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A comparison of quantitative NMR and radiolabelling studies of the metabolism and excretion of Statil™ (3-(4-bromo-2-fluorobenzyl)-4-oxo-3H-phthalazin-1-ylacetic acid) in the rat

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Abstract

The identification and quantitation of the metabolites of Statil™ in rat bile and urine were investigated by ^{1}H- and ^{19}F-NMR spectroscopy and liquid scintillation counting. Male Wistar rats received a single oral dose of 100 mg/kg of radiolabelled Statil™. Statil™ is known to produce glucuronide conjugates which are predominantly excreted into the bile in male rats. The complex multiphasic matrix of bile has been shown to make identification of the resonances by ^{1}H-NMR spectroscopy very difficult as Statil™ appeared to be micellar bound giving rise to very broad signals. This not only impaired unambiguous signal characterisation but also quantification. The partial separation by SPEC-^{1}H-NMR spectroscopy enabled the disruption of the micellar matrices and hence enabled the identification of Statil™ predominantly as aglycone, and to a lesser extent as glucuronide conjugate. In addition, minor acyl migration products of Statil™ glucuronide could also be detected as they were separated during the SPEC-process. ^{19}F-NMR spectroscopic measurements on whole bile confirmed their presence as a number of overlapped signals could be observed. The selectivity, simplicity and signal dispersion characteristic of ^{19}F-NMR spectroscopy also enabled the calculation of dose related recoveries of Statil™ related material in the bile and urine samples without the need for a radiolabel. The aim of this work was to investigate the usefulness and limitations of NMR spectroscopy of intact bile and urine as a means of quantifying levels of drug metabolites. The results obtained from NMR spectroscopy are compared with those obtained using scintillation techniques. Scintillation counting yields unequivocal quantification results, provided the label is preserved in metabolites as has been the case here. In general, quantification by ^{19}F-NMR results similar to those obtained by scintillation counting (in agreement within about 20%). However,

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discrepancies have been observed with very small and broad ^{19}F-NMR signals in bile. Nevertheless, ^{19}F-NMR spectroscopy of bile is a rapid and facile method for assessing metabolite levels of fluorinated drugs.

Keywords: ^{1}H- and ^{19}F-NMR spectroscopy; Statil™; Scintillation counting; Quantitation; Metabolites

1. Introduction

Statil™ (Ponalrestat, ICI 128 436), 3-(4-(bromo-2-fluorobenzyl)-4-oxo-3H-phthalazin-1-yl-acetic acid, underwent clinical trials (some time ago) in insulin-treated diabetics as a potent inhibitor of the enzyme, aldose reductase. Aldose reductase catalyses the formation of sorbitol from glucose, which is found at high levels in plasma of diabetic patients. The metabolism of Statil™ was previously investigated in a number of species and the major route of metabolism was found to be a conjugation of the carboxyl group with glucuronic acid [1–3]. In addition, a pronounced sex difference in the elimination of Statil™ glucuronide had been observed in the rat. Only 5% of the dose was found to be excreted in the urine of male rats, but up to 85% in female rats [2]. A similar observation has been made with the structurally related compound zenarestat (3-(4-bromo-2-fluorobenzyl)-7-chloro-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-1-yl) acetic acid [4].

It was found that Statil™ is excreted in rat urine predominantly as parent compound as a consequence of the high urinary pH (8.5), which caused rapid hydrolysis of the glucuronide ester [2]. The main biliary metabolite was found to be the glucuronide, which appeared to be stabilised by the bile salts despite the alkaline pH of bile [1,2].

The quantitative metabolism and excretion of the model drug, Statil™ has been investigated by means of scintillation counting, ^{1}H- and ^{19}F-NMR spectroscopy. Excretion balance studies have been carried out by scintillation counting relying on the presence of a radiolabel in the parent drug and its metabolites. Although the radiolabel gives ultimate quantitative data, it provides no information on the drug's structure, its stability and mobility, especially in a multiphasic biofluid such as bile. This reflects its limitations and highlights the advantages of alternative ^{1}H- and ^{19}F-NMR spectroscopic approaches. As the synthesis of a radiolabelled drug is costly and time-consuming, quantification by ^{19}F-NMR spectroscopy has been attempted. The reliability of ^{19}F-NMR spectroscopic quantification compared with scintillation counting has been assessed.

2. Experimental

2.1. Bile cannulation

Male rats were fitted with biliary cannulae. The surgical procedure involved an incision across the abdomen just below the liver. After the bile duct was isolated and straightened, a small incision was made and a Portex polyethylene catheter (0.28-mm i.d.) was inserted approximately 5 mm into the bile duct where it was fastened with surgical wire. The abdomen was then closed with surgical clamps. The animals were anaesthetised with Fluothane™ (Zeneca) prior to and during bileduct-cannulation. The body temperature was kept at approximately, 38 °C during the operation by an electrical heating pad.

2.2. Animal dosing

Statil™ was administered orally in aqueous pH 7 solution. The dose level was 100 mg/kg with a dose volume of 5 ml/kg. The final radiochemical dose was 100 μCi/kg. The body weight of each rat was recorded prior to dosing.

2.3. Rats and husbandry

Three female and five bileduct-cannulated male Wistar derived rats with weight of approximately 200–300 g were dosed with Statil™. The rats were

allowed to acclimatise in cages, 48 h before dosing. They were kept on a 12-h dark:12-h light schedule. Feed and water were available ad libitum. Bileduct-cannulated (male) rats were provided with enriched water (glucose, NaCl, KCl), for 24 h prior to the surgery and till the end of the study. The rats were killed by Fluothane™ (Zeneca) inhalation.

2.4. Radiochemical label

^{14}C-labelled Statil™ and the unlabelled compound were obtained form Zeneca Pharmaceuticals. The positions of the radiolabels are indicated by asterisks (Fig. 1). The specific radioactivity of Statil™ was 13.4 μCi/mg with a radiochemical purity of $>98\%$.

2.5. Samples

Bile, urine and cagewash samples were collected into pre-weighed sterile containers. The urine and cagewash samples were centrifuged in order to rid them of solid contaminants. The sample volumes were calculated from their weight, based on a density of 1 g/ml. Bile was sampled after 3, 6, 12 and 24 h urine after 6 and 24 h. The samples were stored frozen (-20 °C) until analysis. The cagewash was obtained by rinsing of the cages with approximately 200 ml of distilled water after completion of the experiment. Cagewash samples were stored at 4 °C prior to measurement by scintillation counting.

