L^{-1} formic acid with ammonium formate [1:4; v/v] (A) and acetonitrile (B). The gradient began with 18% B, was held for 5 min, then was programmed as follows: 5–18 min, gradient to 50% B; 18–19 min, gradient to 60% B; 19–23 min, gradient to 80% B, hold at 80%B; 30–30.5 min, gradient back to 18% B; and 30.5–33 min, hold at 18% B to re-equilibrate the column.

2.3. Mass spectrometry

This study was carried out using a quadrupole mass spectrometer (Applied Biosystems 3200 QTRAP, Applied Biosystems/MDS SCIEX, Canada). The MS/MS analysis in this study was carried out using a TurboIon-Spray ionization source. Zero air was used as the nebulizing gas, and nitrogen was used as the curtain and collision gas. The source temperature was 650 °C, and the ion spray voltage used was 5.5 kV. Acylglycines were analyzed in positive ion multiple-reaction monitoring (MRM) mode. The voltages, collision energies and mass transitions used are listed in Table 1. The mass spectrometer was operated using Analyst software version 1.4. To optimize the compound-dependent mass spectrometer settings, AG standards were infused one by one with a syringe pump at 10 μL min^{-1} in mobile phase.

2.4. Biological samples

Two hundred and four urine samples from apparently healthy subjects aged from 21 days to 17.7 years were analyzed in order to establish reference intervals. Urine samples were stored at −20 °C until assayed. For long term storage, the samples were kept at −70 °C. Clinical samples with known diagnosis, collected in different clinical situations, were studied retrospectively.

2.5. Determination of creatinine in urine samples

Creatinine determination was carried out by a kinetic colorimetric assay based on the modified Jaffe method [18] using the Roche Modular System (Roche Diagnostics, IN, USA), with an analytical range between 360 and 57,500 μmol L^{-1}.

2.6. Solid phase extraction and preparation of acylglycine-butyl esters

The sample cleanup and derivatization procedure was as follows: urine samples were diluted to a creatinine of 1.0 mmol L^{-1}; and 0.1 mL of the diluted urine was added to 0.9 mL water and then mixed with 1 mL 0.5 M phosphate buffer at pH 6.0 followed by addition of 10 μL internal-standard solution (25 μmol L^{-1} of each deuterated internal standard). The sample was passed through a pre-conditioned SAX cartridge. The SAX cartridge was pre-conditioned with 2 mL methanol followed by 2 mL water and 2 mL 0.5 M phosphate buffer at pH 6.0. After complete loading, SAX cartridge was washed with 4 mL water. Acylglycines were eluted by 1 mL 1 M formic acid in absolute methanol. The eluate was evaporated to dryness at 45 °C and added with 100 μL 3 N HCl in n-butanol. Butylation was performed at 65 °C for 15 min in a screw-capped vial. After evaporation of the solvent, the residue was

Table 1
LC–MS/MS settings for all analytes and internal standards.

Acylglycine	Qualifier ion	Q1 mass (Da)	Q3 mass (Da)	DP(V)	EP(V)	CEP(V)	CE(P)	CXP(V)	Associated IS
PG		188.1	76.1	26	3.2	16	20	2.8	d_3-PG
	PG-1	188.1	57.1	26	3.2	16	27	2.4	
IBG		202.1	76.2	25	5.0	13	18	2.0	d_7-IBG
	IBG-1	202.1	128.2	25	5.0	13	12	2.1	
BG		201.9	76.0	25	5.0	11	18	3.0	d_3-BG
	BG-1	201.9	128.2	25	5.0	11	12	2.1	
TG		214.1	83.1	23	4.5	11	18	2.8	d_2-TG
	TG-1	214.1	55.1	23	4.5	11	34	2.0	
2-MBG		215.9	57.2	26	5.0	19	28	2.4	d_9-2MBG
	2-MBG-1	215.9	75.9	26	5.0	19	20	3.4	
IVG		215.9	76.0	22	4.0	11	20	2.2	d_9-IVG
	IVG-1	215.9	57.2	22	4.0	11	32	2.2	
HG		230.2	76.2	27	4.0	21	24	2.2	d_3-HG
	HG-1	230.2	132.1	27	4.0	21	14	2.1	
PPG		264.2	105.1	29	3.7	15	29	3.0	d_2-PPG
	PPG-1	264.2	132.2	29	3.7	15	15	2.9	
SG		344.2	83.2	34	5.0	22	42	3.3	d_2-SG
	SG-1	344.2	168.2	34	5.0	22	23	2.7	

AG, acyl-glycines; PG, propionyl-glycine; IBG, isobutyryl-glycine; BG, butyryl-glycine; TG, tiglyl-glycine; 2-MBG, 2-methylbutyryl-glycine; IVG, isovaleryl-glycine; HG, hexanolyl-glycine; PPG, phenylpropionyl-glycine; and SG, suberyl-glycine.

reconstituted with 100 μL acetonitrile/water (50/50, v/v) containing 0.1% formic acid.

