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^{13}C NMR spectroscopy: a convenient tool for detection of argininosuccinic aciduria

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Abstract

Proton decoupled high resolution ^{13}C NMR spectra of argininosuccinic acid have been measured in a series of dilute water solutions of various acidity. These data have provided a basis for unequivocal determination of the presence of this metabolite in the investigated sample. The method additionally enables simultaneous rough estimation of the metabolite concentration. In order to check the practicability of the usage of this spectroscopy for diagnostic purposes, the spectra of several unprocessed urine samples have been recorded including three from patients with argininosuccinic aciduria. It has been concluded that ^{13}C NMR spectroscopy can be a convenient method of recognising the above syndrome and probably many other inborn metabolic errors which manifest themselves with the excretion of the marker metabolite in amounts comparable to (or larger than) creatinine.

Keywords: ^{13}C NMR; Argininosuccinic aciduria

1. Introduction

Excretion of argininosuccinic acid (ASA) in urine and plasma is a reliable and commonly accepted biochemical marker of argininosuccinic acid lyase deficiency (ASLD) (MIM207900), an inborn error of the urea cycle. Unequivocal detection of ASA in urine or blood plasma by the conventional chromatographic methods of amino acid analysis causes some analytical problems because of coelution with other compounds like leucine, isoleucine or methionine [1–6]. In order to obviate those problems the specific chemical pretreatment of the investigated sample was proposed transforming ASA into its anhydrides [5] or the *o*-phthalaldehyde derivative [6]. These additional operations necessarily add to laboriousness, cost and time for analysis. Another method based on positive-ion thermospray liquid chromatography–mass spectrometry [7] demands costly highly specialized equipment, which is still not com-

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monly accessible in clinical chemistry laboratories. At the same time, the high resolution nuclear magnetic resonance spectroscopy of hydrogen nuclei is more and more frequently used in diagnostics of inborn metabolic diseases [8–20]. Some years ago, Burns et al. proposed using ^{1}H NMR spectroscopy for ASA detection [13,14]. Recently Lindon et al. [17,18] and Wevers [20] have reported a long list of metabolic diseases (including ASLD) which can be diagnosed on the basis of ^{1}H NMR spectra of body fluids. Among chemists ^{1}H and ^{13}C NMR spectroscopy have for a long time been exceptionally popular tools for the structural elucidation of organic compounds as well as for qualitative and quantitative analyses of liquid samples of various complexity of chemical composition. NMR spectrometers are presently standard equipment in the organic chemistry laboratories, yet not so popular in medicine chemistry. The main historical reason for this situation is the fact that biological mixtures such as body fluids are of an extreme chemical complexity and the concentrations of their important components are relatively low. As a result they are especially demanding samples as far as NMR instrumentation is concerned. During recent decades, however, the application of higher and higher magnetic fields and new technologies has enhanced the resolution and sensitivity of NMR spectrometers and the method has become suitable also for the detection of metabolites in body fluids. The experimental procedures recommended in the case of investigating biological samples by NMR spectroscopic method, as well as its advantages and limitations, have been discussed in detail in numerous review articles [15–19,21,22].

In practical applications of ^{1}H NMR, some problems may arise due to frequently encountered rich patterns caused by spin–spin couplings, and because the spectra are spread over a relatively narrow frequency range. In the case of biological samples, numerous weak signals may overlap one another and the spectrum is frequently affected by so-called 'chemical noise' [17,18] in addition to the normal electronic noise. As a result the unambiguous recognition of the complex signal patterns, such as that of ASA, in the ^{1}H NMR spectrum of unprocessed urine can sometimes be troublesome due to the presence of numerous other metabolites.

Chemical laboratory practice shows that such problems can frequently be overcome when the proton decoupled ^{13}C NMR instead of ^{1}H NMR spectra are used. The former type of spectra are composed of the singlet line signals for each stereochemically nonequivalent carbon nucleus rather than of multiplets. Moreover, ^{13}C NMR signals are spread over a ca. five times wider frequency range than ^{1}H NMR signals. Simultaneously, much lower intrinsic sensitivity of ^{13}C NMR spectroscopy remarkably reduces the number of metabolites observable in the spectrum of a given sample. This largely simplified profile still remains informative enough to prove the presence or absence of a given metabolite. ^{13}C NMR spectroscopy has not become very popular in medicinal chemistry applications yet its effectiveness in solving biomedical problems has been evidenced many times and a number of articles pointing out the potential usefulness of carbon NMR spectroscopy in that area have been published [21–25].