2.6. Liquid scintillation measurements

Samples of urine, bile and cagewash (100 μl aliquots, each diluted with 900 μl aliquots of H_2O) were counted after addition of 'Ultima Gold Scintillant' (Packard) scintillation medium (10 ml) on a 'Packard Scintillation Counter' (Model 1600) with Quench (Luminescence) correction.

2.7. Solid phase extraction chromatography (SPEC)

One millilitre aliquots of urine or bile were loaded onto preconditioned C_{18} Bond Elut™ (Jones chromatography, Hengoed, UK, capacity, 3 ml) columns. Prior to loading, the samples were

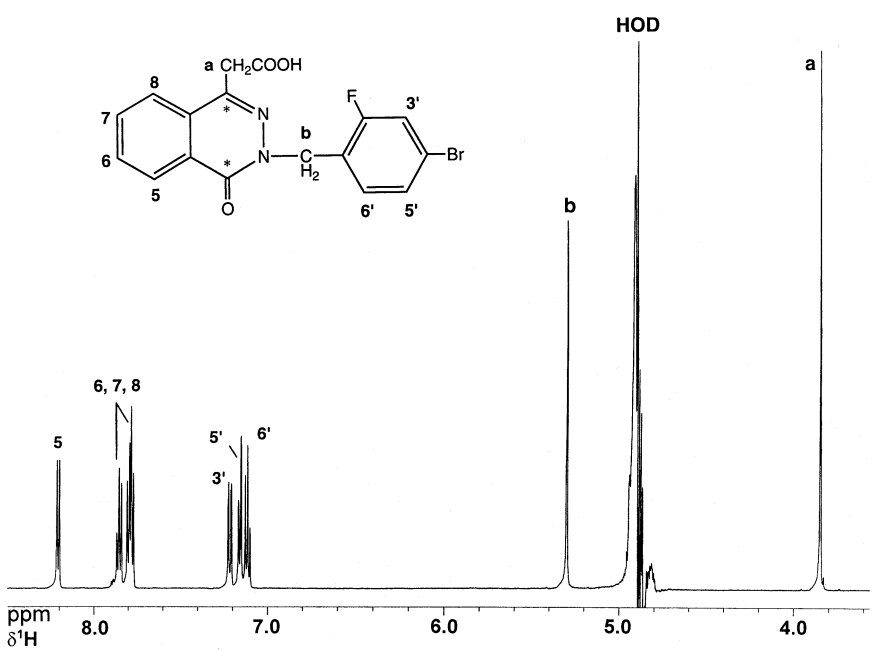

Fig. 1. 400 MHz ^{1}H-NMR spectrum of the dosing solution containing Statil™; HOD, residual water.

acidified to pH 2 (with 0.1 M HCl) in order to suppress ionisation and, hence, ensure retention of the (acidic) analytes. The urine or bile samples were selectively eluted with 1 ml aliquots of methanol/acidified water mixture with increasing elutropic strength (gradient SPEC, 20–100% methanol; Δ20%; v/v) and collected into individual vials. The eluates were then blown by under a stream of N_2 and freeze-dried prior to reconstitution in 500 μl D_2O (deuterium oxide) for ^{1}H-NMR analyses at 600 MHz as described below.

2.8. NMR-spectroscopy

2.8.1. ^{1}H-NMR spectroscopy

^{1}H-NMR spectra of dosing solution, urine and bile were acquired on Bruker AM400 and AMX600 spectrometers operating at 400.13 and 600.13 MHz resonance frequencies, respectively. At 400 MHz, ^{1}H-NMR spectra were acquired with 256 and 512 scans for urine and bile (as a preliminary screen, data not shown), respectively, into 16 K data points over a spectral width of 5000 Hz. The acquisition time was 1.64 s with a relaxation delay of 2.5 s. At 600 MHz, ^{1}H-NMR spectra of bile and urine were acquired with 64–256 scans collected into 32 K, data points over a spectral width of 12 007 Hz and at a constant temperature of 298 K. The spectrometer was retuned between the two types of sample. The acquisition time was 1.36 s followed by a relaxation delay of 2 s resulting in a total pulse recycle time of 3.52 s. Solvent suppression was achieved by applying the NOESYPRESATA pulse sequence [UXNMR, Bruker Spectrospin Ltd.]. ^{1}H-NMR spectra were referenced to the internal reference standard, TSP (sodium trimethylsilylpropionate [$^2H_4$]), at $\delta_{^1H}$ 0.0 in D_2O to provide a field-frequency lock.

2.8.2. ^{19}F-NMR spectroscopy for metabolism and excretion balance studies of Statil™

^{19}F-{^{1}H} bile spectra were acquired on a Bruker AMX600 instrument operating at 564.62 MHz resonance frequency. Decoupling was carried out with composite pulse decoupling (WALTZ-16) using inverse gated decoupling in order to prevent nOe (nuclear Overhauser effect) related signal intensity distortion. The spectra were acquired using 90° pulses with 64–256 scans collected into 130 k data points over a spectral width of 62 500 Hz. The 90° pulse widths were measured to be 15.4 μs for urine and 18.2 μs for bile. The acquisition time was 1.05 s followed by a relaxation delay of 25 s, to ensure full T_1 relaxation, resulting in a total pulse recycle time of 26.05 s (T_1 relaxation values were determined to be 2.5 and 3.6 s for *p*-F-BA, and 0.52 and 0.56 s for Statil™ in urine and bile, respectively). Spectra were acquired at 300 K and externally referenced to trifluoroethanol (TFE)/D_2O at $\delta_{^{19}F}$-77. No linebroadening function was applied prior to Fourier Transformation.

2.9. Calculation of % recoveries

Metabolite concentrations were calculated from ^{19}F-NMR signal integrals compared with that of a standard compound spiked into the sample. The internal standard used was *p*-F-BA (*p*-fluorobenzoic acid) in 1M NaOH at a concentration of 0.3 M. Aliquots of 1 μl of solution were added, containing 0.042 mg of *p*-F-BA.

