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Synthesis and characterisation of acyl glycines Their measurement in single blood spots by gas chromatography–mass spectrometry to diagnose inborn errors of metabolism

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Abstract

Acyl glycines are normally minor metabolites of fatty acids; however, the excretion of certain acyl glycines is increased in several inborn errors of metabolism. Therefore measurement of these metabolites in body fluids can be used to diagnose these metabolic disorders. The chemical synthesis of a range of acyl glycines is described, together with that of their $^{13}C_2$-isotopomers for use as internal standards. An analytical method for the measurement of hexanoyl, octanoyl, 3-phenylpropionyl and suberyl glycines in urine, employing gas chromatography–mass spectrometry with negative-ion chemical ionisation was adapted to measure a larger range of acyl glycines in a single blood spot on a standard Guthrie card. Diagnoses of a case of medium-chain acyl-CoA dehydrogenase deficiency and a case of isovaleric acidaemia were confirmed using a single blood spot from each patient.

Keywords: Acylglycines

1. Introduction

There are several inborn errors of metabolism associated with increased excretion of various acyl glycines. The most common of these is probably medium chain acyl-CoA dehydrogenase (MCAD) deficiency (McKusick 201450), an error in the mitochondrial β-oxidation of straight chain fatty acids which is characterised by increased urinary excretion of hexanoyl glycine (HG) and 3-phenylpropionyl glycine (PPG) [1,2]. Other disorders which produce increased acyl glycine excretion include: isovaleric acidaemia [McKusick 24350, isovaleryl glycine (IVG)]; multiple carboxylase deficiency [McKusick 25327, 3,3-dimethylacryloyl or 3-methylcrotonyl glycine (DMAG) and tiglyl glycine (TG)]; propionic acidaemias I and II [McKusick 23200/23205, TG, propionyl glycine (PG) and 2-methylbutyryl glycine (MBG)]; 3-hydroxy-3-methylglutaric aciduria [McKusick

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24645, DMAG] and 2-methylacetoacetyl CoA thiolase deficiency [McKusick 20375, TG].

Differential diagnosis of these inborn errors of metabolism can be difficult. Clinical presentation is unpredictable and varies from simple hypoglycaemia, intolerance to fasting and lethargy to a Reye-like syndrome, coma and sudden unexplained death. Some are more severe than others and have a high mortality rate, while in MCAD deficiency affected subjects can remain asymptomatic for long periods. Early diagnosis is essential for treatment as in some of these disorders the severe, and potentially fatal, symptoms can be prevented by appropriate dietary measures. In addition, such action is essential to prevent possible developmental retardation.

The method used by Rinaldo et al. [1] and ourselves [2] to diagnose MCAD deficiency by measuring medium chain acyl glycine excretion was gas chromatography–mass spectrometry (GC–MS). More recently Bennett et al. [3] demonstrated that there were increased concentrations of HG and PPG in blood spots from MCAD-deficient subjects. We report the synthesis of PG, IVG, MBG, TG and DMAG together with their $^{13}C_2$-isotopomers for use as internal standards and the development of a method for measuring IVG (to diagnose isovaleric acidaemia), HG, and PPG (to diagnose MCAD deficiency) in a single blood spot (6 mm diameter) collected on a standard Guthrie card, using gas chromatography–negative ion chemical ionisation mass spectrometry (GC–NICI-MS) with a stable isotope dilution technique.

2. Experimental

2.1. Equipment and GC–NICI-MS conditions

The analyses were performed on a Hewlett-Packard capillary gas chromatograph (Model 5890) coupled to a Hewlett-Packard mass spectrometer (Model 5988A) and interfaced with an HP RTE-6/VM data system. The following MS conditions were used: the instrument was tuned in the NICI mode to the ions at m/z 452, 595, and 633 from the perfluorotributylamine calibrant, source temperature was 140°C, electron energy 200 eV and methane gas was introduced to give a source pressure of approximately 1.2 mbar. The gas chromatograph was fitted with an HP-1 (Hewlett–Packard) polyimide clad fused-silica column (12 m × 0.2 mm); the stationary phase of the column was cross-linked methyl silicone gum (film thickness 0.33 μm). Helium carrier gas was used with a head pressure of 35 kPa. The gas chromatograph was programmed as follows: 60°C for 1 min then 10°C min^{-1} to 300°C. Selected ion monitoring (SIM) was also employed to give increased sensitivity and also to reduce the effects of background interference.

