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Short Communication

Proton magnetic resonance spectroscopy of human urine: excretion of 1-(3′-carboxypropyl)-3,7-dimethylxanthine by man after dosing with oxpentifylline

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Introduction

High resolution proton NMR spectroscopy can be used to detect and quantify a wide range of intermediary metabolites present in biological fluids such as urine and plasma [1–8]. With very high field spectrometers this can usually be achieved without extensive physical preparation or chemical pretreatment of the samples. Xenobiotic compounds and their metabolites can also be detected in untreated urine samples by proton NMR, provided they have suitable resonances and are present in the millimolar concentration range [9–12]. The authors have shown for acetaminophen (paracetamol) that the major urinary excretion products (including the glucuronide, sulphate, cysteinyl, *N*-acetyl cysteinyl conjugates as well as the free drug) can be detected and reliably quantified in proton NMR spectra of urine [9]. NMR spectrometers operating at very high magnetic field strengths (e.g. operating at 9.4 or 11.75 T, corresponding to proton resonance frequencies of 400 and 500 MHz) have been used in order to obtain maximum sensitivity and dispersion of signals. This minimises overlap of drug metabolite resonances and those from the more abundant urinary components of endogenous origin. However, such instruments are still not normally available in most analytical laboratories as they are of high capital cost. The authors have previously argued against the use of spectrometers

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operating at lower field strengths for studying the biochemical composition of body fluids by NMR, although this facility would clearly be useful in many laboratories equipped with modest NMR instrumentation. In the present study the authors have used proton NMR to investigate the urinary excretion of 1-(3′-carboxypropyl)-3,7-dimethylxanthine (CPDX), the major metabolite of oxpentifylline in man, comparing quantitative data obtained from 250 and 400 MHz measurements with those of liquid chromatography.

Oxpentifylline (Trental™, pentoxifylline, 1-(5′-oxohexyl)-3,7-dimethylxanthine) is used extensively in the treatment of vascular disease. It has been found to increase blood flow by acting as a vasodilator and by reducing blood viscosity. Studies on the metabolism of the ^{14}C-labelled drug have shown that urinary excretion is rapid (92–96% of dose removed in 24 h) and also the major route of elimination [14]. These studies demonstrated extensive metabolism with no unchanged drug being present in the urine. The major ^{14}C component was the acidic metabolite CPDX, whilst 1-(4′-carboxybutyl)-3,7-dimethylxanthine (CBDX) was also detected as a minor component. The urinary excretion of this class of compound has not previously been the subject of direct investigation by high resolution proton NMR methods. Moreover, the relatively high therapeutic dosage of oxpentifylline (600 mg per dose) together with its rapid urinary excretion as one major metabolite, indicated that it would be suitable for a quantitative NMR study in which 250 and 400 MHz instruments could be compared.

Materials and Methods

Urine samples

Urine samples were collected (and volumes recorded) from a healthy volunteer from 2 h before and 2, 4, 6, 8 and 10 h after an oral 600 mg dose of oxpentifylline. Further 600 mg doses of oxpentifylline were given at 12 and 24 h and urine samples were collected between 12–24 and 24–36 h. All samples were analysed for drug metabolites by high-performance liquid chromatography (HPLC) and proton NMR at two field strengths. Samples were either freeze-dried and redissolved in one fifth of the original volume of ^2H_2O prior to NMR measurement, or were untreated except for the addition of ^2H_2O (final concentration 10% ^2H_2O) to provide an internal field frequency lock. An internal chemical shift reference ($\delta = 0$ ppm) was also present (dissolved in the ^2H_2O) in the form of sodium 3-(trimethylsilyl)[^2H]$_4$ propionate (TSP) at a final concentration of 3.07 mM.

