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Investigation of a wide spectrum of inherited metabolic disorders by ^{13}C NMR spectroscopy

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High-resolution ^{1}H NMR spectroscopy of body fluids has proved to be very useful in diagnostics of inherited metabolic diseases, whereas ^{13}C NMR remains almost unexploited. In this paper the application of ^{13}C NMR spectroscopy of fivefold concentrated urine samples for diagnosis of selected metabolic diseases is reported. Various marker metabolites were identified in test urine samples from 33 patients suffering from 10 different diseases, providing information which could be crucial for their diagnoses. Spectra were accumulated for 2 h or overnight when using spectrometers operating at 9.4 or 4.7 T magnetic fields, respectively. Interpretation of the measurement results was based on a comparison of the peak positions in the measured spectrum with reference data. The paper contains a table with ^{13}C NMR chemical shifts of 73 standard compounds. The method can be applied individually or as an auxiliary technique to ^{1}H NMR or any other analytical method.

Keywords: inherited metabolic disease, ^{1}H NMR, ^{13}C NMR, urine, marker metabolite

INTRODUCTION

Since the discovery of alcaptonuria in 1902 (Markus *et al.*, 2001; Held, 2006) as the first described inherited metabolic disease, a large number of other disorders have been found (Scriver *et al.*, 2001). For a number of this diseases caused by enzyme defects abnormal levels of specific metabolites (markers) in body fluids are symptomatic, providing a convenient way for diagnosis and differentiation of these disorders. Especially the examination of urine is very attractive because it is rich in information and the method is non-invasive. Presently, diverse analytical techniques providing desired information are exploited. The most commonly used are the ones based on various chromatographic methods (Podebrad *et al.*, 1999; Rashed *et al.*, 2000; Kuhara, 2002) such as TLC, HPLC. Various techniques can be used for detection of the metabolites of interest, especially MS. Among other methods in use, CE (Burlina *et al.*, 1999) and NMR (Lehnert & Hunkler, 1986; Zuppi *et al.*, 1997; Moolenaar *et al.*, 2002) of body fluids should be mentioned, the latter becoming more and more popular.

In the past, NMR spectroscopy was considered a technique of rather low sensitivity, tolerable at most by chemists, but it seems that this view ought to be revised. The sensitivity obviously constitutes the limit for every method, but it is noteworthy that modern high-resolution NMR spectrometers enable routine investigations of solutions from the micromolar concentration range upwards. Moreover, NMR allows not only structural but also quantitative analysis to be done. For that reason, NMR spectros-

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Abbreviations: at, acquisition time; CE, capillary electrophoresis; GC, gas chromatography; HPLC, high performance liquid chromatography; HSQC, heteronuclear single quantum correlation; MS, mass spectrometry; NMR, nuclear magnetic resonance; OTC, ornithine transcarbamylase; pw, pulse width; sw, spectral width; TLC, thin layer chromatography; TSP, 3-(trimethylsilyl)-propionic acid-D_4 sodium salt.

copy should clearly be suitable for detection of metabolites or drugs in body fluids. In principle, NMR spectra generated by any of the 1/2-spin nuclei occurring in organic compounds (e.g. ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P) can be used (Lindon *et al.*, 1999), but because of its highest sensitivity, ^{1}H NMR is usually chosen for medical assays (Wevers *et al.*, 1994). Beside the unquestionable advantage of the high resolution ^{1}H NMR over spectroscopy of any other magnetic nucleus, the proton spectra also have some inconvenient features. In the case of urine samples, because of their complex composition, the spectra frequently contain several thousands of resolvable lines. Such spectra potentially provide information on hundreds of low-molecular-mass compounds. In practice, however, full analysis is impossible because of the extensive overlap of multiplets. In addition, the presence of a very strong water signal causes the loss of some metabolite peaks even if the solvent suppression is effective. Those limitations can frequently be overcome by applying ^{13}C NMR technique with ^{1}H decoupling. In this work, we demonstrate that ^{13}C NMR spectroscopy of urine can provide key information for the diagnosis of several inborn errors of metabolism in cases in which high levels of marker metabolites are excreted. The recommended method is simple and can be applied even with only a basic NMR equipment, such as spectrometers operating at 9.4 T.

