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Detection of acylglycines in urine by ^{1}H and ^{13}C NMR for the diagnosis of inborn metabolic diseases

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A range of inborn metabolic diseases result in abnormal accumulation of acylglycines in body fluids. Therefore, detection of these metabolites is important for diagnostic purposes. ^{1}H and ^{13}C NMR spectroscopies have successfully been applied for both qualitative and quantitative determinations of various acylglycines in urine samples from patients suffering from metabolic diseases connected with excretion of these compounds. Various acylglycines were identified in test urine samples from 15 patients suffering from five different metabolic diseases, providing information which could be crucial for their diagnoses. The paper reports complete ^{1}H and ^{13}C NMR data of 11 acylglycines, which is essential for this type of NMR analysis of body fluids. NMR spectroscopy has been proven effective in determining the presence as well as the levels of acylglycines in urine. The proposed method is rapid, simple and requires minimal sample treatment.

Key words: acylglycine, inherited metabolic disease, urinalysis, ^{1}H NMR, ^{13}C NMR

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INTRODUCTION

Determination of the level of various marker metabolites in human body fluids provides basic information required for establishing the diagnosis of inherited metabolic diseases. In general, clinical symptoms of such diseases are insufficient for proper diagnosis and therefore chemical or instrumental methods are used to characterise the metabolic profiles of body fluids (Brusilow, 1991). The examination of urine with respect to acylglycines can be diagnostic for several organic acidurias such as 3-hydroxy-3-methylglutaric aciduria, 3-methylcrotonyl-CoA carboxylase deficiency, beta-ketothiolase deficiency, biotinidase deficiency, isovaleric acidemia, methylmalonic acidemia and propionic acidemia (Brusilow, 1991; Bonafe *et al.*, 2000; Tavazzi *et al.*, 2005). An elevated concentration of acetylglycine, the simplest metabolite in the considered series, as well as of other acylaminoacids, is observed in body fluids in the case of aminoacylase 1 deficiency (Engelke *et al.*, 2008). The urinary excretion of acylglycines is also characteristic for particular fatty-acid diseases (Brusilow, 1991; Kimura & Yamaguchi, 1999; Costa *et al.*, 2000), such as glutaric acidemia type II and medium-chain acyl-CoA dehydrogenase deficiency (MCAD).

So far, various acylglycines have been determined in urine using HPLC (Tavazzi *et al.*, 2005), GC-MS (Aramaki *et al.*, 1991; Kimura & Yamaguchi, 1999; Costa *et al.*, 2000), MS/MS (Bonafe *et al.*, 2000; Pasquali *et al.*, 2006; Waddell *et al.*, 2006) and more complex techniques such as LC-MS/MS (Lewis-Stanislaus & Li, 2010; Ombrone *et al.*, 2011; Fong *et al.*, 2012) or GC-HPLC-MS (La Marca & Rizzo, 2011). Determinations of some of acylglycines in urine using ^{1}H NMR have been reported occasionally (Lehnert & Hunkler, 1986; Moolenaar *et al.*, 2002; Engelke *et al.*, 2008). To our knowledge, however, there are currently no publications addressed specifically to the use of NMR spectroscopy for this purpose. Moreover, spectroscopic data appropriate for medical analyses are not available for acylglycines, except for the ^{1}H NMR spectra of acetylglycine and hippuric acid. It is worth noting that ^{13}C NMR spectroscopy, which is usually very informative, has never been used in such assays, either.

NMR spectroscopy, especially ^{1}H NMR, is an important tool that has found extensive applications in medical diagnostics (Iles *et al.*, 1985; Beckmann, 1995; Fan, 1996; Zuppi *et al.*, 1997; Lindon *et al.*, 1999; Saude *et al.*, 2006; Pinheiro *et al.*, 2009). The method is fast and simple because it does not require pre-processing of the analysed biological sample, necessary, as a rule, before performing chromatographic and/or MS analyses. An important merit of the NMR-based method of analysis of biological samples is that it can provide qualitative and, if necessary, quantitative information about an almost unlimited range of metabolites simultaneously, during one measurement or a measuring series performed for the same sample. Essentially, a high resolution NMR spectral pattern can be theoretically predicted for a given chemical structure, nevertheless, the analysis, as a rule, is based on a comparison of the spectrum recorded for the analysed sample of urine with experimental spectra of model substances. Therefore, in laboratory practice a reference data base collected in the form of chemical shift data tables and/or a reference spectra library is needed.