NMR spectroscopy

Proton NMR spectra were recorded using Bruker WM250 and WH400 spectrometers operating at 250 and 400 MHz proton resonance frequencies, respectively. All spectra were measured at ambient probe temperature (25 ± 1°C) and collected into 16,384 computer points with an acquisition time of 1.7 s. Spectra measured at 250 MHz were the result of between 200 and 700 scans using 90° pulses and a 9 s delay between pulses, including the acquisition time. 400 MHz spectra were obtained by collecting 32–64 free induction decays (FIDs) into 16,384 computer points. Pulse angles of 30° were used with a total pulse recycle time of 5 s to allow full T_1 relaxation. The FIDs were zero-filled to 32,768 data resolution and exponential line broadening functions of 0.2–1 Hz were applied prior to Fourier transformation. The strong water signal was suppressed by a secondary irradiation field applied at the water resonance frequency using the decoupler coils, the power being gated off during acquisition to minimise radio frequency

interference. Quantitative data were obtained by comparing the signal intensity of selected drug metabolite resonances with those of the standard. This was achieved by cutting out and weighing paper traces of the expanded signals of interest and those of the TSP standard rather than by direct computer integration. This method was preferred as some drug metabolite resonances were close in frequency to or partially overlapping with resonances from endogenous urinary metabolites making selection of the baseline difficult with only the standard computer integration software. In the case of overlapping peaks simple Lorentzian lineshapes were assumed. For the C_8H proton resonance of CPDX, peak overlap was small at both 250 and 400 MHz and negligible in resolution enhanced spectra, but for other resonances the degree of overlap was field dependent and this was reflected in the reliability of the concentration data (see results). In ^1H NMR studies of plasma, the authors have previously found that peak area estimation by weighing paper traces gave reliable concentration data for small molecules [2].

Liquid chromatographic identification and quantification of CPDX

Specific analysis of CPDX in urine was obtained using HPLC following solvent extraction as follows: urine samples (1 ml) were pipetted into screw-topped test tubes containing 100 μl of a 1 μg ml^{-1} solution of internal standard (a structural analogue, see ref. 14). 6 ml of dichloromethane and 1 ml of 0.1 M HCl were added to each tube and extractions performed on a rotary inversion mixer for 15 min. After separation of the phases by centrifugation (2000 g, 5 min) the aqueous layer was removed and discarded. The organic layer was transferred to a tapered test tube where the solvent was evaporated under a stream of nitrogen. The residue was then dissolved in the HPLC mobile phase (2 ml) and analysed by HPLC (20 μl aliquots), using the following system: 15 cm × 3 mm (i.d.) stainless steel column, slurry packed with 5 μ ODS spherisorb and a mobile phase of 0.02 M orthophosphoric acid and methanol (71.5:28.5, v/v) at 1 ml min^{-1}. Compounds eluting from the column were detected using a variable wavelength ultraviolet detector (Pye-Unicam) operating at 274 nm. Under these conditions, typical retention volumes were CPDX, 3.4 ml; CBDX, 6.4 ml and the internal standard 11.5 ml. Full details of this method are given elsewhere [14].

Results and Discussion

We have previously shown that with the aid of simple solvent suppression methods, high resolution ^1H NMR spectra of endogenous and drug metabolites in untreated urine samples can be obtained using spectrometers operating at 400 MHz or above [4]. In the present study we were only able to obtain weak spectra from unprocessed urine samples, at 250 MHz even with the aid of a gated secondary irradiation field applied at the water resonance frequency to effect solvent suppression (Fig. 1a). Freeze dried samples redissolved in ^2H_2O with at five times the original concentration gave spectra with greatly improved signal-to-noise ratios on endogenous urinary metabolites (Fig. 1b). Apart from simply increasing metabolite concentrations, this simple procedure overcomes the dynamic range problem caused by the strong water signal and so allows efficient digitization of the metabolite signals and hence better spectral quality. This is particularly important when relatively insensitive low to medium field spectrometers are being used. However, it must be remembered that, whilst not relevant in this case, freeze drying followed by redissolution in ^2H_2O results in the loss of volatile components and the deuteration of acidic or exchangeable protons.

