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^{19}F NMR spectroscopy study of the metabolites of flucloxacillin in rat urine

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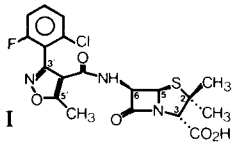
^{19}F NMR spectroscopy was used to monitor the metabolites of flucloxacillin in the urine of a rat dosed with its sodium salt. ^{19}F NMR signals were detected and quantified from flucloxacillin and three metabolites. The ^{19}F NMR method involves minimal sample preparation and is free from interference by endogenous urine components. High-field spin-echo ^1H NMR spectroscopy and HPLC were used to confirm the results.

It has recently been shown by Everett et al (1984) that high-field, spin-echo ^1H NMR spectroscopy is a useful method for studying the metabolites of penicillins in urine, with some advantages over conventional methods. ^1H NMR spectroscopy has also been used recently (Bales et al 1984) to study the urinary metabolites of paracetamol (acetaminophen). The ^1H NMR method is relatively insensitive but it is fast, general, requires minimal sample preparation, yields structural information and also allows the simultaneous monitoring of a wide range of low molecular weight, endogenous urine components. There are two difficulties with the method. Firstly, the observation of proton resonances at possibly sub-millimolar levels in the presence of the huge resonance from water (~100 M), requires the use of selective water suppression techniques. This is normally not a problem unless transfer of saturation from the water to a signal of interest nulls that signal. The second difficulty is caused by the fact that resonances are observed from a wide range of endogenous low molecular weight urine components in the rather narrow chemical shift range (~10 ppm) of the proton. These endogenous component resonances may interfere with the detection of signals from the drug and its metabolites. The purpose of this study was to demonstrate that for fluorinated drugs administered at relatively high doses, such as flucloxacillin (I), ^{19}F NMR spectroscopy can be used to detect and quantify the drug and its metabolites in a biological fluid without interference from endogenous components.



I

* Correspondence.

Since this work was completed Stevens et al (1984) have described the use of ^{19}F NMR spectroscopy to monitor tissue concentrations of 5-fluorouracil and its metabolites in mice and Malet-Martino et al (1983) have described the use of ^{19}F NMR to monitor the blood levels of 5′-deoxy-5-fluorouridine.

MATERIALS AND METHODS

Samples and chemicals

The urine samples for NMR spectroscopy (450 µl) were used neat, unfiltered and untreated except for the addition of D_2O (50 µl) to lock the magnetic field.

The sodium flucloxacillin was Beecham commercial material. The disodium flucloxacillin penicilloate used for spiking purposes had been prepared for previous work (Munro et al 1978). It was shown by ^1H NMR spectroscopy to be a mixture of the 5*R* (72% pure free acid) and 5*S* (14% pure free acid) diastereomers.

Dosage

A male, albino rat (CFY strain, Hacking and Churchill), approximately 450 g, was placed in a metabolism cage with food and water freely available.

On the day of the experiment the animal was given an intravenous bolus injection of 200 mg pure free acid kg^{-1} as sodium flucloxacillin (dose volume: 1 ml sterile water kg^{-1}) and then returned to the metabolism cage.

Urine was collected directly into individual CO_2(s) cooled containers for a period of 24 h before dosing and then at frequent intervals after dosing.

All urine samples were stored at −70 °C until assayed.

Microbiological assay

All samples were assayed microbiologically using a

large-plate agar diffusion technique with *Sarcina lutea* NCTC 8340 as the assay organism.

^{19}F NMR spectroscopy

235 MHz ^{19}F NMR spectra were obtained on a Bruker WM 250. Free induction decays (FIDs) were acquired with a 30° pulse (5 μs), over 10 kHz, into 16384 data points, using a relaxation delay of 3·2 s. Broadband proton decoupling was used to eliminate $^{n}J_{HF}$ splittings. The FIDs were line-broadened by 2 Hz before Fourier transformation. Each spectrum took 14·5 min to acquire and was referenced to external $CFCl_3$. The probe temperature was ~30 °C.

^{1}H NMR spectra

250 MHz Hahn spin-echo ^{1}H NMR spectra were obtained with gated water suppression on a Bruker WM 250. The refocussing time was 60 ms. FIDs were acquired over 3205 Hz into 16384 data points with a relaxation delay of 1·5 s. The FIDs were line-broadened by 1 Hz before Fourier transformation. Each spectrum took 9·6 min to acquire and was referenced to internal water at δ = 4·8 ppm. The probe temperature was 24–25 °C.