MATERIALS AND METHODS

Reference compounds. Most of the standards used in this work were commercially available; some: *N*-acetylaspartic acid (Barker, 1953), 2-hydroxyglutaric acid (Winitz *et al.*, 1956), 3-hydroxyisovaleric acid (Coffman *et al.*, 1958), isovalerylglycine (Carter *et al.*, 1955), 3-ureidoisobutyric acid and 3-ureidopropionic acid (Fink *et al.*, 1956) were synthesized using literature procedures.

Standard solutions. Solutions of standard compounds in H_2O (0.1 M) were prepared and their acidity was adjusted to pH 2.5 ± 0.2 by adding small amounts of 0.5 M HCl. Then 0.5 ml of such solution and 0.05 ml of 0.13 M solution of 3-(trimethylsilyl)-propionic acid-D_4 sodium salt (TSP – chemical shift reference) in D_2O (internal lock signal) were placed in a standard 5 mm o.d. NMR tube, and ^{1}H NMR and ^{13}C NMR spectra of the solution were recorded (Table 1).

Urine sample preparation. Urine samples were collected from 30 patients suffering from one of 10 different metabolic diseases (McKusick, 1994): alcaptonuria (McKusick 203500), Canavan disease (McKusick 271900), glutaric aciduria type I (McKusick 231670), L-2-hydroxyglutaric aciduria (McKusic 236792) and D-2-hydroxyglutaric aciduria (McKusic 600721), isovaleric aciduria (McKusick 243500), lactic aciduria due to combination of mitochondrial energy metabolism defects, orotic aciduria due to OTC deficiency (McKusic 311250), 5-oxoprolinuria (McKusick 266130), phenylketonuria (McKusick 261600), and tyrosinemia type I (McKusick 276700). The collection was performed in the Children's Memorial Health Institute (Warszawa, Poland). The diagnoses had earlier been confirmed at least by urinary organic acid profile using a GC-MS method. All urine samples were stored frozen at –20°C after the collection until required for NMR analysis.

Before NMR measurements a sample of 5 ml of urine was concentrated to 1 ml. Its pH was adjusted to 2.5 ± 0.2 with an appropriate volume of hydrochloric acid and the sample was centrifuged. Then, 0.5 ml of supernatant was transferred to a 5 mm o.d. NMR tube and 0.05 ml of 0.13 M solution of TSP in D_2O was added.

NMR spectroscopy. NMR spectra were measured at room temperature using Varian Mercury 400 or GEMINI 2000 spectrometers operating at 9.4 T and 4.7 T magnetic fields, respectively. The TSP signal (0 ppm) was used as the internal chemical shift reference. For recording ^{13}C spectra the standard measurement parameters were as follows: pulse width pw = 5 μs (pw90 = 15 μs), acquisition time at = 1 s, spectral width sw = 250 ppm. While processing zero filling (fn = 64 K) and exponential filtering (lb = 1) were applied. In order to achieve a satisfactory signal-to-noise ratio the spectra were accumulated for 2 h (Varian Mercury 400) or overnight (GEMINI 2000). For recording ^{1}H spectra the following measurement parameters were used: pw = 6 μs (pw90 = 18 μs), at = 5 s, sw = 15 ppm and pw = 7 μs (pw90 = 23 μs), at = 5 s, sw = 16 ppm when using 400 MHz and 200 MHz spectrometers; 64 to 512 scans were accumulated. The water signal was selectively saturated for 3 s prior to the observing pulse.

RESULTS

The main advantage of ^{1}H NMR over other methods for determination of metabolite urine profiles is avoiding laborious sample preprocessing before analysis. Probably the same applies to ^{13}C NMR when using top class spectrometers. Such NMR equipment is, however, expensive and rarely accessible in medical laboratories. In the current work we demonstrate that even when using a standard basic class 9.4 T NMR spectrometer, ^{13}C NMR analysis is feasible with reasonable acquisition time, provided that urine samples are fivefold concentrated prior to analysis. Such a simple laboratory operation is really effective: let us

Table 1. ^{1}H and ^{13}C NMR chemical shifts of standards in water solutions (pH 2.5 ± 0.2, TSP as an internal standard).