2.7. Standard curves for acylglycines

For every batch of patient samples, a blank and calibrators at 0.5, 1, 5, 10, and 25 $\mu mol\,L^{-1}$ were run. Calibration curves were constructed by linear regression analysis of the ratios of the AGs to the respective internal standard.

2.8. Matrix effect study

In the matrix effect evaluation, two procedures were compared: post-column infusion [19] and post-extraction spike methods [20,21]. The post-column infusion method was performed as follows: ion suppression or enhancement was checked by infusing a 100 $\mu mol\,L^{-1}$ AG at 10 $\mu L\,min^{-1}$ in the mobile phase. At the same time, extracted and derivatized normal urine samples from six different sources were injected into the chromatographic system, and the flows were merged using a polyetheretherketone (PEEK) tee before the source entered, in a post-column infusion system. For the post extraction spike method, samples of normal urine from six different subjects were used. After sample cleanup, aqueous solutions of AG were spiked into the extracted urine (equivalent final concentrations in urine: 0.5, 1, 5, 10, 25, 50 and 100 $\mu mol\,L^{-1}$) followed by derivatization. The matrix effect and the possibility of ionization suppression or enhancement were evaluated by comparing the mean peak area of the AG spiked post-extraction to the mean peak area of the aqueous AG standard multiplied by 100. A value >100% indicated ionization enhancement while a value of <100% indicated ionization suppression [20].

3. Results and discussion

The basic goal of this work was to develop a high-throughput method for the determination of AG in human urine and to set up of pediatric reference intervals in local Chinese using this new method. Unlike other methods [10], we used HPLC to separate the

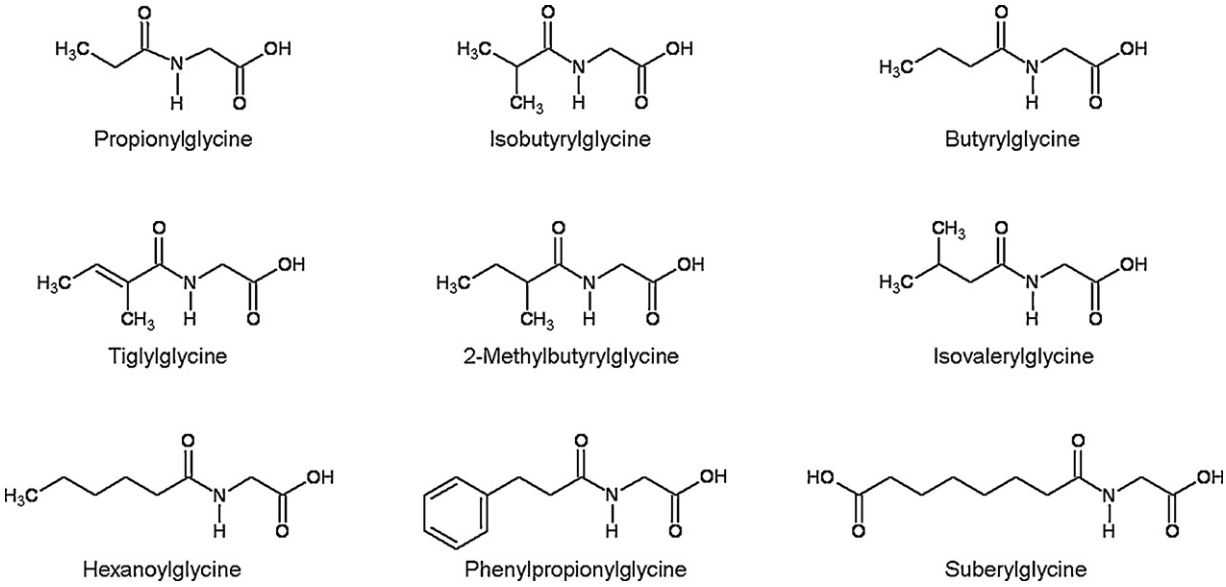

Fig. 1. Chemical structures of acylglycines.

Table 2
Results of quantitative validation (linearity: 1–100 μmol L^{-1}, n = 6; accuracy, n = 10; precision, n = 20; recovery, n = 6).

