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Short Communication

A study of the metabolism of dimethylformamide in the rat by high resolution proton NMR spectroscopy

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Introduction

Dimethylformamide (DMF) is an industrial solvent which may cause liver damage in experimental animals [1] and man [2]. Previous studies have revealed that the major metabolite is *N*-(hydroxymethyl)-*N*-methylformamide (DMFOH) which results from hydroxylation of the methyl group [3–5] (Fig. 1). The formation of *N*-methylformamide (NMF) as a metabolite of DMF has been equivocal due to the possibility that it is simply an artifact arising from breakdown of DMFOH during analysis. In one study, however, DMFOH was administered to rats *in vivo* and NMF was not detected as a metabolite. Hence the rôle of NMF, which is also hepatotoxic, in the toxicity of DMF is uncertain

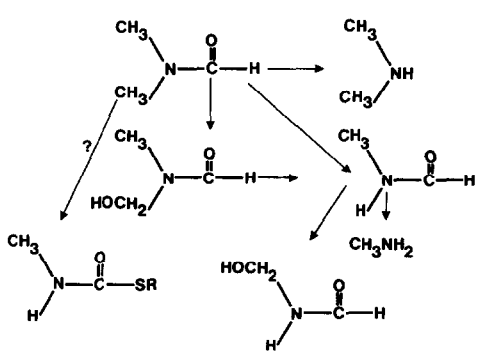

Figure 1
Metabolism of DMF.

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[5]. High resolution proton NMR spectroscopy has been shown to be of considerable utility in the identification of both endogenous and xenobiotic metabolites in biofluids [6–8]. One particular feature, important in the current study, is that it requires little sample pretreatment and does not destroy or chemically alter the sample. Consequently the present NMR urinalysis study was carried out to determine if NMF is a metabolite of DMF in the rat, and whether metabolites of NMF thought to be connected with the hepatotoxicity could also be detected, thus shedding light on the toxicity of DMF.

Experimental

Materials

DMF and NMF were obtained from the Aldrich Chemical Co. Ltd (Gillingham, Dorset, UK). Other chemicals were obtained from Sigma Chemical Co. Ltd (Poole, Dorset, UK) or BDH Ltd (Poole, Dorset, UK).

NMFOH was a gift from Dr A. Gescher, University of Aston. *N*-acetyl-S-(*N*-methylcarbamoyl)-L-cysteine, synthesized as previously described [7], was a gift from Drs J. Troke and I. D. Wilson, Hoechst UK Ltd.

Sample collection and processing

DMF was administered to male Sprague Dawley rats at a dose level of 1000 mg kg^{-1} by i.p. injection in saline (2 ml kg body wt^{-1}).

Rats were housed in metabolism cages arranged for the separate collection of urine and faeces. Urine was collected over ice every 24 h for 72 h after dosing, and samples were kept frozen (−20°C) until analysis. Urine samples (0.45 ml) were diluted with 0.05 ml of ^2H_2O containing sodium tetradeuterotrimethylsilyl propionate (TSP) as an internal chemical shift reference (δ = 0 ppm). Selected urine samples were prepared as described by Van Doorn *et al.* [9]. Samples of urine (2.5 ml) were acidified to pH 2 with 4 M HCl (0.125 ml) and then shaken with ethyl acetate (4 ml) for 15 min. This procedure preferentially extracts thioethers from urine. The ethyl acetate layer was removed after centrifugation and the extraction procedure repeated with another 4-ml aliquot of ethyl acetate. The pooled ethyl acetate layers were evaporated to dryness and the residues dissolved in ^2H_2O (0.5 ml) containing TSP as an internal shift reference, corrected to pH 7 and the NMR spectrum recorded.

NMR spectroscopy

Proton NMR spectra were recorded on a Bruker WH400 spectrometer operating at 400 MHz proton resonance frequency and ambient temperature (25 ± 1°C). For each sample 48–96 free induction decays (FIDs) were collected into 16,384 data points. Typically exponential functions corresponding to a 1 Hz line broadening, were applied to each FID prior to Fourier transformation. A 30° pulse width was used together with a total recycle time of 5.7 s to ensure that the protons were fully T_1 relaxed. The detection of weak NMR signals, such as from urinary metabolites in the presence of the very large signal from water protons causes a dynamic range problem. Consequently, continuous secondary irradiation at the water resonance frequency was used to suppress the intense solvent water peak.

