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Short Communication

A study of flucloxacillin metabolites in rat urine by two-dimensional ^{1}H, ^{19}F COSY NMR

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Introduction

High field ^{1}H NMR spectroscopy has recently been shown to be a powerful tool with which to detect and characterize drugs and their metabolites in intact biofluids [1] The method is fast (<5 min/sample) and involves minimal sample preparation and in contrast to many chromatographic techniques does not require different experimental protocols for the detection of different classes of compounds, i e it is a general method Any drug or metabolite with suitable protons in its structure will be observed when present at concentrations exceeding the detection threshold value of ~50 μM This feature has been very useful in detecting novel metabolites of penicillins [2]

There are, however, some problems associated with the ^{1}H NMR detection of drug metabolites in intact biofluids The first is the observation of metabolite ^{1}H resonances in the presence of the large signal due to water (>100 M) The resulting dynamic range problem necessitates the use of some form of selective water suppression [1] Secondly, the large number of resonances from endogenous biofluid components leads to considerable spectral crowding in the rather narrow chemical shift range (~10 ppm) of the proton This spectral crowding can interfere with the observation and quantitation of drugs and their metabolites in the biofluid

For fluorinated drugs, the dynamic range and spectral crowding can be overcome by using ^{19}F NMR detection rather than ^{1}H NMR as has been recently demonstrated [3–5] These reports highlighted the advantages of ^{19}F NMR in biofluid studies, particularly the high relative sensitivity (83% of the sensitivity of ^{1}H NMR), the large chemical shift range (~800 ppm) and the absence of fluorinated endogenous compounds

However, proton decoupling is often essential for maximizing sensitivity and for proton-decoupled ^{19}F NMR spectra, i e $^{19}F\{^{1}H\}$ NMR, only a single resonance line is observed for each fluorine label in the molecule Even if decoupling is not applied and proton-coupled ^{19}F multiplets are observed the $^{n}J_{H,F}$ data obtained gives only the

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number of protons coupling to the fluorine nucleus and no information on their chemical environment In order to obtain the maximum amount of information on fluorinated metabolites of unknown structure, it is important to know both the number and type of protons in the vicinity of the fluorine label In this study this goal was achieved by the application of two-dimensional (2-D) ^{1}H, ^{19}F correlation spectroscopy (2-D, ^{1}H, ^{19}F COSY) This technique effects the correlation of proton and fluorine-19 nuclei via a scalar coupling interaction, $^{n}J_{H,F}$ The resulting 2-D ^{1}H, ^{19}F COSY NMR spectrum directly gives the chemical shift of each proton coupled to every ^{19}F nucleus The use of ^{19}F detection eliminates the dynamic range and spectral crowding problems of ^{1}H NMR observation as it does for the 1-D ^{19}F NMR experiments A further major advantage results from the application of 2-D ^{1}H, ^{19}F COSY with ^{19}F detection since overlapping ^{1}H signals (coupled to ^{19}F) from corresponding protons in very similar metabolites are resolved, due to the spreading of ^{19}F resonances across the fluorine dimension of the 2-D NMR spectrum

This communication describes the application of the 2-D ^{1}H, ^{19}F COSY experiment to the observation of flucloxacillin (**I**) and its metabolites (**II–IV**, chemical names given in Results and Discussion, structures in Fig 1) in rat urine

Experimental

Four male, albino rats (CFY strain, Charles River), weighing approx 250 g were placed in individual metabolism cages with food and water freely available On the day of the experiment, two of the animals, Nos 1 and 2, were given an intravenous bolus injection of sodium flucloxacillin equivalent to 200 mg kg^{-1} of the pure free acid (dose volume 1 ml sterile water kg^{-1}) The remaining animals, Nos 3 and 4, were given an intravenous injection of sterile water (dose volume 1 ml kg^{-1}) and served as controls All animals were returned to the metabolism cages after dosing Urine was collected directly into individual CO_2(s) cooled containers over periods of 0–6 and 6–24 h after dosing Samples were stored at −70°C until assayed The concentrations of **I–IV** in the 0–6 h post-dose urine of rat No 2 were determined by ^{19}F NMR [3] and microbiological (MB) assay [3] to be **I**, ~1700 (NMR), ~1800 (MB), **II**, ~230 (NMR), **III**, ~140 (NMR), and **IV**, ~100 μg ml^{-1} (NMR)