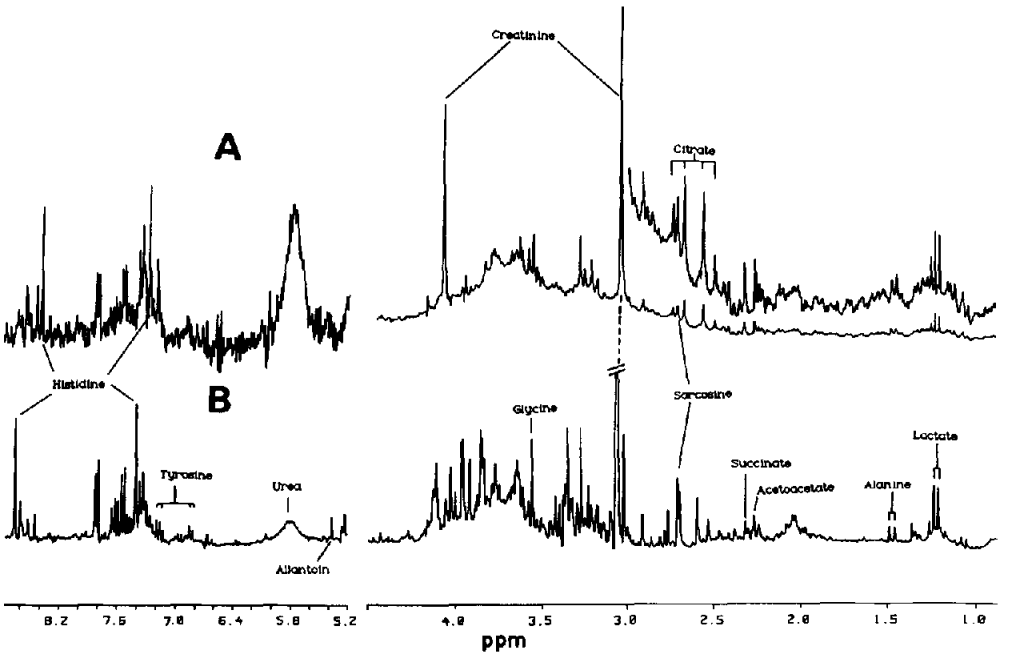

Figure 1
250 MHz ^1H NMR spectra of a control human urine sample (collected prior to treatment with oxpentifylline). (A) Containing 10% ^2H_2O. A total of 2400 FIDs were collected, and an exponential function corresponding to 1 Hz line-broadening was applied prior to Fourier transformation. Solvent suppression was effected using a gated secondary irradiation field applied with the decoupler coils. Digitization of the minor metabolite signals is still poor due to the large dynamic range imposed by the residual water peak; (B) Sample as above but freeze dried and redissolved in ^2H_2O at five times the original concentration. A total of 200 FIDs were collected and a Gaussian resolution enhancement function applied prior to Fourier transformation. Small chemical shift changes are apparent for some signals due to minor pH changes. Selective deuteration of the creatinine CH_2 group has occurred. Digitization of minor signals is greatly improved due to reduction of the dynamic range and increased concentration of metabolites.

The structure of CPDX, the major urinary metabolite of oxpentifylline is shown in Fig. 2. The molecule has several types of proton resonances including singlets and multiplets occurring over a wide range of chemical shifts (i.e. carboxypropyl $CH_2(1)$, δ = 2.23 ppm (triplet); $CH_2(2)$, δ = 1.88 ppm (quintet); $CH_2(3)$, δ = 4.01 ppm (triplet); C_8H, δ = 7.89 ppm (singlet); N_3–CH_3 and N_7–CH_3 (singlets) at 3.54 and 3.96 ppm, respectively). Again it was found that unprocessed urine samples (collected from a subject dosed with oxpentifylline) gave poor quality 250 MHz spectra in which the C_8H and the N-methyl signals of CPDX could barely be discerned even after several thousand FIDs had been accumulated. Therefore, we chose to make all quantitative measurements of CPDX by NMR, using freeze dried urine samples. Typical 250 and 400 MHz

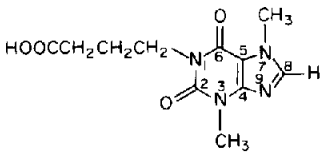

Figure 2
Structure of 1-(3′-carboxypropyl)-3,7-dimethylxanthine (CPDX).

spectra of freeze dried urine samples from a subject treated with oxpentifylline are shown in Fig. 3. In addition to resonances from excreted endogenous compounds, CPDX signals are also observed, those from the C_8H and the $N–CH_3$ protons being particularly strong. No signals from the parent compound or minor metabolites were assigned. Good signal-to-noise ratios could be obtained in less than 5 min with the 400 MHz spectrometer (corresponding to the accumulation of 48–64 FIDs) and in about 10–15 min with the 250 MHz instrument. However, the information content of the 400 MHz spectra was always much higher as more signals could be resolved due to superior signal dispersion. The use of a resolution enhancement technique, e.g. application of a Gaussian function to the FID prior to Fourier transformation, can partially overcome the problem of reduced signal dispersion at 250 MHz, but requires a greater number of FIDs to be effective [15].