NMR spectra were determined with a Bruker 250 MHz instrument using C^2H_3OH as solvent.

2.2. Reagents

All solvents used in extraction for GC–MS analysis were glass-distilled (Rathburn Chemicals, Walkerburn, UK). Chemicals for derivatisation: 2,2,2-trifluoroethanol (TFE) was obtained from Fluorochem (Old Glossop, UK) and pentafluoropropionic anhydride (PFPA) was obtained from Aldrich (Gillingham, UK). Chemicals for synthesis: propionic anhydride, isovaleryl chloride, 2-methylbutyric acid, (*S*)-(+)-2-methylbutyric acid, 3,3-dimethylacryloyl chloride, tiglic acid, thionyl chloride (99 + %) and $^{13}C_2$-glycine were purchased from Aldrich. Glycine was obtained from BDH (Glasgow, UK). HG, octanoyl glycine (OG), PPG and suberyl glycine (SG), together with their respective $^{13}C_2$-isotopomers, had been prepared for our previously reported study [2].

2.3. Synthesis of standard compounds

Propionyl glycine: this was synthesised according to the method of Herbst and Shemin [4] for the synthesis of acetylglycine, by stirring a slight excess of propionic anhydride (62.5 m*M*) with glycine (50 m*M*) in 1 *M* sodium hydroxide (160 ml) on ice for 1.5 h. The reaction mixture was neutralised with 6 *M* hydrochloric acid, evaporated to dryness on a rotary evaporator and the resultant solid dried completely in a vacuum

oven overnight. This was subjected to Soxhlet extraction, using sodium-dried diethyl ether, affording PG as a white precipitate (yield 47.3%; m.p. 128–130°C: lit. [5–8] 126–127°C, 122–126°C, 124–125°C, 122–123°C) which was removed by filtration, washed with cold ether and dried in a vacuum oven. Found: C, 45.76%; H, 6.98%; N, 10.75%; calculated for $C_5H_9NO_3$; C, 45.79%; H, 6.92%; N, 10.68%. The ^{1}H NMR spectrum exhibited the following signals: 1.14 (3H, t, CH_3CH_2, $J = 7.63$ Hz), 2.27 (2H, q, CH_3CH_2, $J = 7.63$ Hz), 3.89 (2H, s, CH_2COOH). Propionyl $^{13}C_2$-glycine was prepared in the same way but on a smaller scale (5 m*M*) (yield 36.8%; m.p. 125–130°C).

Isovaleryl glycine: this was synthesised according to the procedure described by Bondi and Eissler [9]. Isovaleryl chloride (100 m*M*) and 1 *M* sodium hydroxide (150 ml) were added dropwise to a solution of glycine (100 m*M*) in 1 *M* sodium hydroxide (100 ml) cooled in an ice bath and the mixture stirred for a further 48 h. Then it was acidified with 6 *M* hydrochloric acid and the water removed on a rotary evaporator. The resultant solid was extracted with several portions (each of ca. 100 ml) of hot diethyl ether and the solvent removed from the extract, giving a yellow oil. Petroleum ether (60–80°C) was added and the solution was maintained at 4°C when some small, pink elongated rhombs of IVG formed. The product was re-crystallised from hot ether, washed with petroleum ether (60–80°C) and dried in a vacuum oven overnight. A pale pink solid was obtained (yield 10%; m.p. 108–110°C: lit. [6–10] 98–101°C, 105–106°C, 103–105°C, 106°C). Found: C, 52.56%; H, 8.28%; N, 8.65%; calculated for $C_7H_{13}NO_3$; C, 52.81%; H, 8.23%; N, 8.80%. The ^{1}H NMR spectrum exhibited the following signals: 0.97 (6H, d, $(CH_3)_2CH$, $J = 6.52$ Hz), 2.11 (3H, m, $CHCH_2CO$), 3.89 (2H, s, CH_2COOH). Isovaleryl $^{13}C_2$-glycine was made in the same way on a smaller scale (5 m*M*), but the product was extracted in a Soxhlet apparatus using dry ether as solvent (yield 4.6%, m.p. 106–108°C).

