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Accelerated metabolite identification by "Extraction-NMR"

Ute Gerhard *, Steven Thomas, Russell Mortishire-Smith

Merck Sharp and Dohme Research Laboratories, The Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR, UK

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Abstract

Examples of the use of extraction-NMR, an efficient and rapid method to obtain structural information on metabolites without prior separation, are described. Crude ethyl acetate extracts of in vitro microsomal incubations were analysed by NMR spectroscopy. The region downfield of 5.5 ppm in the proton spectra of these crude extracts was found to be essentially clear of endogenous resonances. As a consequence, sites of aromatic hydroxylation can often be determined without prior separation of the crude extracts. Sites of metabolism close to the aromatic region may also be accessible via two-dimensional (2D) homonuclear experiments (e.g. COSY, NOESY, TOCSY). One-dimensional (1D) and 2D fluorine experiments also can provide additional information on the structure of metabolites. Depending on the complexity of the aromatic region of the parent compound, signal overlap and the relative abundance of the individual components, extraction-NMR has the potential to provide information for unambiguous structure elucidation of two or three major metabolites. Should extraction-NMR produce inconclusive results, i.e. too many metabolites are present or metabolism occurred exclusively on aliphatic regions, it is possible to re-use the extraction-NMR sample and proceed with traditional methods of analysis.

Keywords: Drug metabolism; Drug discovery; Metabolite structure determination; In vitro and in vivo samples; 1D proton and fluorine NMR; 2D NMR; Mixture analysis

1. Introduction

The assessment of metabolic stability and the determination of routes of metabolism has become a significant aid in the compound optimisation process in drug discovery [1–3]. Historically, extensive studies on routes of metabolism have been performed on relatively few compounds, often with the help of radiolabelled compounds. Knowledge of metabolic stability and information on routes of metabolism, in parallel to SAR studies, today influence compound selection at an early stage of the drug discovery process [4]. With the advent of high-throughput drug metabolism and pharmacokinetic (DMPK) methodologies, more compounds can be assessed for favourable DMPK parameters [5]. In this context, the requirement for timely delivery of information on metabolite structures has presented new challenges to NMR spectroscopy, which is arguably

* Corresponding author. Tel.: +44-1279-44-0335.
E-mail address: ute_gerhard@merck.com (U. Gerhard).

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the most comprehensive tool for chemical structure elucidation. Historically, analytical scientists have generated metabolites in vivo or in vitro, acquiring NMR data on the isolated compounds. Recent advances have bypassed the preparative step with LC-NMR [6–9]. In particular, hyphenated techniques such as LC-NMR-MS have proved to be powerful tools in the analysis of metabolite structures from complex matrices [10].

While all these techniques are designed to isolate metabolites prior to NMR analysis, it was also demonstrated that a complete separation of metabolites from the surrounding matrix (e.g. urine) and other metabolites is not always required to determine their structures. Stepwise elution from a solid phase extraction (SPE) cartridge can yield selective recovery of metabolites from urine samples [11–14]. These fractions were shown to be of sufficient purity to allow rapid analysis of metabolite structures by NMR spectroscopy. Still simpler is the conventional ethyl acetate extraction, which has been shown to be sufficiently effective to allow NMR structure determination of a metabolite using a crude urine extract [15].

This paper describes the application of ''extraction-NMR'' to a variety of drug-like compounds. We present four examples where the structures of major metabolites of compounds in discovery were determined through either one or two-dimensional (1D or 2D) homonuclear experiments, or heteronuclear fluorine-proton correlation experiments, with no recourse to sample separation.

2. Experimental

2.1. Chemicals and reagents

Dulbecco's phosphate buffered saline (PBS) was purchased from Life Technologies and NADPH was supplied by Calbiochem. Rat liver microsomes were prepared in-house by the method of Lu and Levin [16]. Microsomal protein activity and P450 content were determined following the protocols of Lowry [17] and Omura [18]. Dimethyl sulfoxide-d_6 (100% D) and methyl-d_3 alcohol-d (99.95% D) were purchased from Aldrich.