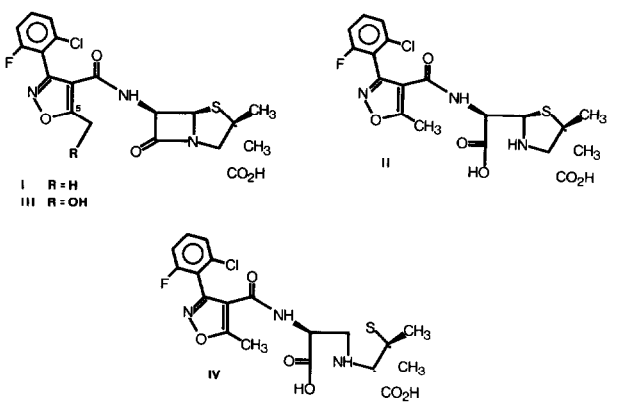

Figure 1
The structures of compounds **I–IV**

The urine samples for NMR spectroscopy (450 μl) were used neat and unfiltered D_2O (50 μl) was added to act as an internal lock of the magnetic field All NMR spectra were acquired on a Bruker AM400 spectrometer equipped with a 5 mm ^1H, ^{19}F dual probe ^1H and ^{19}F NMR spectra were obtained at resonance frequencies of 400 13 and 376 50 MHz, respectively One-dimensional ^{19}F free induction decays (FIDs) were collected into 32K data points with a sweep width of 7042 Hz A 0 5 Hz line-broadening function was applied prior to Fourier transformation (FT) One-dimensional ^1H FIDs were collected into 32K data points with a sweep width of 5208 Hz, using gated irradiation water suppression A 0 3 Hz line-broadening function was applied prior to FT

The ^1H, ^{19}F heteronuclear shift correlated 2-D NMR spectra were obtained using the polarization transfer experiment of Bax and Morris [6] with broad-band ^1H decoupling during ^{19}F acquisition Sweep widths of 7 kHz for ^{19}F (F_2) and 2 kHz for ^1H (F_1) were employed, the ^{19}F FIDs being acquired into 4K data points Thirty-two scans and two dummy scans were collected for each of 128 increments (0 125 ms) of the evolution time A polarization transfer time of 84 ms, optimized for $^nJ_{H,F}$ 6 Hz, and a relaxation time of 1 s were used The 90° ^{19}F and ^1H pulses were 14 0 and 9 9 μs, respectively and the total acquisition time was 97 min The raw data was zero-filled, and processed using unshifted sine–bell functions in both dimensions, to give a 2K × 1K matrix after double FT

Results and Discussion

The metabolism of flucloxacillin and the identification of the resultant metabolites have been studied by a number of groups [3, 7, 8] The three major metabolites identified by these workers were (5R)-flucloxacillin penicilloic acid (**II**) 5′-hydroxymethylflucloxacillin (**III**) and (5S)-flucloxacillin penicilloic acid (**IV**)

Figure 2 shows the ^1H NMR spectra of the 0–6 h post-dose urine of rat No 2 and that of a 0–6 h control urine sample from rat No 3 The additional high field resonances in the post-dose spectrum (δ_H ~1 6–1 0 ppm) have been unambiguously assigned to the gem-dimethyl groups of **I**, **II** and **IV** [3] Even at the higher field strength of this study (400 vs 250 MHz), resolution of the gem-dimethyl resonances of **III** was incomplete The aromatic region of the post-dose urine ^1H NMR spectrum (rat No 2) also showed additional resonances (Fig 2) due to **I–IV** However, these resonances were subject to severe overlap by one another and by the resonances of endogenous compounds such as hippurate and assignment was therefore not possible

Figure 3 shows the ^{19}F {^1H} NMR spectrum of the 0–6 h post-dose urine of rat No 2 Four resonances are clearly observed, free of overlap by any endogenous components and of any dynamic range problem (the assignments were confirmed previously by "spiking" experiments with authentic materials [3]) The presence of discrete resonances for **I–IV** is a good indication of the sensitivity of ^{19}F chemical shifts to remote structural change, even 8–10 bonds distant This ^{19}F chemical shift dispersion allows the separate characterization of the proton resonances connected to each fluorine atom (via scalar coupling) by the use of the 2-D ^1H, ^{19}F COSY experiment From such data the structural environment of the connected protons in each metabolite can be determined