Compound	^{1}H (δ/ppm)	^{13}C (δ/ppm)	
		Protonated carbons	Unprotonated carbons
Acetic acid	2.08	23.32	179.52
Acetone	2.22	32.96	218.10
Acetylacetic acid	2.23 3.66	32.54 52.58	174.53 210.13
N-Acetylaspartic acid	2.03 2.95 4.72	24.45 38.56 52.17	176.72 177.41
N-Acetylcitrulline	1.56 1.75 2.04 3.12 4.31	24.34 28.17 30.57 42.05 55.69	164.29 177.02 178.90
Adipic acid	1.63 2.41	26.37 36.16	181.460
Alanine	1.47 3.79	18.90 53.31	178.39
β-Alanine	2.71 3.24	34.63 38.52	178.57
3-Aminobutyric acid	1.35 2.71 3.75	20.43 40.95 47.37	177.50
4-Aminobutyric acid	1.95 2.50 3.05	24.90 33.49 41.70	179.96
4-Aminohex-5-enoic acid (vigabatrin)	1.88 2.40 3.81 5.42 5.76	29.91 32.54 56.31 124.28 135.07	179.78
2-Aminoisobutyric acid	1.52	26.14	60.55 179.58
3-Aminoisobutyric acid	1.26 2.85 3.16	16.99 40.05 44.28	180.89
Arginine	1.71 1.91 2.87 3.24	24.52 28.14 41.24 54.74	157.58 174.42
Ascorbic acid	3.76 4.08 5.02	64.89 71.69 78.96	120.45 158.99 176.18
Asparagine	2.89 2.95 4.09	37.12 53.66	175.44 176.92
Aspartatic acid	3.01 4.13	37.22 53.25	175.28 176.77
Betaine	3.25 3.93	56.08 68.49	171.60
Citric acid	2.80 2.97	46.20	76.22 176.59 180.13
Citrulline	1.57 1.88 3.14 3.88	27.61 30.23 41.95 56.64	164.28 176.40
Creatine	3.05 4.11	39.72 55.58	160.18 176.16
Creatinine	3.14 4.31	33.62 56.85	159.89 175.68

5,6–Dihydrouracil	2.67 3.45	32.16 38.27	158.80 177.40
N,N-Dimethylglycine	2.92 3.75	46.32 62.11	172.65
Ethylmalonic acid	0.94 1.88 3.36	13.60 25.14 56.24	177.67
Fumaric acid	6.82	136.98	172.04
α-Glucose	3.18–3.87[1] 5.22	63.36 72.36 74.13 74.28 75.56 94.84	
β-Glucose	3.18–3.87[1] 4.68	63.52 72.36 76.95 78.57 78.64 98.66	
Glutamic acid	2.10 2.58 3.88	28.15 32.52 56.32	176.09 179.53
Glutamine	2.07 2.41 3.78	28.94 33.56 56.91	176.60 180.39
Glutaric acid	1.88 2.44	22.33 35.57	180.83
Glycine	3.56	44.25	175.10
Guanidinacetic acid	3.93	46.21	159.97 176.29
Homogentisic acid	3.64 6.72–6.85	38.49 118.13 119.51 120.80	125.51 150.32 151.63 179.40
2-Hydroxybutyric acid	0.94 1.75 4.24	11.15 29.40 74.21	181.03
3-Hydroxybutyric acid	1.22 2.50 4.19	24.57 45.84 67.29	178.96
4-Hydroxybutyric acid	1.79 2.42 3.61	24.24 30.65 73.28	185.78
2-Hydroxyglutaric acid	1.96 2.11 2.50 4.26	31.49 32.38 72.48	180.64 180.90
2-Hydroxyisovaleric acid	0.89 0.98 2.08 4.11	18.43 20.72 34.16 78.02	180.60
3-Hydroxyisovaleric acid	1.33 2.55	30.86 49.89	72.55 178.58
2-Hydroxyphenylacetic acid	3.69 6.91–6.99 7.20–7.29	38.41 118.28 123.36 131.80 134.23	124.20 156.85 179.68
4-Hydroxyphenylacetic acid	3.65 6.86 7.18	42.38 118.34 133.50	128.79 157.35 180.07
4-Hydroxyphenyllactic acid	2.90 3.07 4.46 6.85 7.17	41.45 74.22 118.12 133.48	131.34 157.04 180.12