HPLC

Two M600A pumps (Waters) were used together with an M660 programmer (Waters), a model 7125 injector (Rheodyne) and a model 212 detector (Cecil). Chromatograms were produced using a C-18 μBondapak column (Waters 300 × 4 mm) by linear programmed elution from 90% eluent A, 10% eluent B to 20% eluent A, 80% eluent B over 10 min. Eluent A was 0·05 M sodium dihydrogen phosphate, pH 7·0, containing 5% v/v acetonitrile and 0·1% v/v ethanolamine. Eluent B was 0·05 M sodium hydrogen phosphate, pH 7·0, containing 40% v/v acetonitrile and 0·1% v/v ethanolamine. The injection volume was 20 μl, the detection wavelength was 240 nm and the urine samples were diluted seven-fold with eluent A before injection. The flow rate was 1 ml min^{-1}.

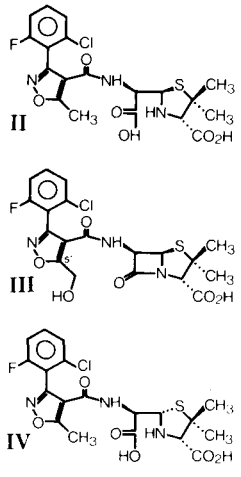
RESULTS

Table 1 gives the concentrations of flucloxacillin (I), (5*R*)-flucloxacillin penicilloic acid (II), 5′-hydroxymethylflucloxacillin (III) and (5*S*)-flucloxacillin penicilloic acid (IV) in the rat urine as determined by ^{19}F NMR, ^{1}H NMR and microbiology. Table 2 gives the ^{1}H and ^{19}F NMR parameters of the standards I, II, III and IV in D_2O. Table 3 gives the ^{1}H and ^{19}F NMR parameters of I, II, III and IV in three of the urine samples.

Table 1. The concentrations (μg ml^{-1}) of I, II, III and IV in the urine of a rat dosed with sodium flucloxacillin[a,b].

Sample		Compound I	II	III	IV
0–2 h urine: (2·8 ml)	^{19}F NMR	870	200	~60	~40
	^{1}H NMR	910	170	—	~40
	MB[f]	910	—	—	—
2–4 h urine: (1·4 ml)	^{19}F NMR	1910	510	140	110[e], 90
	^{1}H NMR	[d]	[d]	—	[d]
	MB	1940	—	—	—
4–6 h urine: (0·9 ml)	^{19}F NMR	630	210	~50	110
	^{1}H NMR	710	170	—	120
	MB	740	—	—	—
6–8 h urine: (1·0 ml)	^{19}F NMR	480	160	~40	100
	^{1}H NMR	520	120	—	90
	MB	660	—	—	—
8–24 h urine: (8·9 ml)	^{19}F NMR	80	~50[e], ~50	~30	150[e], 110
	^{1}H NMR	120	—	—	~60
	MB	130	—	—	—

[a] For ^{19}F NMR, all results by peak heights relative to spiking II into the 2–4 h urine.
[b] For ^{1}H NMR, all results at 250 MHz by peak heights relative to spiking II into the 0–2 h urine. The peak chosen for quantitation was the high field *gem*-CH_3 resonance as this was least interfered with by the other resonances.
[c] Independent results based on peak height increases from the spiking of IV into the 2–4 h urine.
[d] No sample available for ^{1}H NMR.
[e] Independent result based on peak height increases on spiking II into the 8–24 h urine.
[f] MB = microbiology.



DISCUSSION

Flucloxacillin has been reported (Murai et al 1983; Thijssen 1980) to be metabolized in man to the penicilloic acid, the antibacterially active 5′-hydroxymethyl derivative (III) and the penicilloic acid of the 5′-hydroxymethyl derivative (V). The metabolism of flucloxacillin in the rat has been

Table 2. A table of ^1H and ^{19}F NMR chemical shifts δ in ppm and coupling constants ^nJ in Hertz for I, II, III and IV in D_2O (≈0·05 M).

