Spin-echo ^{1}H N.M.R. Spectroscopy: a New Method for studying Penicillin Metabolism

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High-field, spin-echo ^{1}H n.m.r. spectroscopy has been used to detect the diketopiperazine, (**1**), a new metabolite of ampicillin, (**2**), in rat urine.

Conventional detection and assay methods for penicillins and their metabolites in biological fluids include various microbiological[1] and chromatographic methods.[2] The former methods are sensitive but imprecise and give no information on metabolites. The chromatographic methods, especially h.p.l.c., can give information on metabolites but are subject to interference from endogeneous fluid components. Precipitation, solvent extraction, and pre- and post-column derivatisation may be necessary to remove this interference. In addition, each chromatographic or microbiological method may be specific for only one penicillin. We have found that high-field, spin-echo ^{1}H n.m.r. spectroscopy is a powerful method for detecting and quantitating penicillins and their metabolites in urine. The n.m.r. method is relatively insensitive (detection limits[3] *ca.* 50 nmol ml^{-1}) but it is fast, requires minimal sample preparation, is general for all penicillins, and does give information on metabolites. In addition, because of the dependence of ^{1}H n.m.r. spectral parameters on structure, the detection of n.m.r. signals from a metabolite gives at least some indication of structure. We have used the n.m.r. method, for the first time, to study the metabolites of ampicillin, an important aminopenicillin, in the rat.†

Figure 1 shows part of the 400 MHz spin-echo ^{1}H n.m.r. spectrum of the 0—2 h, post-dose [intravenous, 700 mg kg^{-1}

† For a biochemical application of this method, see J. K. Nicholson, M. P. O'Flynn, P. J. Sadler, A. F. Macleod, S. M. Juul, and P. H. Sönksen, *Biochem. J.*, 1984, **217**, 365.

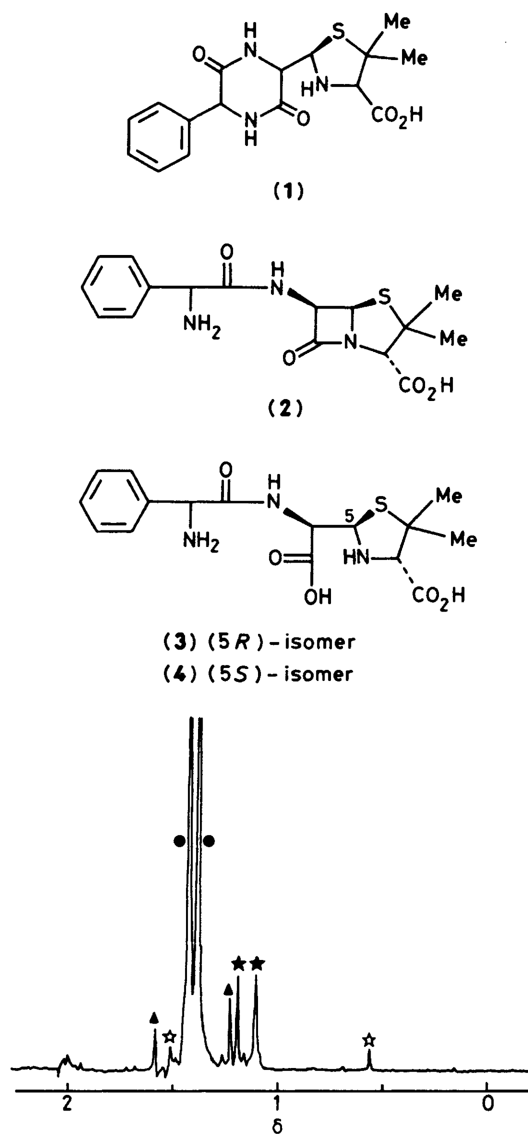

Figure 1. The high-field region of the 400 MHz Hahn spin-echo ^{1}H n.m.r. spectrum of the 0—2 h post-dose rat urine (450 μl plus 50 μl D_2O). Free induction decays (144) were acquired into 16K points, over 4807.7 Hz, with a re-focussing time of 60 ms and a delay of 2.4 s. The water signal was partially saturated by homodecoupling. A line-broadening of 1 Hz was applied prior to Fourier transformation. See text for signal assignments.