In this work, the practicability of using ^{13}C NMR spectroscopy for the direct detection of argininosuccinic aciduria in ASLD-affected patients was proved and chemical shift data necessary for application of this method were collected.

2. Materials and methods

Measurements were performed for samples of unprocessed urine or 0.15 M water solution of ASA disodium salt (Sigma). The 0.5 ml of urine or the ASA solution was transferred to a 5-mm o.d. high precision NMR tube. Then, 50 μl D_2O and 10 μl dioxane were added as the spectrometer lock and chemical shift reference ($\delta(^{13}C)$, 67.19 ppm [26]). The sample pH was controlled directly in the NMR tube using a pH electrode and pH-meter (Cole-Parmer Instrument Co.). This measuring system was standardised using pH 4, pH 7, pH 10 (Cole-Parmer Instrument Co.) buffers. The pH of the reference ASA solution was adjusted by adding small amounts of 1 M HCl or 0.55 M NaOH H_2O solutions.

The proton decoupled ^{13}C NMR spectra were recorded using a Varian ULTRAplus 500 spectrometer operating at 11.7 T magnetic field. The standard measurement parameter set: pulse width, 7 μs (PW90, 12.5 μs), acquisition time 1 s, spectral window 200 ppm, and WALTZ 16 decoupling mode were applied. In total, 4000–8000 scans were accumulated and after zero-filling to 64 K FID signals were subjected to Fourier transformation using 3 Hz line broadening. Similar measurement and processing conditions were applied when using a GEMINI 2000 spectrometer operating at 4.7 T. The only essential difference was in the accumulation time which in this case was as long as 16 h.

The measurement temperature was 30°C, except for the measurements aimed at checking the influence of temperature on the signal positions.

Three urine samples from affected patients and a number of control samples were examined. The first was from patient PK from Utrecht, who did not have neonatal hyperammonemia, but was referred for non-specific mental retardation at the age of four. Two other urine samples, MD and PM, came from two Polish infants for which ASA in the urine sample was independently detected by ^{1}H NMR and TLC amino acid analysis.

3. Results and discussion

The proton decoupled ^{13}C NMR spectrum of ASA in the water solution of pH 6.45 is shown in Fig. 1. The signal at 67.19 ppm originated from dioxane being the chemical shift reference. The signals lying upfield (20–60 ppm) were from methylene and methine carbons of ASA, while those lying downfield (150–180 ppm) were due to guanidine and carbonyl carbons. The latter signals were of the characteristic low intensities because of the long relaxation times and reduced nuclear Overhauser enhancement factors. The detailed assignment of all the ^{13}C NMR signals of ASA in water solution was established previously [23] on the basis of two-dimensional NMR correlation techniques with the abbreviations COSY ($^{1}H-^{1}H$ correlation spectroscopy), HSQC (proton detected $^{1}H-^{13}C$ heteronuclear single quantum correlation spectroscopy) and HMBC (proton detected $^{1}H-^{13}C$ heteronuclear multi-bond correlation spectroscopy) [27].

It is well known that some proton, as well as carbon, chemical shifts happen to be solvent-, temperature- and pH-dependent. Such effects, though usually small, can sometimes cause assignment problems, especially in the case of rich spectra of biological samples. In view of diagnostic