2.2. In vitro incubations

Compounds were incubated in rat liver microsomes at substrate concentrations of 1 and 10 μM to establish their metabolic profiles. Incubations were performed in 9 ml polypropylene round bottom vials with 17 mm push caps (SARSTEDT). The total incubation volume of each sample was 1 ml, comprising of 0.5 ml of a microsome suspension (1 mg/ml), 0.39 ml Dulbecco's PBS and 10 μl of a substrate solution in DMSO (100 μM for a 1 μM incubation and 1 mM for a 10 μM incubation). After a preincubation period of 5 min in a water bath at 310 K, 0.1 ml of 10 mM solution of NADPH in Dulbecco's PBS was added and the suspensions were shaken in a water bath for a further 15 min. The incubations were quenched with an equal volume of acetonitrile, centrifuged for 5 min at 3000 rpm, and the supernatant was screened by LC-MS for turnover and presence of metabolites.

For NMR analysis, the incubations were scaled up to substrate concentrations of either 50 or 100 μM. For a typical incubation, 6.40 ml Dulbecco's PBS, 2.5 ml of 4 mg/ml rat liver microsomes and 100 μl of a 10 mM solution of substrate in DMSO (for a 100 μM incubation) were mixed in a 100 ml conical flask and shaken at 310 K for 5 min in a water bath. After the pre-incubation period, 1 ml of a 10 mM solution of NADPH in Dulbecco's PBS solution were added. The mixture was shaken at 310 K for a further 2 h to maximise the yield of metabolites. Incubations of basic compounds were quenched through addition of 5 ml of 1 M aqueous sodium hydroxide solution and subsequently extracted with 20 ml of ethyl acetate. After evaporating the organic layer, the residue was dissolved in an appropriate solvent and profiled by LC-MS analysis for the presence of metabolites and parent. The samples were then dried again and redissolved in an appropriate deuterated NMR solvent.

2.3. Rat bile preparation

Rat bile was sampled from a bile duct cannulated rat over 24 h after dosing of Compound **4**. Bile (5 ml) was loaded onto a SUPELCOSIL™

ABZ-Plus HPLC column (250 mm × 10 mm i.d., 5 μm particle size). The hydrophilic constituents of the bile were washed off for 5 min at 5 ml/min with 100% ammonium formate buffer (pH 3; 25 mM). This was followed by elution with 95% acetonitrile/5% ammonium formate buffer (pH 3; 25 mM) for a further 10 min (flow rate 5 ml/min). The organic eluent was collected and evaporated to a minimum volume (ca. 5 ml) under a nitrogen stream at 50 °C. The residue was acidified with 1 M hydrochloric acid and extracted twice with 10 ml of ethyl acetate. The organic layers were combined and evaporated under a nitrogen stream at 50 °C. The residue was dissolved in 200 μl of methyl-d_3 alcohol-d.

2.4. NMR

Samples were dissolved in either dimethyl sulfoxide-d_6 (100% D) or methyl-d_3 alcohol-d (99.95% D). All experiments were acquired on a Bruker AMX 500 NMR instrument equipped with either a 5 mm inverse broadband probe with Z gradients, a 5 mm proton fluorine dual probe or a 2.5 mm dedicated proton probe. A temperature of 300 K was maintained throughout the data acquisition.

2.5. LC-MS

LC-MS data were acquired on a Micromass Platform I spectrometer equipped with a HP1090 HPLC system. Acetonitrile and ammonium formate buffer (pH 3; 25 mM) were used as a mobile phase with a Kromasil C18 (250 mm × 2.1 mm i.d.) column at a flow rate of 0.2 ml/min. Gradients used for profiling microsomal incubates and extracts are summarised in Table 1. Positive ion mass spectral data were obtained with electrospray ionisation (ESI). Peaks were detected either by single ion recording (SIR) or by scanning over an appropriate mass range. The data were collected and analysed with MASSLYNX 3.0 software.