Assignments of resonances were confirmed by a combination of chemical shifts, coupling constants, pH dependencies of chemical shifts and standard additions of candidate compounds.

Two-dimensional (2-D) homonuclear proton–proton COSY NMR spectra were recorded using the pulse sequence:

$$[90° - t_1 - 90° - \text{collect}(t_2)] - \text{relaxation delay.}$$

The parameters used for the collection of data were essentially the same as those previously described [10].

Results

Examination of the urine of rats collected 0–24 h after dosing with DMF by high resolution NMR, showed a number of metabolites (Fig. 2). The most prominent resonance occurred at $\delta = 2.93$ and was consistent with the literature value [5] for the *N*-methyl group of the *syn* isomer of DMFOH. There was another resonance at $\delta = 3.085$ corresponding to the resonance from the *N*-methyl group of the *anti* isomer of DMFOH. Further downfield, the resonances, consistent with those from the formyl proton of DMFOH, were detected at $\delta = 8.198$ (*anti*) and $\delta = 8.060$ (*syn*) (Fig. 3). The other prominent resonances were in accordance with those expected from DMF itself (*N*-methyl *syn*, $\delta = 2.885$; *anti*, $\delta = 3.03$; formyl $\delta = 7.96$). Resonances at $\delta = 2.75$, 2.64

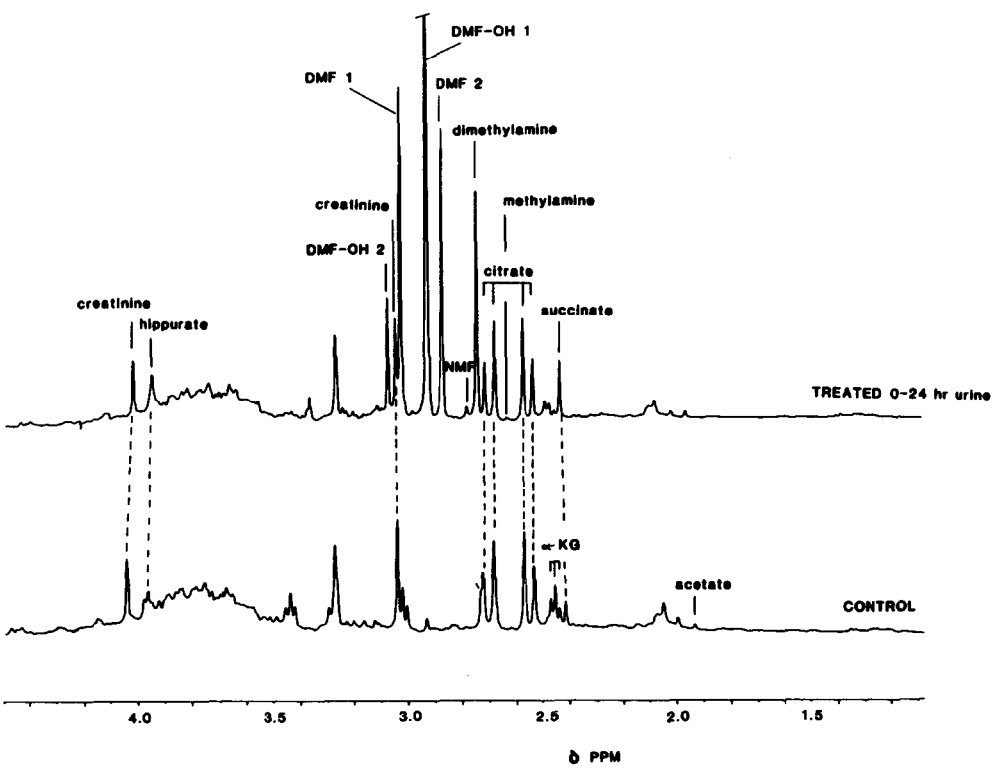

Figure 2
Partial 400-MHz proton NMR spectra of control rat urine and urine collected from a rat 0–24 h after dosing with DMF ("aliphatic region").