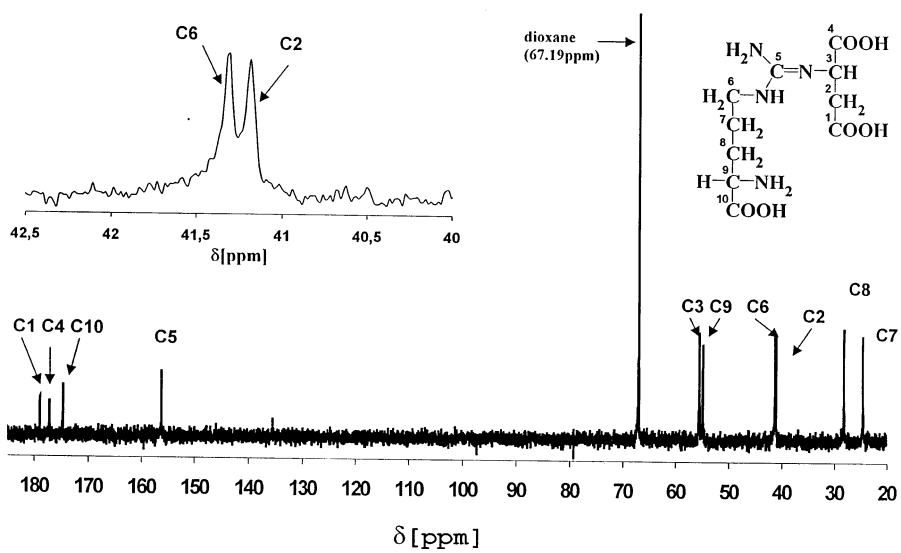

Fig. 1. ^{13}C NMR spectrum of argininosuccinic acid (ASA) in water solution of pH 6.45.

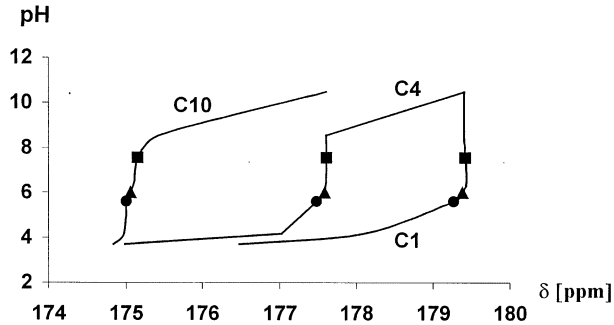

Fig. 2. pH dependence of ^{13}C chemical shifts, δ [ppm], for carbonyl carbons of argininosuccinic acid in water solutions. The symbols ■, ▲, ●, denote data for urine samples from patients PK, MD, PM, respectively.

applications, the pH dependence of the ^{13}C chemical shift of ASA has been investigated in more detail and the results are given in Table 1. The sensitivity of particular carbons in argininosuccinic acid to pH variation were represented by the chemical shift difference ($\Delta\delta$) between the solutions of pH 3.7 and 9.2. These ranges are given in the last row of Table 1. The observed sensitivity order was as follows:

$$\Delta\delta_{C\text{-}4} > \Delta\delta_{C\text{-}1} > \Delta\delta_{C\text{-}10} > \Delta\delta_{C\text{-}2} > \Delta\delta_{C\text{-}8},\ \Delta\delta_{C\text{-}3} > \Delta\delta_{C\text{-}9} > \Delta\delta_{C\text{-}7},\ \Delta\delta_{C\text{-}6},\ \Delta\delta_{C\text{-}5}$$

It is noteworthy that the chemical shift variation for carbonyl carbons C-4, C-1 and C-10 was remarkably larger than that observed for the remaining ones. It was obvious that this enhanced sensitivity was related to the association–dissociation phenomena of their carboxylic protons. Indeed, the chemical shift versus solution pH relationship for the carboxylic group carbons had a form of the typical titration curve (Fig. 2). All the above results indicated that the acidity of the sample had to be taken into account when searching for ASA.

With the purpose of excluding any unusual temperature influences that might affect the ^{13}C chemical shifts of ASA in water solution, the additional spectra were measured at temperatures of 25 and 35°C for the solution of pH 6.45. It was found that the chemical shift changes caused by a 5°C deviation from the standard temperature were not larger than 0.04 ppm, so they were meaningless from the analytical point of view.

^{13}C NMR spectra were measured for a number of control urine samples and for urine samples of three patients with argininosuccinic aciduria. The signals of ASA in all three latter cases dominated those of any other urine component and were very easily recognisable. The spectrum of the urine sample of PK (pH 7.55) is shown in Fig. 3. Keeping in mind the pH dependence of the spectrum of ASA, the chemical shifts were in perfect agreement with the data collected in Table 1. This agreement is illustrated in Fig. 2 for the signals of C-1, C-4 and C-10. The additional signals with low intensities present in this spectrum originated