Table 1
Gradient LC methods used for the analysis of microsomal incubates for metabolite profiling

Method 1		Method 2	
Time (min)	% Acetonitrile	Time (min)	% Acetonitrile
0	25	0	5
2.0	25	2.0	5
22.0	60	23.0	95
25.0	60	26.0	95
25.1	90	26.5	5
28.0	90	35.0	5
28.1	25		
35.0	25		

3. Results and discussion

A proton NMR spectrum of the ethyl acetate extract of a microsomal incubation is shown in Fig. 1. Aliphatic endogenous signals obscure the proton spectrum between 0.7 and 5.5 ppm rendering this region of the spectrum unusable for analysis. Metabolic transformations on aliphatic regions of the parent compound are, therefore, relatively difficult to identify on the basis of such a spectrum. In contrast, the aromatic region is free of resonances originating from protein and lipid contamination. Resonances observed in this region are mainly due to resonances of the parent compound and its metabolites. The distinct upfield chemical shifts of aromatic metabolite protons are characteristic of common metabolic transformations on the aromatic moieties of substrates.

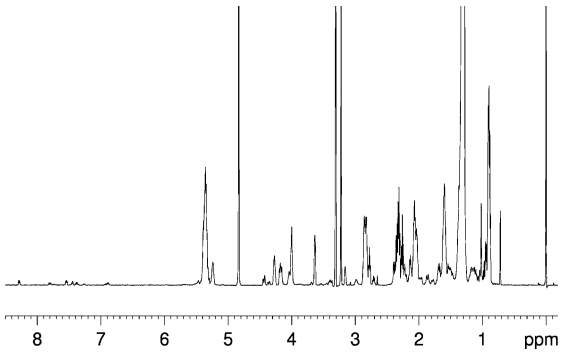


Fig. 1. Proton NMR spectrum of the crude ethyl acetate extract of a microsomal incubation.

3.1. Example 1

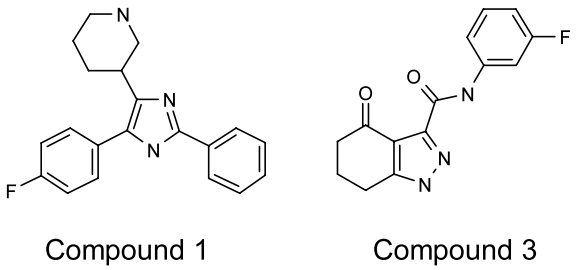
LC-MS analysis showed that Compound **1** (Scheme 1) was metabolised by rat liver microsomes predominantly to a single M+16 metabolite. The aromatic region of the proton spectrum of the extract (Fig. 2a) consists of three sets of resonances with different intensities. Direct comparison with a spectrum of parent (Fig. 2b) reveals that the parent molecule is one of the components present in the mixture. Also present is nicotinamide (Fig. 2c) which is likely to be introduced through NADPH and readily extracts into ethyl acetate. After excluding those signals, the remaining resonances include two doublets of equal intensity. Their coupling pattern and chemical shifts are characteristic of 4-hydroxylation. Thus, the major in vitro metabolite in rat liver microsomes of Compound **1** was determined to be the 4-hydroxyphenyl analogue.

3.2. Example 2

Compound **2** (Fig. 3) was found to produce one major M+16 metabolite in rat liver microsomes. In the proton spectrum of the microsomal extract of Compound **2** (Fig. 3), no obvious chemical shift differences indicative of aromatic hydroxylation were observed between metabolite and parent.

Protons 5, 6 and 8 of the parent were identified through a correlation spectroscopy (COSY) spectrum (Fig. 4). The COSY experiment also showed long range couplings from proton 8 to the methyl signal (2.45 ppm, indicated by *).

This coupling was mirrored in the metabolite by a coupling from the corresponding aromatic resonance to a downfield methylene group, at 4.71 ppm, (indicated by •). This supported the hypothesis that the hydroxymethyl compound was the major in vitro M+16 metabolite of Compound **2**.



Scheme 1. Structures of Compound **1** and **3** investigated in this study.

The above examples demonstrate that extraction-NMR is an efficient tool for the rapid determination of the site of aromatic hydroxylation. Aliphatic metabolism is not so easily discerned from 1D 1H spectra but the appropriate use of 2D NMR techniques can give insight into informative signals that would otherwise be obscured by endogenous resonances in the 1D spectrum.

