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Deuterium NMR spectroscopy of biofluids for the identification of drug metabolites: application to *N,N*-dimethylformamide

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Abstract: The metabolism of *N,N*-dimethylformamide in the Sprague–Dawley rat has been reinvestigated using NMR spectroscopy of urine. In particular, through the use of *N,N*-dimethylformamide-d_7 (DMF-d_7) and ^{2}H-NMR spectroscopy, the principal metabolites of this compound have been confirmed in a direct manner. The use of inverse-detected two-dimensional ^{2}H–^{13}C correlation with ^{13}C decoupling aided metabolite identification through the provision of ^{13}C chemical shifts.

Keywords: *^{2}H-NMR; metabolism; urine; DMF; ^{2}H–^{13}C correlation.*

Introduction

NMR spectroscopy of biofluids, in particular urine and bile, has for the last few years proved to be a useful adjunct to the traditional techniques for identifying drug metabolites. It has the advantages of being rapid and non-destructive and does not rely on the use of radioactive species [1]. In combination with simple solid-phase extraction techniques for concentrating metabolites, it has proved to be a general and versatile approach [2]. Recently, it has been suggested [3] in the light of observations of bacterial incorporation of deuterium into some endogenous substances in rat urine, that deuterium NMR of deuterated drugs might provide a facile method for identifying metabolites in view of the high sensitivity of high-field NMR spectrometers and the low natural abundance of deuterium, thereby minimizing background problems. In addition, ^{2}H-NMR lines are relatively sharp for small molecules, T_1s are short because of the quadrupolar nature of ^{2}H and the inherent sensitivity of ^{2}H-NMR is nearly as good as ^{13}C for equal numbers of nuclei. On the other hand, the chemical shift range for ^{2}H in frequency terms is rather small such that high magnetic fields are an advantage.

This approach has now been pursued in a preliminary manner through the use of the fully deuterated model drug *N,N*-dimethylformamide (DMF-d_7), which has been undergoing trials as a potential anti-cancer agent, and the range of information available using ^{2}H-NMR spectroscopy has been investigated. In a previous ^{1}H-NMR study of DMF metabolism [4], elevated levels of certain metabolites were noted. These included methylamine which is known to be an endogenous metabolite but also could be a possible DMF metabolite. Thus it was of particular interest to search for signals from deuterated methylamine which could only come from the exogenous source.

Experimental

A Sprague–Dawley rat weighing approximately 250 g was dosed intraperitoneally with a single administration of DMF-d_7 (Aldrich, Gillingham, Dorset, UK, 99.5 atom %^{2}H) at 1 g kg^{-1}. The animal was placed in a metabolism cage 24 h before dosing for acclimatiz-

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ation and the collection of a pre-dose urine sample. Urine was collected over ice during four time points, namely predose (−24–0 h), and 0–8, 8–24 and 24–48 h after dosing, with the pH of each sample measured as 7.22 (predose), 7.24 (0–8 h), 7.42 (8–24 h) and 7.11 (24–48 h). All NMR spectra were measured on a Bruker AMX-600 spectrometer at 303 K. ^{1}H-NMR spectra were measured unlocked at 600.14 MHz using a one-dimensional NOESY sequence for water suppression [5]. No chemical shift reference compound or D_2O was added. Typical parameters were spectral width, 18518 Hz, time domain points, 128 K, acquisition time (AQ), 3.54 s, saturation time, 3.0 s, 90° pulse, 64 scans, linebroadening of 0.3 Hz. ^{2}H-spectra were measured at 92.13 MHz using an inverse detection 5 mm broadband probe in an unlocked mode. Typical parameters were spectral width, 2778 Hz, 16 K time domain points, acquisition time 2.95 s, relaxation delay, 0.65 s, 65° pulses, 64 scans, linebroadening of 0.3 Hz. ^{2}H spin-lattice relaxation times (T_1) were measured using the standard inversion–recovery method. ^{13}C-NMR spectra were measured at 150.92 MHz with power-gated composite pulse decoupling (WALTZ-16). Typical parameters were 64 K time domain points, spectral width 83333 Hz, acquisition time 0.39 s, relaxation delay 2.3 s, 30° pulse, 27,000 transients and an exponential line-broadening of 1.3 Hz prior to FT. ^{2}H–^{13}C correlation spectra were measured using the HMQC technique [6] with GARP decoupling [7] of the ^{13}C during acquisition. Typical parameters were, time domain points 1024, acquisition time 0.78 s, 2300 scans per increment, 64 F1 increments zero-filled to 512 before Fourier transformation. The data were weighted before Fourier transformation using the shifted sine-bell squared function $[\sin(\pi - \pi/a)(t/AQ) + \pi/a]^2$ with values of a of 2.5 and 2.4, respectively in the F2 and F1 domains. As no chemical shift reference material was added to the urine, ^{1}H chemical shifts were referenced to that of creatinine at δ3.04, ^{2}H chemical shifts were referenced to that of the higher frequency methyl resonance of DMF-d_7 at δ2.92 and ^{13}C shifts were relative to the ^{13}C resonance of the higher frequency methyl group of DMF-d_7 at δ34.89.