In this work, we describe an approach that involves the use of ^{1}H and ^{13}C NMR techniques in the analysis of urine for the detection of a selected group of clinically important acylglycines. The investigated urine samples were taken from independently diagnosed patients suffering from metabolic diseases connected with excretion of acylglycines. In this way the practical utility and credibility of the proposed analytical method have been checked and confirmed. Furthermore, we report ^{1}H and ^{13}C NMR data for selected acylglycines, which can be useful in similar analyses.

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Abbreviations: ACY1D, aminoacylase 1 deficiency; at, acquisition time; Cr, creatinine; IVA, isovaleric aciduria; IVGly, isovalerylglycine; MCAD, medium-chain acyl-CoA dehydrogenase deficiency; MCG, 3-methylcrotonylglycinuria; NMR, nuclear magnetic resonance; PA, propionic aciduria; PGly, propionylglycine; pw, pulse width; sw, spectral width; TSP, 3-(trimethylsilyl)-propionic acid-D4 sodium salt.

MATERIALS AND METHODS

Reference compounds. Most of acylglycines used as standards in this work were synthesised by acylation of glycine (Sigma-Aldrich) with appropriate anhydride or acid chloride, using the literature procedure (Carter *et al.*, 1955). Hippuric acid was a commercial product (Sigma-Aldrich).

Creatinine NMR chemical shift in ppm: ^{1}H at pH 2.5: 3.14 (s, CH_3), 4.31 (s, CH_2); at pH 7.0: 3.04, 4.05; ^{13}C at pH 2.5: 33.62 (CH_3) , 56.85 (CH_2), 159.89, 175.68; at pH 7.0: 32.93, 59.11, 171.98, 191.57.

Standard solutions. Solutions of standard compounds were prepared at a concentration of 0.1 M in H_2O, their acidity was adjusted to pH 2.5 or 7.0±0.2 by adding small amounts of 0.5 M HCl or NaOH and controlled with a pH-meter. Then, 0.5 ml portion of such solution and 0.05 ml of 0.13 M solution of 3-(trimethylsilyl)-propionic acid-D4 sodium salt (TSP) in D_2O (internal lock signal) were placed in a standard 5-mm o.d. NMR tube. Further pH adjustment was unnecessary as the investigated solutions exhibited a remarkable buffering capacity.

Urine sample preparation. Urine samples were collected from 18 patients suffering from one of five different metabolic diseases. The urine collection was performed and the diagnoses were established in The Children's Memorial Health Institute in Warsaw. All urine samples were stored frozen at –20ºC after the collection until required for NMR analysis. The samples for NMR measurements were prepared directly in NMR tubes. A 0.5-ml portion of unprocessed urine and 0.05 ml of 0.13 M solution of TSP in D_2O were placed in a standard 5-mm o.d. NMR tube and their acidity was adjusted to pH 2.5 or 7.0 ± 0.2 by adding small amounts of 0.5 M HCl or NaOH. In order to detect metabolites at low levels some of our samples were concentrated approximately fivefold before measurements. During the standard procedure a 5-ml portion of urine was placed in a rotary evaporator, heated to 40ºC and concentrated to a volume of ca. 1 ml. Then the pH of the concentrate was adjusted and the sample for NMR measurements was prepared as described above.

NMR spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded at 25ºC using Varian 400 or 500 MHz spectrometers operating at 9.4 T or 11.7 T magnetic fields, respectively, and equipped with temperature controllers. The TSP signal (0.0 ppm) was used as the internal chemical shift reference. For recording ^{13}C NMR spectra the standard measurement parameters were as follows: pulse width pw=5 μs, pw90=15 μs (pulse angle 30º), repetition time=acquisition time at ≈1 s, spectral width sw=250 ppm. In order to achieve a satisfactory signal/noise ratio the spectra were accumulated for 2 hours or overnight. For recording ^{1}H NMR spectra the following measurement parameters were used: pw=6 μs (pw90=18 μs), at=5 s, sw=15 ppm. The water signal was saturated for 3 s prior to the observing pulse and 64 to 512 scans were accumulated.