AG	Aqueous calibrator linearity: R^2 (slope, intercept)	Urine-based calibrator linearity: R^2 (slope, intercept)	Slope ratio (urine/aqueous)	Mean value in % of target value	
				Accuracy, level I	Accuracy, level II
PG	0.9968 (0.0119, 0.0053)	0.9994 (0.0119, 0.0057)	1.08	93.1	92.7
IBG	0.9975 (0.0165, 0.1395)	0.9919 (0.0176, 0.1351)	0.97	91.3	90.8
BG	0.9996 (0.0102, 0.0079)	0.9988 (0.0101, 0.0083)	1.05	90.4	108.4
TG	0.9971 (0.0286, 0.3351)	0.9966 (0.0264, 0.3246)	0.97	109.0	104.6
2MBG	0.9931 (0.0193, 0.0901)	0.9985 (0.0182, 0.0847)	0.94	91.1	107.2
IVG	0.9994 (0.0100, 0.0639)	0.9966 (0.0093, 0.0594)	0.93	92.2	96.5
HG	0.9944 (0.0271, 0.0989)	0.9913 (0.0257, 0.0944)	0.95	94.7	104.9
PPG	0.9915 (0.0206, 0.0367)	0.9982 (0.0211, 0.0378)	1.03	104.2	96.1
SG	0.9911 (0.0171, 0.0085)	0.9947 (0.0179, 0.0893)	1.05	97.0	101.2

AG	Within-run imprecision (μmol L^{-1})		Between-run imprecision (μmol L^{-1})		LLOD (μmol L^{-1})	LLOQ (μmol L^{-1})
	Level I: mean (CV, %)	Level II: mean (CV, %)	Level I: mean (CV, %)	Level II: mean (CV, %)		
PG	0.79 (3.54)	1.45 (3.03)	0.78 (4.23)	1.39 (5.68)	0.0006	0.002
IBG	5.64 (4.59)	8.57 (8.40)	5.48 (6.41)	8.48 (5.70)	0.0045	0.015
BG	1.24 (4.31)	2.32 (2.60)	1.20 (4.67)	2.25 (4.09)	0.0018	0.006
TG	5.23 (6.90)	7.97 (7.90)	5.15 (8.89)	7.97 (8.21)	0.0013	0.004
2MBG	3.64 (5.60)	6.16 (9.59)	3.52 (6.19)	6.07 (9.31)	0.0022	0.007
IVG	7.37 (7.69)	11.58 (8.10)	7.24 (9.63)	11.03 (9.30)	0.0045	0.015
HG	1.30 (4.23)	2.29 (3.89)	1.26 (6.51)	2.10 (6.10)	0.0020	0.007
PPG	0.85 (2.94)	1.48 (2.30)	0.83 (3.25)	1.44 (5.42)	0.0003	0.001
SG	4.00 (4.20)	7.13 (4.70)	4.09 (4.50)	7.29 (5.40)	0.0032	0.011

AG	Concentration (μmol L^{-1})							CV range (%)
	0.5	1	5	10	25	50	100	
Aqueous calibrators (mean recovery in %)								
PG	98.9	101.2	94.8	103.7	106.9	101.2	97.6	3.66–10.35
IBG	104.4	105.9	101.8	106.2	99.6	100.1	96.7	1.85–4.63
BG	98.5	102.7	101.2	95.7	97.4	98.9	104.7	1.65–4.14
TG	106.0	99.5	98.8	102.0	100.7	101.6	98.4	2.08–6.25
2-MBG	102.4	103.9	102.6	105.5	101.5	101.9	102.0	1.46–5.09
IVG	101.0	104.5	104.7	101.0	101.5	102.8	94.7	5.14–10.21
HG	97.2	99.6	105.0	101.9	102.4	101.7	97.2	2.98–11.04
PPG	101.3	99.8	102.7	100.9	104.5	100.1	95.9	1.98–5.38
SG	101.3	98.7	100.5	95.7	100.8	101.5	101.7	1.48–6.85
Spiked urine based calibrators								
PG	97.0	97.2	98.1	102.5	99.8	96.9	103.6	5.63–11.25
IBG	90.5	92.6	96.2	91.1	90.2	98.6	99.5	2.15–5.64
BG	97.3	97.3	101.9	91.6	97.9	98.0	100.5	2.98–6.18
TG	109.3	96.9	98.1	98.9	108.0	100.6	103.4	4.08–8.73
2-MBG	102.3	105.2	105.7	98.2	101.0	101.3	95.4	4.61–12.47
IVG	90.6	91.1	98.0	94.5	90.5	96.6	105.7	4.15–13.58
HG	94.0	96.8	98.6	98.7	107.0	100.0	97.0	3.25–11.08
PPG	95.2	97.9	95.1	97.2	104.0	101.3	105.0	1.98–6.58
SG	96.1	92.3	105.9	95.8	101.0	102.7	102.2	2.48–7.55
Post SPE spiked urine based calibrators								
PG	93.3	101.1	105.3	101.6	98.8	107.3	94.3	5.65–9.35
IBG	91.4	109.7	92.5	102.0	107.3	100.6	102.9	5.81–7.85
BG	93.3	108.1	100.7	96.9	96.0	99.5	95.9	1.74–7.43
TG	104.0	104.6	101.6	104.7	101.5	97.9	99.7	5.16–8.61
2-MBG	102.6	98.5	105.4	94.5	94.5	101.8	93.3	4.98–10.48
IVG	95.7	103.8	93.1	92.0	94.4	98.5	96.1	5.95–11.02
HG	99.4	107.2	106.1	109.1	95.5	93.8	98.3	6.48–11.31
PPG	93.6	97.7	105.9	101.8	101.0	99.5	106.7	1.98–6.58
SG	90.6	101.1	103.6	98.7	95.3	96.8	94.0	3.11–7.12