The % recovery of the Statil™ metabolites was calculated according to the following procedure:

$$\text{No. of moles}_{\text{(Statil dosed)}} = \frac{\text{Vol.}_{\text{(dose)}}}{1000} \times \text{conc.}_{\text{(Statil dose-solution)}}$$

$$\text{No. of moles}_{\text{(Statil/NMR-tube)}} = \frac{\text{integral}_{\text{(Statil)}}}{\text{integral}_{(p\text{-F-BA})}} \times \frac{\text{no. of nuclei}_{(p\text{-F-BA})}}{\text{no. of nuclei}_{\text{(Statil)}}} \times \text{no. of moles}_{(p\text{-F-BA/Vol. spiked})}$$

$$\text{No. of moles}_{\text{(Statil/Total Vol.)}} = \frac{\text{Total Vol.}_{\text{(excreted)}}}{\text{Total Vol.}_{\text{(NMR-tube)}}} \times \text{no. of moles}_{\text{(Statil/NMR-tube)}}$$

$$\text{No. of moles}_{\text{(Statil/Total Vol.)}} = \frac{\text{Total Vol.}_{\text{(excreted)}}}{\text{Vol.}_{\text{(NMR-tube)}}} \times \text{no. of moles}_{\text{(Statil/NMR-tube)}}$$

Table 1
% Recovery of Statil™ by Scintillation Counting [^{14}C]

Matrix	Rat 1 male	Rat 2 male	Rat 3 male	Rat 4 male	Rat 6 male	Rat 7 female	Rat 8 female	Rat 9 female
Cagewash	0.14	0.24	1.98	0.13	1.88	16.78	15.84	36.64
3 h bile	4.54	1.38	0.00	4.69	0.00	n.a.	n.a.	n.a.
6 h bile	18.43	9.84	0.24	19.26	0.01	n.a.	n.a.	n.a.
12 h bile	38.42	34.09	5.33	45.30	0.33	n.a.	n.a.	n.a.
24 h bile	24.67	32.85	13.74	21.51	35.97	n.a.	n.a.	n.a.
6 h urine	0.41	0.18	0.30	0.35	1.71	7.54	8.75	10.39
12 h urine	0.53	0.84	3.51	0.98	6.07	n.c.	n.c.	n.c.
24 h urine	0.55	1.03	11.21	0.79	6.32	40.88	37.90	24.24
Total %	87.69	80.45	36.31	93.01	52.29	65.20	62.49	71.27

n.c., not collected, therefore, 12 h urine samples were pooled with the 24 h samples; n.a., not applicable, as female rats were not bile cannulated. Rat 5 died during the post-operative recovery period possibly as a consequence of the bile cannulation.

where concentration of Statil™ in dosing solution = 20 mg/ml.

^{19}F-NMR spectra were acquired with 90° pulse angles, ensuring a sufficient relaxation delay of $5 \times T_1$. The use of 90° pulse angles reduced the spectral excitation width. However, uniform excitation was achieved as the signals were within ± 10 ppm from the offset. Spectra were baseline corrected manually. Typically, spectra were phased and integrated 5 times sequentially, and the mean integral value was used for the calculation of the % recoveries. The signals were separated by approximately, 5 ppm, ensuring integration over ± 2 ppm around *p*-F-BA and ± 3 ppm for Statil. This would ensure $> 99\%$ of the peak area to have been integrated.

3. Results and discussion

3.1. Excretion balance studies with radiolabelled Statil™ by scintillation counting

The quantitative liquid scintillation counting data on Statil™ and its metabolites are shown in Table 1. Male rats 1, 2 and 4 showed consistency with $> 80\%$ total recovery, with between 92 and 96% excreted into the bile. Male rats 3 and 6 showed much lower total recovery (ca. 45%), with only 50–60% of that in the bile. An average total recovery of approximately, 70% was observed with the male rats, which was slightly lower than stated in the literature [1–3]. Female rats were not bile cannulated as excretion was established to be primarily via the urine [1–3]. Total excretion showed $> 60\%$ of Statil™ of which 48–75% was excreted into the urine of female rats.

3.2. ^{1}H-NMR spectroscopy of biliary and urinary metabolites following Statil™ dosing

The spectrum from the dosing solution of Statil™ (pH 7) is shown in Fig. 1 with the assignments marked. Statil™ is known to be excreted predominantly into the bile of male rats, a finding confirmed here (see Table 1) [1–3]. The ^{1}H-NMR spectra of bile from rat 4 collected 3, 6, 12 and 24 h after dosing are shown in Fig. 2. The signals at δ_{1_H} 8.4, 8.05, 7.95, 7.4 (overlapped with endogenous biliary metabolites) and 5.35 (labelled b) were likely to be related to Statil™, but unambiguous assignment was very difficult. The peaks could be tentatively assigned as H5–H8 of Statil™, however, there appeared to be a significant chemical shift change compared with the dosing solution. This was probably due to the compartmentalisation of the compound in micelles. Thus in bile, the resonances were very broad compared with free solution as a consequence of restricted molecular mobility. On the contrary, some endogenous metabolites were easily identified and assigned, as their resonances were sharp. These resonances were due to the presence of extra-micellar aromatic amino acids contained in rat bile. The intensity of

Statil™ signals shows clear time dependence as expected from the ^{14}C excretion data. Maximum excretion in the bile was observed in the 12-h bile sample, however, based on the poor peak shapes of the postulated Statil™-related signals, quantification by ^{1}H-NMR in neat bile would not have been reliable.