Figure 4 shows a contour plot of the 2-D ^1H, ^{19}F COSY NMR spectrum of the 0–6 h post-dose urine of rat No 2, together with the corresponding F_2 (^{19}F) projection and ^1H NMR spectrum Each of the four fluorine resonances exhibited correlations to two coupled protons These correspond to the protons *ortho* and *meta* to the fluorine nuclei ($^3J_{H,F}$ ~9 Hz, $^4J_{H,F}$ ~6 Hz) The COSY experiment was tuned for $^nJ_{H,F}$ values of ~6 Hz

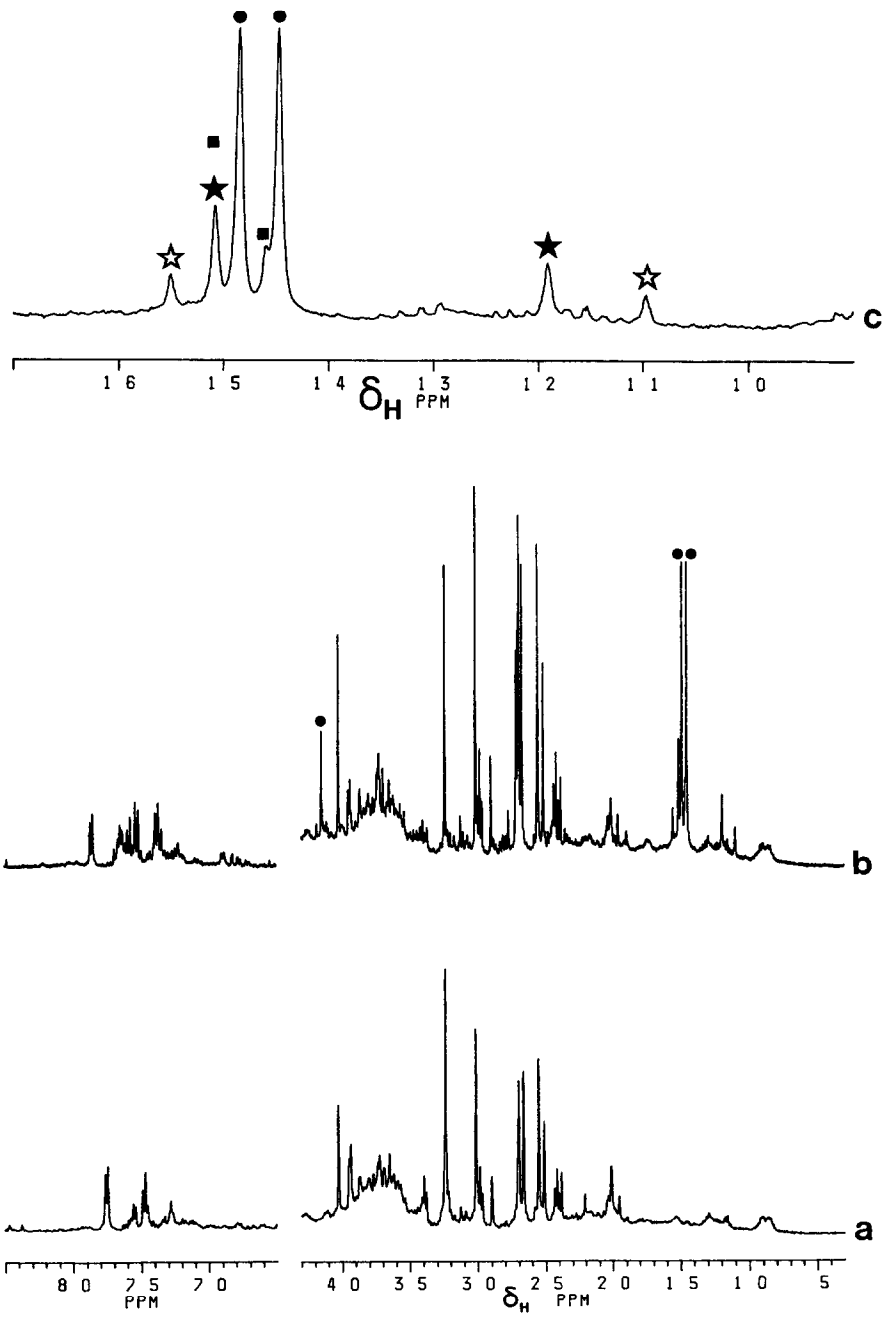

Figure 2
The 400 MHz ^{1}H NMR spectra of (a) the 0–6 h urine of rat No 3 (control), (b) the 0–6 h post-dose urine of rat No 2, (c) the high field expansion of (b) Key ●, I, ★, II, ■, III, ☆, IV