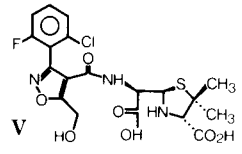
	Parameter									
	δF	$^nJ_{HF}$			δH					$^nJ_{HH}$
Compound		^3J	^4J	^5J	*gem*-CH_3	5′-CH_3	H-3	H-5,6	Aromatics	$^3J_{5,6}$
I	−110·37	9·3	6·0	0·7	1·47, 1·43	2·66	4·12	5·55, 5·47	≈7·6–7·2	4·1
II[b]	−110·59	9·3	5·9	[a]	1·51, 1·20	2·70	3·39	4·90, 4·25	≈7·7–7·2	6·7
III	−110·67	9·3	6·0	[a]	1·51, 1·46	[d]	4·18	5·57, 5·51	≈7·7–7·2	4·0
IV[c]	−110·26	9·3	6·0	0·7	1·55, 1·10	2·77	3·37	4·96, 4·76	≈7·7–7·2	3·0

[a] Not resolved. [b] Initial product of mild basic hydrolysis of I. [c] formed from II on standing in mild base. [d] δ 5′-CH_2 = 5·01 ppm, singlet.

Table 3. A table of ^1H and ^{19}F NMR chemical shifts δ in ppm for I, II, III and IV in urine.

	Parameter						
	δF				δH (*gem*-CH_3)		
Sample	I	II	III	IV	I	II	IV
0–2 h rat urine	−110·36	−110·42	−110·53	−110·07	1·49, 1·45	1·51, 1·19	1·56, 1·10
0–2 h rat urine spiked with II and IV	—	—	—	—	1·50, 1·46	1·52, 1·20	1·56, 1·11
2–4 h rat urine	−110·35	−110·42	−110·51	−110·08	—	—	—
2–4 h rat urine spiked with II and IV	−110·35	−110·41	−110·51	−110·08	1·49, 1·45	1·51, 1·19	1·55, 1·10

reported (Murai et al 1983) to be similar to that in man and III has been isolated from rat urine (Thijssen 1979).



V

Murai et al (1983) and Thijssen (1980) did not specify the stereochemistry of the flucloxacillin penicilloic acid metabolite which they identified solely by co-elution with an authentic sample on HPLC. In our hands, the methods of preparation of the penicilloic acid which they used as the chromatography marker gave a mixture of the 5*R* (II) and 5*S* (IV) isomers. We therefore conclude that the HPLC systems used by these authors did not resolve the 5*R* and 5*S* isomers of the penicilloic acid and that both isomers could have been present in their flucloxacillin penicilloic acid metabolite peak. The 5*S* isomer arises from the natural 5*R* isomer via epimerization at C-5 in aqueous solution, as occurs with other penicilloic acids (Bird et al 1983; Ghebbre-Sellasie et al 1984). Consequently it is to be expected that both isomers would be present in biological fluids containing the penicilloic acid as a metabolite. The two isomers are readily distinguished by their ^1H NMR spectra as shown by the shifts in Table 2, and they are resolved by the HPLC system used in this work.

The ^{19}F NMR spectrum of sodium flucloxacillin in D_2O showed an 8-line multiplet due to coupling of the fluorine to the three aromatic protons. All ^{19}F NMR spectra of urine samples were therefore acquired with broadband proton decoupling in order to eliminate the splittings. These ^{19}F $\{^1H\}$ NMR spectra had much enhanced sensitivity, relative to the coupled spectra, due to the multiplet collapse and the nuclear Overhauser effect (Noggle & Schirmer 1971).

Fig. 1 shows a series of ^{19}F $\{^1H\}$ NMR spectra of the urines of a rat dosed with the sodium salt of flucloxacillin, I. The signals of four fluorinated species can be observed in this series of spectra, corresponding to the signals from I and three metabolites. The assignments of I, II, III and IV were made by the observation of coincident resonances upon spiking authentic samples into the urines (Fig. 2). Table 3 lists some representative data. Differences in chemical shifts between the D_2O solutions (Table 2) and the urines (Table 3) are due to pH, concentration and temperature variations. The penicilloic acid of III, V, occurs at very low levels (Murai et al 1983) and was not expected to be observed in the spectra.

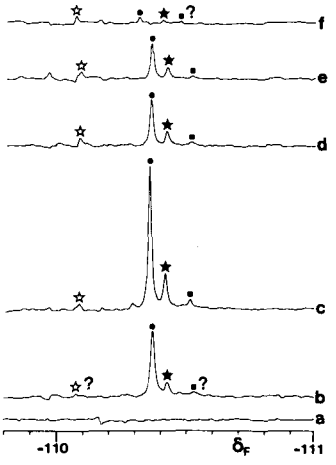


FIG. 1. A series of ^{19}F {^1H} NMR spectra of the urines from a rat dosed with sodium flucloxacillin; (a) control urine, (b) 0–2 h urine, (c) 2–4 h urine, (d) 4–6 h urine, (e) 6–8 h urine, (f) 8–24 h urine. Key: ● I, ★ II, ■ III, ☆ IV.

