APPLICATION OF HIGH RESOLUTION ^{1}H-NMR SPECTROSCOPY TO THE DETECTION OF PENICILLAMINE AND ITS METABOLITES IN HUMAN URINE

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SUMMARY

Preliminary studies on the use of high resolution ^{1}H-NMR spectroscopy for the detection of the thiol drug penicillamine and its metabolites in human urine are described. The technique is rapid, simple and requires minimal sample pretreatment. Application of NMR to the qualitative analysis of penicillamine in urine is illustrated by penicillamine disulphide formation from penicillamine following spiking into human urine and the detection of penicillamine, penicillamine disulphide and penicillamine-cysteine disulphide (following oral administration of the drug to patients).

I. INTRODUCTION

Whilst penicillamine has been in clinical use for some time in the treatment of a number of clinical conditions, especially rheumatoid arthritis and Wilson's disease, it is only within the last decade that suitable analytical methods have been developed to enable its determination in biological fluids /1/. The majority of these methods have been based on high performance liquid chromatography (HPLC), generally, though not exclusively, using electrochemical detection (review see Perrett and Rudge, 2). Although this approach is more than adequate for the determination of unchanged penicillamine, with good sensitivity and high specificity, the methods rely on the presence of a free sulphydryl group and are consequently less satisfactory for the measurement of thiomethyl metabolites of penicillamine or disulphides formed metabolically or chemically by oxidative reactions in the samples themselves. Information on the quantities and types of penicillamine derived materials present in biological samples is, consequently, difficult to obtain and this limits the understanding of the pharmacokinetics, pharmacology and toxicology of the drug. Currently, strategies aimed at determining "total" penicillamine depend on the chemical or electrochemical reduction of any disulphides present, assaying the liberated thiol, and determining the free and total by difference. In the case of penicillamine in particular this methodology suffers from the problem that the disulphides of this compound are somewhat refractory to reduction because of steric hindrance. The mixed penicillamine-cysteine (cys-pen) disulphide is significantly easier to

reduce than the homodisulphide (pen-pen). In addition the very act of reducing the disulphides results in the loss of information on the metabolic fate of the drug and its reaction with endogenous cysteine and possibly other thiols, an interaction which may have toxicological significance. Reduction methods give no information on the amount of thiomethyl or other possible S derivatised moieties.

High resolution ^{1}H-NMR spectroscopy can be used to detect and quantify a wide range of endogenous and xenobiotic metabolites present in biological fluids such as urine and plasms /3-10/. With very high field spectrometers (400-500MHz) this can usually be achieved without extensive physical preparation or chemical pre-treatment of the samples. Indeed all that is required to enable the detection, and if desired the quantification, of xenobiotics and their metabolites is that they have suitable resonances and are present in the millimolar concentration range. For example we 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 /6/. In the present study we have applied proton NMR urinalysis techniques to the assay of penicillamine and its mixed and homo-disulphides in human urine.

II. EXPERIMENTAL

2.1 Chemicals

Penicillamine, cysteine and penicillamine disulphide were obtained from the Sigma Chemical Co., (Poole, U.K.), Ellman's reagent (5,5-dithiobis-2-nitrobenzoic acid, DTNB) was purchased from Aldrich Ltd. (Gillingham, U.K.). All chemicals were used without further purification.

2.2 Samples

Control urine samples were provided by healthy volunteers. Urine samples, from subjects undergoing treatment for Wilson's disease and receiving 500mg of D-penicillamine a day, were collected and

frozen without further treatment or collected into vessels containing 1 mgml^{-1} of DTNB (to derivatise any free penicillamine in order to prevent further oxidation to pen-pen). Samples were then frozen at −20°C until analysed by proton NMR as described below. Penicillamine was also spiked into control urine samples at a concentration of 400μg/ml to allow the formation of the homodisulphide to be observed. All samples were otherwise untreated except for the addition of 10% of D_2O.

2.3 NMR Spectroscopy

400MHz proton NMR spectra were measured on a Bruker WH 400 spectrometer operating at 25°C in quadrature detection mode. All spectra were the result of 96 free induction decays (FID's) which were collected into 16,384 data points which were then zero filled to 32,768 points and multiplied by an exponential weighting factor corresponding to 0.5Hz prior to Fourier transformation. A pulse angle of 45 (4μs) was employed with a total recycle time of 5s to ensure full T1 relaxation of sample protons. The water signal was suppressed by application of a secondary irradiation field at the water resonance frequency. Spectra were referenced internally to sodium trimethylsilyl (D4) propionate (δ=0ppm) which was present at final concentration of 1mM and added with the D2O.

III. RESULTS AND DISCUSSION

As we have discussed in previous papers on NMR urinalysis /3,6,9/ there are many regions of the typical urinary proton NMR spectrum that are relatively free of "chemical noise" i.e. interference from the overlapping signals of those endogenous metabolites present at the millimolar or submillimolar level. In such spectral regions xenobiotic metabolites may be detected and measured with comparative ease. In figure 1a the spectral region covering the range 1 to 3.5ppm of a control human urine is shown. Relatively few signals from endogenous molecules are present, those from a creatinine and citrate being the most prominent. In figure 1b the

