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Investigation of the feasibility of directly-coupled HPLC-NMR with ^2H detection with application to the metabolism of *N*-dimethylformamide-d_7

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Abstract

The use of ^2H NMR spectroscopy as a detector for HPLC has been investigated using the continuous flow method in which rat urine containing metabolites of *N*-dimethylformamide-d_7 was employed as a test case. Three xenobiotic-related species, including DMF-d_7 itself, were detected. It is shown that for small molecules which give relatively sharp ^2H NMR resonances, ^2H HPLC-NMR spectroscopy is a feasible technique. For larger molecules, the resulting broad lines are likely to preclude the determination of detailed structural information. However, extension of the approach is possible by the use of selectively ^2H-labelled xenobiotics to determine HPLC retention times of metabolites with continuous-flow ^2H NMR spectroscopy detection, followed by stop-flow ^1H HPLC-NMR spectroscopy for structural characterisation. © 1997 Elsevier Science B.V.

Keywords: HPLC-NMR; ^2H NMR; Metabolism; Urine; DMF

1. Introduction

NMR spectroscopy of biofluids, in particular of urine and bile, has for the last few years proved to be a useful adjunct to the traditional techniques for identifying drug metabolites and in combination with simple solid-phase extraction techniques for concentrating metabolites, it has proved to be a general and versatile approach [1]. The methodology available has been augmented by the use of directly-coupled HPLC-NMR which has been shown to be a powerful and efficient technique for separation of mixtures and identification of their components [2,3]. Applications have included chemical mixtures [4], natural products [5] and peptide libraries [6]. However, the methodology has been used most extensively for the identification of drug metabolites [7–12] and related model compounds [13,14].

Although the most sensitive NMR nuclei, ^1H and ^{19}F with spin $I = 1/2$, have been used so far in HPLC-NMR, one study has employed ^{31}P NMR detection [15] and it should be possible in princi-

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ple to use other nuclei such as ^{13}C. In addition, deuterium (^{2}H) NMR detection in HPLC-NMR of deuterated drugs might provide a useful method for identifying metabolites in view of the high sensitivity of high field NMR machines and the low natural abundance of deuterium thereby minimising background problems. The spin of the ^{2}H nucleus is $I = 1$ and this study is the first use of a quadrupolar nucleus for HPLC-NMR detection. 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. All NMR spectrometers use ^{2}H NMR for provision of a field-frequency lock and hence, with appropriate signal routing, have facilities for ^{2}H NMR detection. 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 and for larger molecules the short T_2s can give broad NMR resonance line widths. An additional advantage of the use of ^{2}H NMR for HPLC detection is the removal of the requirement to use deuterated HPLC solvents.

The use of ^{2}H NMR detection for HPLC-NMR has now been investigated in a preliminary manner through the use of the fully deuterated model drug *N*-dimethylformamide-d_7 (DMF-d_7), which has aroused interest as a model anti-cancer agent. In a previous ^{1}H NMR study of DMF metabolism [16] several xenobiotic metabolites were identified but other metabolites of DMF which could also appear in urine are indistinguishable from endogenous metabolites. These include methylamine which is known to be an endogenous metabolite but which could also be derived from the xenobiotic source.

The metabolism of *N*-dimethylformamide in the Sprague-Dawley rat has been re-investigated recently using NMR spectroscopy of urine [17]. In particular, through the use of DMF-d_7 and ^{2}H NMR spectroscopy, the principal metabolites of this compound were confirmed in a straightforward manner. These were confirmed as the *E*- and *Z*-isomers of *N*-hydroxymethyl-*N*-methylformamide-d_6 with the *E*-isomer being predominant. Thus it was of particular interest to search for signals from deuterated methylamine which could only come from the exogenous source. 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. Samples from this earlier study were therefore used to provide a suitable test for ^{2}H-detected continuous-flow directly-coupled HPLC-NMR.