of (**2**)] urine of a rat dosed with the sodium salt of (**2**). The spin-echo excitation serves to eliminate broad resonances and phase sorts[4] multiplet resonances (with J_{av} *ca.* 8 Hz) into positive (singlets, triplets) and negative (doublets, quartets) phase. The penicillin and its metabolites are easy to detect since their sharp, paired, *gem*-dimethyl resonances occur at high-field (δ 1.8—0.3), in a region of the spectrum which we have found to be clear of endogenous component resonances in all the rat and human urine samples we have examined so far. The *gem*-CH_3 resonances of (**2**) are marked with solid circles (●). The other three pairs of *gem*-CH_3 resonances are due to three metabolites. The resonances of the natural (5*R*)-isomer of the penicilloic acid (**3**) are shown with solid stars (★) and those of the epimerised (5*S*)-isomer (**4**) with open stars (☆). These are known[5,6] mammalian metabolites of (**2**). The signals marked with the solid triangles (▲) are due to the *gem*-CH_3 groups of (**1**), the new metabolite. The

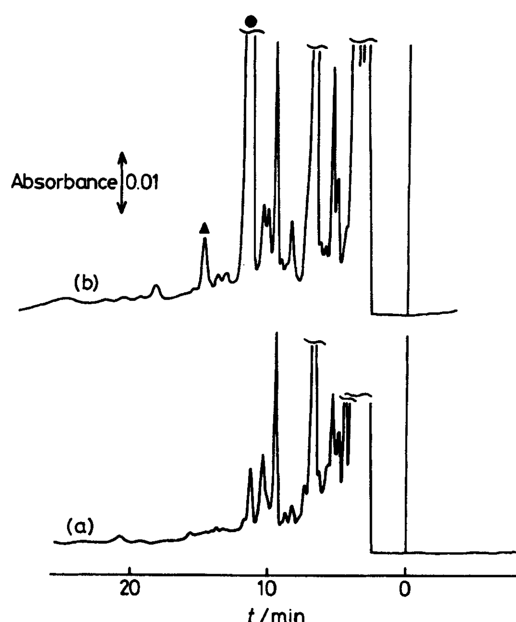

Figure 2. (a) The chromatogram of the control rat urine; (b) that of the 0—2 h post-dose rat urine. The chromatograms were produced by linear programmed elution from 90% eluent A, 10% eluent B to 75% A, 25% B over 5 min. Eluent A was 0.5 M sodium formate, pH 5. Eluent B was 0.05 M sodium formate, pH 5, containing 37.5% v/v acetonitrile. The flow rate was 1.0 ml min^{-1}. The samples (20 μl) were loaded onto a C-18 μBondapak column (Waters, 300 × 4 mm) and the u.v. detection wavelength was 240 nm. The elution time *t* is in minutes. The peak marked with the solid circle (●) is due to ampicillin.

assignments were initially made on the basis of the ^{1}H n.m.r. chemical shifts[6,7] and spiking experiments with authentic (**1**), (**2**), and (**3**). The assignment of (**1**) was confirmed by h.p.l.c. Figure 2b shows the h.p.l.c. trace of the dosed rat urine. The peak marked with the solid triangle (▲) coeluted with authentic (**1**) and was absent from the chromatogram of control urine, Figure 2a. The concentration of (**1**) in the urine was *ca.* 400 μg ml^{-1} (n.m.r.) and *ca.* 315 μg ml^{-1} (h.p.l.c.).

Compound (**1**) has not previously been reported as a metabolite of (**2**) although it is known to be an *in vitro* product of the incubation of (**2**) with serum albumin.[8] The discovery of (**1**) extends current knowledge of the mammalian metabolism of aminopenicillins and demonstrates the power of the n.m.r. method.

We thank Mr. A. E. Bird for encouragement and advice, Mr. E. A. Cutmore for carefully checking the manuscript, Dr. G. E. Hawkes (U.L.I.R.S.) for making n.m.r. facilities available, and Mr. B. P. Hatton for supplying authentic (**1**).

Received, 26th April 1984; Com. 589

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