2-Methylbutyryl glycine: this was synthesised in a manner essentially the same as that used for IVG with a crude preparation of 2-methylbutyryl chloride, made by refluxing 2-methylbutyric acid (100 m*M*) with a slight excess of thionyl chloride (120 m*M*) at 80°C for 30 min. In this case the product was successfully extracted with hot diethyl ether, the solvent removed and the resultant small, creamy white elongated rhombs washed with petroleum ether (60–80°C) before drying in a vacuum oven (yield 27%; m.p. 117–119°C: lit. [7,8] 114–115°C, 113–115°C). Found: C, 52.76%; H, 8.31%; N, 8.61%; calculated for $C_7H_{13}NO_3$; C 52.81%; H, 8.23%; N, 8.80%. The ^{1}H NMR spectrum exhibited the following signals: 0.92 (3H, t, CH_3CH_2, $J = 7.42$ Hz), 1.12 (3H, d, $CH_2CH(CH_3)CO$, $J = 6.88$ Hz), 1.42 (1H, m, $CH_2CH(CH_3)$), 1.62 (1H, m, $CH_2CH(CH_3)$), 2.29 (1H, m, $CH_2CH(CH_3)$), 3.89 (2H, s, CH_2COOH). (*S*)-(+)-MBG and 2-methylbutyryl $^{13}C_2$-glycine were made in the same way on smaller scales, 25 m*M* (yield 13.6%; m.p. 98–102°C) and 5m*M* (yield 4.2%; m.p. 116–118°C), respectively.

Tiglyl glycine: after several unsuccessful attempts to prepare TG via a mixed anhydride of tiglic and acetic acids [6] and glycine methyl ester [7,11], TG was prepared essentially according to Bondi and Eissler [9]. Tiglyl chloride was synthesised by refluxing tiglic acid (100 m*M*) with a slight excess of thionyl chloride (120 m*M*) at 80°C for 30 min. The resultant crude acid chloride was used in the reaction with glycine, which was carried out in the manner described for the preparation of IVG. Acidification with 6 *M* hydrochloric acid afforded tiglic acid, a white precipitate, which was extracted with diethyl ether. The solvent was removed from the remaining aqueous phase on a rotary evaporator and the residue extracted with hot diethyl ether. The extract was evaporated to dryness, yielding a pale yellow oil which eventually crystallised by triturating it with a small amount of diethyl ether. The product was dried in a vacuum oven overnight and a small amount of contaminating tiglic acid was removed by sublimation. A cream coloured solid was obtained (yield 7.6%; m.p. 85–90°C: lit. [6–8] 83–86°C, 86–87°C, 76–78°C). Found: C, 53.24%; H, 7.33%; N, 8.73%; calculated for $C_7H_{11}NO_3$; C, 53.49%; H, 7.06%; N, 8.91%. The ^{1}H NMR spectrum gave the follow-

ing signals: 1.79 (3H, d, $CH_3CHC(CH_3)$, $J = 6.93$ Hz), 1.85 (3H, s, $CH_3CHC(CH_3)$), 3.89 (2H, s, CH_2COOH), 6.49 (1H, dq, $CH_3CHC(CH_3)$). Tiglyl $^{13}C_2$-glycine was synthesised in the same way on a smaller scale (5 m*M*), but the product could not be crystallised and remained as a viscous, yellow oil (yield, 6.6%).

3,3-Dimethylacryloyl glycine: 3,3-dimethylacryloyl chloride reacted with water immediately in aqueous solution giving 3,3-dimethylacrylic acid (senecioic acid) and so the synthesis of DMAG was carried out in dry pyridine. Glycine (25 m*M*) was suspended in dry pyridine (240 ml, distilled from and stored over sodium hydroxide pellets) with stirring, at 0–4°C, whilst 3,3-dimethylacryloyl chloride (25 m*M*) was added dropwise. The reaction mixture was allowed to stand overnight at 0–4°C, with stirring. The pyridine was removed on a rotary evaporator and the remaining brown oil extracted with several volumes of hot diethyl ether. The extracts were combined, the solvent removed and the resultant creamy, white solid extracted in a Soxhlet apparatus with dry diethyl ether (yield 6.8%; m.p. 149–151°C: lit. [6–8] 150–152°C, 150–151°C, 144–146°C). Found: C, 53.60%; H, 7.11%; N, 8.87%; calculated for $C_7H_{11}NO_3$; C, 53.49%; H, 7.06%; N, 8.91%. The ^{1}H NMR spectrum gave the following signals: 1.87 (3H, s, $(CH_3)C$), 2.11 (3H, s, $(CH_3)C$), 3.91 (2H, s, CH_2COOH), 5.75 (1H, s, $CHCO$). 3,3-Dimethylacryloyl $^{13}C_2$-glycine was synthesised in the same way on a smaller scale (5 m*M*) (yield 11.1%; m.p. 125–150°C).