2. Experimental

Compound administration and urine collection were as described previously [17]. In the earlier study, a small amount of D_2O was added to the urine samples to provide a field-frequency lock for ^{1}H NMR studies [17]. Directly coupled HPLC-^{2}H NMR was conducted on freeze dried samples of the urine reconstituted into 70% H_2O–30% acetonitrile. The HPLC system comprised a Bruker LC22C pump and a Bischoff 1000 Lambda variable wavelength UV detector. The UV detector was connected to the NMR probe via a 0.25 mm i.d. PEEK capillary line. HPLC analysis was carried out using a 10 μl injection on to a 250 × 4.6 mm i.d. Spherisorb ODS-2 column. After 5 min of isocratic flow of 100% H_2O, linear gradient elution using H_2O–acetonitrile was employed with UV monitoring of the eluent peaks at 254 nm, ending after 50 min at H_2O–acetonitrile, 1:1. All NMR spectra were measured on a Bruker AMX-600 spectrometer at 303 K. No chemical shift reference compound or D_2O was added. Single pulse ^{2}H spectra were measured on the whole reconstituted urine at 92.1 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 degree pulses, 64 scans, line broadening of 0.3 Hz. As no chemical shift reference material was added to the urine, ^{2}H chemical shifts were referenced to that of the high frequency methyl resonance of DMF-d_7 at δ2.92. The continuous-flow HPLC ^{2}H NMR spectrum was acquired by collecting 16 transients using an acquisition time of 0.93 s giving a chromatographic time resolution of 15 s.

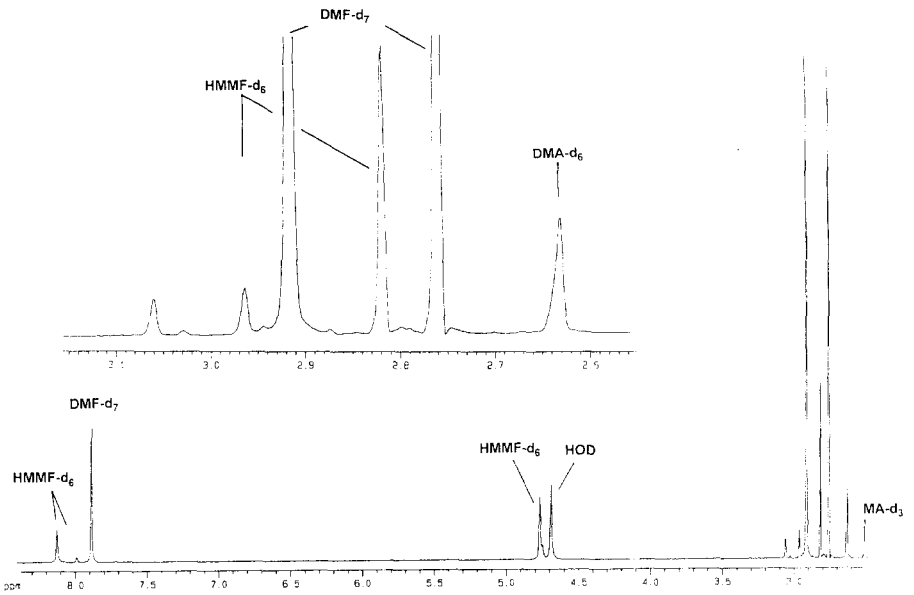

Fig. 1. 92.13 MHz ^{2}H NMR spectra of whole urine from a rat collected 0–8 h after dosing with DMF-d_7. The assignments are as marked.

3. Results

Fig. 1 shows the 92.1 MHz ^{2}H NMR spectra of the 0–8 h post-dose urine. The spectrum of the pre-dose urine contained only a small peak due to the natural abundance of HOD. The two non-equivalent methyl signals of the parent compound DMF-d_7 can clearly be seen at δ2.76 and δ2.92 in Fig. 1, and these were observed to 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 have been reported previously [17] 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 not equally populated. 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. The use of ^{2}H NMR without water suppression enabled the CD_2 signals to be clearly resolved with the *E*- and *Z*-isomer resonances at δ4.78 and δ4.75 respectively just to a high field of the frequency of the HOD signal at δ4.68; these resonances serve to confirm the identity of the major metabolite. Other ^{2}H signals were seen at δ2.63 and δ2.52 arising from dimethylamine-d_6 (DMA-d_6) and methylamine-d_3 (MA-d_3) respectively [14]. In the 0–8 h urine an additional peak was observed at δ3.06 and which had disappeared in the 8–24 h urine and this was not assigned.

The continuous flow HPLC-NMR spectrum with ^{2}H detection is shown in Fig. 2. Fig. 2a depicts the single pulse ^{2}H NMR spectrum of the whole urine after D_2O addition showing the DMF-d_7 metabolite resonances and peaks from D_2O and from deuterated urea at δ5.7. D_2O had been added previously to the whole urine sample to provide a field lock for ^{1}H NMR spectroscopic studies. Fig. 2b shows the continuous flow HPLC-NMR spectra with ^{2}H detection as a function of time as a contour plot, with the horizontal axis representing the ^{2}H chemical shift and the vertical axis being time. The large peak arises from D_2O and the deuterated urea elutes with a retention time of 4.1 min. Three DMF-d_7 metabolites can be observed at retention times of 4.5, 4.9 and 5.8 min.