Results

Figure 1 shows the 600 MHz ^{1}H-NMR spectrum of the rat urine obtained 24–48 h after dosing and is typical of a normal rat urine with many of the peaks assigned to known

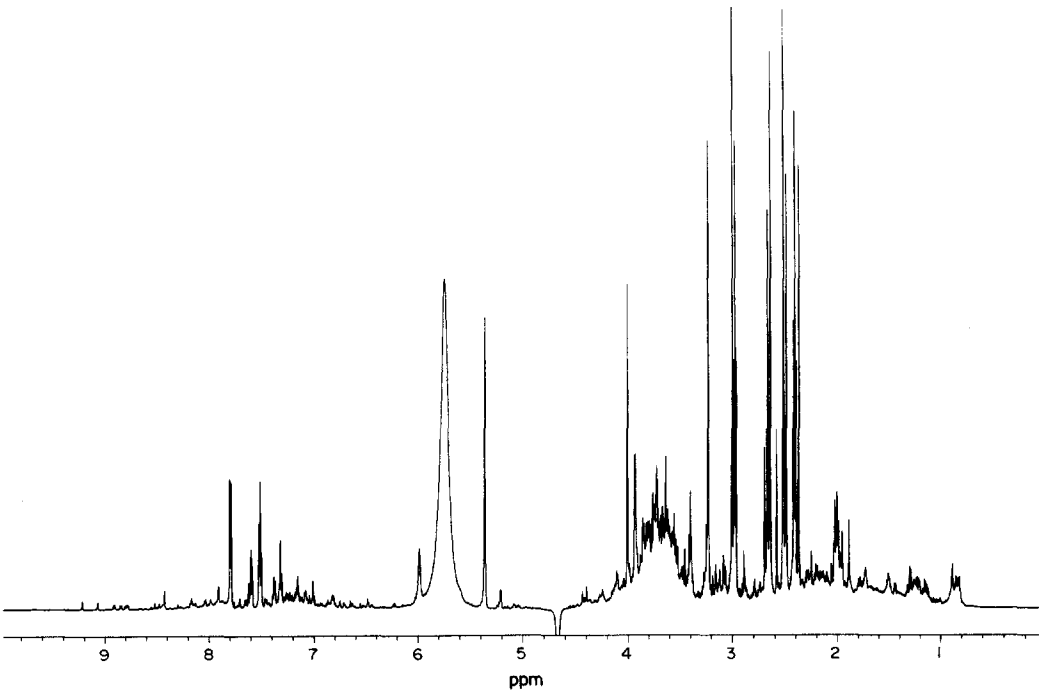

Figure 1
600.14 MHz ^{1}H-NMR spectrum of urine from a rat dosed with DMF-d_7 collected 24–48 h after dosing.

endogenous metabolites [8]. The spectra from the earlier time points were very similar and apart from a variable level of acetate, which may be formed because of bacterial contamination of the urine, there were no obvious changes due to a toxic effect of the administered compound using the known NMR markers for liver, kidney or reproductive organ damage [9]. The only significant change from the control, pre-dose, urine is the appearance of a singlet at δ2.02, most probably due to an acetyl resonance and from a previous study using non-deuterated DMF [4], this can be assigned to the *N*-acetylcysteine adduct of *N*,*N*-methylformamide (*vide infra*).