RESULTS AND DISCUSSION

Standards

As it was mentioned in the Introduction, in the course of the analytical procedure the presence or absence of a given acylglycine in the examined urine has been established by a simple comparison of the ^{1}H or ^{13}C NMR spectrum of the investigated sample with the reference spectrum of the marker, recorded at exactly the same conditions (temperature, pH, D_2O content). Unfortunately, there was a general lack of information in the literature regarding the NMR data suitable for analytical purposes for this group of metabolites. Therefore, we decided to build a library of spectra of the majority of acylglycines occurring in urine as normal or abnormal metabolites. We measured ^{1}H and ^{13}C NMR spectra for these compounds in aqueous solutions of pH 2.5 and 7.0. These two pH values were chosen to follow the literature recommendations (Moolenaar *et al.*, 2002) and to reproduce the conditions usually encountered in urine. The chemical shift data obtained in the current study are collected in Table 1. In the case of ^{1}H NMR signals their multiplicities and spin-spin coupling constants are also given.

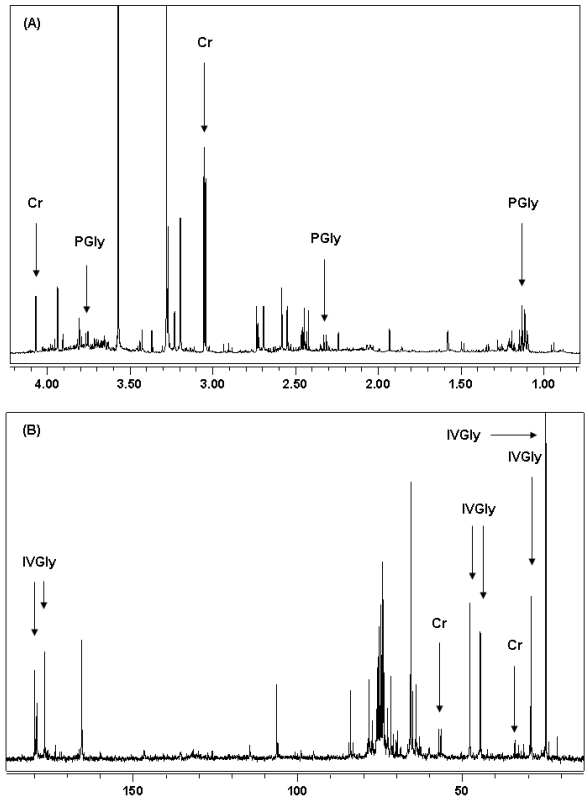

Figure 1. NMR spectra of urine samples of patients suffering from metabolic diseases
(A) ^{1}H NMR, propionic aciduria; **(B)** ^{13}C NMR, isovaleric aciduria. Cr, creatinine; IVGly, isovalerylglycine; PGly, propionylglycine.

Urine analysis

The determination of urine acylglycines was made without any pre-treatment (except for occasional fivefold concentration) of the biological samples; in particular, neither derivatisation nor isolation of the metabolites of interest were performed. In the first step of the procedure, ^{1}H NMR spectrum of the examined urine sample was measured. Usually, such a measurement takes only a few minutes when using a 500 MHz spectrometer. The resulting spectrum was then compared with the reference spectra of individual standard substances in order to recognise particular metabolites and assign all their signals. The signal positions (chemical shifts) in the reference and investigated spectra being compared are expected to agree within 0.02 ppm in the case of ^{1}H

Table 1. Reference ^{1}H and ^{13}C NMR spectral data for acylglycine standards in aqueous solutions