Isoleucine	0.89 0.99 1.26 1.87 3.74	13.69 16.01 28.08 38.28 60.96	176.29
Isovaleric acid	0.92 2.02 2.23	24.21 28.08 45.93	181.54
Isovalerylglycine	0.92 1.97 2.16 3.94	24.19 28.80 44.00 47.35	176.65 179.73
2-Ketoisocapronic acid	0.91 2.09 2.60	24.47 26.81 50.79	173.27 209.71
Lactic acid	1.40 4.33	22.11 69.28	181.47
Leucine	0.95 1.72 1.75 3.84	24.60 26.77 42.24 55.57	177.36
Lysine	1.49 1.72 1.89 3.04 3.83	24.08 29.17 32.51 41.99 56.95	176.75
Malonic acid	3.46	43.75	174.90
Mannitol	3.62–3.89	66.01 72.04 73.61	
Methylmalonic acid	1.36 3.47	16.05 49.12	178.07
Myoinositol	3.26 3.49 3.57 4.05	73.93 74.99 75.22 77.15	
Ornithine	1.67 1.87 3.04 3.84	25.53 30.07 41.80 56.65	176.37
Orotic acid	6.22	104.52	149.29 160.90 164.78 187.05
5-Oxoproline	2.16 2.42 2.55 4.36	27.58 32.27 59.50	180.26 184.88
5-Oxotetrahydrofurane-2-carboxylic acid (lactone of 2-hydroxyglutaric acid)	2.23 2.63 4.97	28.61 30.45 81.67	179.40 184.14
Phenylalanine	3.18 3.28 4.16 7.34 7.40 7.44	38.89 58.31 130.48 131.83 132.07	137.47 175.95
3-Phenyllactic acid	2.97 3.15 4.51 7.26–7.43	42.32 74.17 129.63 131.30 132.15	139.59 180.21
Proline	2.03 2.35 3.38 4.16	26.39 31.54 48.95 63.69	176.85

Propionic acid	1.08 2.33	11.12 29.99	182.74
Sarcosine	2.74 3.65	35.48 53.34	173.77
Serine	3.84 3.98	59.20 62.96	175.00
Succinic acid	2.66	31.61	179.93
Thymine	1.86 7.35	13.99 112.77 141.89	155.98 170.38
Trimethylamine	2.90	47.47	
Trimethylamine *N*-oxide	3.53	60.58	
Tyrosine[2]	3.15 3.28 6.90 7.20	37.54 57.07 118.70 133.55	128.35 157.92 174.37
Urea			165.60
3-Ureidoisobutyric acid	1.12 2.67 3.27	16.59 42.84 45.26	164.10 182.00
3-Ureidopropionic acid	2.57 3.37	37.14 38.51	164.09 179.38
Valine	1.00 1.04 2.29 3.75	19.37 20.45 31.81 62.51	176.09

Similar spectroscopic data as well as data for many other compounds can be found, e.g., in references (Beckmann, 1995; Lindon *et al.*, 1999). [1] Measured for mixture of both α- and β-glucose, [2] pH = 1.0.

remind that a 5-fold increase of the solute concentration results in a 25-fold reduction of measurement time for the same s/n ratio. The basis for the analysis was a comparison of the ^{13}C spectrum of the investigated sample and the spectroscopic data coming from the reference spectra of standards, recorded in exactly the same conditions (Table 1). As the limiting criterion of the presence of a given metabolite in the analysed urine we assumed the presence of the signals of all the metabolite proton-bearing carbons in the examined spectrum. The peak positions (chemical shifts) in the spectra to be compared were expected to agree within 0.1 ppm. Also a qualitative agreement of the relative signal intensities in these spectra was controlled every time.

In the test investigations reported below, normal and abnormal levels of various metabolites were identified in urine samples from 33 patients suffering from 10 different inherited metabolic diseases. Thus, samples from 10 patients with disorders of tyrosine catabolism were analysed: seven with alcaptonuria and three with tyrosinemia type I. Also samples from patients with five other organic acidurias were examined: glutaric aciduria type I, D- or L-2-hydroxyglutaric aciduria, isovaleric aciduria, lactic aciduria and orotic aciduria. Finally, three disorders that result in abnormal urine amino acid excretion were investigated, namely: Canavan disease, 5-oxoprolinuria, and phenylketonuria. In the samples tested only the signals of abundant metabolites were interpreted and the presence of markers expected on the basis of the initial diagnosis was checked. All the results are summarised in Table 2, and the details are commented upon below. Additionally, normal urine concentrations of the compounds considered, taken from the literature (Sweetman, 1991; Blau *et al.*, 1996), are given in Table 2.