2.4. Subjects

Blood samples were obtained from five control subjects, one subject with MCAD deficiency and one with isovaleric acidaemia. They were collected as spots on standard Guthrie cards and stored at room temperature, but in the absence of light, until analysis.

2.5. Extraction of blood spots

A standard solution of the $^{13}C_2$-labelled acyl glycines (equivalent to 400 pg–10 ng or 1.7 pmol–0.08 nmol of each, depending on the type of sample) in acetonitrile was added to the blood spot (6 mm in diameter on a standard Guthrie card). This was eluted with water (0.5 ml) by sonification for 10 min, shaking for 10 min and allowing it to stand at room temperature for a further 30 min. Then 2 *M* hydrochloric acid (0.1 ml) was added, followed by extraction with ethyl acetate (2 × 1.5 ml). The extracts were dried by separate passage through a column of anhydrous sodium sulphate and the solvent removed with a stream of nitrogen prior to derivatisation with PFPA and TFE as described below. A blank sample (consisting of a paper spot, 6 mm in diameter, punched from a blank Guthrie card and spiked with the same amounts of $^{13}C_2$-labelled acyl glycines) was analysed by the same procedure with every batch of blood spots, to check for contamination of the labelled standards and reagents with endogenous acyl glycines. Likewise a 1:1 standard, a paper spot containing equal amounts of $^{13}C_2$-labelled and non-labelled acylglycines, was analysed with every batch of samples to compare extraction, derivatisation, and instrumental response of the endogenous compounds and their respective internal standards.

2.6. Derivatisation procedure

Pentafluoropropionyl–trifluoroethyl (PFP–TFE) derivatives of the standard acyl glycines or biological extracts were prepared in the same manner. TFE (10 μl) and PFPA (40 μl) were added and the mixture was heated at 60°C for 10 min, allowed to cool and stand at room temperature for a further 20 min. Toluene (10 μl) was added before removal of the excess reagents under nitrogen and the derivatives were reconstituted in ethyl acetate (20–100 μl) prior to analysis by GC–NICI-MS.

2.7. Calibration curves

Calibration curves were constructed for the three acyl glycines, IVG, HG and PPG, used to demonstrate clinical diagnosis of a case of isovaleric acidaemia and a case of MCAD de-

ficiency in this study. $^{13}C_2$-Labelled acyl glycines (1 ng: 6.3 pmol IVG; 5.8 pmol HG and 4.8 pmol PPG) were added to paper spots (6 mm in diameter) punched from blank Guthrie cards together with a range of the unlabelled acyl glycines (100 pg–10 ng: 0.63 pmol–0.063 nmol for IVG, 0.58 pmol–0.058 nmol for HG and 0.48 pmol–0.048 nmol for PPG). These concentrations were within the ranges measured in the blood spots from the affected individuals. Extraction, derivatisation and GC–MS analysis was carried out in duplicate as described above.

3. Results and discussion

Melting points of these acyl glycines have been reported previously by several different groups [5–8,10], accompanied by limited descriptions of synthetic procedures and structural data. We report the synthesis of PG, IVG, MBG, TG and DMAG, together with that of their $^{13}C_2$-isotopomers, and their unequivocal characterisation by spectral and analytical means.

Table 1 summarises the chromatographic data for the PFP–TFE derivatives of this series of acyl glycines and gives the principal ions in their NICI mass spectra. None of the derivatives examined afforded a detectable molecular ion. The base peak for most of these derivatives of the acyl glycines resulted from the loss of hydrogen fluoride (HF) from the molecular ion; except in the case of DMAG, where the base peak resulted from the loss of TFE. In all cases the most abundant ion was the only ion monitored in SIM. A typical mass spectrum, that of IVG–PFP–TFE, is shown in Fig. 1. The fragmentation patterns in the NICI mass spectra of all the derivatised $^{13}C_2$-isotopomers (used as internal standards) were identical to those of the corresponding $^{12}C_2$-derivatives. The ion monitored in SIM was two mass units higher than that of the corresponding $^{12}C_2$-acyl glycine.