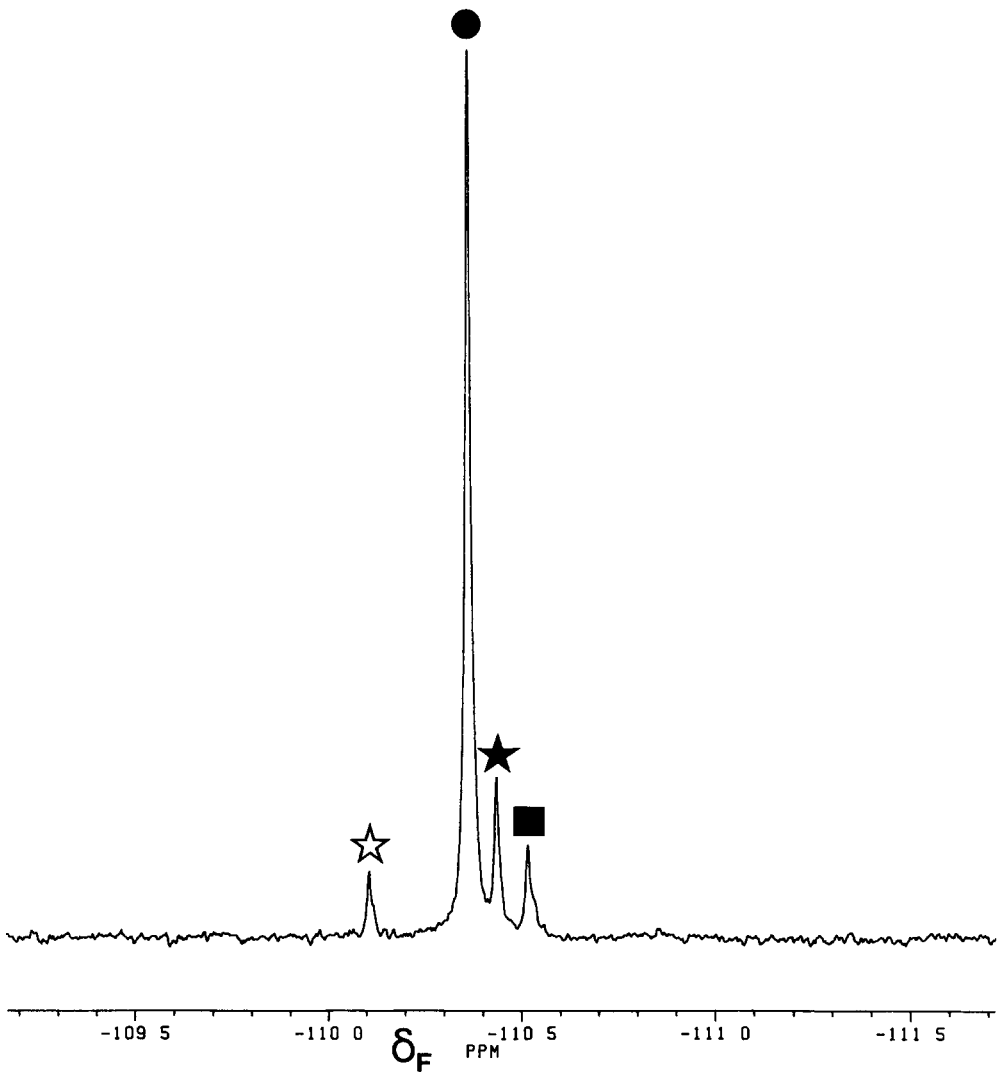

Figure 3
The 1-D ^{19}F {^{1}H} NMR spectrum of the 0–6 h post-dose urine of rat No 2 Key ●, I, ★, II, ■, III, ☆, IV

(D2 = 84 ms) and thus no correlations were either expected or observed to the *para*-protons ($^{5}J_{H,F}$ ~0 7 Hz) The contour plot clearly shows that as a result of the ^{19}F chemical shift dispersion the otherwise overlapped resonances of the *ortho*- and *meta*-aromatic protons of **I–IV** are resolved *and* freed from interference by endogenous component resonances The proton chemical shifts of each proton coupled to each ^{19}F nucleus in **I–IV** could thus be obtained In this particular case, the known metabolites **II–IV** are all similar in structure to the drug flucloxacillin (**I**) in the fluorine-containing aromatic ring Thus, the protons coupled to the ^{19}F nucleus exhibit similar chemical shifts For unknown metabolites the ability to obtain information on the chemical environment of protons in the vicinity of the fluorine label could be crucial in a structure determination Two-dimensional ^{1}H, ^{19}F COSY NMR is a powerful tool with which to