Alcaptonuria

The ^{13}C NMR spectra of all urine samples from patients with alcaptonuria revealed the presence of very large concentrations of homogentisic acid (7 to 18 mol/mol creatinine), the marker metabolite of this disease (Markus *et al.*, 2001; Held *et al.*, 2006). Urine samples from various patients showed a variable pattern of metabolites. Creatinine peaks were not always visible in our ^{13}C NMR spectra because of low concentrations of this metabolite. This situation was uncomfortable since creatinine is usually used as a concentration reference when analysing urine. In consequence, only in two samples was the quantitative information obtained by ^{13}C NMR and in three samples by ^{1}H NMR. In most of the samples large amounts of citric acid were visible. Other metabolites, namely creatine, glycine, betaine

Table 2. Results of analysis of urine samples from patients with metabolic diseases by ^{13}C NMR spectroscopy

Metabolic disease	Number of urine samples	Metabolite	Detected urine levels (mol/mol creatinine)	Normal urine levels (mmol/mol creatinine)
Alcaptonuria	7	Homogentisic acid	7–18	<2
Tyrosinemia type I[1]	3	Tyrosine	0.5–1.5	2–55
		4-Hydroxyphenylacetic acid	0.6	6–28
		4-Hydroxyphenyllactic acid	0.7	<2
Glutaric aciduria type I	2	Glutaric acid	1.5	<2
2-Hydroxyglutaric aciduria	2	2-Hydroxyglutaric acid	2–3	1–20
Isovaleric aciduria	4	3-Hydroxyisovaleric acid	5	0–46
		Isovalerylglycine	2–11	0–10
Lactic aciduria	2	Lactic acid	18	0–25
Orotic aciduria	3	Orotic acid	0.1–1	0–11
Canavan disease	4	*N*-Acetylaspartic acid	1–3	<2
5-Oxoprolinuria	2	5-Oxoproline	14	42–115
Phenylketonuria	4	Phenylalanine	0.2–0.8	4–32
		3-Phenyllactic acid	0.6–3	<2
		2-Hydroxyphenylacetic acid	<0.2	<2

[1]pH = 1.0

and trimethylamine *N*-oxide, were also detected in some of the samples.

Tyrosinemia type I

In general, urine from patients with tyrosinemia type I has complex composition as a result of liver and kidney dysfunctions. Numerous characteristic metabolites, such as tyrosine, 4-hydroxyphenylpyruvic acid, 4-hydroxyphenyllactic acid, and 4-hydroxyphenylacetic acid, succinylacetone and succinylacetoacetic acid accumulate in blood and urine (van Dyk & Pretorius, 2005), but usually succinylacetone is used as the diagnostic marker of this disease (Tanguay *et al.*, 1996; Held *et al.*, 2006). Because in the case of tyrosinemia type I the urine level of succinylacetone ranges from 15 to 520 mmol/mol creatinine (Al-Dirbashi *et al.*, 2006), detection of this metabolite by ^{13}C NMR can be difficult, if at all possible. In all three investigated cases the presence and concentration of tyrosine was determined. Additionally, in the analysed samples elevated amounts of secondary markers: 4-hydroxyphenyllactic and 4-hydroxyphenylacetic acids were determined, as well as of some non-specific compounds: alanine, citric acid, α-glucose, β-glucose, glutamic acid, or lactic acid.

Glutaric aciduria type I

According to literature sources, glutaric aciduria type I is characterized by accumulation of glutaric acid and 3-hydroxyglutaric acid in urine (Iles *et al.*, 1985; Basinger *et al.*, 2006). In this study we analysed two samples of patients with glutaric aciduria type I. In both cases glutaric acid was detected and its level relative to creatinine was determined using ^{13}C NMR. On the other hand, no signals from the second marker were detected, either by ^{13}C NMR or by ^{1}H NMR.

2-Hydroxyglutaric acidurias

Two different disorders, L-2-hydroxyglutaric aciduria and D-2-hydroxyglutaric aciduria connected with the excretion of the L and D enantiomers of 2-hydroxyglutaric acid are known (Duran *et al.*, 1980; Chalmers *et al.*, 1980). We examined samples of urine of two patients with one of these acidurias. In the first sample signals of 2-hydroxyglutaric acid were found in both ^{13}C and ^{1}H NMR spectra. In the second case, however, only the ^{1}H NMR spectrum contained well recognisable signals of the expected metabolite. The failure of ^{13}C NMR analysis in this case was caused by a low concentration of the marker in the investigated sample.