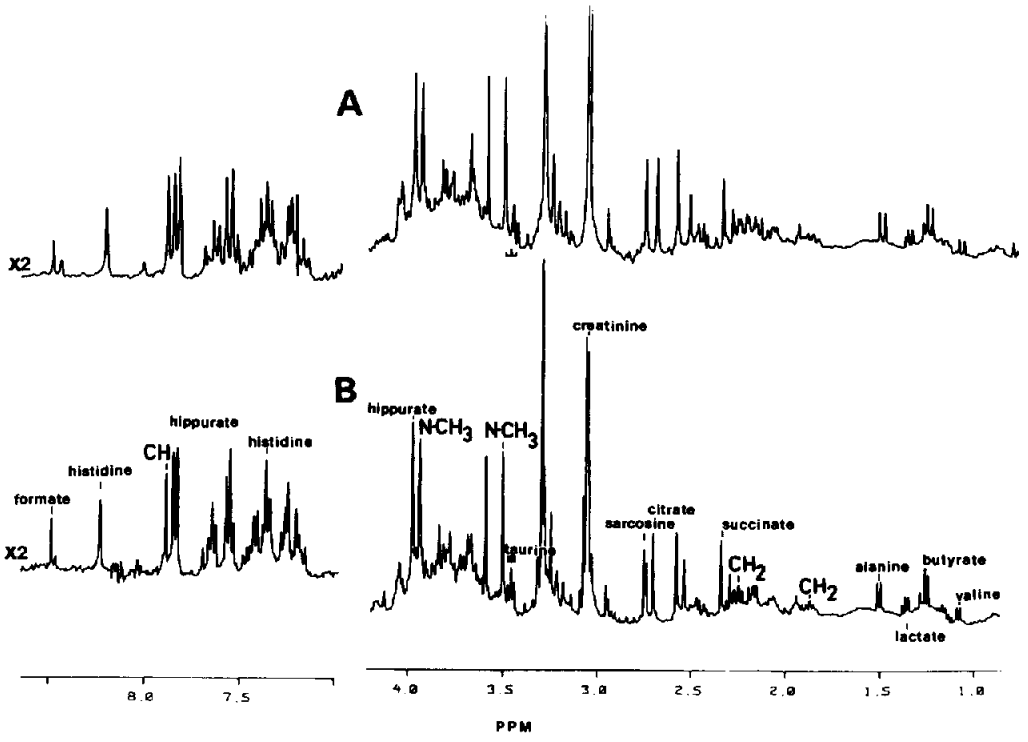

Figure 3
^1H NMR spectra of a freeze dried urine sample (redissolved in ^2H_2O at five times original concentration) collected 0–2 h after a 600 mg dose of oxpentifylline, containing a final concentration of 16.05 mM CPDX. (A) Measured at 250 MHz, the result of 200 FIDs; (B) measured at 400 MHz, the result of 32 FIDs.

Quantification of CPDX from NMR spectra was based on area measurements of the singlet C_8H and N_3-methyl (at 7.89 and 3.54 ppm in Fig. 3) resonances obtained by cutting out and weighing paper traces. The results of these measurements together with those obtained by HPLC are shown in Table 1. Statistical analysis of these data showed that the values for NMR and HPLC concentrations were strongly correlated (all correlation coefficients were >0.99, Table 1). Student's *t* tests for paired samples were performed, comparing HPLC results with those for 250 and 400 MHz NMR data (Table

Table 1
Quantitation of 1-(3'-carboxypropyl)-3,7-dimethylxanthine (CPDX) in urine samples, based on proton NMR and HPLC measurements