AG before MS/MS analysis which enabled the identification and quantification of AG isomers which are important in some IEM cases [22,23]. Fig. 1 shows the AG structures and a typical chromatogram of AG standards is shown in Fig. 2.

3.1. Ion suppression or enhancement

Severe ion suppression was encountered in the initial phase of the study, especially for the early eluting peaks, namely, propionylglycine, butylglycine and isobutylglycine. We considered using various preparation procedures including SPE with different compositions of adsorption materials, Oasis MCX, HLB, MAX and Agilent SAX, varied wash and eluting solutions in preparation steps, etc. (data not shown). Although modifications of mobile phase and elution program were attempted, the ion suppression phenomenon was always encountered. In light of this, derivatization was attempted to increase the efficiency in removing ion suppression. Better observations were made after derivatization with both Oasis-MAX and Agilent-SAX. Ion enhancement was not found. In our work, the best recovery was obtained for Agilent SAX, and therefore it was used in subsequent method development.

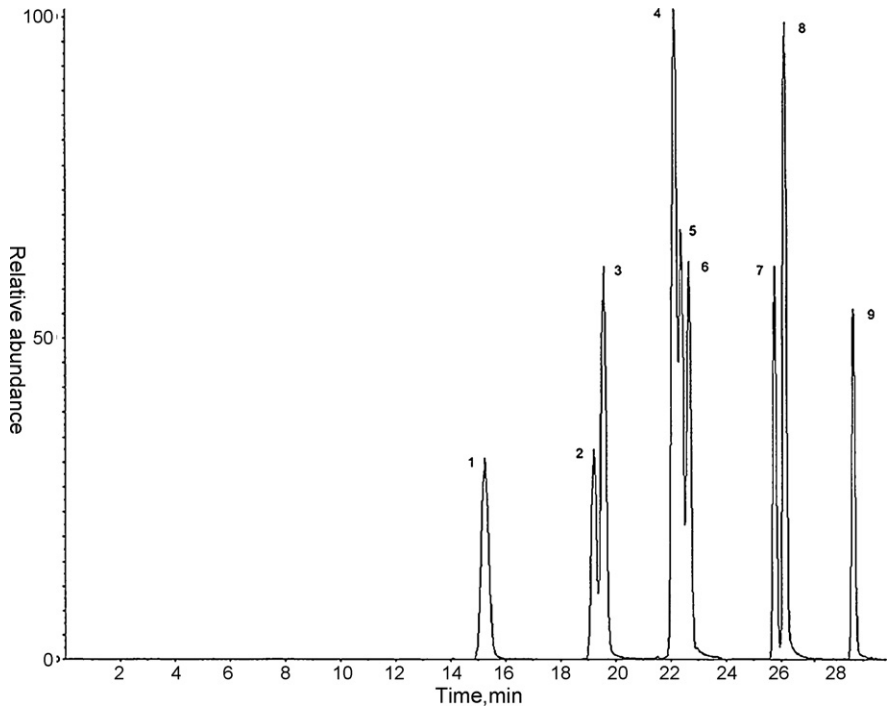

Fig. 2. Acylglycine standards (10 μmol L^{-1}). Peak identities are as follows: 1: propionyl-; 2: iso-butyryl-; 3: butyryl-; 4: tiglyl-; 5: 2-methylbutyryl-; 6: isovaleryl-; 7: hexanoyl-; 8: phenylpropionyl-; and 9: suberyl-glycine. Running condition: please refer to Section 2.2 of the text.