C_{18} Bond Elut™ solid-phase extraction chromatography (SPEC) has been shown to be a very useful approach for the separation and isolation of endogenous and exogenous metabolites in biofluids prior to NMR analysis (SPEC-NMR) [5–8]. A step-gradient was employed with the aim of disrupting the biliary micelles, hence, releasing the Statil™-related compounds and simplifying the resultant ^{1}H-NMR spectra. The spectra of the 12 h bile sample from rat 4 after being subjected to gradient SPEC greatly aided the signal assignment as shown in Fig. 3 Statil™-related material eluted in the 80 and 100% MeOH fractions predominantly as the aglycone and glucuronide (δ_{1_H} 5.5, anomeric proton doublet overlapped with Statil-b). The 80% fraction revealed the presence of minor acyl migration products as indicated by two small triplets at δ_{1_H} 5.02 and 5.2 and two doublets at 4.65 and 4.7 (part of spectrum not shown), presumably from 3- and 4-*O*-acyl glucuronides. The micellar compartmentalisation of Statil™ seemed to be verified by the co-elution with two bile-salts in the 80 and 100% MeOH fractions, which were readily identifiable by the presence of two prominent singlets at δ_{1_H} 0.7 and 0.66 representing the axial methyl group at the

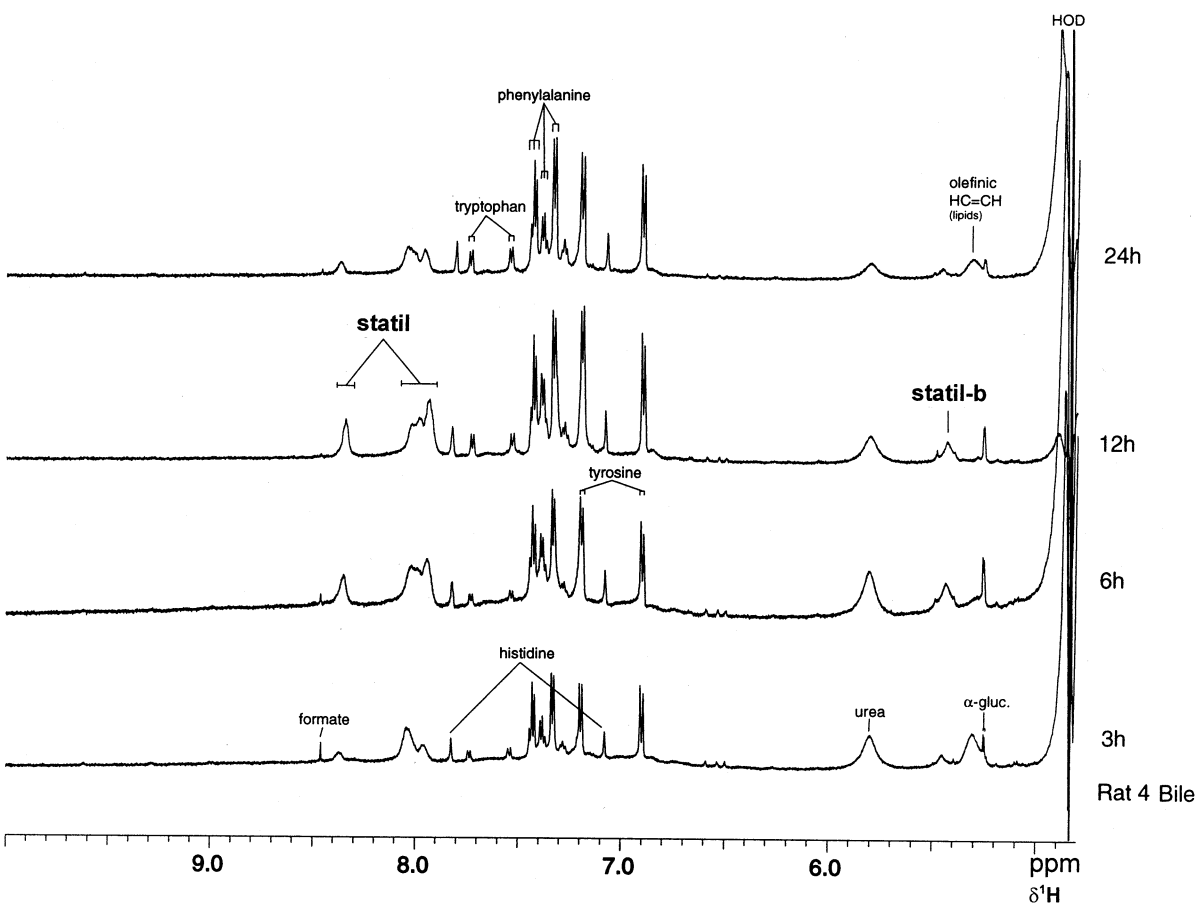

Fig. 2. 600 MHz ^{1}H-NMR spectra of the aromatic region of the bile samples from rat 4 collected at 3, 6, 12 and 24 h after dosing with Statil™. α-glucose represents the anomeric proton of α-D-glucose/-glucuronic acid; HOD, residual water.

