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^{19}F and ^{1}H magnetic resonance strategies for metabolic studies on fluorinated xenobiotics: application to flurbiprofen [2-(2-fluoro-4-biphenylyl)propionic acid]

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Abstract: Strategies for the use of ^{1}H and ^{19}F nuclear magnetic resonance (NMR) spectroscopy as an aid to the study of the metabolic fate of fluorinated drugs are discussed with reference to the application of these methods to flurbiprofen metabolism in man. ^{1}H and ^{19}F NMR analysis of untreated urine enabled the detection of two major and eight minor metabolites of the drug. The two major metabolites were identified using a combination of NMR spectroscopy, solid-phase extraction chromatography with ^{19}F and ^{1}H NMR detection and chemical hydrolysis to a flurbiprofen glucuronide and the glucuronide of the 4-hydroxy metabolite. ^{1}H–^{19}F 2D shift correlated spectroscopy and spin–echo difference experiments are discussed in relation to their use in the structural identification of drug metabolites.

Keywords: *^{19}F NMR; ^{1}H NMR; biofluid analysis; metabolic profiling; flurbiprofen.*

Introduction

High resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy can be applied to determine the metabolic fate of foreign compounds both *in vivo* and *in vitro* [1–8]. ^{19}F NMR has also proved to be effective in studies on the metabolism of fluorinated xenobiotics in man and animals [9–19]. As an NMR probe for fluorinated drug metabolism, ^{19}F is particularly useful as it has a nuclear spin of $I = 1/2$ giving favourable line width and relaxation properties. The isotope is present at 100% natural abundance, and has a high magnetogyric ratio, hence it is very sensitive (83% of ^{1}H). The ^{19}F chemical shift range is large (>200 ppm) and consequently resonance frequencies of ^{19}F nuclei are exquisitely sensitive to changes in chemical environment that occur when fluorinated xenobiotics are metabolized. Indeed it is possible to use ^{19}F NMR to detect structural changes in a molecule (e.g. due to metabolism) at sites at least eight bonds distant to the ^{19}F atom. Hence the presence of a single fluorine atom can provide a useful NMR "handle" with which to follow the biotransformations of the molecule as a whole [5]. In addition, the absence of endogenous fluorinated compounds in body fluids eliminates any interference problems that may be inherent to biological ^{1}H NMR spectroscopic detection of xenobiotic metabolites. In order for signals to be detectable the concentration of an ^{19}F species needs to be not <5–10 μM using a 9.4T spectrometer (376 MHz) with a 5-mm probe. Although this may not compare favourably with the sensitivity of many chromatographic methods used in drug analysis, a wide range of fluorinated drugs may be present in this concentration in body fluids following normal therapeutic doses. Information on the number and proportion of major metabolites may be obtained from ^{19}F NMR measurements of urine with relatively simple sample handling and data processing procedures. Quantitative information on fluorinated drugs in body fluids can be easily obtained by spiking samples with a known concentration of an internal standard and integrating relative peak areas [9]. In favourable cases excretion balance studies may be performed without the need for expensive radiochemicals [9, 10]. Very little sample pre-

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treatment is normally necessary to detect and quantify metabolites using ^{19}F NMR, and structural information can be obtained after simple work up procedures such as NMR monitored solid-phase extraction [20, 21] and NMR monitored enzymatic and chemical hydrolysis experiments. Various multipulse ^{1}H–^{19}F NMR experiments can also be used as an aid to structural identification of metabolites, for example ^{1}H–^{19}F 2-dimensional correlation experiments [10] allow spectral association of ^{19}F and ^{1}H nuclei that are spin–spin coupled giving information on the number of protons near to the ^{19}F label. With a combination of ^{19}F, ^{1}H and ^{1}H–^{19}F NMR, information on the sites of metabolism and structure of the metabolites of a drug can be obtained. Here we report the combined use of multipulse ^{19}F and ^{1}H NMR, solid-phase extraction chromatography (SPEC–NMR) and chemical modification of the sample monitored by NMR, which together constitute an analytical strategy for the elucidation of the metabolic fate of fluorinated xenobiotics. We have chosen flurbiprofen [2-(2-fluoro-4-biphenylyl)propionic acid] to illustrate the use of these techniques as its metabolic fate is well known (Fig. 1) [22, 23], it is used in moderate therapeutic doses (50–200 mg/day in man) and it contains only a single ^{19}F nucleus which is heavily proton coupled in both the parent compound and its metabolites. The latter allows the use of ^{19}F–^{1}H correlation experiments and other NMR techniques that can exploit heteronuclear couplings for identification purposes.