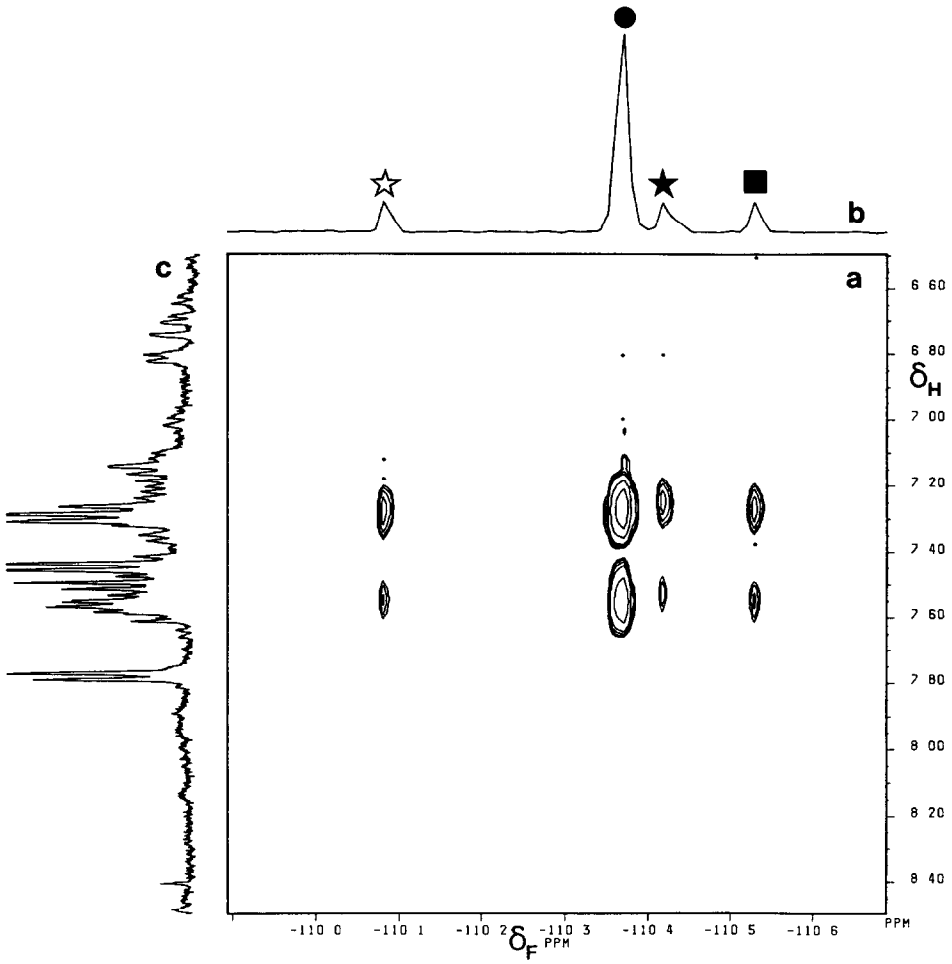

Figure 4
(a) A contour plot of the 2-D ^1H, ^{19}F COSY NMR spectrum of the 0–6 h post-dose urine of rat No 2 The dotted lines trace out the connectivity between each fluorine and the *ortho*- (δ_H ~7 25) and *meta*- (δ_H ~7 55 ppm) protons to which it is coupled (b) The ^{19}F (F2) projection of the 2-D spectrum (c) The corresponding 400 MHz 1-D, ^1H NMR spectrum Key ●, I, ★, II, ■, III, ☆, IV

obtain this information since metabolites can be observed with good sensitivity (w r t ^1H NMR) in complex biofluids with no interference from endogenous component ^1H resonances

Conclusion

Two-dimensional ^1H, ^{19}F correlation spectroscopy has been shown to be a powerful technique for the detection and characterization of flucloxacillin and its metabolites in rat urine The methodology described has considerable potential in studies of the metabolism of other fluorinated drugs

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