3.2. Linearity and recovery

Linearity and recoveries were evaluated by fortifying normal urine with AG. The urine was diluted to 1 mmol L^{-1} creatinine, at various concentrations (equivalent spiked final concentrations in urine: 0.5, 1, 5, 10, 25, 50, and 100 μmol L^{-1}). Altogether six runs, each using a different source of urine, were performed. After sample cleanup and derivatization, peak areas of the spiked samples were compared to those of aqueous standards that had been simply evaporated and derivatized. Analyte peak area was plotted against concentration using linear regression. The slope of the aqueous calibrators and that of urine-based calibrators were compared, as shown in Table 2. The assay was linear up to 100 μmol L^{-1} for all AG. The calibration curves showed correlation with R^2 greater than 0.99 and was considered linear [24]. Ratios of slopes for aqueous calibrators to urine-based calibrators were in the range of 0.93–1.08 for all AG, which is within ±10%; therefore aqueous calibrators were used for subsequent experiments. With the optimized conditions, the recovery of urinary AG ranged from 90.2 to 109.3% (CV: 1.98–13.58%).

3.3. LLOD, LLOQ, accuracy and imprecision assessment

The lower limit of detection (LLOD) was defined as three times the baseline noise ($S/N \geq 3$), and the lower limit of quantification (LLOQ) was defined as the concentration when the S/N ratio is 10 with less than 20% variation in precision. For accuracy study, because a commercial quality control sample for AG was not available, in-house spiked samples at two different levels (levels I and II: normal and high concentrations) were used; these two sets of samples were also used for imprecision study. Accuracy of the method was expressed as [(mean observed concentration with blank substraction)/(spiked concentration)] × 100. For within-run imprecision, samples were run 20 times in a single batch. For between-run imprecision, samples were run once a day for 20 days.

The LLOQ were in the range of 0.001–0.015 μmol L^{-1} (Table 2); this is generally better than other methods [16,25]. For accuracy, both levels I and II were within ±10% of expected values. Inter-assay CVs range from 3.3 to 9.6%, while intra-assay CVs ranged from 2.6 to 9.6%.

Table 3
Reference intervals of urine acylglycines from apparently healthy children. Values are presented as μmol $mmol^{-1}$ creatinine.

	Mean	Mean ± 1.96 SD[a]	Median	2.5–97.5%[b]
Age (years)			4.85	0.76–17.2
Propionylglycine	0.017	0.003–0.109	0.016	0.004–0.096
Isobutyrylglycine	0.853	0.191–3.810	0.894	0.150–2.813
Butyrylglycine	0.052	0.009–0.290	0.056	0.009–0.230
Tiglylglycine	1.955	0.566–6.760	2.103	0.593–5.857
2-Methylbutyrylglycine	0.317	0.083–1.208	0.321	0.081–0.948
Isovalerylglycine	0.871	0.179–4.243	0.897	0.152–3.698
Heaxanoylglycine	0.257	0.057–1.145	0.249	0.049–1.131
Phenylpropionylglycine	0.011	0.002–0.059	0.010	0.003–0.059
Suberylglycine	0.087	0.022–0.352	0.085	0.026–0.326

[a] Reference intervals were determined after logarithmic transformation of the data. After transformation, the data were normally distributed (Anderson–Darling test $p > 0.05$ in all instances).
[b] The non-parametric 95% interval was determined from untransformed data and is presented for illustrative purposes.

3.4. Sample stability

The stability of samples was assessed in two ways: post sample preparation and placed on the auto-sampler (on board) for a prolonged period; and stability of untreated samples stored in the freezer.

For post sample preparation on board stability, pooled samples sufficient for 50 injections were prepared and run in a single run. Responses of internal standard, AG and their ratio were monitored. The changes in response were compared with the initial signal; all were within ±10% and considered acceptable [24].

For stability of samples held in the freezer, urine samples from 3 different subjects were divided into aliquots and stored at −20 °C. Samples were thawed after 1 week, one month and 3 months' storage. Results were compared with the initial determination made when samples were fresh. The changes of all AG were within ±10%. This suggests that acylglycines are stable in human urine for at least 3 months when stored at −20 °C.