Materials and Methods

Dosing and sample collection

On separate occasions an adult male ingested 150 and 800 mg of flurbiprofen [froben^{TM} (trademark) and froben^{SR} (slow release)]. Urine was collected 2 and 6 h after ingestion of 150 mg froben^{TM} and at various time points up to 38 h after 800 mg froben^{SR} (75 min, 195 min, 405 min, 515 min, 590 min, 790 min, 22 h 20 min, 26 h 35 min and 38 h). Urinary volumes and pH were recorded and samples stored at −40°C until analysis.

NMR spectroscopic measurements

Samples (0.4 ml) containing 0.1 ml of $^{2}H_2O$ added as an internal field frequency lock were placed into 5-mm glass NMR tubes. Alternatively, urine samples were freeze-dried and redissolved in $^{2}H_2O$ to allow concentration of the samples before acquiring the spectra. Typically 5–10 ml of urine was freeze-dried. Measurements were made on Varian VXR400 and Bruker AM400 spectrometers operating at 9.4 T field strength (400 MHz ^{1}H frequency or 376 MHz ^{19}F frequency).

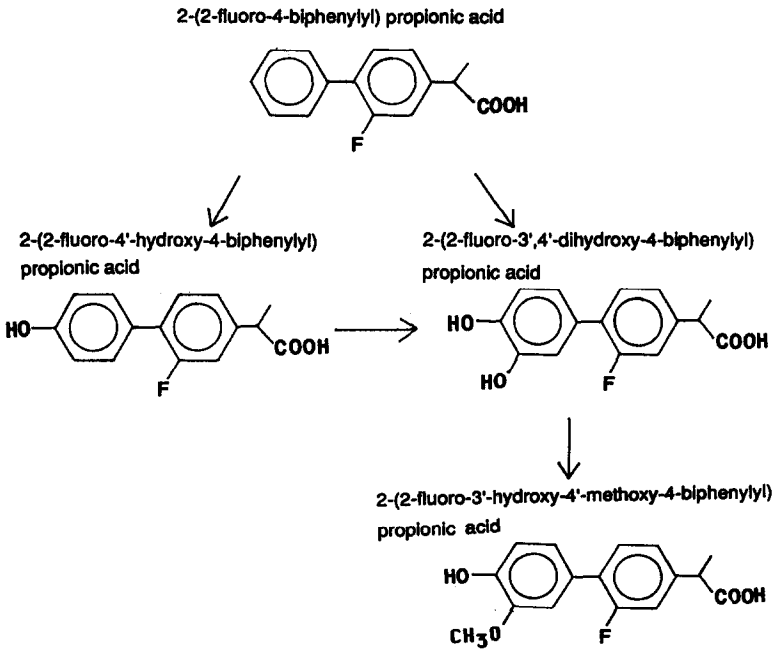


Figure 1
The main metabolic routes of flurbiprofen.

^1H NMR spectroscopy

Proton spectra were measured using a 52° (6 μs) pulse angle, 16,384 computer points, a spectral width of 6000 Hz, an acquisition time of 3.2 s, and an additional delay between pulses of 2.5 s to ensure full T_1 relaxation, and ambient probe temperature (25°C). The FIDs were multiplied by an exponential function corresponding to 0.5 Hz line broadening prior to Fourier transformation. A continuous secondary irradiation field at the water resonance frequency was applied in order to suppress its intense signal. Chemical shifts were referenced internally to sodium 3-(trimethylsilyl)-1-propanesulphonate (δ = 0 ppm) added in the ^2H_2O.

^{19}F NMR spectroscopy

^{19}F NMR spectra (376 MHz) were obtained using a dedicated 5-mm $^{19}F/^1H$ probe and a ^{19}F transmitter and preamplifier. $^{19}F\{^1H\}$ spectra were obtained using continuous broadband ^1H irradiation. ^1H coupled and decoupled ^{19}F spectra were measured at 376 MHz using a flip angle of 28°, sweep width 6000 Hz, 16,384 computer points, and a delay of 2.5 s to ensure T_1 relaxation. Shifts were referenced externally to $CFCl_3$ (δ = 0 ppm). Signal/noise ratios were improved by an exponential multiplication function corresponding to 2 Hz line broadening prior to FT.