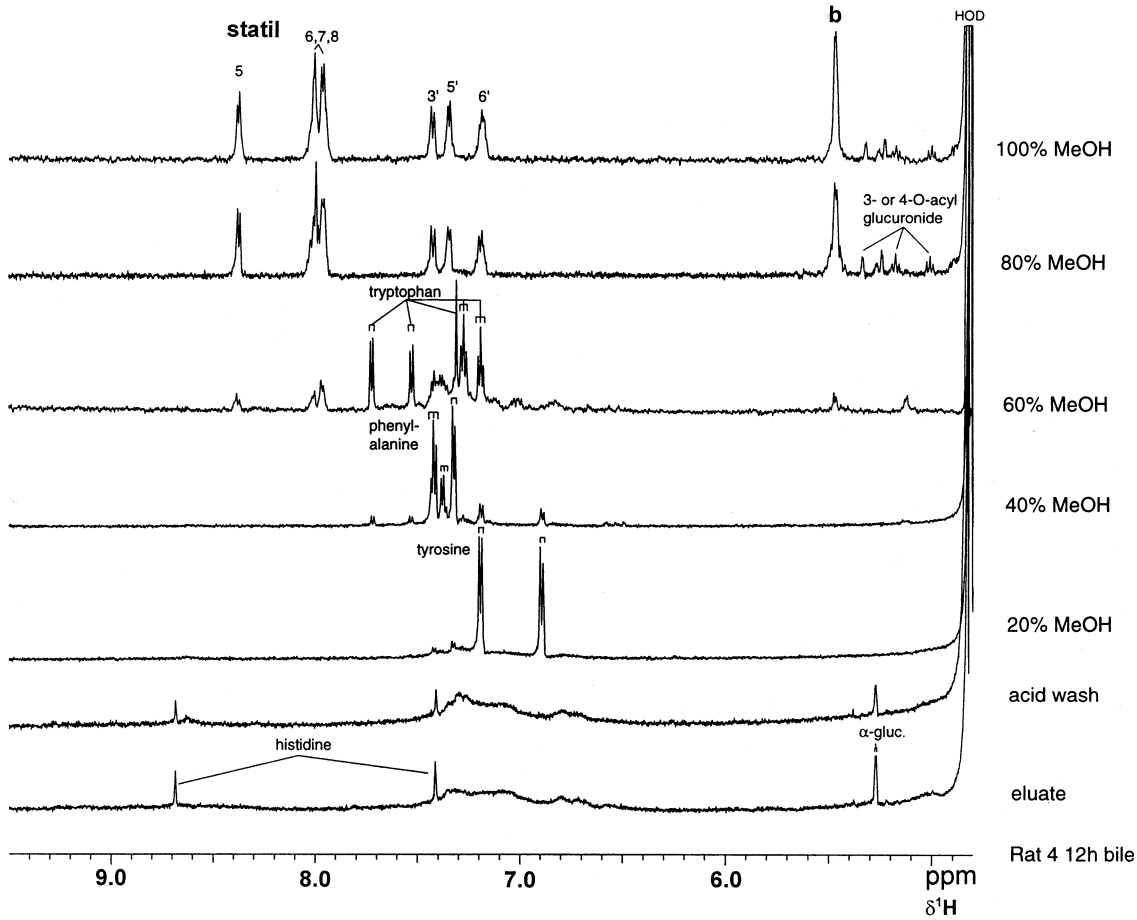

Fig. 3. Gradient SPEC-NMR spectroscopy as a sample-clean-up and Statil™ isolation procedure. 600 MHz ^{1}H-NMR spectra of the aromatic region of the C_{18} Bond–Elut™ solid phase extracts of bile collected from rat 4, 12 h after dosing with Statil™; α-glucose represents the anomeric proton of α-D-glucose/-glucuronic acid, HOD = residual water.

C-18 position of the steroid back-bone (part of spectrum not shown).

It is a common practice in the SPEC-analysis to blow- and freeze-dry the samples after elution with methanol/water, prior to reconstitution in D_2O for ^{1}H-NMR spectroscopy. However, there was some water-insoluble residue left in the 80–100% vials, which was combined and taken up in a deuterated organic solvent (MeOH-d_4), and monitored by ^{1}H-NMR spectroscopy. This revealed that it mainly comprised of Statil™, bile acids and lipids (data not shown). This gave reason to believe that Statil™ was either still contained in a micellar structure which rendered it insoluble in D_2O or that the acidic pH required for the SPEC-analysis caused precipitation of Statil™ and made it insoluble in D_2O. However, the precipitated aglycone would have escaped detection in the conventional SPEC-NMR approach, thus, precluding quantification by ^{1}H-NMR spectroscopy.

Resonances from Statil™ or Statil™-glucuronide were not apparent in urine samples of the female rats at either of the collection points (data not shown). It is stated in the literature that Statil™ is mainly found as the parent compound in urine as rapid hydrolysis appears to occur at the slightly alkaline pH of the urine samples [2]. As the Statil™ resonances were not clearly detectable in the rat urine, it had to be assumed that, Statil™ and its metabolites were either severely motionally re-

stricted or in an environment of intermediate exchange causing severely broadened lines, as precipitation was not obvious. For that reason, separation and characterisation of Statil™ and its metabolites was attempted by gradient C_{18} Bond Elut™ SPEC-^{1}H-NMR spectroscopy. However, the gradient SPEC-fractions did not reveal the drug/compound or its conjugate (data not shown).

Statil™ was in fact found to have precipitated during the SPEC-process. The ^{1}H-NMR spectrum of the hydrophobic residue in an organic solvent (CAN-d_3) confirmed the structure of Statil™ as the aglycone and seemed to account for all the Statil™ (as aglycone) present in the urine sample (data not shown).

In accordance with findings in the literature, Statil™ was found to be present as aglycone in urine and to a lesser extent as glucuronide (and acyl-migration products) in bile. The urine and bile samples were not collected on ice, and hydrolysis and acyl-migration may, therefore, have occurred at room temperature. Alternatively, hydrolytic cleavage of the ester link or the formation of glucuronide positional isomers could have taken place in vivo (37 °C) before excretion.

3.3. ^{19}F-NMR spectroscopy of biliary and urinary metabolites after Statil™ administration

The 564.62 MHz ^{19}F{^{1}H}-NMR spectra of the bile from rat 4 collected 3, 6, 12 and 24 h after