3.3. Example 3

Fluorine NMR (^{19}F-NMR) has found numerous applications in drug metabolism [19–23]. Several fortuitous properties of fluorine make this nucleus an ideal tracer for drug material in complex biological matrices. Organic fluorine is not a component of biological systems but is often contained in drug substances. Hence, any fluorine signals observed are likely to be due to either drug material or its metabolites. Due to the 100% natural abundance of the ^{19}F nucleus and its magnetic properties, ^{19}F-NMR is almost as sensitive as ^{1}H-NMR.

LC-MS analysis showed that Compound **3** (Scheme 1) was transformed to a single M+16 metabolite by rat liver microsomes. The 1D ^{19}F-NMR spectrum (Fig. 5) of the crude extracted incubate showed two fluorine signals with a 24 ppm difference between the chemical shifts of parent and metabolite, suggesting that metabolism had occurred in the 2- or 4-position of the fluorophenyl ring.

This conclusion was supported by 1D ^{1}H-NMR spectra of this sample (Fig. 6).

The fluorine decoupled ^{1}H-NMR spectrum of the extract (Fig. 6a) contained resonances consistent with the presence of parent, nicotinamide and a 1,3,4 trisubstituted benzene ring. This was consistent with the metabolite being hydroxylated either in 4- or 6-position. The precise regiochemistry was determined from the fluorine coupled proton spectrum (Fig. 6b). The lack of ^{19}F–^{1}H

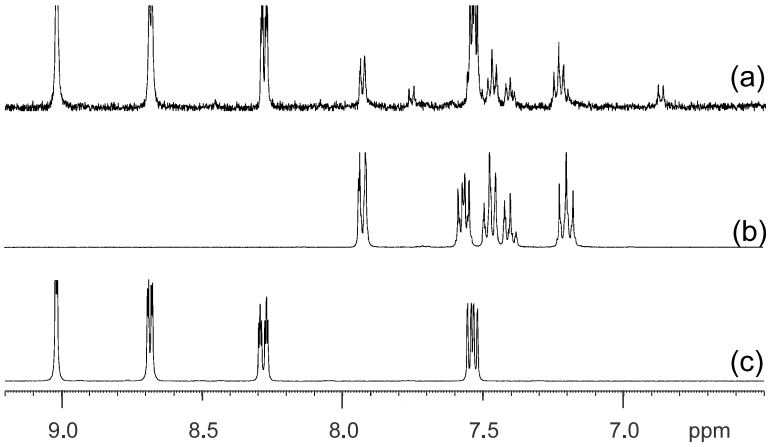


Fig. 2. The aromatic region of a proton NMR spectrum of (a) the ethyl acetate extract of a microsomal incubation of Compound **1**, (b) Compound **1**, (c) nicotinamide.

coupling for the metabolite resonance at 7.25 ppm (d, 8.1 Hz) was consistent with its assignment as metabolite proton 6′ (Fig. 6). This unambiguously proved the metabolite to be the 3-fluoro-4-hydroxy species, a conclusion, which is also consistent with the chemical shifts observed in the 1D ^{19}F-NMR spectrum (Fig. 5).

3.4. Example 4

In an elegant piece of work published in 1989 [19], a ^{1}H–^{19}F COSY was used to obtain structural information of metabolites from urine samples. Despite the increased use of fluorine in new chemical entities (NCE's) and improved NMR hardware and pulse programs, this approach has since found little application in the structure identification of metabolites.

Bile is a very complex biological matrix and even after solid phase and liquid–liquid extractions, a proton spectrum of bile (Fig. 7) which was collected over 24 h after dosing Compound **4** (Fig. 8) is extremely convoluted.

Even the aromatic region of such a spectrum is unusable due to endogenous components, which are present at far higher concentrations than the dosed material.