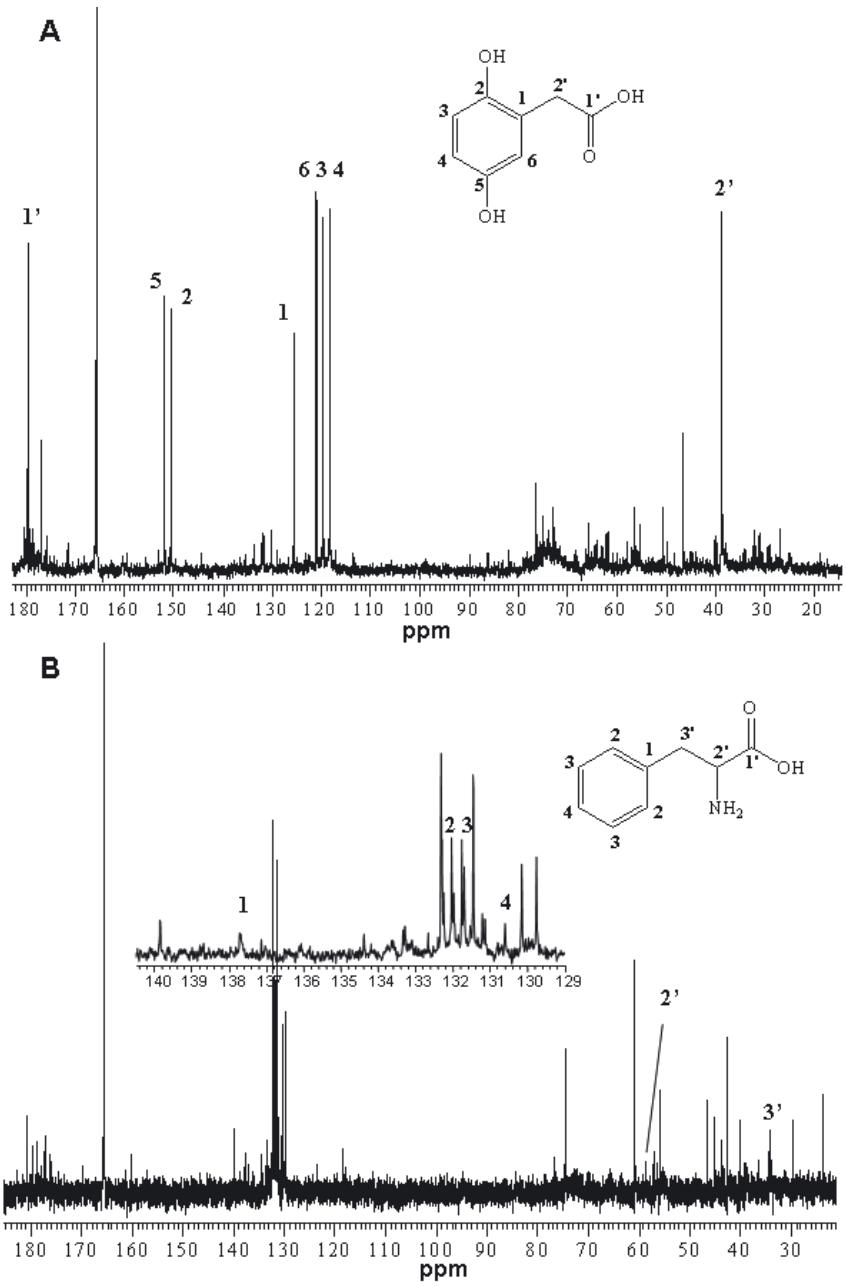

Figure 1. ^{13}C NMR spectra of urine with different amounts of marker metabolites.
A. Alcaptonuria, high concentration of homogentisic acid. B. Phenylketonuria, very low concentration of phenylalanine — too low to determine all ^{13}C signals.

Because the enantiomeric structures are in general indistinguishable by NMR, the method tested in this work is by definition not able to differentiate between the two acidurias.

Isovaleric aciduria

The ^{13}C NMR spectra of all the investigated urine samples from patients with this disorder revealed the presence of large concentrations of isovalerylglycine, which is a marker of this inborn error (Tanaka & Isselbacher, 1967; Dionisi-Vici *et al.*, 2006). In one of the investigated samples the presence of 3-hydroxyisovaleric acid was also observed.

Lactic aciduria

In both investigated samples elevated lactic acid (Robinson, 2006) was evident, but the low concentration of creatinine made the determination of the marker metabolite concentration by ^{13}C NMR impossible. In one of the samples remarkable amounts of mannitol and β-glucose were additionally detected.