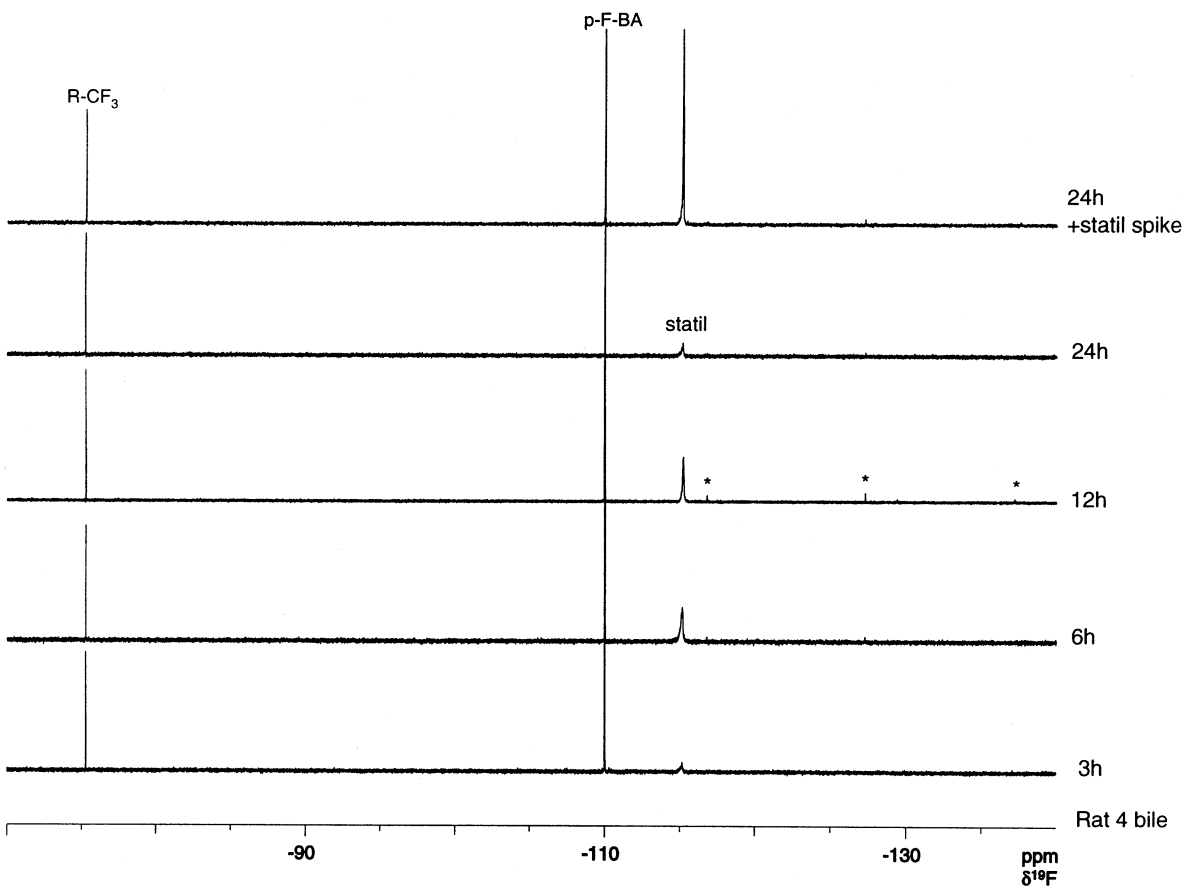

Fig. 4. 564.62 MHz ^{19}F{^{1}H}-NMR spectra of bile from rat 4, at 3, 6, 12 and 24 h after dosing with Statil™. The spectra contain signals from the internal standard *p*-fluorobenzoic acid (*p*-F-BA, δ_{19_F}-110); *, describes minor metabolites and/or artefacts.

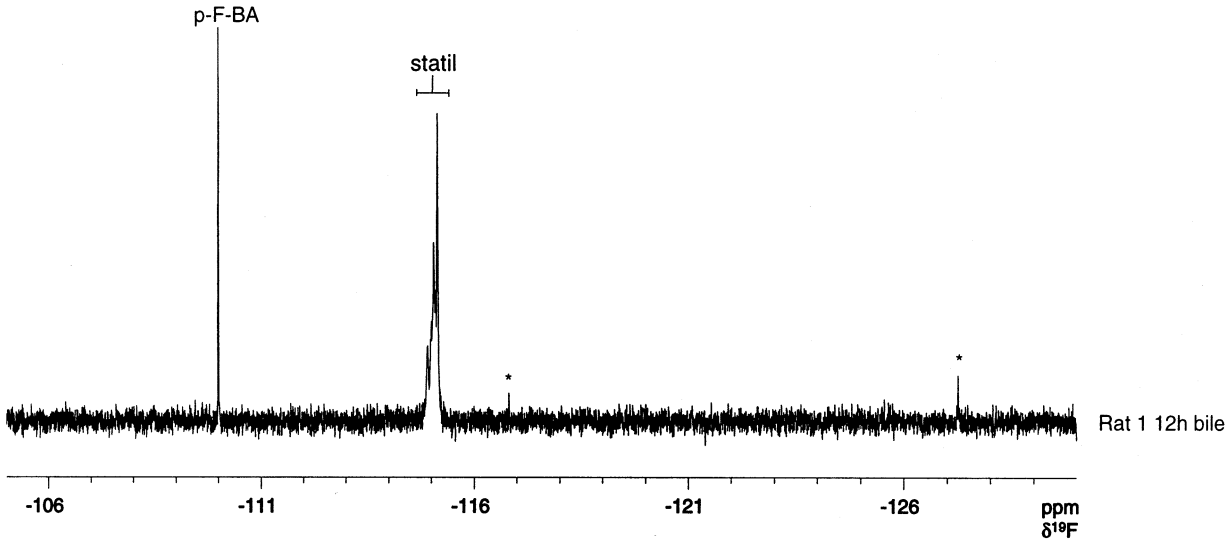

Fig. 5. 564.62 MHz $^{19}F\{^{1}H\}$-NMR spectrum of bile from rat 1, collected 12 h post-dose. The spectrum contain signals from the internal standard *p*-fluorobenzoic acid (*p*-F-BA, δ_{19_F}-110); *, describes minor metabolites and/or artefacts.

Statil™ administration are shown in Fig. 4. Spiking of Statil™-parent into the 24 h bile sample confirmed its chemical shift at δ_{19_F}-115.27. The broadening of the Statil™ signal in the ^{19}F-NMR spectrum reflected the micellar compartmentalisation as observed in the corresponding ^{1}H-NMR spectra.

The sharp signal at δ_{19_F}-75.4 is typical for CF_3 groups and appeared to be a contaminant from the anaesthetic (2-bromo-2-chloro-1,1,1-trifluoroethane, Fluothane™, Zeneca) administered during the operation. It was visible in bile spectra probably due to its lipophilicity. Contamination with anaesthetics in bile does not seem to be uncommon, signals from the dosing vehicle of Sagatal™ have previously been reported [9]. The signal at δ_{19_F}-110 is due to the internal standard (*p*-F-BA) which was used for integration purposes. Further minor signals (indicated by asterisks, e.g. at δ_{19_F}-127 and -137) were observed which may account for the hydroxy-metabolite(s), suggested as minor metabolite(s) in the literature [1–3].

The $^{19}F\{^{1}H\}$-NMR spectrum (Fig. 5) of bile from male rat 1, 12 h after dosing, clearly showed the presence of a number of overlapped signals at around δ_{19_F}-115, which revealed/indicated the presence of closely structurally related metabolites. The formation of acyl migration products could not be ruled out supporting the observations made in the corresponding ^{1}H-NMR spectra. The sit of conjugation in Statil™ is eight bonds away from the fluorine nucleus, resulting in very minor but distinct chemical shift differences of the positional glucuronide isomers. Acyl migration and hydrolysis have been observed and monitored by ^{19}F-NMR spectroscopy previously with trifluoromethylbenzoic acids [10,11] and flurbiprofen [12], respectively.

