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Short Communication

NMR of biofluids: detection of ^{2}H-acetate and ^{2}H-formate in urine as an indicator of microbiological contamination

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Introduction

^{1}H-NMR spectroscopy of biofluids and in particular urine, has been shown to be of value in characterizing endogenous biochemistry [1] and how it can be perturbed by disease [2], effects of drug therapy [3], surgery [4] and xenobiotic toxicity [5]. Recently, the classification of the complex spectra arising from urine and other biofluids has been aided by the use of pattern recognition methods [6] including the development of an automatic routine for generating descriptors directly from the spectra [7]. For this to work effectively, knowledge is required of contributions to the spectra of such factors as drug metabolites and contamination or degradation on storage.

NMR spectra are usually measured either after freeze-drying and reconstitution into D_2O or by adding an aliquot of D_2O for field-locking requirements. This note reports investigation of changes that may occur in the ^{1}H-NMR spectra of such urine samples after storage at room temperature and shows that certain endogenous metabolites can be increased in concentration at the expense of others. In addition it has been found that ^{2}H atoms can be incorporated into acetate and formate; it is suggested that the resulting NMR signals can be used as markers for the level of bacterial contamination. The presence of ^{2}H incorporation has been confirmed through the use of ^{2}H-NMR spectroscopy and this leads to the suggestion that ^{2}H-NMR spectra may also be useful for identifying metabolites of ^{2}H-labelled drugs as an alternative to the current practice of using radioactive tritiated materials for drug metabolism studies.

Experimental

Urine was collected over 5 h periods from control Wistar rats housed in metabolite cages and stored at −20°C until analysed. Aliquots (300 μl) of urine were placed in 4 mm o.d. NMR tubes and 100 μl of D_2O containing the partially deuterated reference compound sodium trimethylsilyl-(2,2,3,3-$^2H_4$)-propionate, TSP [1] was added to each tube. ^{1}H-NMR spectra were recorded using water suppression at 600.14 MHz using a Bruker AMX-600 instrument. Typical acquisition parameters were: 90° pulse, 128 K data points, spectrum width 18518 Hz, acquisition time 3.53 s, relaxation delay 3.0 s, 1024 transients. Chemical shifts were referenced to the internal TSP at δ0.000. ^{2}H-NMR spectra were measured at 92.13 MHz on the same spectrometer using the receiver coil of a dedicated ^{1}H probe which is also double-tuned to ^{2}H for the lock channel signal. The drift on the 14.1 T magnet is less than 1 Hz per hour for ^{1}H spectroscopy which corresponds to only about 0.15 Hz h^{-1} for ^{2}H-NMR and therefore the ^{2}H spectra were measured unlocked. Typical parameters were pulse width 60 μs (~45°), spectral width

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2008 Hz, 16 K data points zero-filled to 32 K prior to FT, 1024 transients. Resolution enhancement was by the Lorentzian–Gaussian method. The ^1H spectrum of the deuterated TSP contains resonances at δ0.74 and δ2.13 arising from residual protons at the otherwise fully deuterated propionate methylene groups. The ^2H spectrum shows resonances from the deuterated form of the molecule and ^2H chemical shifts have been referenced to the methylene resonance taken as δ2.13 which yields a consistent shift of the other methylene group of δ0.75.

Results

Figure 1(a) shows the 600 MHz ^1H-NMR spectrum of a typical control fresh urine. Many of the peaks have been assigned previously [1] and in total about 40 endogenous metabolites may be identified and quantified. Figure 1(b) shows the region of the spectrum between δ1.88 and 1.94, the largest signal at δ1.924 arising from acetate.