Figure 2 shows the 92.1 MHz ^{2}H-NMR spectra of the three post-dose urines. Not shown is the spectrum of the pre-dose urine which only contains a small peak due to the natural abundance of HOD. From the top, the figure shows, respectively, the ^{2}H-NMR spectra from urines after 0–8, 8–24 and 24–48 h post-dosing. The two non-equivalent methyl signals of the parent compound DMF-d_7 can clearly be seen at δ2.76 and δ2.92, and these decrease progressively with time. The formyl deuteron resonance of DMF-d_7 is visible at δ7.89 and this also shows the same decrease. The other major signals which appear and which increase with time are due to the major metabolite *N*-hydroxymethyl-*N*-methylformamide-d_6 (HMMF-d_6). Because of the slow rotation about the *N*-formyl bond, two distinct rotamers appear in the spectrum and unlike DMF because of the asymmetric nature of the N-substitution the two forms are

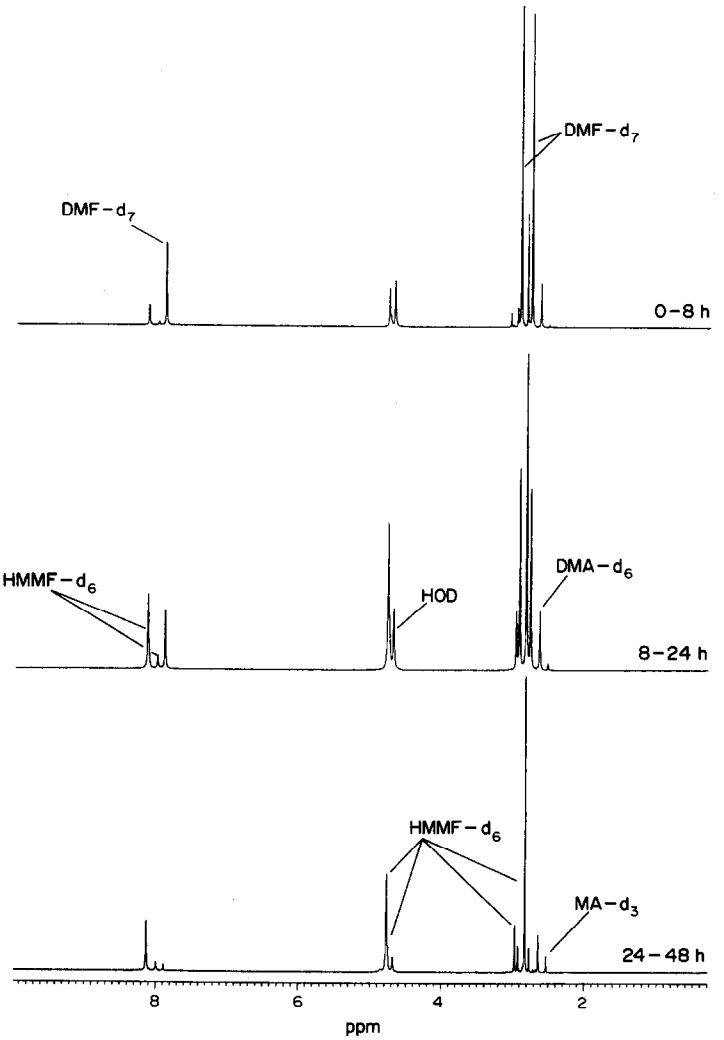

Figure 2
92.13 MHz ^{2}H-NMR spectra of urine from a rat dosed with DMF-d_7 showing results after urine collection from 0–8, 8–24 and 24–48 h after dosing.