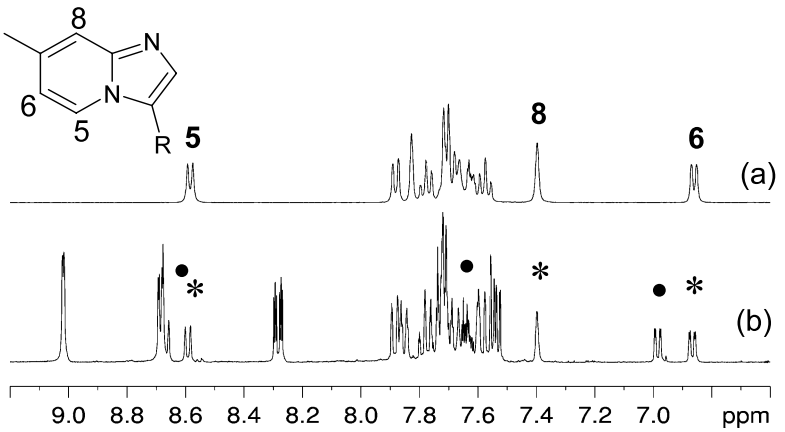


Fig. 3. The aromatic region of the proton NMR spectra of the ethyl acetate extract of (a) a reference spectrum of Compound **2** and (b) the microsomal extract of Compound **2** (* parent signals, • metabolite signals).

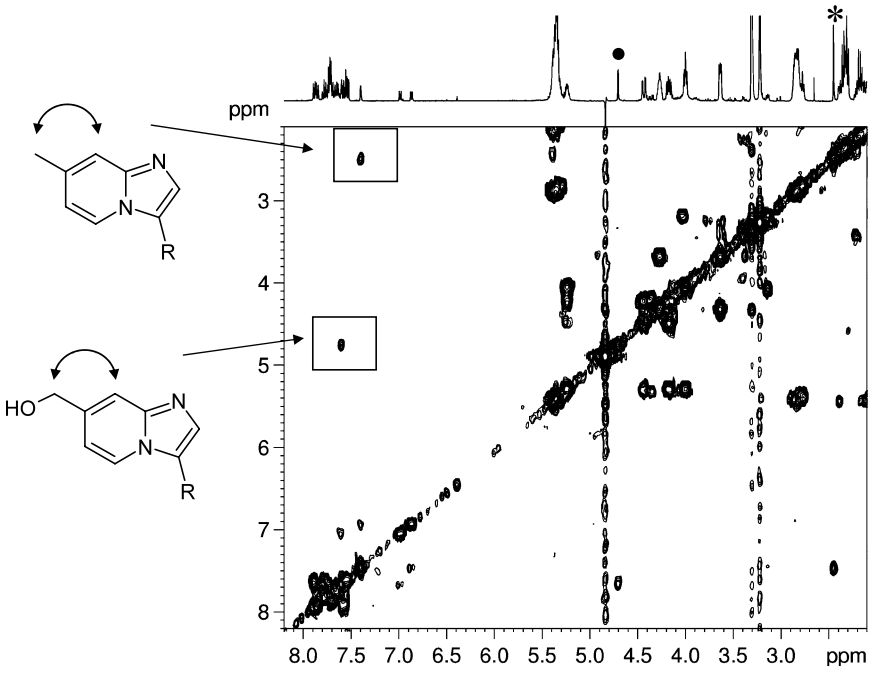


Fig. 4. COSY spectrum of the microsomal extract of Compound **3** (* parent methyl group, • metabolite methylene group).

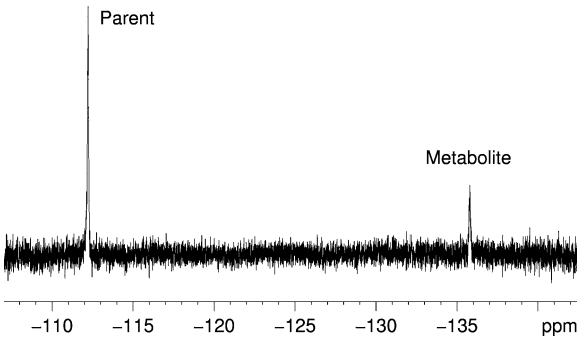


Fig. 5. 1D ^{19}F-NMR spectrum of the crude extracted incubate of Compound **3**.