Figure 2(a) shows the ^1H-NMR spectrum of the same sample which had been allowed to remain at room temperature (21°C) for 48 h before measurement. Detailed examination of the spectrum reveals substantial increases relative to TSP in the levels of acetate (singlet at δ1.924), formate (singlet at δ8.461), β-hydroxybutyrate (doublet at δ1.188), lactate (doublet δ1.338) and a small singlet at δ2.900, possibly trimethylamine. There are decreases in the levels of succinate (singlet at δ2.409), citrate (AB pattern at δ2.552 and δ2.716) and fumarate (singlet at δ6.535). Figure 2(b) shows the region between δ1.88 and 1.94 and the increase in acetate is obvious. Also centred at δ1.910 is a 1:1:1 triplet arising from CH_2DCOO^-, the splitting of 2.21 Hz being due to the one-bond ^1H-^2H spin-coupling. The low frequency chemical shift of δ0.014 is consistent with the isotope effect of ^2H on ^1H shielding [8]. There is evidence of a further resonance at δ1.896 which, on resolution enhancement, consists of a 1:2:3:2:1 quintet indicating the presence of CHD_2COO^-, i.e. the incorporation of two ^2H atoms. The measured proportions of CH_3COO^- to CH_2DCOO^- to CHD_2COO^- are 22.5:4.7:1.0, compared to the expected 9:3:1 for random incorporation suggesting a preference for ^1H rather than ^2H incorporation from the D_2O–H_2O solvent. The ^1H-NMR spectrum could, of course, provide no evidence of ^2H incorporation into formate while it also gave no evidence of any incorporation of ^2H into any of the other endogenous species which increased on storage. In addition, as a control, a sample of acetate in phosphate buffer at pH 7.4 containing 25% v/v D_2O showed no deuterium exchange after 48 h at 21°C.

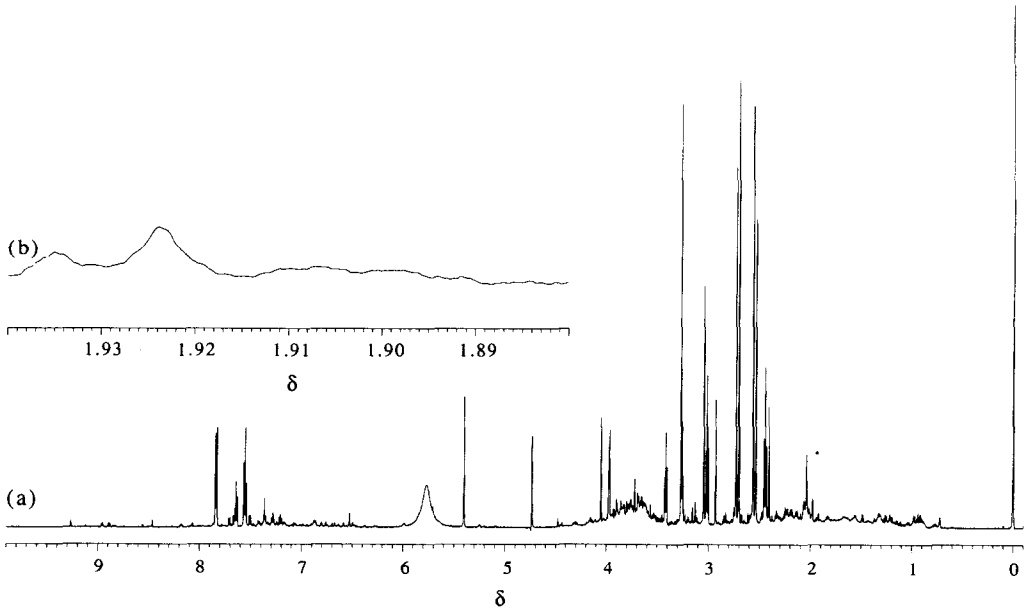

Figure 1
(a) 600 MHz ^1H-NMR spectrum of fresh rat urine with added D_2O. (b) Expansion of region δ1.88–1.94.