not equally populated. In fact the proportions are approximately 6.4:1 with the *E* isomer predominant, the assignments having been made on authentic material [10]. Thus the CD_3 signal of E-HMMF-d_6 appears at δ2.82 and the corresponding signal for the *Z* isomer is at δ2.97. The formyl resonances are at δ8.13 and δ7.99 for the *E* and *Z* isomers, respectively. In an earlier study on the metabolism of DMF using ^{1}H-NMR it was not possible to observe the methylene resonances of HMMF because they were obscured by the water resonance and were lost when using water suppression techniques. In this study the CD_2 signals are clearly resolved with the *E* and *Z* isomer resonances at δ4.78 and δ4.75, respectively, just to higher frequency of the HOD signal at δ4.68; these resonances serve to confirm the identity of the major metabolite. Other ^{2}H signals are seen at δ2.63 and δ2.52 arising from dimethylamine-d_6 (DMA-d_6) and methylamine-d_3 (MA-d_3), respectively [4] and which will be protonated at the urine pH. This proves that the elevation in urinary methylamine following DMF administration [4] is from the xenobiotic rather than from endogenous sources. Using ^{1}H-NMR with non-deuterated DMF, these xenobiotic metabolites would be indistinguishable from the endogenous forms of the two molecules. In the 0–8 h urine an additional peak was observed at δ3.06, which disappeared in the 8–24 h urine and has not been assigned. A small resonance was observed in the 24–48 h urine at δ4.86 as a doublet, and this is most likely due to *N*-hydroxymethylformamide-d_3 (HMF-d_3) in which a spin coupling can be resolved between the CD_2 spins and the NH proton. In addition in the 8–24 h urine, and more clearly in the 24–48 h urine, small peaks were observed at δ2.67 arising from the methyl resonance of *N*-methylformamide-d_4 (NMF-d_4) and at δ2.71 arising from the *N*-methyl group of the *N*-acetylcysteine aduct, *N*-acetyl-*S*-(*N*-methylcarbamoyl)-cysteine (NASC-d_3) previously identified [4]. This peak probably arises from the same species as the *N*-acetyl resonance observed in the ^{1}H-NMR spectrum. A small peak at δ8.22 may be assigned to the formyl deuteron of *N*-hydroxymethylformamide-d_3 on intensity grounds. The proportions of the rotamers in NMF-d_4 are about 9:1 (J.K. Nicholson, unpublished results) and it is therefore unlikely that the minor rotamer will be observed. It is expected that similar considerations will apply to HMF-d_3. The structures of the metabolites are given in Fig. 3.