Isolation and identification of flurbiprofen metabolites

Flurbiprofen was identified by standard addition of the parent compound to urine in the NMR tube. Glucuronides in whole urine were subjected to alkaline hydrolysis by addition of sodium hydroxide to pH 6, 7.5, 8, or 9 and then reanalysed by ^{19}F NMR. Alkaline hydrolysis was used rather than β-glucuronidase for simplicity. Solid-phase extraction chromatography with NMR detection (SPEC–NMR) [20, 21] was performed on the urine samples using a C_{18} Bond Elut™ column (Analytichem International). Urine was loaded onto the preconditioned column at pH 2 and the metabolites were eluted by a step-gradient of methanol (acidified with 1% formic acid to pH 2) from 0 to 100% in 20% subfractions. Solvent was removed using a stream of nitrogen, freeze-drying and then redissolving in ^2H_2O. The samples were then analysed by ^{19}F and ^1H NMR spectroscopy. The 60 and 80% methanolic eluates were evaporated to dryness, reconstituted with ^2H_2O, acidified and reapplied to the C_{18} column and eluted as follows. The 60% fraction was eluted with 50, 55, 60 and 80% methanol; and the 80% fraction was eluted with 60, 70 and 80% methanol. Similar fractions as detected by ^1H NMR were combined and subjected to a final clean-up on a C_{18} with 60 and 100% methanol. Fast atom bombardment mass spectrometry (Kratos MS80) was performed on selected fractions to confirm the NMR spectroscopic identifications.

(a) *^1H–^{19}F shift correlated NMR spectroscopy*. Spectra were obtained using the polarization transfer experiment of Bax and Morris [24] with broadband ^1H decoupling during ^{19}F acquisition. The pulse sequences used were as follows:

^1H channel: D0[90°–D0–90°–D3–180°–D3–90°–D0–D3–90°–D4–broadband decoupling]

^{19}F channel: D0[——D1————180°————D1————90°–D4–collect FID],

where D1 is 1–5 × T_1 (the relaxation time for protons, 2 s), D0 is the incremented evolution time, D3 is the polarization time of 1/2 J (FH) (50 ms) and D4 is the delay needed for refocusing ^{19}F resonances equal to 1/4J (FH). The parameters used were 90° ^{19}F and ^1H pulse angles of 13 and 10.4 μs, respectively, 128 acquisitions and two dummy scans were collected for each of 256 increments of the evolution time. Prior to Fourier transformation the FIDs were multiplied by a shifted sine-bell function to enhance the resolution. The Fourier transformed 2D spectrum was phased using a magnitude calculation routine in both directions.

(b) *$^1H\{^{19}F\}$ spin–echo difference experiments (SED)*. In this experiment proton NMR difference spectra are observed and ^{19}F decoupled with an 180° ^{19}F broadband pulse irradiation applied during the 180° ^1H pulse. The operation of the ^1H and ^{19}F spectrometer channels in the two experiments that make up the SED experiment are as follows:

^{1}H channel: D0 [90°x–τ–180°y–τ–acquire FID]

^{19}F channel: D0 [——2τ——decouple during ^{1}H acquisition]

^{1}H channel: D0 [90°x–τ–180°y–τ–acquire FID]

^{19}F channel: D0 [———τ–decouple during 180° ^{1}H pulse].

The first leads to a normal spectrum, since the ^{1}H–^{19}F coupling is refocused together with everything else. In the second, decoupling during the 180° ^{1}H pulse stops the coupling from being refocused. Thus the signals from the protons coupled to the ^{19}F atom are inverted in the second experiment. Subtracting the two spectra reinforces the labelled protons while cancelling all other proton resonances. The advantages of this experiment is that it combines the selective nature of ^{19}F labelling with the sensitivity of ^{1}H detection. Thus these spectra only contain signals from those ^{1}H s which are coupled to ^{19}F nuclei. The conditions used were sweep width 500 Hz, acquisition time 1 s, recycle time 2 s, spin–echo delay (30 ms), 90° ^{19}F and ^{1}H pulse angles and 32 scans. A range of ^{19}F–^{1}H coupling constants can be found in the flurbiprofen parent molecule and its metabolites and hence it is not possible to select a value for τ which produces perfect phase modulation for all spins. The expected ratio of flurbiprofen and metabolites in urine was calculated by comparison of signal areas.