Acylglycine		Chemical shift in ppm (splitting behaviour, spin-spin coupling constant in Hz, assignment)	
		pH 2.5	pH 7.0
Acetylglycine	^{1}H	2.05 (s, CH_3), 3.94 (d, 6.0, CH_2), 8.22 (bs, NH)	2.04 (s, CH_3), 3.74 (d, 5.9, CH_2), 8.00 (bs, NH)
	^{13}C	24.63, 44.42, 176.92, 177.70	24.76, 176.91, 46.26, 179.71
Butyrylglycine	^{1}H	0.91 (t, 7.3, CH_3), 1.61 (sxt, 7.3, CH_2), 2.28 (t, 7.3, CH_2), 3.96 (d, 5.9, CH_2), 8.25 (bs, NH)	0.92 (t, 7.3, CH_3), 1.62 (sxt, 7.3, CH_2), 2.28 (t, 7.3, CH_2), 3.76 (d, 5.9, CH_2), 7.95 (bs, NH)
	^{13}C	15.67, 21.75, 40.31, 44.28, 183.92, 194.19	15.65, 21.63, 40.43, 46.13, 175.30, 179.81
Hexanoylglycine	^{1}H	0.86 (t, 6.6, CH_3), 1.29 (m, 2xCH_2), 1.60 (m, CH_2), 2.31 (t, 7.2, CH_2), 3.94 (d, 5.9, CH_2) , 8.28 (bs, NH)	0.88 (t, 6.4, CH_3), 1.30 (m, 2xCH_2), 1.61 (m, CH_2), 2.30 (t, 7.2, CH_2), 3.74 (d, 5.9, CH_2), 7.97 (bs, NH)
	^{13}C	16.15, 24.59, 27.81, 33.34, 38.35, 44.20, 176.73, 180.88	16.08, 24.54, 27.79, 33.37, 38.59, 46.17, 179.65, 180.06
Hippuric acid	^{1}H	4.18 (s, CH_2), 7.54 (m, 1H), 7.63 (m, 2H), 7.83 (m, 2H)	3.96 (s, CH_2), 7.53(m, 1H), 7.59 (m, 2H), 7.82 (m, 2H)
	^{13}C	42.56, 127.83, 129.47, 133.09, 133.52, 174.50, 178.77	42.96, 127.82, 129.45, 133.04, 133.63, 171.59, 175.06
Isobutyrylglycine	^{1}H	1.10 (d, 6.8, 2xCH_3), 2.56 (m, CH), 3.93 (d, 5.9, CH_2), 8.19 (bs, NH)	1.12 (d, 6.8, 2xCH_3), 2.56 (m, CH), 3.74 (d, 5.7, CH_2), 7.89 (bs, NH)
	^{13}C	21.49, 37.76, 44.25, 176.88, 184.63	21.45, 37.82, 46.14, 179.70, 183.72
Isovalerylglycine	^{1}H	0.93 (d, 6.8, 2xCH_3), 2.00 (m, CH), 2.18 (d, 7.3, CH_2), 3.96 (d, 5.9, CH_2), 8.29 (bs, NH)	0.94 (d, 6.8, 2xCH_3), 2.01 (m, CH), 2.18 (d, 7.3, CH_2), 3.76 (d, 5.9, CH_2), 8.01 (NH)
	^{13}C	24.42, 28.99, 44.16, 47.56, 176.59, 179.96	24.38, 28.89, 46.12, 47.80, 179.25, 179.59
2-Methylbutyrylglycine	^{1}H	0.87 (t, 7.4, CH_3), 1.08 (d, 6.8, CH_3), 1.48 (m, CH_2), 2.34 (m, CH), 3.94 (d, 5.9, CH_2), 8.26 (bs, NH)	0.88 (t, 7.6, CH_3), 1.10 (d, 6.8, CH_3), 1.49 (m, CH_2), 2.33 (m, CH), 3.77 (d, 5.7, CH_2), 7.91 (bs, NH)
	^{13}C	13.99,19.62, 29.74, 44.22, 45.16, 176.84, 184.04	13.94, 19.48, 29.63, 45.20, 46.09, 179.59, 183.11
3-Methylcrotonylglycine	^{1}H	1.87 (d, 1.2, CH_3), 2.04 (d, 1.0, CH_3), 3.98 (d, 5.6, CH_2), 5.78 (m, CH), 8.03 (bs, NH)	1.88 (d, 1.2, CH_3), 2.04 (d, 1.0, CH_3), 3.79 (d, 5.9, CH_2), 5.79 (bs, CH), 7.75 (bs, NH)
	^{13}C	22.33, 29.19, 44.17, 119.72, 156.57, 173.37, 177.10	22.18, 29.01, 45.96, 120.21, 155.20, 172.79, 179.89
Phenylpropionylglycine	^{1}H	2.62 (t, 7.3, CH_2), 2.94 (t, 7.3, CH_2), 3.87 (d, 5.9, CH_2), 7.27 (m, 3H), 7.36 (m, 2H), 8.15 (bs, NH)	2.62 (t, 7.4, CH_2), 2.95 (t, 7.4, CH_2), 3.68 (d, 5.7, CH_2), 7.29 (m,3H), 7.37 (m, 2H), 7.90 (bs, NH)
	^{13}C	33.97, 39.99, 44.38, 129.33, 131.32, 131.59, 143.51, 176.82, 179.18	33.99, 40.21, 46.12, 129.24, 131.23, 131.52, 143.57, 178.45, 179.51
Propionylglycine	^{1}H	1.11 (t, 7.8, CH_3), 2.32 (q, 7.8, CH_2), 3.95 (d, 5.9, CH_2), 8.17 (bs, NH)	1.13 (t, 7.8, CH_3), 2.32 (q, 7.8, CH_2), 3.76 (d, 5.9, CH_2), 7.89 (bs, NH)
	^{13}C	12.19, 31.76, 44.41, 177.03, 181.47	12.17, 31.87, 46.18, 179.69, 180.66
Suberylglycine	^{1}H	1.34 (m, 2xCH_2), 1.60 (m, 2xCH_2), 2.31 (t, 7.3, CH_2), 2.37 (t, 7.3, CH_2), 3.96 (d, 5.8, CH_2), 8.24 (bt, NH)	1.32 (m, 2xCH_2), 1.57 (m, 2xCH_2), 2.18 (t, 7.5, CH_2), 2.30 (t, 7.5, CH_2), 3.75 (d, 5.9, CH_2), 7.99 (bt, NH)
	^{13}C	27.03, 27.89, 30.61, 30.67, 36.73, 38.27, 44.26, 176.77, 180.71, 182.31	27.85, 28.61, 30.67, 31.33, 38.48, 40.42, 46.16,179.63, 179.85, 187.00