3.5. Application to biological samples

We determined reference intervals for AGs by analyzing samples collected from 204 apparently healthy individuals aged 21 days to 17.7 years (median, 4.9 years) with 110 males and 94 females. Reference intervals were established according to CLSI guideline C28-A3c [26]. The distributions of AG in the "normal" urine were skewed towards the right. After log transformation, all the results were normally distributed as assessed by the Anderson–Darling test ($p > 0.05$). Partitioning into age group reference intervals was not indicated according to the Harris and Boyd approach [27] (data not shown). In this context, a single reference interval for each acylglycine could be used (Table 3). Unlike our study, propionyl-, phenylpropionyl-, and suberyl-glycine were never detected in control urine samples in the study of Bonafe et al. [10]. In our study, all these 3 AGs were detected in the normal urine samples. This may be due to different ethnic groups selected or assay sensitivity. The other study conducted by Liang Li's team showed that no propionylglycine was detected in all six healthy adult volunteers [28]. But in the present work on urine samples collected from Chinese pediatric population, propionylglycine was detected in all normal urine samples. This difference may be due to differences in race and age.

For the application of this method to patient samples, Table 4 shows a summary of AG levels detected in patients' urines.

The following cases illustrate the complementary role of determining urine AGs to organic acid analysis for the diagnosis of some IEMs, especially when the patient is doing well or specimens cannot be collected during acute episode.

The first case was a 3-year girl suffering from holocarboxylase synthetase (EC 6.3.4.10) deficiency (samples 1–3). Qualitative organic acids analysis of sample 1, which was collected during acute episode, revealed lactic acidosis, ketosis, presence of propionyl-CoA and 3-methylcrotonyl-CoA metabolites. Holocarboxylase synthetase deficiency [29] was genetically confirmed later. The LC–MS/MS acylglycine analysis on sample 1 showed grossly increased levels of propionylglycine and tiglylglycine, elevated isobutyrylglycine, butyrylglycine, isovalerylglycine and 2-methylbutylylglycine (Fig. 3 lower panel). In both samples 2 and 3, collected at 1 week and 2 weeks post biotin therapy, urine organic acid showed a normal pattern; however, mildly elevated amount of propionylglycine and tiglylglycine were found in sample 2 and sample 3, respectively, by using this method.

The second illustration is an 11-year girl who was a proven case of β-ketothiolase (EC 2.3.1.9) deficiency [30]. Sample 4

Table 4
Acylglycine in patients' urines of different disorders and clinical conditions.

Sample no.	Urine acylglycines (μmol mmol^{-1} creatinine)									Diagnosis and clinical condition
	PG	IBG	BG	TG	2-MBG	IVG	HG	PPG	SG	
1	1621	56.8	20.5	890	23.0	22.5	4.89	0.18	1.67	Case 1: holocarboxylase synthetase deficiency in acute episode
2	0.49	3.29	0.27	3.38	0.76	0.93	0.52	0.02	0.14	Case 1: 1 week post biotin therapy
3	0.10	3.63	0.11	8.73	1.03	2.25	0.56	0.01	0.16	Case 1: 2 weeks post biotin therapy
4	0.10	2.86	0.14	43.1	10.9	2.35	0.27	0.01	0.07	Case 2: β-ketothiolase deficiency on carnitine supplement
Reference interval	0.003–0.109	0.191–3.81	0.009–0.29	0.566–6.76	0.083–1.208	0.179–4.243	0.057–1.145	0.002–0.059	0.022–0.352	