Time (h)*	HPLC (mM/l)	NMR data 400 MHz C_8H	N_3–CH_3	250 MHz C_8H	N_3–CH_3
−2/0	ND	ND	(0.1)†	ND	(0.3)†
0–2	3.21	3.09	3.03	3.11	3.56
2–4	7.34	7.24	7.28	7.15	7.54
4–6	4.18	3.98	4.24	3.80	4.76
6–8	1.92	2.01	2.11	2.12	2.66
8–12	2.54	2.40	2.49	2.76	2.97
24–36	5.83	5.56	5.95	5.96	6.46
36–48	6.46	6.61	6.71	6.76	6.92
Student's‡					
t	—	−1.43	−0.846	−0.29	−7.01
P	—	NS	NS	NS	$P<0.001$
Pearson's§					
r	—	0.998	0.995	0.996	0.997

*600 mg doses of oxpentifylline given at 0, 12 and 24 h.
†Due to endogenous urinary components.
‡Paired *t* test, comparisons with HPLC data.
§HPLC versus NMR data, $P<0.001$, all values.

1). Only the 250 MHz NMR data derived from measurements of the N_3–CH_3 signals proved to be unreliable (i.e. significantly different from the HPLC data). The N_7–CH_3 and propyl signals (except C_1) of CPDX could be detected in 250 and 400 MHz spectra of freeze dried urines (Fig. 3), but were too extensively overlapped with endogenous components for quantification to be attempted. In the present case the C_8H signal of CPDX gave the most reliable quantitative results (as judged by HPLC criteria) as this signal was in a region of the urine NMR spectrum where there were few signals from endogenous metabolites. This can be conveniently thought of as a spectral region low in "chemical noise", i.e. where there is little background interference from other molecules. This is distinct from the electronic or instrumental noise that is normally thought of as limiting the sensitivity of NMR experiments. The problem of "chemical noise" is more severe when spectra are measured on lower field strength spectrometers, e.g. at 250 MHz, where (in frequency terms) the signals are not so well dispersed as they are at 400 MHz. This is well illustrated by comparing the closeness to the HPLC data of 250 MHz measurements based on the C_8H resonance of CPDX and that of the less reliable N_3–CH_3 signal (Table 1). In contrast, the 400 MHz spectra gave CPDX concentrations (based on either the C_8H or the N_3–CH_3 signal) that were not significantly different to those given by HPLC. In NMR spectra of urine, the region from 3.0 to 4.1 ppm contains many resonances from endogenous compounds such as CH and some CH_2 signals from simple sugars and amino acids [4]. At 250 MHz, the extensive overlap of the N_3–CH_3 resonance of CPDX with the triplet signal from endogenous taurine, appears to be the main reason that this signal affords an unreliable indication of CPDX concentration (Fig. 3A). These signals are well resolved at 400 MHz (Fig. 3B). In dilute solutions, and in the absence of interfering compounds, it is clearly easier to detect the N–CH_3 signals (three magnetically equivalent protons) of CPDX than that of the

single C_8H proton. However, in urine, the stronger signals are less useful for quantitative purposes, particularly at lower field strengths, as they occur in a band of high chemical noise.

Clearly ^1H NMR spectral measurements can give reliable quantification of drug metabolites in urine samples, even at 250 MHz, provided that the molecules give signals in spectral regions of low chemical noise. At 250 MHz within the normal chemical shift range for protons in small organic molecules, these "windows" are from 0 to 1 ppm, 1.5 to 1.9 ppm, 5 to 7 ppm and from 7.8 to 11 ppm. In 400 MHz spectra, drug metabolite resonances occurring in other spectral regions may be measured with caution, excepting the region from 3.5 to 4.1 ppm which contains many overlapped resonances from endogenous molecules. These can only be simplified in untreated urine samples with the aid of two-dimensional (e.g. shift–shift correlation, COSY) NMR methods [8]. However, it should be noted that drugs which are used in large therapeutic doses such as penicillins [10, 16] or drugs taken in overdose cases (unpublished), can often be readily detected in NMR spectra of urine irrespective of their proton chemical shifts, because concentrations greatly exceed those of endogenous metabolites.

This study demonstrates that widely available NMR spectrometers operating at or about 250 MHz can be usefully applied in the analysis of drug metabolites in freeze dried urine samples, provided that the compounds under study are cleared rapidly in the urine and that they have proton signals in spectral regions of low chemical noise.

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