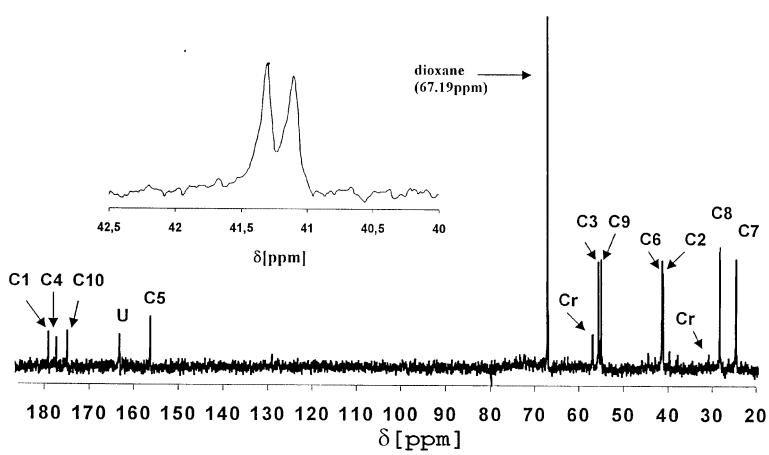

Fig. 3. ^{13}C NMR spectrum of urine sample of PK (pH 7.55). C-1 to C-10 – ASA; Cr, creatinine; U, urea.

Table 1
pH dependence of ^{13}C chemical shifts, δ [ppm], of ASA in water solution (No. 1–6) and of urine of patients (No. 7–9)

No.	pH	^{13}C chemical shifts δ [ppm] of carbon number									
		1	2	3	4	5	6	7	8	9	10
1	3.70	176.48	39.88	55.12	174.99	156.55	41.44	24.62	28.29	55.02	174.84
2	4.17	178.09	40.03	55.11	177.02	156.55	41.44	24.62	28.30	55.11	174.98
3	5.85	179.36	41.41	55.80	177.56	156.61	41.41	24.63	28.32	55.13	175.02
4	6.45	179.43	41.28	55.77	177.60	156.61	41.41	24.62	28.33	55.12	175.11
5	8.55	179.41	41.31	55.81	177.60	156.62	41.44	24.65	28.52	55.20	175.43
6	9.20	179.41	41.31	55.80	179.41	156.61	41.50	24.75	29.10	55.35	177.60
7	Urine of patient PM 5.60	179.27	41.20	55.74	177.48	156.60	41.41	24.62	28.31	55.12	175.01
8	Urine of patient MD 6.00	179.38	41.26	55.75	177.58	156.61	41.41	24.62	28.32	55.12	175.06
9	Urine of patient PK 7.55	179.42	41.27	55.74	177.61	156.58	41.43	24.63	28.36	55.13	175.16
10	$\Delta\delta$	2.95	1.53	0.70	4.42	0.07	0.09	0.13	0.81	0.33	2.76

from other metabolites (e.g. urea, 163.40 ppm; creatinine, 30.83 and 57.04 ppm). The spectra of the urine samples from the other two patients were very similar. On the other hand, none of the control samples exhibited the set of signals characteristic for ASA.

When using modern spectrometers, the precision of measurements of ^{13}C NMR signal positions is determined by the digital resolution, signal-to-noise ratio and spectral linewidth, which in routine spectra usually yields precision higher than 0.02 ppm. The reproducibility of the chemical shift measurements of nuclei for a given chemical species is in practice determined by the reproducibility of the measurement conditions and careful referencing of the frequency scale. Taking into account the spectroscopic properties of ASA as well as general features of ^{13}C NMR spectra of similar organic compounds one may propose a criterion on the basis of which the presence of ASA in the investigated sample can be recognised. In our opinion it should be documented by the presence of all six signals of protonated carbons of ASA in the spectrum in positions which deviate by less than $0.1 + \Delta$ ppm from those predicted from the data in Table 1, where Δ is the interpolation error. It is to be stressed that if the criterion is not fulfilled, one may only state that the concentration of the marker is lesser than the detection limit, which is in turn determined by the experimental s/n ratio.