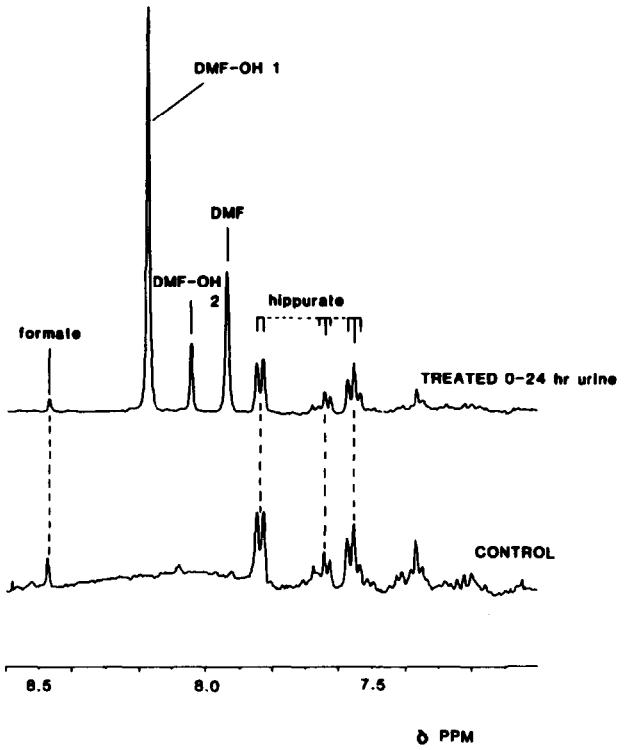

Figure 3
Partial 400-MHz proton NMR spectra of control rat urine and urine collected from a rat 0–24 h after dosing with DMF ("aromatic region").

and 2.802 were also detected, consistent with those from the *N*-methyl groups of dimethylamine, methylamine and of NMF standards, respectively. When the urine collected 24–48 h after dosing was examined, resonances believed to be due to NMF (*N*-methyl, *syn* isomer, $\delta = 2.802$), were more prominent. The resonances from the *N*-methyl (*anti* isomer) and the formyl proton resonance were obscured by the large resonance from DMFOH.

When the urine collected 48–72 h after dosing was examined by NMR, resonances identical to those from *N*-acetyl-S-(*N*-methylcarbamoyl)-cysteine were observed (*N*-acetyl, $\delta = 2.03$; *N*-methyl, $\delta = 2.82$; β-CH_2, $\delta = 3.28$ and 3.49; α-CH, $\delta = 4.44$). Extraction of urine samples with ethyl acetate under acidic conditions afforded a clearer spectrum as shown in Fig. 4. In this extract, the resonances from the conjugate could be clearly seen (a, b, c and d) and resonances at $\delta = 2.09$ (A_1) and 2.04 (A_2) were also detected. These resonances had also been detected in urine from animals dosed with NMF. Again resonances consistent with those from the methyl groups of NMF, DMF (both isomers), dimethylamine and methylamine, were observed. The identity of the dimethylamine NMR signal has been confirmed independently by HPLC [5]. As the extract was dissolved in ^2H_2O, the resonances due to the CH_2 protons of DMFOH and NMFOH could also be clearly seen at $\delta = 4.892$ and 4.958, respectively, as well as those from the *N*-methyl group of these metabolites (Fig. 4).