Thus the spectrum which corresponds to a retention time of 5.8 min shows two methyl resonances and a formyl deuteron resonance and the chemical shifts (methyl peaks at δ2.77 and δ2.92 and deuteron resonance at δ7.89) identify this as the parent compound DMF-d_7 [17].

The spectrum corresponding to a retention time of 4.5 min also shows two methyl resonances. One of these is at δ2.82 and corresponds to the *E*-isomer of HMMA-d_6. Although the *E*- and *Z*-isomers of HMMA-d_6 are in slow exchange on the NMR time-scale and give separate resonances, they will exchange many times during the chromatographic separation and would be expected to appear at the same retention time. Even though the *Z*-isomer is detectable in the whole urine, it was not possible to identify it in the HPLC-NMR spectra, probably because of its low level, at approximately 15% of the *E*-isomer. The formyl deuteron resonances are also not detected, again probably because they are below the detection threshold. The other methyl resonance which appears at this retention time has a chemical shift of δ2.63 and corresponds to DMA-d_6.

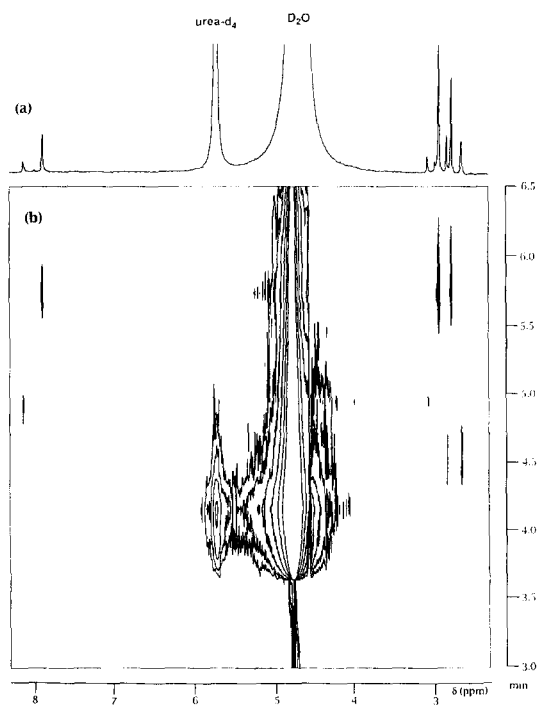

Fig. 2. ^{2}H NMR detection in continuous-flow HPLC-NMR. The contour plot from rat urine collected 0–8 h after dosing with DMF-d_7. The horizontal axis corresponds to ^{2}H NMR chemical shifts and the vertical axis is chromatographic retention time.

Finally small peaks can be seen at a retention time of 4.9 min in the contour plot shown in Fig. 2. These arise from a metabolite detected in the previous study but not identified [17]. This has a single methyl resonance at δ3.06 and a formyl resonance at δ8.13. This is probably *N*-methylformamide-d_4.

In summary, therefore, ^{2}H NMR spectroscopic detection in coupled HPLC-NMR has been shown to be potentially useful for the detection and characterisation of small molecule drug metabolites in intact biofluids. Although it will be rare that a drug is synthesised with complete deuteration, there may be circumstances where the expense is justified. Nevertheless, incorporation of ^{2}H into selected parts of a drug molecule would still provide diagnostic ^{2}H HPLC-NMR spectra and allow the determination of retention times of the deuterated species for stop-flow ^{1}H HPLC-NMR studies for metabolite identification. Stop-flow or time-slice ^{2}H HPLC-NMR spectroscopy itself would be useful for structural purposes with chemical shifts which are likely to be diagnostic for functional group modifications. In addition, the use of a fully deuterated molecule and ^{2}H NMR detection removes the need for the use of D_2O as an HPLC eluent. The use of ^{2}H NMR spectroscopy is acceptable for very small molecules which, because of the relatively long ^{2}H T_1 values, give sharp lines. However, for medium sized molecules, the ^{2}H NMR line widths rapidly become broad, thereby limiting the signal:noise values in the spectra. This might be alleviated by measuring the spectra at elevated temperatures or in low viscosity solvents if possible. Because of the small ^{1}H–^{2}H spin-coupling, the information content in ^{2}H NMR spectra is limited to that provided by diagnostic chemical shifts and therefore ^{2}H-detected HPLC-NMR would be enhanced considerably by additional on-line coupling to mass spectrometry [18,19].

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