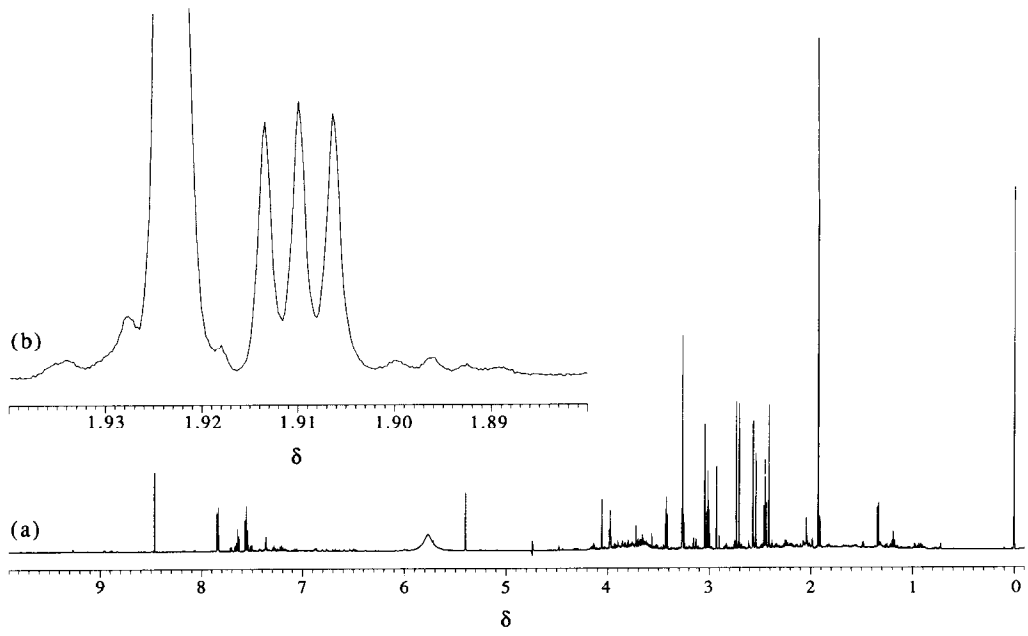

Figure 2
(a) 600 MHz ^{1}H-NMR spectrum of rat urine with added D_2O stored at 21°C for 48 h. (b) Expansion of region δ1.88–1.94.

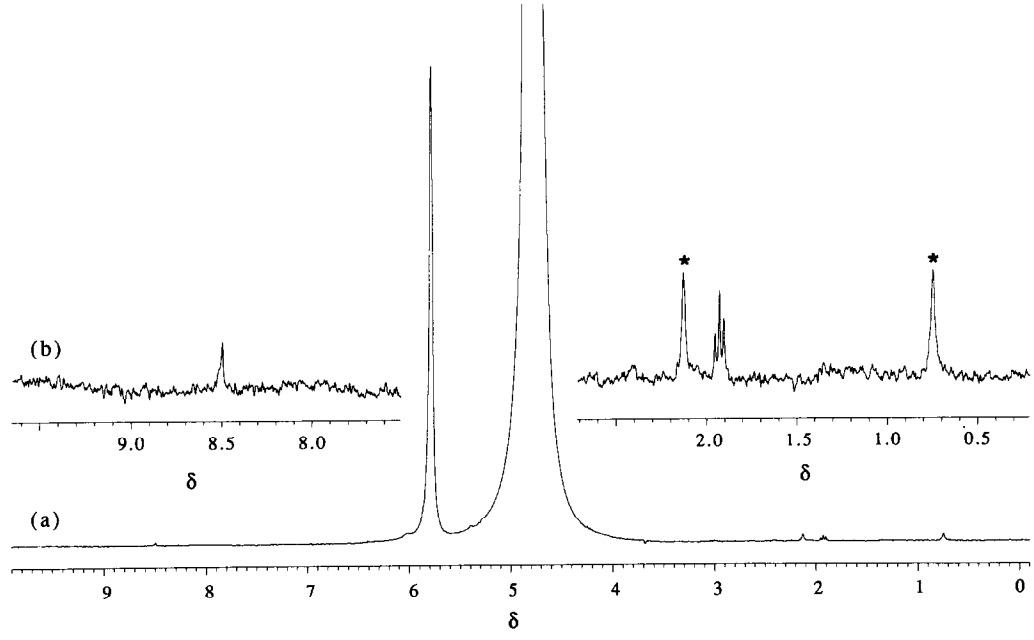

Figure 3
92 MHz ^{2}H-NMR spectrum of rat urine as used for Fig. 2. The resonance marked * are from the deuterated chemical shift reference substance TSP.