Results and Discussion

^{1}H and ^{19}F NMR analysis of urine

The 400 MHz ^{1}H NMR spectra of flurbiprofen, control urine and urine from a human treated with flurbiprofen are shown in Fig. 2. A large number of resonances from endogenous metabolites can be detected but few clearly identifiable signals can be seen from the drug or its metabolites, except for the CH_3

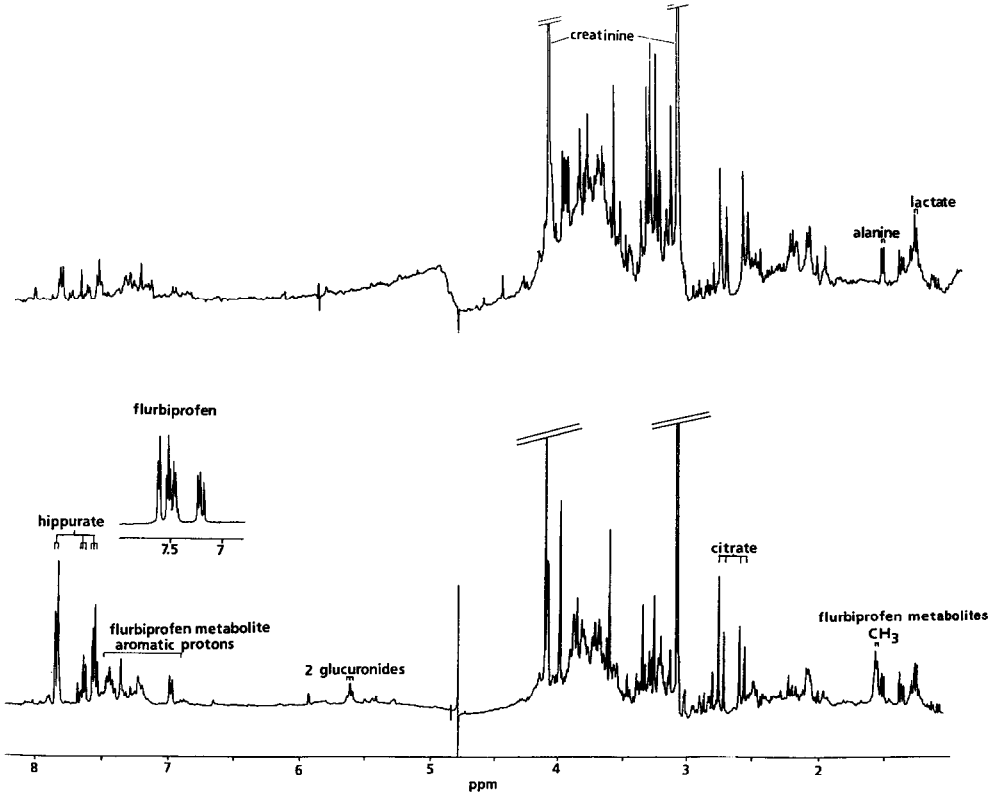


Figure 2
400 MHz ^{1}H NMR spectra of (A) control human urine and (B) human urine 195 min after dosing with 800 mg flurbiprofen (number of scans = 64).