The PFP–TFE derivatives of the unsaturated compounds, TG and DMAG, were thermolabile and so the derivatisation conditions had to be optimised. Heating the mixture at 60°C for 10 min and then allowing it to stand at room temperature for 20 min was the best compromise, but SG was not derivatised completely under these conditions. In addition, the PFP–TFE derivatives of the shorter chain compounds, especially PG, were very volatile and could be completely lost when the excess reagents were removed in a stream of nitrogen, even at room temperature [12]. Addition of toluene (10 μl) prior to removal of excess PFPA and TFE helped

Table 1
Kovats indices and intensities of relevant ions of PFP–TFE derivatives of acyl glycines

Acyl glycine	Kovats Index	M^- (Rel. intens.)	Major ions (Rel. intens.)
PG	1073	359 (0%)	339 (100%)
IVG	1170	387 (0%)	367 (100%)
MBG (peak 1)	1160	387 (0%)	347 (100%)
			367 (59%)
MBG (peak 2)	1175	387 (0%)	367 (100%)
MBG (peak 3)	1185	387 (0%)	367 (100%)
TG	1228	385 (0%)	365 (100%)
			345 (25%)
DMAG	1247	385 (0%)	302 (100%)
			365 (48%)
			345 (48%)
HG	1293	401 (0%)	381 (100%)
OG	1470	431 (0%)	411 (100%)
PPG	1598	435 (0%)	415 (100%)
SG	1697	541 (0%)	521 (100%)

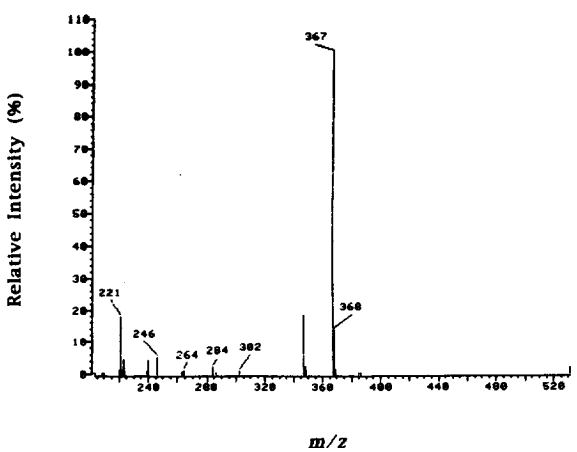

Fig. 1. NICI mass spectrum of the PFP–TFE derivative of isovaleryl glycine (IVG–PFP–TFE).

to reduce losses but, nevertheless, a great deal of care was necessary during this procedure.

When the PFP–TFE derivative of MBG was chromatographed and monitored in SIM (*m/z* 367 *or* 369) three peaks were observed; the first small, the second and third much larger and of equal size. The spectral and analytical data indicated that the substance, which was synthesised from the racemic acid, was homogeneous and pure. However, it was clear from the NMR spectrum that the protons attached to C3 were in different environments (affording separate multiplets of 16 lines), which is consistent with the molecule being disastereotopic. In order to gain further information the synthesis was repeated using (*S*)-(+)-methylbutyric acid as starting material; the resultant PFP–TFE derivative also gave three peaks (corresponding to the same ion, *m/z* 367) and with the same retention times as before.

Coefficients of variation for the measurement of standard acyl glycines (applied to Guthrie cards) were as follows: PG, 4.03%; IVG, 4.70%; MBG, 8.16%; TG, 6.58%; DMAG, 10.96%; HG, 4.61%; OG, 5.97%; PPG, 2.97% and SG, 8.54%.

Acyl glycines were measured in single blood spots (6 mm diameter) punched from a standard Guthrie card from five control subjects, one MCAD-deficient subject and one subject with isovaleric acidaemia. In all these blood spots the concentrations of PG, MBG, TG, and DMAG were below the limits of detection (i.e. the intensity of the signal was less than twice that of the background). In some of the samples the peak for OG was obscured by another, much larger, peak and results for SG were not very consistent, probably because of the derivatisation problem mentioned above; however, it is not such a reliable diagnostic marker for MCAD deficiency as HG and PPG [1]. Calibration curves were linear over the range 100 pg–10 ng (0.63 pmol–0.063 nmol for IVG, 0.58 pmol–0.058 nmol for HG and 0.48 pmol–0.048 nmol for PPG), giving correlation coefficients of 0.9990 for IVG, 0.9997 for HG and 0.9997 for PPG. Limits of detection were: IVG, 0.012 nmol/g dried blood spot; HG, 0.03 nmol/g dried blood spot and PPG, 0.006 nmol/g dried blood spot. Limits of detection for the remaining six acyl glycines were as follows: PG, 0.06 nmol/g dried blood spot; MBG, 0.018 nmol/g dried blood spot; TG, 0.03 nmol/g dried blood spot; DMAG, 0.06 nmol/g dried blood spot; OG, 0.015 nmol/g dried blood spot and SG 0.05 nmol/g dried blood spot.