Orotic aciduria

Pathognomonic concentrations of orotic acid, the main marker metabolite in this disease (Loffler *et al.*, 2005), happen to be low in urine (Brusilow, 1991). So we were not surprised that when using our standard procedure, the ^{13}C NMR signals of orotic acid were visible only in one of the three investigated samples from patients suffering from orotic aciduria. In two other samples concentrations as low as 0.3 mol/mol creatinine and 0.1 mol/mol creatinine were determined by ^{1}H NMR. In the latter case the presence of orotic acid was confirmed also by ^{1}H-^{13}C HSQC spectrum (see Discussion). The dominant signals in the investigated samples were assigned to acetic acid, lactic acid and 3-hydroxybutyric acid, which, however, were not diagnostic metabolites.

Canavan disease

In three of the four investigated urine samples from independently diagnosed patients suffering from Canavan disease (Burlina *et al.*, 1999) the primary marker metabolite, *N*-acetylaspartic acid, was determined by ^{13}C NMR using our standard procedure. The failure in the fourth case was caused by a remarkable sample dilution (no creatinine ^{13}C signals) and simultaneously a low relative marker concentration which amounted to 1 mol/mol creatinine as was found by ^{1}H NMR.

5-Oxoprolinuria

The dominant and characteristic peaks in the spectra of 5-oxoprolinuria (Mayatepek, 1999) samples originated from 5-oxoproline. The presence of this metabolite was evident in both investigated urine samples, but in one of them a quantitative analysis was not possible. The reason was that in the ^{1}H NMR spectrum the signals overlapped extensively and simultaneously the concentration of creatinine was too low for a ^{13}C NMR-based analysis. Again, the problem could probably be overcome by using a higher-field spectrometer.

Phenylketonuria

As expected, abnormally high concentrations of phenylalanine, 3-phenyllactic acid and 2-hydroxyphenylacetic acid were determined in urine originating from most of the patients with phenylketonuria (Constantinou *et al.*, 2005) using ^{13}C NMR. It was interesting that in one of the samples phenylalanine signals could not be recognised in the ^{1}H NMR spectrum due to an extensive signal overlap. In the urine sample of another phenylketonuria patient only one of the characteristic metabolites, 3-phenyllactic acid, was detected using both ^{13}C NMR and ^{1}H NMR.

DISCUSSION

The advantage of the presented method is its simplicity. Sample preparation is easy because no derivatisation or extraction is required. Moreover, ^{13}C NMR spectra of urine samples are usually much more transparent than ^{1}H NMR spectra, as it is illustrated in Fig. 1, especially when the marker concentration is high. ^{1}H NMR spectra are sometimes difficult to interpret. In samples of complex composition the overlapping of ^{1}H NMR multiplets may prevent signal assignments, whereas overlapping in ^{1}H decoupled ^{13}C NMR spectra takes place only occasionally. Indeed, the absence of splittings due to scalar couplings and the wider chemical shift range in ^{13}C NMR diminish the probability of an accidental signal overlap. Additionally, the water signal suppression before recording carbon NMR spectra is no longer required.

On the other hand, there is a limitation to this technique associated with the low natural abundance and low gyromagnetic ratio of ^{13}C nuclei, which results in poorer sensitivity than that of ^{1}H NMR (Beckmann, 1995; Decking, 1998; Krawczyk *et al.*, 2001). In consequence, when using a 9.4 T spectrometer and fivefold concentrated urine samples the marker concentration in urine should be at least of the order of 0.5 mol/mol creatinine, if the measurement is expected to last about 2 h. For most of the interesting metabolites in non-concentrated urine samples the recording of individual ^{13}C NMR spectrum usually has to last 10 h or more. This problem could be substantially diminished by using spectrometers operating at higher magnetic fields, equipped with a cryogenic probe (Keun *et al.*, 2002) or allowing measurements of samples of higher volumes and exploiting indirect detection of carbon spectra using two-dimensional correlated spectroscopy (Moolenaar *et al.*, 2001; Gerhard *et al.*, 2003).