In order to provide additional NMR evi-

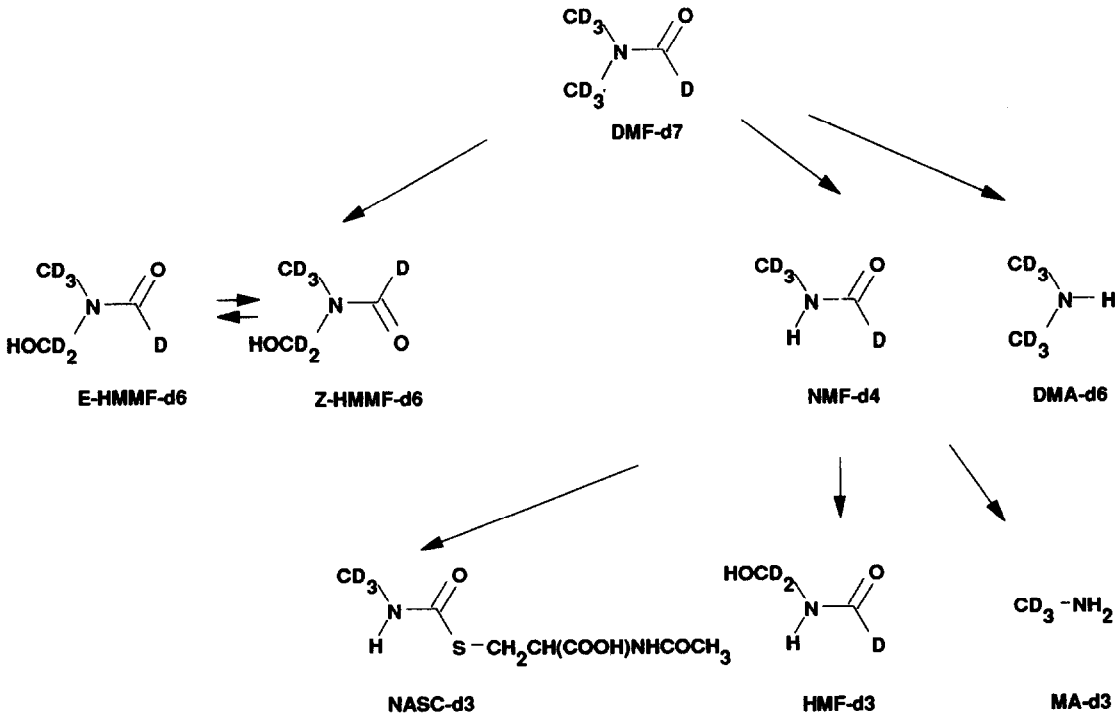

Figure 3
The structures of the metabolic products of DMF-d_7.

dence for the identity of the metabolites the ^{2}H–^{13}C heteronuclear correlation spectrum of a urine sample was measured using the HMQC technique. The results of this experiment can be visualized as a two-dimensional contour map whose axes are the ^{13}C and ^{2}H chemical shifts and showing peaks with coordinates at the ^{13}C and ^{2}H chemical shifts of each directly bonded ^{13}C–^{2}H moiety (i.e. CD_3, CD_2 and CD groups). A refocusing delay of $1/2*J_{CD}$ of 25 ms corresponding to $^{1}J_{CD}$ of 20 Hz and carbon decoupling using the GARP method was employed. This refocusing delay was optimized for methyl and methylene groups but is not optimal for the formyl resonances where $^{1}J_{CD}$ is about 29 Hz. The ^{2}H T_1s were measured in order to allow an estimate of the optimum recycle delay. Thus the formyl deuterons had T_1s around 420 ms, the CD_2 groups of HMMF-d_6 had T_1s of about 390 ms, whilst the methyl group T_1s were typified by values of 1200 ms for the CD_3 groups of DMF-d_7 and 1130 ms for the CD_3 groups of DMA-d_6. A ^{2}H–^{13}C correlation contour map is shown in Fig. 4 for the 24–48 h urine. The highly truncated nature of the time-domain data on the ^{13}C axis produces lobes on the sides of the peaks after Fourier transformation and this results in the appearance of a triplet nature to the peaks in the contour plot. This figure shows only the CD_3 region where the ^{13}C chemical shifts of the two methyl groups of DMF-d_7 appear at δ29.6 and δ34.9 and the ^{13}C resonances of HMMF-d_6 appear at δ26.5 and δ31.8 for the *E* and *Z* isomers, respectively. In addition, the ^{13}C resonance correlating with