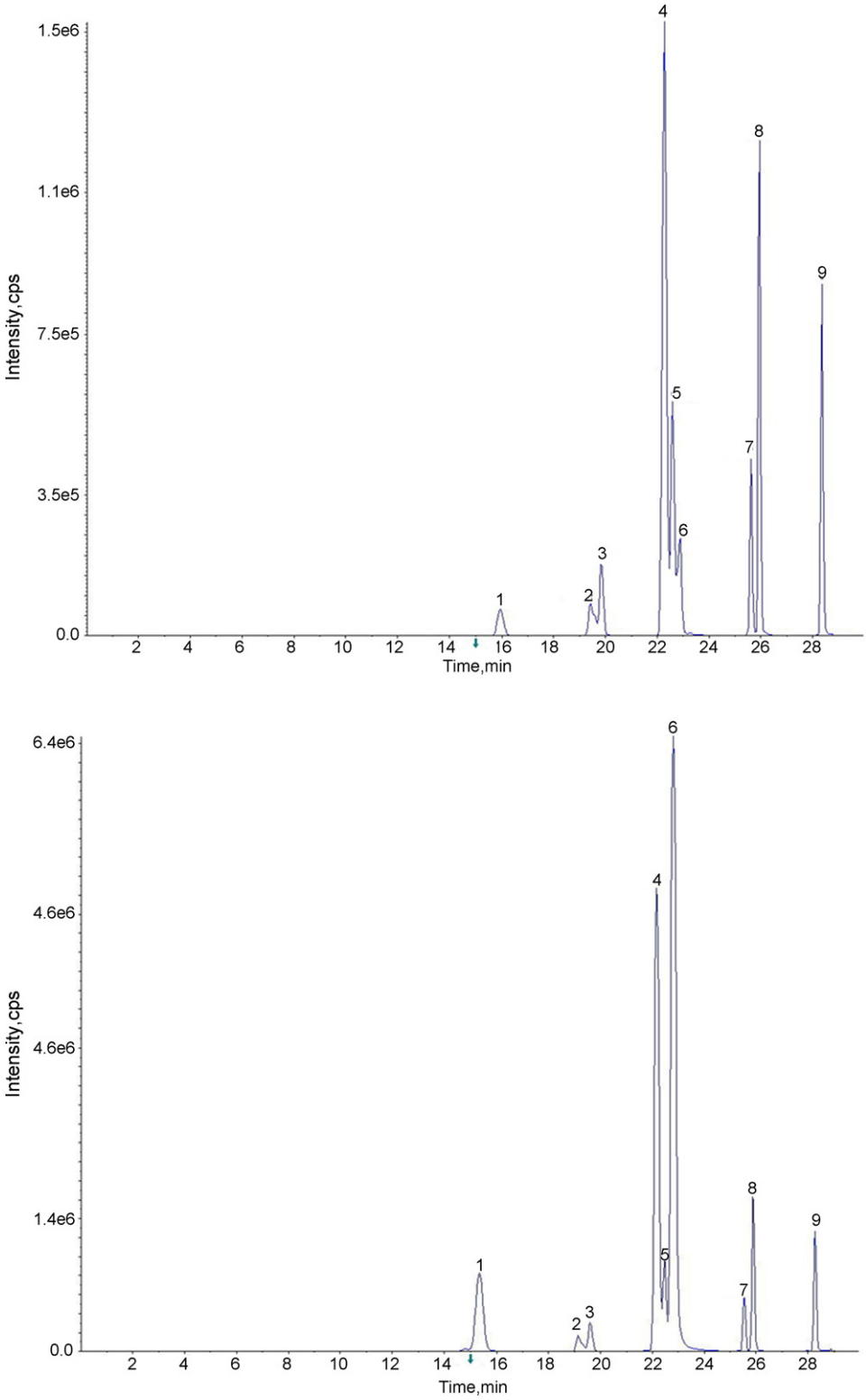

Fig. 3. A typical acylglycines TIC chromatogram of a patient's urine sample. Upper panel: normal urine; lower panel: patient sample 1 diluted 100 times.

was collected while the patient was on carnitine supplement. Urine organic acid qualitative analysis revealed gross excretions of tiglylglycine and 2-methyl-3-hydroxybutyrate with small amounts of 2-methylacetoacetate, 2-methylglutaconate and 3-hydroxybutyrate. The LC–MS/MS acylglycine method also revealed increased tiglylglycine in this specimen.

4. Conclusion

In conclusion we have developed a high-throughput method for the determination of AG in human urine and set up the pediatric reference intervals in local Chinese using this new method. The above cases illustrate how LC–MS/MS analysis can complement and refine

biochemical analysis for IEM diagnosis. Although several analytical methods for AG have been published, the present method requires a simple and straight forward sample preparation procedure; it also offers a better limit of quantification. The ability to identify and quantify AG isomers is critical for diagnosing some IEMs. With this method, it is possible to diagnose some IEMs even the patient is in remission or specimens cannot be collected during acute episode.