To illustrate the effectiveness of such an approach we investigated once again the sample containing orotic acid at about 0.1 mol/mol creatinine, which could not be determined by the standard method used in this work. Application of 2-dimensional ^{1}H-^{13}C HSQC technique and a 16.3 T spectrometer yielded a good result within 30 min. It is noteworthy that correlated ^{1}H-^{13}C 2D spectroscopy highly increases the credibility of the analysis and is strongly recommended especially when the proton spectrum of a crucial metabolite contains very few peaks. In general, we suggest the use of ^{13}C NMR spectroscopy especially in cases when ^{1}H NMR gave doubtful results or no diagnostic information was

achieved. Such a situation was observed in the sample from a patient with phenylketonuria, in which the huge signal overlap in ^{1}H spectra prevented the determination of phenylalanine. The ^{13}C spectrum did evidence the presence of the marker metabolite in this case.

The next aspect to be considered is quantitative analysis. It is to be stressed that interpretation of signal intensities in standard ^{13}C NMR spectra is difficult and has to be done with great caution because of non-uniform saturation and different nuclear Overhauser enhancements of particular signals when standard measurement conditions are used. It is especially true for quaternary carbons possessing long relaxation times. For such nuclei reduced signal intensities are observed. Quantitative ^{13}C NMR measurements in which signal intensities are exactly proportional to solute concentrations are essentially feasible, although completely impractical in the case of samples of interest in this work. On the other hand, it is to be realized that even very crude estimations of metabolite concentrations relative to a reference compound, normally creatinine, are usually quite satisfactory. This is because normal and pathologic concentrations of marker metabolites usually differ very much, often by orders of magnitudes. Thus, the information contained in a standard ^{13}C NMR spectrum can be sufficient for semi-quantitative analyses. Actually, a rough proportionality between ^{13}C NMR signals and solute concentration might be expected in our samples since molecules of most of the metabolites of interest are of similar size and are flexible. Indeed, in this work it was shown that if only signals of proton-bearing carbons are considered, some rough estimation of the relative metabolite concentrations can be achieved. Usually peaks of both protonated carbons of creatinine (CH_3 and CH_2 at 33.62 ppm and 56.85 ppm, respectively) and frequently even more peaks of the metabolite of interest are present in the spectrum. This fact allows elimination of some accidental (but not systematic) errors of the relative concentration determination. In the present study these estimations were checked whenever possible by integration of the appropriate signals in ^{1}H NMR spectra (the method is accurate within 15% in the case of resolvable signals). Without any exception the divergence between the ^{13}C NMR- and ^{1}H NMR-based estimations was smaller than 50%. Thus, keeping in mind the purpose of urine analysis, it may be concluded that ^{13}C NMR spectroscopy can be applied for rough concentration evaluation provided that the signals of protonated carbons are used. Obviously, for semi-quantitative urine analysis not only the marker metabolite but also the reference compound have to be present in the sample in sufficient concentrations, which was not always the case in the investigated samples when using the 9.4 T spectrometer.

In this work creatinine was used as the reference compound and the metabolite levels were reported as relative concentrations in mole per mole of creatinine. It is the most widely used way of expressing metabolite levels in medical urine analysis. Creatinine is excreted by humans in urine in approximately constant amounts proportional to the total muscle mass. That is why creatinine is commonly used as the reference. Metabolite levels in urine are usually reported as relative concentrations in weight (or moles) of an analyte per gram (or mole) of creatinine, sometimes in such peculiar units as micrograms of the analyte per milligrams of creatinine. Absolute concentrations are considered to be less useful as being dependent on urine dilution. Unfortunately, some relatively small variations of creatinine concentrations are observed related to age and sex. Moreover, kidney malfunctions and some other disease states result in abnormal urinary creatinine levels. Nevertheless, no better reference substance has been proposed until now.

Finally, it may be concluded that urine analysis performed by ^{13}C NMR spectroscopy is potentially a useful method for diagnostics of inborn errors of metabolism and can be exploited either separately or as an auxiliary technique together with ^{1}H NMR. All the comparisons of the results obtained by the proposed method with those obtained by ^{1}H NMR have shown full agreement between the outcomes of these two techniques. The method is simple, although limitations tightly connected with the class of the NMR equipment in use have to be realised. No preliminary extraction or derivatisation of samples is needed. In most cases semiquantitative estimation of the relative metabolite concentration can also be achieved.

The results reported illustrate that ^{13}C NMR of fivefold concentrated urine samples can be used for direct determination of the presence and concentration of their major components. Thus the technique is suitable for detection of marker metabolites and can deliver information crucial for diagnosis of numerous metabolic diseases.

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