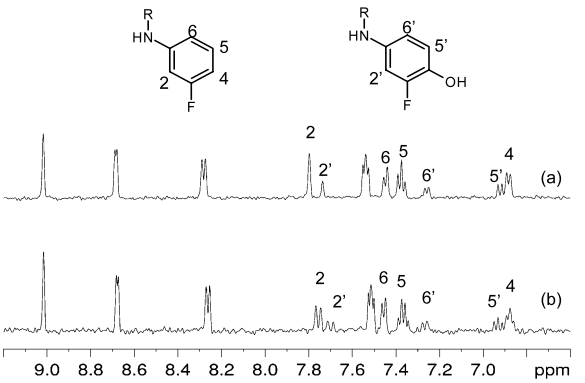


Fig. 6. The aromatic region of the proton NMR spectrum of the ethyl acetate extract of a microsomal incubation of compound **3** (a) fluorine decoupled, (b) fluorine coupled.

Compound **4** (Fig. 8) contains two fluorine atoms which are coupled to each other (^{19}F–^{19}F ^{5}J coupling constant of 9.4 Hz). This is reflected in the ^{19}F-NMR spectrum of extracted rat bile where two major and a number of smaller sets of doublets are present. An LC-MS analysis confirmed that parent and one major hydroxylated metabolite were present in the bile sample. Through spiking of the parent compound, the parent signals were identified as the resonances at −115.4 (F2) and −112.9 ppm (F2′). The resonances at −113.0 and −112.0 ppm belong to the major metabolite, however, the precise assignment of the metabolite fluorine resonances cannot be deduced from this experiment. Through a 2D ^{1}H–^{19}F COSY (Fig. 9), it was possible to extract the chemical shifts of the protons which are coupled to the individual fluorine resonances,

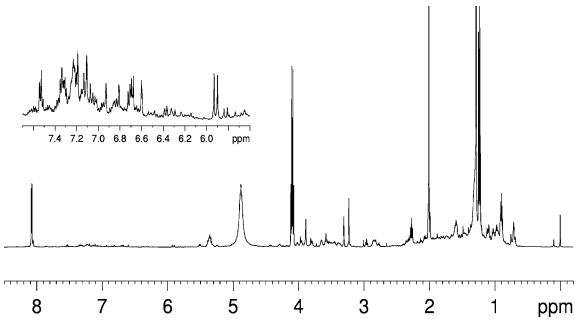


Fig. 7. Proton NMR spectrum of an ethyl acetate extract of a rat bile sample.

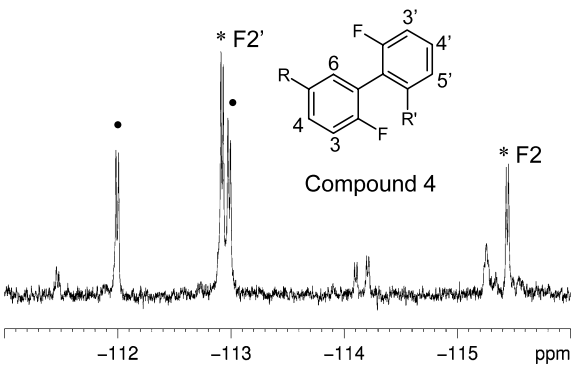


Fig. 8. Fluorine NMR spectrum of an extracted rat bile sample after dosing Compound **4** (* parent signals, • signals due to a major hydroxylated metabolite).

regardless of the complexity of the proton spectrum of the sample. The fluorine resonances of parent at −112.9 and metabolite at −113.0 ppm are each coupled to two proton resonances with similar chemical shifts at 7.64 and 7.69 ppm (parent) and 7.59 and 7.63 ppm (metabolite), respectively. This indicates that no metabolic transformation has occurred on this ring system.