In order to visualize independently the level of ^{2}H incorporation into formate, as well as acetate, a ^{2}H-NMR spectrum of the same solution was measured. This is shown in Fig. 3, and clearly indicates the presence of ^{2}H-formate at δ8.50. Also the ^{2}H resonances of CH_2DCOO^- can be seen as the expected 1:2:1 triplet at δ1.93. The signal–noise ratio did not allow the detection of the less abundant CHD_2COO^- and CD_3COO^- species. The ^{2}H-NMR spectrum shows no evidence of incorporation of ^{2}H into lactate or β-hydroxy-

butyrate which also are present at increased levels in the sample. Obviously ^2H is incorporated into NH and OH groups of molecules found in rat urine, such as the NH_2 groups of urea, by a simple chemical rather than enzymatic mechanism and peaks from such relatively abundant molecules can also be observed. There was no evidence from either the ^1H- or ^2H-NMR spectra of chemical exchange of the aromatic protons of amino acids which could occur.

The incorporation of ^2H into acetate and formate can only be as a result of enzymic action and the presence of deuterated species is therefore indicative of a biochemical mechanism of incorporation which is most likely as a consequence of a bacterial presence in the urine. The enzymic increase in acetate, formate, lactate and β-hydroxybutyrate in this case is at the expense of citrate, succinate and fumarate, all Krebs' cycle intermediates, bearing in mind that the glucose level in the urine was low at the outset. On the other hand there was no change in the concentration of α-ketoglutarate, another Krebs' cycle molecule. It may be that different types of contamination may result in different profiles of metabolite change and hence the use of pattern recognition methods coupled with NMR [6] may allow a way of identifying the source of contamination.

As well as providing the ability to investigate the biochemical incorporation of ^2H into endogenous metabolites, high field ^2H-NMR spectroscopy opens up the possibility of a facile way of identifying drug metabolites and their excretion time-courses through the use of deuterated drug molecules and the measurement of ^2H-NMR spectra of untreated biofluids because of the lack of background signals and the avoidance of the radioactive samples normally used in ^3H-labelled drug studies.

References

[1] J.K. Nicholson and I.D. Wilson, *Prog. NMR Spectrosc.* **21**, 449–501 (1989).
[2] R.E. Iles, A.J. Hind and R.A. Chalmers, *Clin. Chem.* **31**, 1795–1801 (1985).
[3] J.R. Bales, J.D. Bell, J.K. Nicholson, P.J. Sadler, J.A. Timbrell, R.D. Hughes, P.N. Bennett and R. Williams, *Magn. Reson. Med.* **6**, 301–309 (1988).
[4] P.J.D. Foxall, G.J. Mellotte, M.R. Bending, J.C. Lindon and J.K. Nicholson, *Kidney Internat.*, in press.
[5] K.P.R. Gartland, F.W. Bonner and J.K. Nicholson, *Mol. Pharmacol.* **35**, 242–250 (1989).
[6] K.P.R. Gartland, C.R. Beddell, J.C. Lindon and J.K. Nicholson, *Mol. Pharmacol.* **39**, 629–642 (1991).
[7] R.D. Farrant, J.C. Lindon, E. Rahr and B.C. Sweatman, *J. Pharm. Biomed. Anal.* **10**, 141–144 (1992).
[8] P.E. Hansen, *Prog. NMR Spectrosc.* **20**, 207–255 (1988).

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