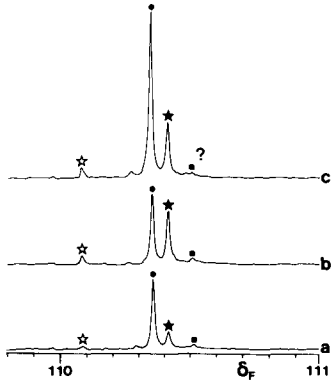


FIG. 2. A series of ^{19}F {^1H} NMR spectra of the 2–4 h rat urine; (a) 2–4 h urine, (b) 2–4 h rat urine spiked with a mixture of II (★) and IV (☆), (c) as (b) but with an additional spike of I (●).

The ^{19}F {^1H} NMR spectra of the rat urines are simple. Each fluorinated compound in the urine gives rise to just one singlet resonance. Since there are no significant fluorinated endogenous urine components there are no interfering background signals and no dynamic range problems. The chemical shift dispersion of the ^{19}F resonances is due to remote structural changes, 6 to 8 bonds from the fluorine nucleus, thus demonstrating the great sensitivity of the latter to molecular environment.

250 MHz spin-echo ^1H NMR spectra (Everett et al 1984) were run on all the rat urine samples to provide an independent check on the ^{19}F NMR assignments (Fig. 3). The sharp, paired, singlet, three-proton

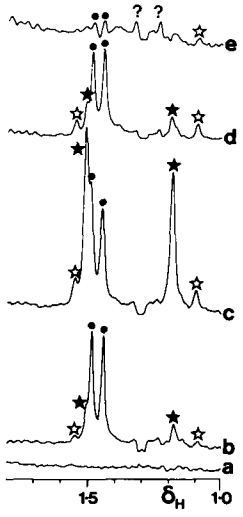


FIG. 3. A series of Hahn spin-echo ^1H NMR spectra of the urines from a rat dosed with sodium flucloxacillin; (a) control urine, (b) 0–2 h urine, (c) 0–2 h urine spiked with a mixture of II and IV, (d) 4–6 h urine, (e) 8–24 h urine. Key: ● I, ★ II, ☆ IV.

resonances seen in the high-field expansions of Fig. 3 are due to the *gem*-dimethyl protons of I, II and IV. All assignments were made by spiking with authentic I, II and IV. No signals that could be conclusively assigned to III or V were seen in these ^1H NMR spectra.

The spin-echo ^1H NMR spectra shown in Fig. 3 clearly confirm the presence of II and IV in the rat urines. In addition to the *gem*-CH_3 resonances, a sharp, singlet resonance was observed at δ2·78–2·77 in the spectra of the 0–2, 2–4, 4–6 and 8–24 h urines. This resonance was absent from the spectrum of the control urine and was coincident with the resonance of the 5′-methyl protons of IV upon spiking. Similarly, the H-3 resonance of I (δ ~ 4·15) and the coincident 5′-CH_3 resonances of I and II were observed in all the urine samples up to 8 h.

A further check on the ^{19}F NMR results was provided by HPLC analysis which confirmed the presence of I, II, III and IV in the 0–2 h urine with retention times of 18·3, 12·1 16·6 and 12·3 min, respectively.

Table 1 lists the concentrations of I, II, III and IV found in the rat urine samples by ^{19}F NMR spectroscopy, ^1H NMR spectroscopy and microbiology. In general, the quantitative agreement between all the techniques is quite good considering that the figures are all single determinations. Agreement is poorer at lower concentrations, as would be expected, since the NMR techniques are inherently insensitive.

Conclusions

^{19}F NMR spectroscopy has been shown to be a powerful tool with which to investigate the metabolism of fluorinated drugs in urine. The method is fast (<15 min per sample), requires minimal sample preparation (the addition of one drop of D_2O) and suffers no interference from endogenous urine components. The utility of the method was demonstrated by the detection and quantitation of I, III and both C-5 isomers of flucloxacillin penicilloic acid, in the urine of a rat dosed with sodium flucloxacillin.

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