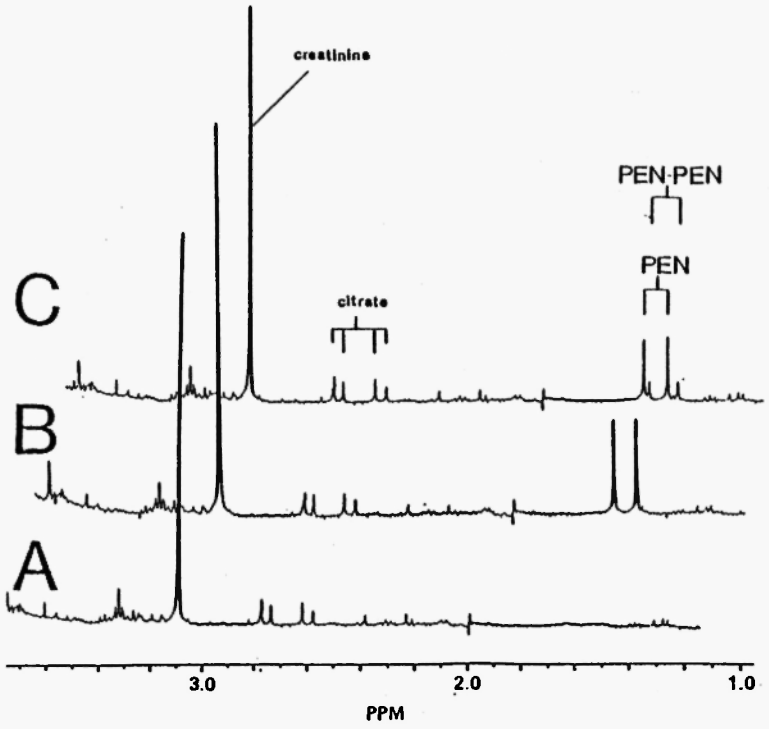

Fig. 1: ^{1}H-NMR spectra of:

a) control human urine showing the region 1.0 to 3.6ppm.

b) control human urine into which penicillamine had been added at a final concentration of 400 g/ml.

c) the same sample as in spectrum b) after standing at room temperature for 120 minutes.

same sample is shown following a standard addition of D-penicillamine (400μg/ml). A prominent pair of singlets (δ≐1.55 and 1.65ppm) due to the presence of two non-equivalent methyl groups on the β-carbon adjacent to the thiol group can be seen. After this sample had been aged at ambient temperature for 120 minutes at pH6.6, the NMR spectrum was remeasured and is shown in figure 1c. An extra pair of doublets was observed (δ≐1.52 and 1.63ppm) due to the formation of the internal disulphide (pen-pen, confirmed by standard addition of the authentic compound). This reaction is favoured at higher pH's as a greater proportion of the thiol is deprotonated.

In figure 2 the spectrum of a urine sample obtained from a subject undergoing treatment with 500mgs per day of D-penicillamine is shown. This urine was treated with DTNB on collection in order to prevent the loss of penicillamine to the homodisulphide on storage. This treatment was necessary as it was not possible in this instance to measure NMR spectra of these samples immediately on collection. Where immediate analysis can be performed, sample pretreatment can be limited to acidification and/or the addition of EDTA. In addition to the two prominent singlet signals from the homodisulphide methyl protons (from pen-pen previously formed in the body or the bladder urine) a further pair of singlets from the cysteine-penicillamine disulphide was present. In some samples signals from the DTNB-penicillamine derivative, which represented

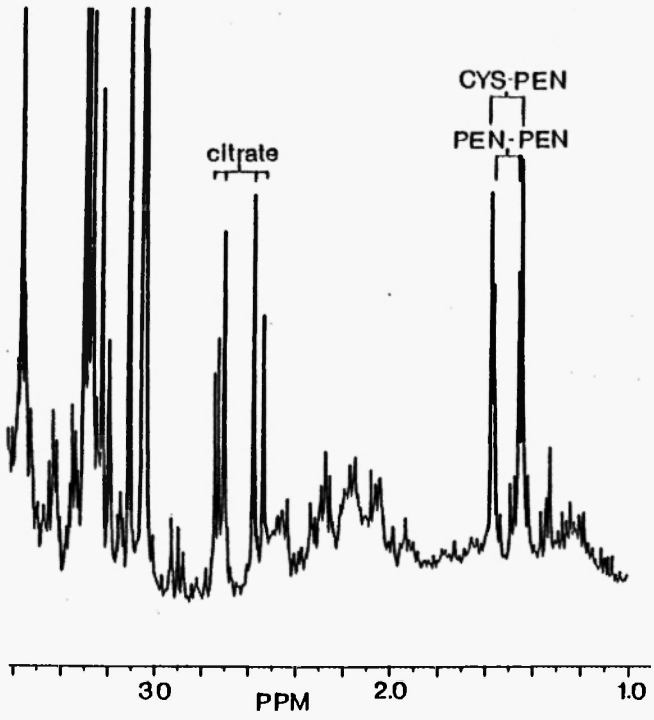

Fig. 2: ^{1}H-NMR spectrum showing the region 1.0 to 3.6ppm obtained for the 3 to 6h urine sample from a patient receiving a single 500mg dose of penicillamine.

the relatively small amount of free drug present in the newly voided urine were also detectable. The signals from these penicillamine related materials are sufficiently well resolved to allow quantitative measurements to be made. The total analysis time for this procedure was 5 minutes per sample and each of the metabolites was detected with equal sensitivity. This compares favourably with HPLC techniques where it is likely that considerable sample pretreatment would be required in order to obtain data on the homo and mixed disulphides.

We have now conducted preliminary studies on urines from a number of subjects treated with penicillamine. These experiments have been very promising in showing that proton NMR is sufficiently sensitive and specific to enable the qualitative analysis of penicillamine and related compounds, following administration at therapeutic doses, in human urine without difficulty. In future studies we will extend this work to the quantitative analysis of these compounds in urine and other biofluids.

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