In general, interpretation of the signal intensities in routine ^{13}C NMR spectra has to be done with caution. The quantitative ^{13}C NMR measurements, aimed at determining relative concentrations of solution components, though feasible, are impractical for dilute samples. Nevertheless, a semiquantitative information can be gained provided that the relaxation times and NOE enhancement factors are not much varied for the carbon signals being compared. In fact, in our case these conditions seem to be fulfilled, at least when comparing signals of protonated carbons. The validity of such an approach was checked by the quantitation based on the signal integration in ^{1}H NMR spectra, the procedure usually ensuring accuracy on the level of $\pm 10\%$. Obviously, the procedure based on signal integration can only be applied when the signals of interest are sufficiently separated from other signals occurring in the spectrum. One may expect that ^{1}H NMR spectra of urine samples do not always fulfil the above requirement, whereas such a complication is very unlikely in the case of ^{13}C NMR spectra. To sum up, carbon NMR spectra usually provide only a rough estimate of ASA concentration relative to the reference substance, most frequently creatinine. Fortunately, such information is frequently simultaneously sufficient for diagnostic purposes.

The striking feature of the spectrum shown in Fig. 3 as well as the spectra of urine from two other patients was that the intensities of the ^{13}C signals of ASA were many times higher than those of creatinine. It reflected a much higher concentration of the former metabolite in the investigated samples. The relative concentrations of the ASA marker in the urine samples of PK, MD and PM amounted to 3.6, 18 and 19, respectively, and were higher than those reported by Burns et al. (3.1–4.7 [13]). On the other hand, no signals of meaningful intensity attributable to ASA anhydrides were found in the investigated samples. Actually, on the basis of investigations by Burns and Iles [14], anhydride concentrations on the level of a few percent of ASA could be expected, which meant that no signals of those compounds should be visible in more or less standard ^{13}C spectra. The inspection of ^{1}H NMR spectra also confirmed the conclusion about the absence or low level of ASA anhydrides in the investigated samples.

In our opinion, ^{13}C NMR and complementary ^{1}H NMR spectroscopy are potentially powerful and convenient tools in diagnosing argininosuccinuria. It is, however, to be remembered that the time required for the analysis depends critically on the concentration of the metabolite being determined. This study indicated that a sufficient quality ^{13}C NMR spectrum of the urine sample from an ASLD affected patient was achievable using even a simple high resolution spectrometer operating at 4.7 T magnetic field. In such a case the analysis required overnight measurement. Using an instrument working at B0, 11.7 T, even better results were obtained within a couple of hours. Further significant measurement time re-

duction could be gained using ^{13}C dedicated NMR probes adapted to larger sample volumes and/or by applying the indirect detection scheme, e.g. two-dimensional HSQC spectroscopy [27]. The latter method restricts information to protonated carbons only and, in practice, lowers resolution, which, however, is overcompensated by the information concerning correlation of ^{1}H and ^{13}C chemical shifts. Finally, in difficult cases, one may consider concentrating the sample as a remedy, resigning from the important advantage of the NMR spectroscopic approach which usually allows avoiding all the laborious pre-processing procedures. An additional effort might pay back, as the n-fold increase of the solute concentration allows for an $n/2$-fold reduction of the measurement time necessary for gaining a given s/n ratio in the spectrum.

It was mentioned that ^{1}H NMR spectroscopy has proved many times its exceptional usefulness in diagnostics of various inborn genetic errors. The only weakness of this method is the complexity of the obtained profiles which sometimes makes their analysis difficult. Even the computer assisted analysis using the pattern recognition algorithms encounters some problems due to variability of the urine composition caused by the differences in nutrition and by the excretion of drugs and other xenobiotics. From this point of view the ^{13}C NMR spectroscopy frequently offers the unique solution. The ^{13}C spectra of the urine samples, recorded within a reasonable time, are simple enough not to cause interpretation problems and simultaneously rich enough to provide a reliable fingerprint. The method based on ^{13}C NMR spectroscopy is equally suitable for diagnosing numerous inborn error diseases other than ASLD, which manifest themselves with the appearance of abnormal metabolites in the patient's urine. The obvious limitation of this method is its inherent sensitivity. It seems that nowadays commonly available instrumentation enables routine determinations when the characteristic marker molecules appear in urine in amounts comparable to or higher than creatinine. Actually, this method was exploited in the studies on galactosemia [24] and in the detection of the defect in dimethylglycine dehydrogenase [25]. Moreover, our preliminary results pointed out its usefulness in diagnostics of aspartylglycosaminuria, 5-oxoprolinuria, Canavan's disease and 2-hydroxyglutaric aciduria. One may thus expect that '^{13}C NMR of body fluids will be increasingly important both as a complement to in vivo measurements and as an auxiliary tool for the characterisation of metabolic diseases' [22].

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