The parent fluorine signal (F2) at −115.4 ppm had shifted downfield to −112.0 ppm in the metabolite which is consistent with hydroxylation occurring in the 3- or 5-position relative to a fluorine atom. The 2D $^{1}H-^{19}F$ COSY revealed that the parent fluorine resonance at −115.4 ppm is coupled to protons at 8.35 and 7.50 ppm. The corresponding metabolite fluorine resonance at −112.0 ppm is coupled to protons with chemical shifts of 8.37 and 6.98 ppm. The proton chemical

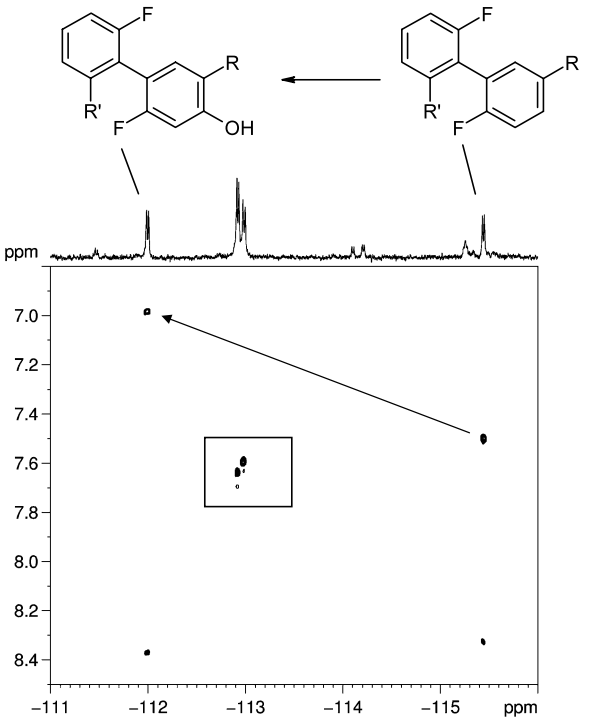


Fig. 9. 2D $^{1}H-^{19}F$ COSY of an extracted rat bile sample after dosing Compound **4**.

shift changes in the metabolite suggested that a hydroxyl group was introduced in the *ortho* and *meta* position of these ring protons. Taken together, these observations lead to the conclusion that the metabolite is the 2-fluoro-4-hydroxy derivative (Fig. 9).

4. Summary

The examples shown here demonstrate clearly that extraction-NMR has a place in the spectroscopic strategies for the structure identification of metabolites produced in vitro (i.e. microsomal and hepatocyte incubations), especially where a metabolic transformation has occurred in the aromatic regions of a molecule. In some special cases, where the aliphatic region of a molecule is metabolised in the vicinity of an aromatic ring (e.g. a benzyl group), 2D experiments such as NOESY, TOCSY or COSY offer opportunities to obtain structural information on the metabolite. If a parent mole-

cule contains fluorine atoms, fluorine chemical shifts and proton–fluorine coupling can be useful probes to determine metabolite structures. 2D $^{1}H-^{19}F$ correlations provide further means to access structural information on metabolites in extremely complex biological matrices such as extracted bile.

Particular attention must be paid to the scale-up conditions and extraction procedures. In this context, the value of an LC-MS analysis is unrivalled. The LC-MS analyses of the crude incubates are used to assess whether the desired metabolites were produced in the scaled up incubations. LC-MS analyses also confirm whether the metabolites were extracted efficiently from the crude incubates. Most importantly, the MS analysis yields the molecular weights of the metabolites, which is critical information for the subsequent NMR analysis. Depending on the complexity of the aromatic region of the parent compound, signal overlap and the relative abundance of the individual components, extraction-NMR has potential to provide information for unambiguous structure elucidation of two or three major metabolites using 1D and 2D NMR experiments.

The major advantage of this approach lies in the time and resource saving. The analysis time is dramatically reduced since no chromatographic methods need to be developed either for preparative LC or LC-NMR. Should extraction-NMR produce inconclusive results, i.e. too many metabolites are present or metabolism occurred exclusively on aliphatic regions, it is also possible to use the extraction-NMR sample and proceed with traditional methods of analysis. Extraction-NMR is not designed to replace LC-NMR. By performing a relatively quick experiment, it can, however, reduce the number of cases where metabolite structure identification requires an extensive LC-NMR analysis.

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