Table 2. Summary of results of urine sample analyses for various diseases connected with elevated acylglycine concentrations

Disease	Abbreviation	Acylglycine	Patient	Acylglycine [mol/mol of creatinine]
Aminoacylase 1 deficiency	ACY1D	Acetylglycine	1	0.3
Isovaleric aciduria	IVA	Isovalerylglycine	2	Not detected (less than 0.1)
			3	1.0
			4	3
			5	4
			6	4
			7	4.5
			8	9
			9	10
			10	11
Medium-chain acyl-CoA dehydrogenase deficiency	MCAD	Hexanoylglycine	11	0.5
			12	0.8
			13	1.6
		Phenylpropionylglycine	11	Not detected (less than 0.15)
			12	Not detected (less than 0.1)
			13	Not detected (less than 0.1)
		Suberylglycine	11	0.15
			12	0.3
			13	0.9
3-Methylcrotonylglycinuria	MCG	3-Methylcrotonylglycine	14	0.2
			15	0.9
Propionic aciduria	PA	Propionylglycine	16	Not detected
			17	Not detected (less than 1)
			18	0.7

NMR, whereas the appropriate ^{1}H–^{1}H coupling constants should differ by less than 0.2 Hz. Finally, the metabolite levels (relative to creatinine) were evaluated by comparing the integral intensities of the metabolite signals with the intensities of the creatinine resonances. Owing to the proportionality of the signal intensity to the number of protons yielding the signal (when observing well known experimental conditions), the metabolite level determination accuracy could be, in most cases, cross-checked many times in one spectrum. It should be noted that the minimum detection limit is about 0.1 mol/mol of creatinine in the case of our 500 MHz system, but in particular urine spectra it strongly depends on sample composition because of possible signal overlapping.