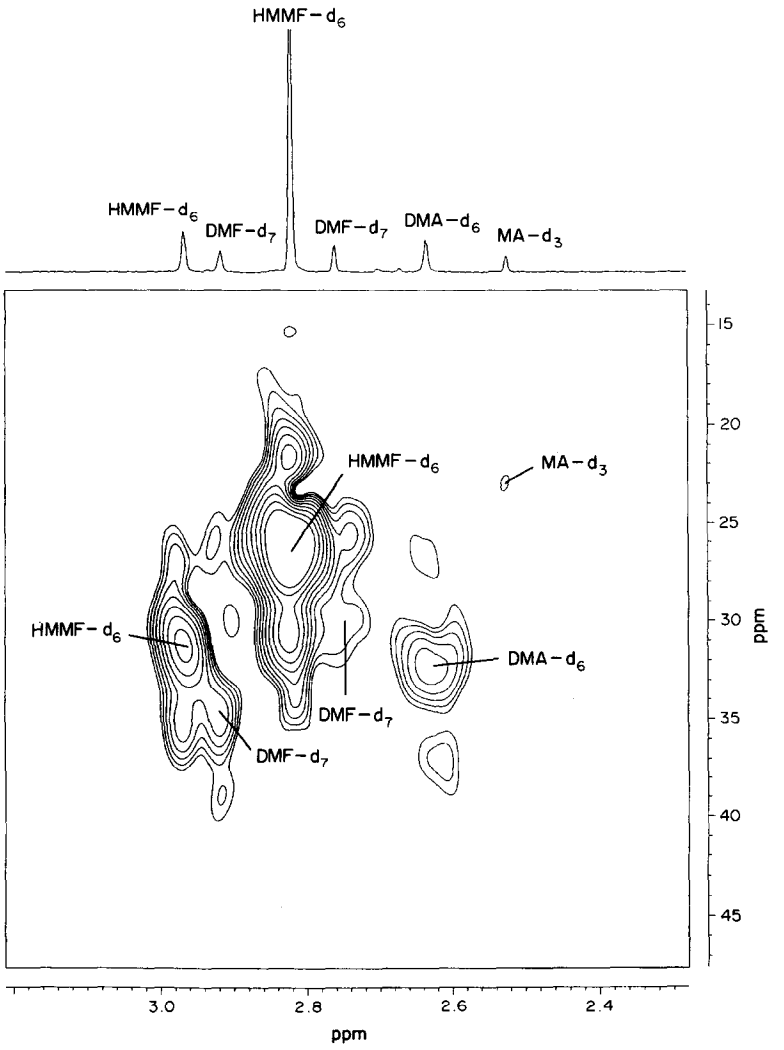

Figure 4
^{2}H–^{13}C HMQC contour plot (methyl chemical shift region) from a rat urine collected 24–48 h after dosing with DMF-d_7. The assignments of the resonances are marked.

the ^{1}H resonance at δ2.62 is at δ31.9 in agreement with the assignment to DMA-d_6. The resonance at δ2.52 in the ^{1}H spectrum correlates with a peak in the ^{13}C spectrum at δ20.4 also in agreement with its assignment to MA-d_3. Not shown is the CD_2 region, where the ^{13}C resonances for HMMF-d_6 appear close together on both the ^{2}H and ^{13}C axes at δ71.4, thus providing evidence from their chemical shifts of the hydroxy substitution. The formyl resonance ^{2}H signals also correlate in the ^{13}C spectrum with peaks at δ163.8 and δ163.2 for the *E* and *Z* isomers of HMMF-d_6, respectively.

Additional confirmatory evidence for the identity of HMMF-d_6 comes from the measurement of a ^{13}C spectrum of the urine both with and without ^{2}H decoupling. The 1:2:3:4:3:2:1 septet structure of the CD_3 group signals and the 1:2:3:2:1 quintet structure of the CD_2 group signals are detectable, with the splitting collapsing on ^{2}H decoupling.

Conclusions

^{2}H-NMR spectroscopy at high field has been shown to be potentially useful for the detection and characterization of drug metabolites in intact biofluids. In combination with solid-phase extraction techniques or through the use of coupled HPLC–NMR it could provide a new approach to drug metabolism without the need for radio-labelling.

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References

[1] J.K. Nicholson and I.D. Wilson, *Prog. Drug Res.* **31**, 427–479 (1987).
[2] I.D. Wilson and J.K. Nicholson, *Anal. Chem.* **59**, 2830–2832 (1987).
[3] B.C. Sweatman, R.D. Farrant and J.C. Lindon, *J. Pharm. Biomed. Anal.* **11**, 169–172 (1993).
[4] K. Tulip, J.K. Nicholson and J.A. Timbrell, *J. Pharm. Biomed. Anal.* **7**, 499–505 (1989).
[5] D.J. States, R.A. Haberkorn and D.J. Ruben, *J. Magn. Reson.* **48**, 286–292 (1982).
[6] A. Bax and S. Subramanian, *J. Magn. Reson.* **67**, 565–569 (1986).
[7] A.J. Shaka, P.B. Barker and R. Freeman, *J. Magn. Reson.* **64**, 547–552 (1985).
[8] J.K. Nicholson and I.D. Wilson, *Prog. NMR Spectrosc.* **21**, 449–501 (1989).
[9] K.P.R. Gartland, C.R. Beddell, J.C. Lindon and J.K. Nicholson, *Mol. Pharmacol.* **39**, 629–642 (1991).
[10] P. Kestell, M.H. Gill, M.D. Threadgill, A. Gescher, O.W. Haworth and E.H. Curzon, *Life Sciences* **38**, 719–724 (1986).

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