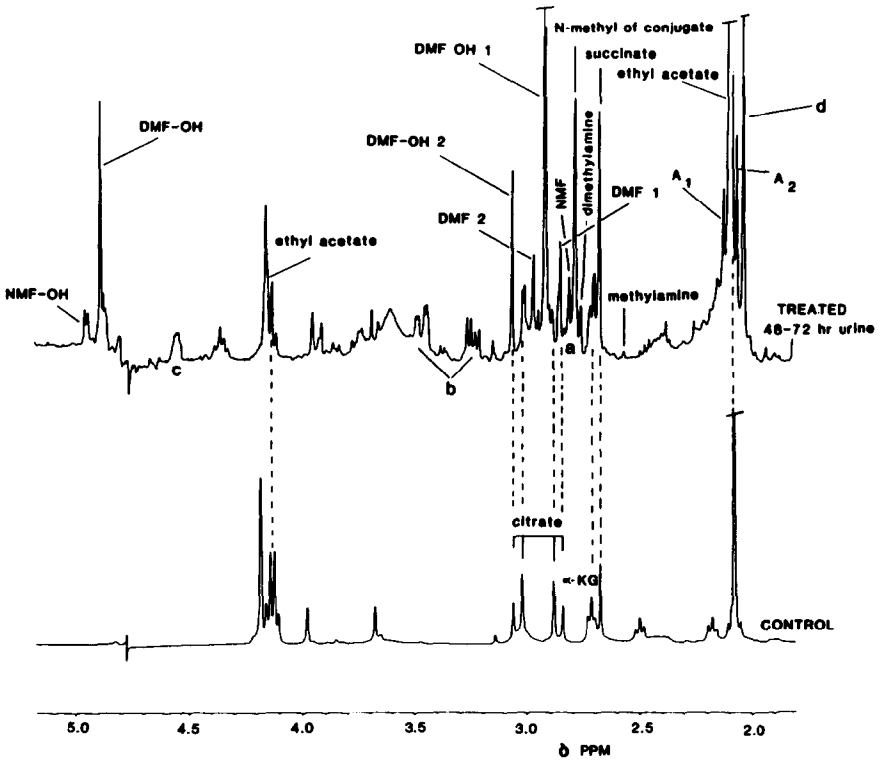

Figure 4
Partial 400-MHz proton NMR spectrum of an ethyl acetate extract of control rat urine and urine collected from a rat 48–72 h after dosing with DMF. A_1 and A_2 refer to acetyl signals from acetylated DMF metabolites; a, b, c and d refer to the *N*-methyl, cysteinyl β-CH_2, cysteinyl α-CH and cysteinyl *N*-acetyl signals of *N*-acetyl-S-(*N*-methylcarbamoyl)cysteine, respectively.

A 2-D COSY spectrum of the ethyl acetate extract of the 24–48-h urine sample also confirmed the presence of the *N*-acetyl cysteine conjugate. Cross peaks from the coupled cysteinyl CH_2CH protons forming an (ABX) spin system could be clearly observed.

Discussion

The results of metabolic studies in rats presented in this paper are consistent with previous work utilizing other techniques [4, 11, 12] and are similar to those reported for mice using NMR [5]. Although metabolites have not been quantitated, from the intensity of the resonances observed in the NMR spectra the major metabolite of DMF appears to be the hydroxymethyl derivative, DMFOH (Fig. 1). This metabolite is detectable in urine and unequivocally assigned from its methyl and formyl proton resonances. It is also detectable in the ethyl acetate extract of urine where, due to the absence of an interfering water signal, the CH_2 resonance is also detectable. There are therefore three resonances present in the correct intensity ratios that are consistent with this structure (Fig. 1). The other metabolites previously detected in the mouse after DMF treatment are NMF, methylamine and dimethylamine and these have now also been shown to be present in the rat.

Particularly important are the strong signals due to *N*-acetyl-S-(*N*-methylcarbamoyl)-cysteine in the urine. It has previously been reported that this conjugate was not a metabolite of DMF in mice [13]. This conjugate has the same structure as that detected by NMR in urine from rats and mice dosed with NMF [7, 14]. This conjugate has also recently been detected in a human subject exposed to DMF [15]. The fact that both NMF and the conjugate are excreted at the later time periods, is consistent with the suggestion that at least some of the routes of DMF metabolism may be saturated or inhibited by the presence of metabolites [4].

The presence of the conjugate is a particularly significant finding as it has been suggested that it results from the reaction of a reactive intermediate with glutathione [16]. This reactive intermediate might, therefore, be involved in the hepatotoxicity of DMF. The conjugate could arise either by metabolism of DMF to NMF and then subsequent metabolism to the presumed reactive intermediate which reacts with glutathione to give rise to the conjugate, or by direct metabolic activation of DMF followed by reaction with glutathione (Fig. 1). There are several pieces of evidence that make the first route credible. The presence of NMF in urine indicates that DMF is metabolized at least in part to NMF, perhaps via DMFOH. The presence of methylamine, similarly, is consistent with NMF as an intermediate. However, from previous studies in both the mouse and the rat, the amounts of NMF excreted are small and therefore unlikely to account for the hepatotoxicity. The observations in the current study are consistent with this. However, when DMFOH was administered to rats, no NMF was detected in the urine [11], indicating that the main metabolite is probably not the intermediate for the production of NMF. Some authors have previously suggested that the hepatotoxicity of DMF is unlikely to be due to DMFOH [5] although Scailteur and Lauwerys [11] have shown that DMFOH is more hepatotoxic than DMF while NMF is more hepatotoxic than DMFOH. One suggestion [12] is that NMF may arise from DMF by a pathway involving hydroxyl radicals rather than demethylation via DMFOH. Perhaps such a pathway is responsible for an intermediate which could give rise to methylisocyanate and hence the *N*-acetylcysteine conjugate as postulated for NMF. Methylisocyanate could also account for the methylamine in the urine. Clearly, therefore, there are several questions arising from the discovery of a *N*-acetylcysteine conjugate in the urine which have a bearing on the heptotoxicity of DMF and which remain to be resolved.

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