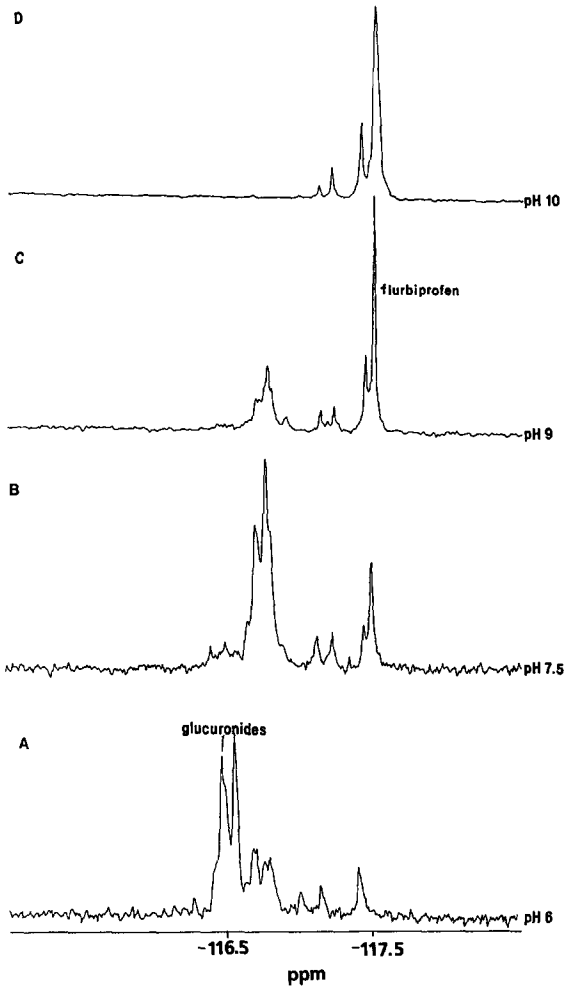


Figure 3
376 MHz ^{19}F NMR spectra of alkaline hydrolysed flurbiprofen metabolites in human urine. (Sodium hydroxide was used to pH the urine from pH 6 to 7.5, 8, 9 and 10.)

resonance at 1.5 ppm. The $^{19}F\{^1H\}$ decoupled spectrum of treated urine is shown in Fig. 3 (spectrum A). Signals from two major metabolites are observed at $\delta = -116.248$, $\delta = -116.315$ ppm, and signals from eight minor metabolites are also detected in this region of the spectrum. The $^{19}F\{^1H\}$ spectra of alkaline hydrolysed urine are shown in Fig. 3 (spectra B, C and D). It can be seen from this that the two major metabolite peaks decreased in intensity with increasing pH and the two peaks at $\delta = -117.19$ (flurbiprofen) and $\delta = -117.24$ ppm increased. This type of behaviour is typical of that expected of ester glucuronides.

Solid-phase extraction chromatography with ^1H and ^{19}F NMR detection

In order to characterize further and identify unambiguously the major metabolites, we attempted to isolate and purify them. In the past we have found the use of solid-phase extraction, generally on C_{18} bonded silica gel, followed by step-wise gradient elution (SPEC–NMR) [22, 23] to be a useful method for effecting rapid purification. In this instance, the bulk of the drug related material, partially fractionated with respect to the two major metabolites, eluted in the 60 and 80% methanol washes following the application of acidified urine to the cartridge. These fractions were then re-extracted and eluted using a shallower methanolic gradient, with the 60% fraction eluted with 50, 55, 60 and 80% washes; and the 80% fraction with 60, 70 and 80% methanol. Fractions containing spectroscopically similar material were combined prior to mass spectrometric analysis. The 60% eluates consisted of a mixture of parent compound as its ester glucuronide (^1H NMR, ^{19}F NMR, FAB–MS) and a hydroxy glucuronide (^1H NMR, ^{19}F NMR, FAB–MS) with hydroxylation having occurred in the 4′ position [2-(2-fluoro-4′-hydroxy-biphenylyl) proprionic acid]. The material eluting in the 80% fraction was essentially flurbiprofen glucuronide (^1H NMR, ^{19}F NMR, FAB–MS). ^1H resonances were assigned by consideration of their chemical shifts and coupling constants (Table 1). The remaining eight metabolites are probably sulphate and glucuronide conjugates of the metabolites shown in Fig. 1 [20].

^{19}F–^1H NMR experiments

The studies described above illustrate the way in which relatively simple experiments using ^1H and ^{19}F NMR, either alone or in combination with a simple procedure such as SPEC–NMR can provide information on the metabolic fate of a fluorinated xenobiotic like flurbiprofen. Further information can also be obtained using more advanced NMR experiments. The ^{19}F–^1H 2D shift correlated experiment shows correlations between ^{19}F nuclei and coupled protons, hence there is a reduction in the spectral crowding that is present in ^1H NMR detection of drug metabolites in body fluids. The number of protons near the ^{19}F atom is determined and overlapping protons in structurally similar metabolites are resolved because of dispersion of ^{19}F resonances in the ^1H frequency domain. The ^{19}F–^1H 2D-COSY spectrum of flurbiprofen metabolites in whole urine is shown in Fig. 4. In the case of flurbiprofen a total of 10 metabolites can be