The ^{19}F-NMR spectra of the urine samples, on contrary, showed a single signal corresponding to the aglycone at δ_{19_F}-115.27 as shown in the 564.62 MHz $^{19}F\{^{1}H\}$-NMR spectrum in Fig. 6 *p*-F-BA (δ_{19_F}-110) was again contained as internal reference for quantification purposes. The linewidth of the ^{19}F-NMR resonance of Statil™, however, was still much larger compared with that of the internal standard ($\nu_{1/2}$ = ca. 17 Hz for Statil™ c.f. 3 Hz for *p*-F-BA), indicating that the molecule must have been motionally constrained due to aggregation or complexation. Alternatively, the broadening could be due to intermediate H-exchange as a function of pH.

The theory of restricted mobility or intermediate H-exchange of Statil™ in urine was supported

by the proton-coupled ^{19}F-NMR spectrum clearly showing the AA′BB′X pattern of *p*-F-BA, yet failing to display fine splittings for the Statil™ contained as the parent Fig. 7. The same increased the linewidth of Statil™, confirmed that the line-broadening was not simply due to inefficient decoupling.

3.4. Excretion balance studies by quantitative 19F-NMR spectroscopy

Results of the % recoveries calculated from the integral values from the Statil™ and *p*-F-BA signals in the 564.62 MHz ^{19}F{^{1}H}-NMR spectra are shown in Table 2. The % recoveries based on ^{19}F-NMR spectroscopy were, in general, in good agreement with the results obtained by scintillation counting with [^{14}C] (within ca. 10–20% recovery by [^{14}C]). More severe differences were observed for individual samples, e.g. rat 4; 3, 12 and 24 h bile (within ca. 30–50% recovery by [^{14}C]).

The ^{19}F-NMR resonance integration values were expected to be more accurate from the urine samples as the Statil™ signals are (relatively) sharp and since a micellar submatrix is absent. This contrasts with the situation in the bile samples which were expected to give rise to some problems as a series of overlapped and broad (w.r.t. internal standard) lines were encountered as a consequence of micellar compartmentation and, hence, restrained mobility, and the presence of acyl-migration products.

3.4.1. Factors affecting integration and quantification results

The problems encountered in the quantification procedure as outlined in this study also gave reason to highlight a number of NMR criteria to be considered for successful quantification by NMR spectroscopy.

Apart from the problems presented by the biological matrix itself (e.g. biliary micelles) and the

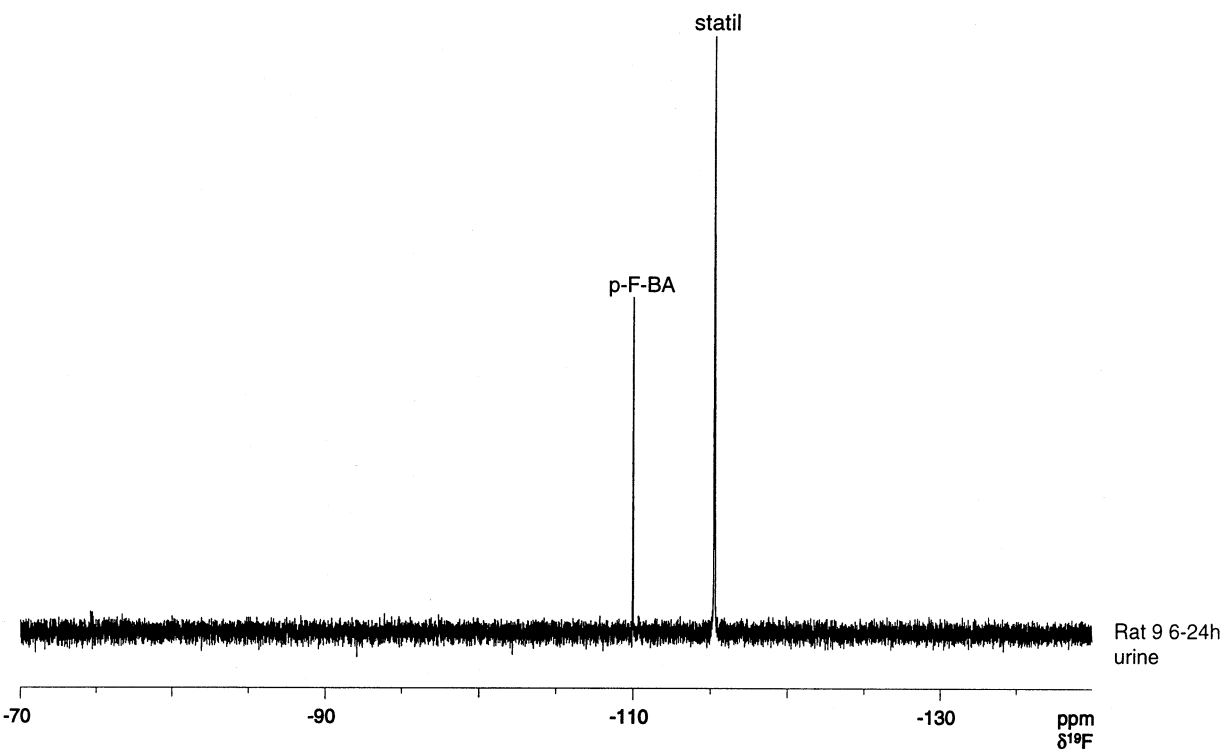

Fig. 6. 564.62 MHz ^{19}F{^{1}H}-NMR spectrum of a urine sample from a female rat (rat 9) collected 24 h after dosing with Statil™. The spectrum contain signals from the internal standard *p*-fluorobenzoic acid (*p*-F-BA, δ_{19_F}-110).