Table 2 gives the concentrations of IVG, HG and PPG in blood spots. The concentrations of HG and PPG were significantly higher in the blood spots from the MCAD-deficient subject than those from the control subjects. However, they were 10–50 fold lower than those reported by Bennett et al. [3], despite the fact that the method reported here is more sensitive. Bennett and his colleagues had to use more than one blood spot for each analysis and could not detect HG or PPG in any of the control blood samples. The concentration of IVG in the blood spot from the subject with isovaleric acidaemia is certainly diagnostic; IVG was below the limit of detection in all the control blood spots.

Fig. 2 shows the total ion chromatogram (monitored in SIM) of the derivatised extract from a blood spot obtained from the subject with isovaleric acidaemia. It shows clearly only one major peak (due to IVG–PFP–TFE). Fig. 3 shows traces for the two ions monitored in SIM for the PFP–TFE derivative of (A) IVG (*m/z* 367) and (B) IV–$^{13}C_2$-glycine (*m/z* 369) in the same chromatogram.

The method reported here is more sensitive

Table 2
Concentration (nmol/g dried blood spot) of IVG, HG, and PPG in blood spots

Subject (*n*)	IVG	HG	PPG
Controls (5)	N.D.	N.D.–0.10	N.D.–0.03
MCAD-deficient (1) mean ± S.D.	N.D.	0.79 ± 0.14	0.30 ± 0.06
Isovaleric acidaemia (1) mean ± S.D.	7.5 ± 0.44	N.D.	N.D.

N.D. = below limit of detection (intensity of the signal less than twice that of the background; actual values in text).

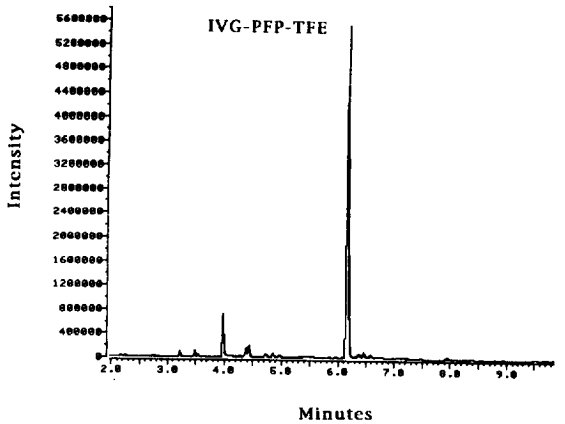

Fig. 2. Total ion GC–NICI-MS trace, monitored in SIM, of PFP–TFE derivatives of an extract of a single blood spot from a patient with isovaleric acidaemia. Isovaleryl $^{13}C_2$-glycine (5 ng) was added as internal standard prior to extraction.

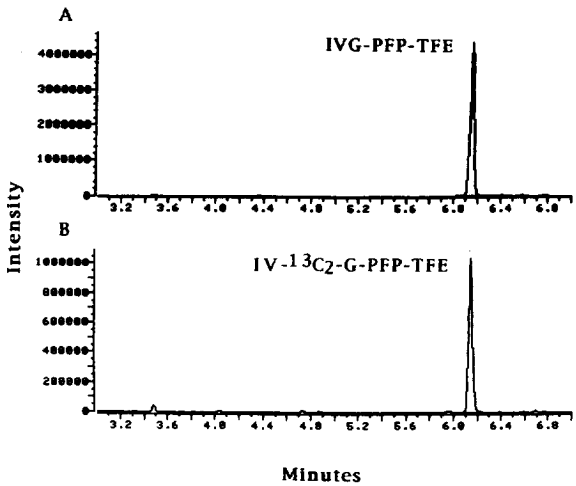

Fig. 3. SIM trace (A) the PFP–TFE derivative of endogenous isovaleryl glycine (IVG–PFP–TFE, m/z 367) extracted from a single blood spot from a patient with isovaleric acidaemia and (B) the PFP–TFE derivative of isovaleryl $^{13}C_2$-glycine (IV–$^{13}C_2$-G–PFP–TFE, m/z 369), added to the blood spot prior to extraction, as internal standard.

than that previously described [3] and has great potential for development as a screening technique for those patients (listed in the introduction) at risk of the inborn errors of metabolism which are associated with increased production of acyl glycines.

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