Table 1
NMR parameters of flurbiprofen metabolites

Metabolite		^{1}H Chemical shift/ppm in ^2H_2O								^{19}F Chemical shift/ppm
		CH_3	CH	G	2	5,6	2′, 6′	3′, 5′	4′	
2-(Fluoro-4-biphenylyl)propionic acid glucuronide	J^3_{HH}	1.45 (4) 7.33	3.6 (q) 7.93	5.62	—	7.07, 7.35	7.96 (d) 7.93	7.48 (t)	7.53 7.32	−116.4
2-(Fluoro-4-biphenylyl)propionic acid	J^3_{HH}	1.40 (d) 7.33	3.66 (q) 7.93		7.22	7.24, 7.435	7.59 (d) 7.32	7.49 (t) 7.32	7.48 7.32	−117.4
2-(2-Fluoro-4′-hydroxy-4-biphenylyl) propionic acid glucuronide	J^3_{HH}	1.52 (d) 7.33	3.6 (q) 7.93	5.68	—	—	—	—	—	−116.5
2-(2-fluoro-4′-hydroxy-4-biphenylyl) propionic acid		—	—	—	—	—	—	—	—	−117.5

(Where d = doublet, t = triplet, q = quartet, J^3_{HH} = 3 bond ^{1}H–^{1}H coupling constant in Hz and G = glucuronide.)
Other flurbiprofen metabolites giving ^{19}F NMR signals have the following chemical shifts: −116.461, −116.651, −116.712, −116.756, −116.883 and −116.991 ppm.

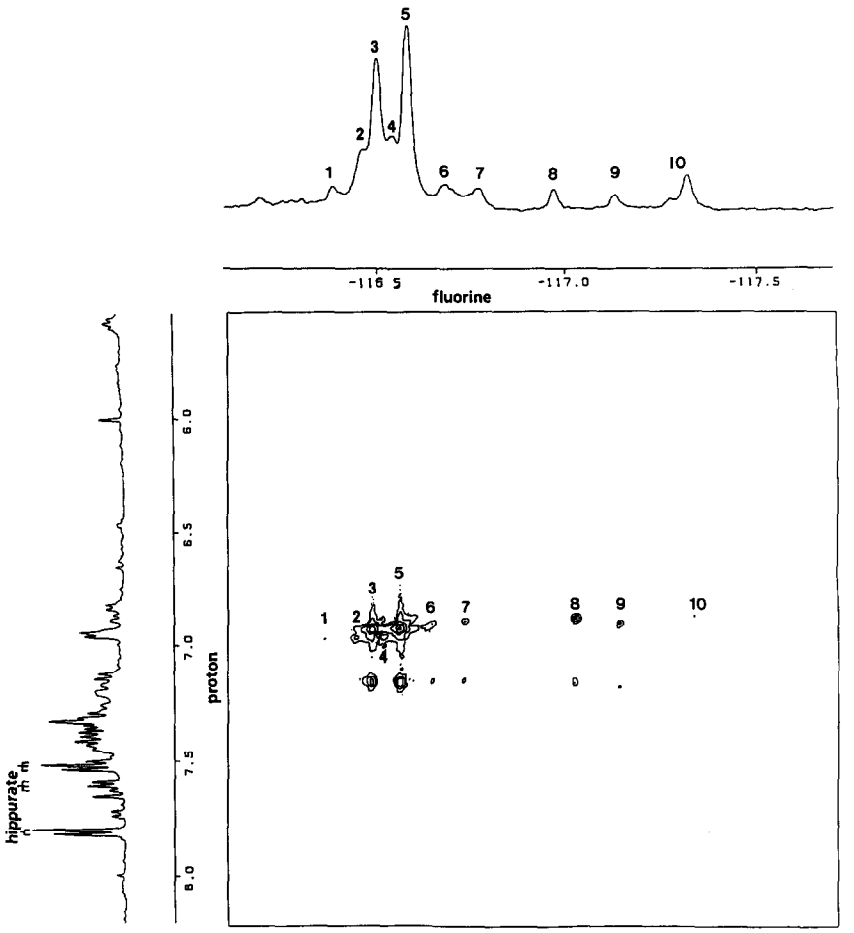


Figure 4
The ^{1}H–^{19}F 2D shift correlated spectrum of flurbiprofen metabolites in whole human urine. (Metabolite 3 is flurbiprofen glucuronide, metabolite 5 is a hydroxy 4′ flurbiprofen glucuronide, compound 10 is flurbiprofen.)

detected by ^{19}F NMR in untreated urine but this experiment reveals only the most concentrated metabolites (only six are observed). The strength of the cross-peak is proportional to concentration as well as coupling constants. The metabolites have two protons coupled to the ^{19}F atom as determined by the observation of two cross peaks.