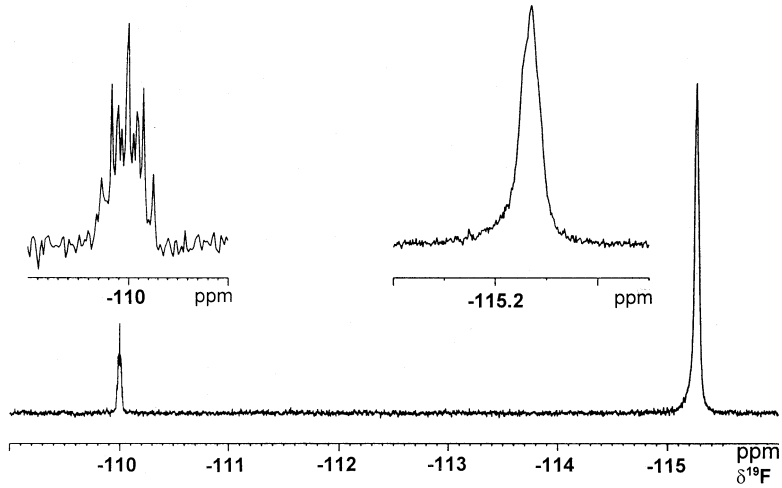

Fig. 7. 564.62 MHz ^{19}F-NMR spectrum of 24 h urine from rat 8. The *p*-F-BA signal (δ_{19_F}-110) displays its characteristic AA′BB′X splitting. The Statil™ signal (δ_{19_F}-115.27) appears to have lost its triplet splitting due to motional restriction.

hydrogen-bonding/-exchanging capacity of Statil™ (as revealed in the urine samples), it should be noted that reliable and accurate integral values for quantification require:

1. an internal standard of high purity, chemical inertness, easy solubility, low volatility, which should be well resolved from the analyte signals.
2. A relaxation delay to be sufficiently long to ensure full T_1-relaxation using a 90 °C pulse width to ensure 99.3% of the true signal intensity.
3. Adequate digitisation of the signals in question.
4. Uniform excitation especially for heteronuclear NMR experiments where large excitation bandwidths are required.
5. A reasonably short receiver dead time to prevent *loss in signal intensity*.
6. Phase correction considering that a 5° *phase error* causes a 1% integral-error.
7. Well resolved signals, since, to achieve integration of 99.9% of the signal area, integration of $\pm$318 times the linewidth would be necessary.
8. nOe effects to be counteracted by applying a gated decoupling sequence.

4. Conclusions

NMR spectroscopy, is a non-selective and non-destructive technique which can provide a unique understanding of the metabolism and dynamics of a drug within a biofluid, Micellar compartmentalisation of Statil™ in bile could be easily confirmed by the ^{1}H-NMR spectra showing broad unassignable resonances of Statil™ next to the sharp signals of the extra-micellar aromatic amino

Table 2
Quantification results and % recoveries of Statil™ and metabolites using 564.62 MHz ^{19}F{^{1}H}-NMR spectroscopy

Sample	% Recovery ^{19}F	% Recovery ^{14}C
Rat 8, 24 h urine	35.5	37.9
Rat 9, 24 h urine	19.2	24.2
Rat 1, 12 h bile	37.8	38.4
Rat 4, 3 h bile	2.3	4.7
Rat 4, 6 h bile	14.8	19.5
Rat 4, 12 h bile	59.0	45.3
Rat 4, 24 h bile	27.1	21.5

^{19}F{^{1}H}-NMR experiments were carried out with inverse gated decoupling (coupled spectra and power gated spectra provided results within $\pm$5% error). % Recoveries were calculated based on the number of moles of the internal standard (*p*-F-BA) as described in the Section 2.

acids. SPEC-NMR achieved the clear separation of the aglycone and the glucuronide metabolites from other endogenous material and confirmed the occurrence of acyl-migration products in bile. Statil™ signals were not observed in whole urine, and, again, the SPEC-NMR approach had to be relied on for its detection. The effect of the biological matrix on the metabolites appeared to be quite different: glucuronidation was probably the primary pathway, but hydrolysis must have occurred at a much faster rate in urine, despite the lower pH (7 and below) compared with bile. This finding confirms the statement made in the literature that bile acids have a stabilising effects on glucuronides.

^{19}F-NMR spectroscopy confirmed the observations made initially by ^{1}H-NMR spectroscopy. The bile spectra revealed a number of metabolites very close to the chemical shift of the aglycone, indicating that metabolism must have occurred at a position distant to the fluorine. The peaks were still broad confirming the restricted mobility of the molecules. In urine, one major peak was observed at the exact resonance of the parent consistent with the rapid hydrolysis theory. The peak, again, was relatively broad (ca. six times wider than the internal standard) indicating possible aggregation and reduced molecular mobility. Aggregation of Statil™ could be visualised in the fully coupled ^{19}F-NMR spectrum containing Statil™ aglycone confirming motional constraint of the compound due to a lack of fine splitting of the Statil™ peak.

Quantification by ^{1}H-NMR spectroscopy appears to be a suitable analytical technique for motionally unconstrained molecules yielding sharp and well dispersed lines and, hence, reliable integration values. Bile samples, containing motionally constrained and, therefore, broad resonances are not suitable for integration by ^{1}H-NMR spectroscopy. The degree of sample preparation required for detection and potential quantification by ^{1}H-NMR spectroscopy has been demonstrated.

^{19}F-NMR spectroscopy provided the solution to this problem. ^{19}F is not naturally present in biofluids, hence, the ^{19}F-NMR spectra of bile and urine samples containing Statil™ and its metabolites were greatly simplified in comparison to the corresponding ^{1}H-NMR spectra. Signal overlap was additionally minimised by the wide range of fluorine chemical shifts and, hence, the large spectral width over which ^{19}F-NMR spectra were typically acquired. The concomitant signal dispersion inherent to ^{19}F-NMR spectroscopy, enabled precise integration of the signal areas and, therefore, quantification. Although resonances from Statil™ (and its acyl migration products in bile) were still broad due to micellar binding, there was no overlap with endogenous metabolites and integration of the (total) peak-areas could be carried out. Comparison of the results obtained from ^{19}F-NMR spectroscopy and the use of radiolabelling showed relatively good correlation ($R^2 = 0.92$), although radiotracer techniques are expected to be inherently more accurate and sensitive than NMR spectroscopy. However, a radiolabel is required on the xenobiotic to be studied. Discrepancies in the % recoveries from ^{19}F-NMR spectroscopy compared with scintillation counting appear to describe a bio-physical (matrix effect) rather than an analytical problem.

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