References

[1] T.S. Raghuveer, U. Garg, W.D. Graf, Am. Fam. Physician 73 (2006) 1981–1990.
[2] N. Gregersen, Scand. J. Clin. Lab. Invest. 44 (Suppl. 174) (1985) 1–60.
[3] B.J. Wilcox, K.J. Ritenour-Rodgers, A.S. Asser, L.E. Baumgart, M.A. Baumgart, D.L. Boger, J.L. DeBlassio, M.A. DeLong, U. Glufke, M.E. Henz, L. King, K.A. Merkler, J.E. Patterson, J.J. Robleski, J.C. Vederas, D.J. Merkler, Biochemistry 38 (1999) 3235–3245.
[4] D.S. Millington, K.M. Gibson, O.N. Elpeleg, D.H. Morton, R.S. Wappner, W.L. Nyhan, K.M. Gibson, M. Duan, in: N. Blau, M. Duran, M.E. Blaskovics, K.M. Gibson (Eds.), Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases, 2nd ed., Springer, New York, 2003, pp. 57–76, 165–190, 309–334.
[5] B. Wilcken, C.A. Stanley, M. Bennett, E. Mayatepek, in: J. Fernandes, J.M. Saudubray, G. van den Berghe, J.H. Walter (Eds.), Inborn Metabolic Diseases, 4th ed., Springer, New York, 2006, pp. 55–56, 175–190.
[6] W.L. Nyhan, P.T. Ozand, in: W.L. Nyhan, P.T. Ozand (Eds.), Atlas of Metabolic Diseases, Chapman & Hall, London, 1998, pp. 4–12, 27–32.
[7] I. Sahai, D. Marsden, Crit. Rev. Clin. Lab. Sci. 46 (2009) 55–82.
[8] K.G. Sim, J. Hammond, B. Wilcken, Clin. Chim. Acta 323 (2002) 37–58.
[9] S.H. Korman, B.S. Andresen, A. Zeharia, A. Gutman, A. Boneh, J.J. Pitt, Clin. Chem. 51 (2005) 610–617.
[10] L. Bonafe, H. Troxler, T. Kuster, C.W. Heizmann, N.A. Chamoles, A.B. Burlina, N. Blau, Mol. Genet. Metab. 69 (2000) 302–311.
[11] S.M. Carter, J.M. Midgley, D.G. Watson, R.W. Logan, J. Pharm. Biomed. Anal. 9 (1991) 969–975.
[12] M. Kimura, S. Yamaguchi, J. Chromatogr. B 731 (1999) 105–110.
[13] K.A. Kouremenos, J. Pitt, P.J. Marriott, J. Chromatogr. A 1217 (2010) 104–111.
[14] C.G. Costa, W.S. Guerand, E.A. Struys, U. Holwerda, H.J. ten Brink, I. Tavares de Almeida, M. Duran, C. Jakobs, J. Pharm. Biomed. Anal. 21 (2000) 1215–1224.
[15] T. Hagen, M.S. Korson, M. Sakamoto, J.E. Evans, Clin. Chim. Acta 283 (1999) 77–88.
[16] D.S. Millington, N. Kodo, N. Terada, D. Roe, D.H. Chace, Int. J. Mass Spectrom. Ion Processes 111 (1991) 211–228.
[17] M.S. Rashed, J. Chromatogr. B 758 (2001) 27–48.
[18] H. Bartels, M. Bohmer, C. Heierli, Clin. Chim. Acta 37 (1972) 193–197.
[19] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175–1185.
[20] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019–3030.
[21] B.K. Matuszewski, J. Chromatogr. B 830 (2006) 293–300.
[22] D. Matern, M. He, S.A. Berry, P. Rinaldo, C.B. Whitley, P.P. Madsen, S.C. van Calcar, R.C. Lussky, B.S. Andresen, J.A. Wolff, J. Vockley, Pediatrics 112 (2003) 74–78.
[23] M. Pasquali, G. Monsen, L. Richardson, M. Alston, N. Longo, Am. J. Med. Genet. C Semin. Med. Genet. 142C (2006) 64–76.
[24] J.W. Honour, Ann. Clin. Biochem. 48 (2011) 97–111.
[25] P. Rinaldo, S.H. Hahn, D. Matern, in: C.A. Burtis, E.R. Ashwood, D.E. Bruns (Eds.), Tietz Textbook of Clinical Chemistry and Molecular Diagnostics, 4th ed., Saunders, St. Louis, 2005, pp. 2207–2247.
[26] G.L. Horowitz, S. Altaie, J.C. Boyd, F. Ceriotti, U. Garg, P. Horn, A. Pesce, H.E. Sine, J. Zakowski, Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory, Approved Guideline, CLSI Document C28-A3c, vol. 28, no. 30, third ed., 2011, ISBN 1-56238-682-4, ISSN 0273-3099.
[27] E.K. Harris, J.C. Boyd, Clin. Chem. 36 (1990) 265–270.
[28] A.E. Lewis-Stanislaus, L. Li, J. Am. Soc. Mass Spectrom. 21 (2010) 2105–2116.
[29] L. Sweetman, W.L. Nyhan, N.A. Sakati, A. Ohlsson, M.S. Mange, R.B. Boychuk, R. Kaye, J. Inherit. Metab. Dis. 5 (1982) 49–53.
[30] L.H. Ngu, M.Y. Zabedah, B. Shanti, S.H. Teh, Malays. J. Pathol. 30 (2008) 109–114.