The ^{19}F–^{1}H SED spectra of flurbiprofen and post-dose flurbiprofen urine are shown in Fig. 5. The SED spectrum edits out all interfering resonances from endogenous urinary metabolites, thus simplifying the spectra. The signals on SED spectra are complex since multiplets become phase-modulated depending on the relaxation delays used, however, each has a characteristic intensity and modulation pattern and hence can be interpreted in terms of metabolism. Metabolic information can be obtained by looking at the shape, pattern, intensity and chemical shift of the signals. If no metabolism has occurred all these factors remain as for the parent compound. The signals from 2′,6′ protons changed noticeably in chemical shift indicating metabolism has occurred at a position close on the ring. This signal was reduced in intensity by approximately 75% relative to the signal from the protons at ring position 2,5 indicating either that hydroxylation has occurred at the 2′ or 6′ position or that there is a significant reduction in the T_2 relaxation times across the whole molecule as would occur on glucuronidation. The signals from protons at ring positions 3′,5′,4′,6 have reduced by 63% relative to the signal from the protons at ring position 2,5, also indicating metabolism has occurred on this ring. Apparently no metabolism has occurred at sites 2 or 5 because the resonances from these protons remain at the same chemical shift in the parent compound and the drug metabolites. This experiment shows metabolism of

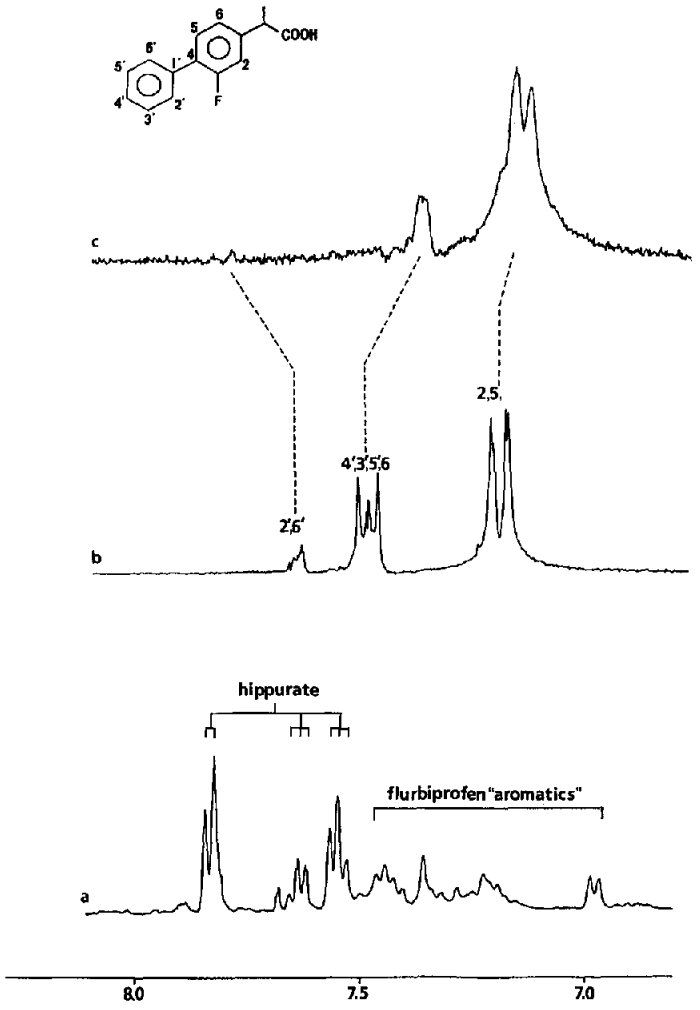
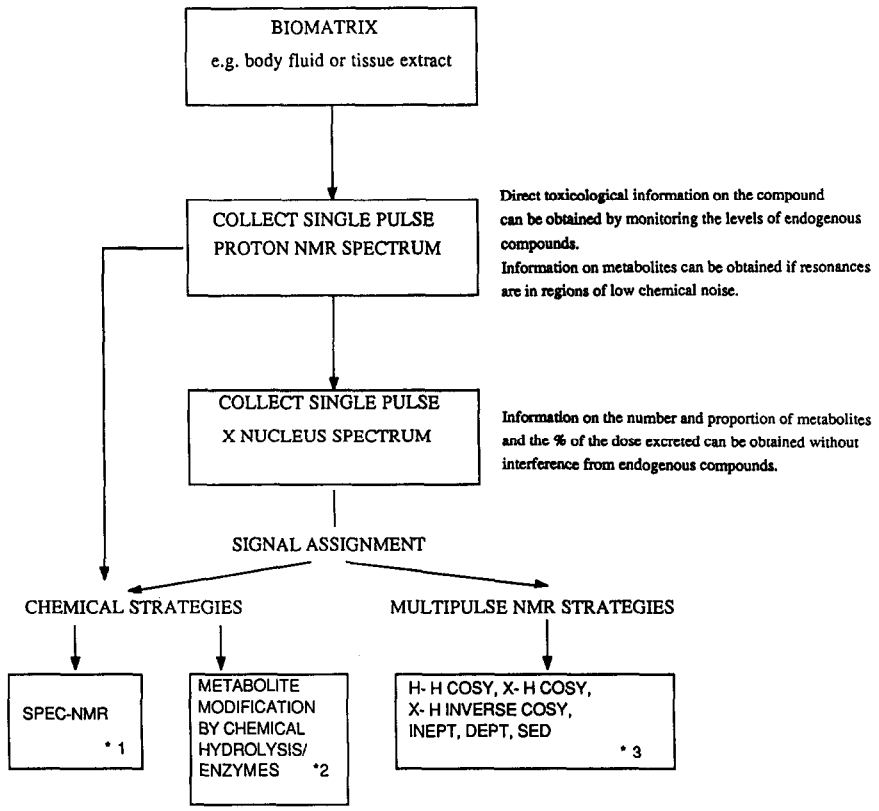