Figure 1A shows an example of a typical ^{1}H NMR spectrum of urine with elevated acylglycine. In the sample the presence of propionylglycine was confirmed and its concentration was determined to be 0.7 mol/mol of creatinine. It is to be stressed that the doublet (J=5.9 Hz) at 3.76 ppm (at pH 7.0) or 3.95 ppm (at pH 2.5), originating from the methylene protons of the -$NHCH_2COOH$ group, is characteristic for most acylglycines. This signal, however, cannot be used for differentiation between particular acylglycines, since the values of chemical shift and also the coupling constant for this signal are very similar for all acylglycines except for hippuric acid (see Table 1). As the limiting criterion proving the presence of a given acylglycine in the analyzed sample we assumed the ascertainment of at least two well-recognisable ^{1}H NMR signals of the metabolite, i. e., signals that are not overlapped by the signal of water or signals of other metabolites in the examined spectrum. Therefore, in the case when a strong signal overlap occurred in the spectrum another technique had to be additionally employed.

In order to solve the above problem we applied ^{13}C NMR spectroscopy, which seems to be a method of choice in such circumstances. As it was discussed in more detail elsewhere (Bal *et al.*, 2008), the distinctive advantage of proton decoupled ^{13}C NMR spectra is their much higher transparency as compared to ^{1}H NMR spectra. On the other hand, there is a limitation to this technique associated with its much poorer sensitivity than that of ^{1}H NMR. This limitation can be largely overcome by the fivefold concentration of urine before measurements or by application of two-dimensional ^{1}H-^{13}C correlation NMR experiments. Actually, both ^{1}H NMR and ^{13}C NMR methods can complement each other to provide an unambiguous result of analysis. It has to be remembered, however, that due to relaxation phenomena, the signal intensities in standard ^{13}C NMR spectra should always be interpreted with some caution and compared only for protonated carbons and only within the same substitution type, i.e., separately for the carbons in methyl groups and for the carbons in methylene and methine groups. Figure 1B shows a ^{13}C NMR spectrum of urine of a patient suffering from isovaleric

aciduria (IVA). The relative concentration of isovalerylglycine could be estimated to be 4 mol/mol of creatinine.

Results of analysis of test samples

Eighteen urine samples of patients suffering from one of the diseases quoted in Table 2, were analysed in this study. All the patients had been independently diagnosed in the Children's Memorial Health Institute in Warsaw using in all cases the GC-MS method to establish the urinary organic acid profile and, if necessary, by using additional diagnostic methods. In the course of our analyses the appropriate acylglycines were discovered in urine samples of the patients with ACY1D, MCAD and MCG in all investigated cases. However, in the case of MCAD, connected with excretion of three different acylglycines, only two of them, hexanoylglycine and suberylglycine, were detected in all samples, whereas phenylpropionylglycine was not observed. In the case of two other diseases, IVA and PA, the NMR urine analysis showed the presence of acylglycines in 8 of 9 cases and 1 of 3 cases, respectively. In all the investigated samples the acylglycine concentration relative to creatinine was determined. Furthermore, in the case of the apparent absence of a given marker metabolite in the investigated sample its threshold concentration (maximum possible concentration) could be estimated.

In conclusion, we have shown that the high resolution ^{1}H NMR and ^{13}C NMR techniques, widely accessible in physicochemical laboratories, can be used either separately or in combination for qualitative and quantitative determination of acylglycines in urine. The method is simple as neither preliminary extraction nor derivatisation of metabolites are needed. It can be used for the simultaneous screening for all the metabolic diseases connected with excretion of acylglycines. The ^{1}H NMR and ^{13}C NMR chemical shifts and signal multiplicities reported above provide a database which can readily be used in performing similar analyses by other diagnostic laboratories equipped with NMR instruments.

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