Figure 5
(a) The ^{1}H NMR spectrum of human urine 195 min after dosing with 800 mg flurbiprofen (aromatic region). (b) The ^{1}H–^{19}F spin–echo difference spectrum of flurbiprofen IN $^{2}H_2O$. (c) The ^{1}H–^{19}F spin–echo difference spectrum (with magnitude calculation) of human urine containing metabolites of flurbiprofen.

flurbiprofen to be on the non-fluorinated ring, consistent with previous studies using conventional methods alone [22, 23]. These spectral editing techniques are potentially of great value in drug metabolism studies for compounds with ^{19}F nuclei or possibly other NMR active nuclei. Much structural information on the metabolism can be obtained very rapidly.

In conclusion these techniques can be used as part of a general strategy for metabolic studies of fluoroxenobiotics as illustrated in Fig. 6. Initially ^{1}H NMR spectra of the whole biofluid should be obtained to give an overall picture of the toxicological effects of the drug (if any), and possibly to identify the drug metabolites. If the drug metabolites do not have resonances in clear windows of the spectrum and a ^{19}F nucleus is present then single pulse ^{19}F NMR can be performed. Both quantitative and qualitative information can be obtained from ^{19}F NMR spectra. Quantitative information is obtained by addition of a known concentration of a standard fluorinated compound and the subsequent integration of metabolites, whilst identification of metabolites is based on chemical shifts and coupling constants or from the standard addition of suspected drug metabolites. The chemical and multipulse NMR branches of the strategic tree come into effect if these identification techniques fail. Solid-phase extraction chromatography, enzymic/chemical hydrolysis as monitored by NMR, ^{1}H–^{19}F COSY and ^{1}H–^{19}F SED have proved very useful in this case. This strategy can be extrapolated to drugs containing nuclei other than ^{19}F, for example ^{15}N and



*1 Solid-phase extraction is used to separate and purify metabolites followed by identification using NMR and / or mass spectrometry.

*2 Chemical hydrolysis using alkali or enzymes (for example B-glucuronidase) is used to deconjugate metabolites. The change in structure of metabolites is monitored by NMR.

*3 Various multipulse NMR techniques can be used to give information on the number and structure of metabolites. The most useful experiments are those which correlate X nuclei with protons.
[^{1}H-^{1}H COSY = ^{1}H-^{1}H shift correlation spectroscopy, ^{1}H-X COSY = ^{1}H-X shift correlation spectroscopy ^{1}H-X inverse COSY = ^{1}H-X inverse shift correlation spectroscopy, SED = spin-echo difference spectroscopy.]

Figure 6
Strategy for investigating the metabolism of xenobiotics.

^{13}C and indeed other body fluids (bile, plasma), cells and cell extracts. Using this type of NMR approach to the identification of fluorinated drug metabolites offers an efficient and rapid means of collecting data on novel therapeutic agents on which there is little biochemical data.

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