Utilizing $^{19}$F NMR to investigate drug disposition early in drug discovery

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Abstract

1. Nuclear magnetic resonance (NMR), a non-selective and inherently quantitative method, has not been widely used as a quantitative tool for characterizing the disposition of lead molecules prior to clinical development. As a test case, we have chosen a fluoroypyrimidine compound in lead optimization phase and evaluated its disposition following oral administration to rats using $^{19}$F NMR.

2. Urine, bile and feces from individual rats were profiled and the amount of dose eliminated in each matrix was calculated. The results indicated that, in male rats, the mean dose eliminated over 0–48 h was 40%, with 28% in urine, 9% in bile and 3% in feces. In female rats, the mean dose recovered in excreta over the same period was 55%, with 40% in urine, 8% in bile and 7% in feces.

3. In addition, plasma from rats and plasma from toxicology study in dogs were also profiled and exposure of circulating entities was determined. Plasma exposure determined by $^{19}$F NMR was in good agreement with those determined by conventional LC-MS/MS method, suggesting quantitative $^{19}$F NMR can be reliably used to estimate single dose or steady-state systemic exposure of circulating entities in animals and humans.

Keywords

Drug disposition, fluorine NMR, fluoroypyrimidine, mass balance, metabolite profiling

Introduction

Understanding disposition of drug candidates is vital in the drug development process. Regulatory guidance recommends that a human disposition study be conducted early in the development program such that the elimination pathways of drug candidate and its active metabolite(s), if any, can be identified early to determine the need to assess the effect of organ impairment on their systemic exposures. Conventionally, disposition studies are conducted using appropriately radiolabeled substances of drug candidates and the objectives include (1) assessment of mass balance by quantifying radioactivity excretion in urine, feces and breath (if applicable), (2) determination of the pharmacokinetics (PK) of radioactivity in plasma and whole blood as well as the PK of the drug candidate in plasma and (3) identification of the major metabolites in plasma, urine and feces. The design, advantages and potential limitations of these studies have been reviewed (Beumer et al., 2006; Penner et al., 2009; Roffey et al., 2007).

It is equally important to understand the disposition of lead molecules or the optimized candidates before one is selected for clinical development to reduce attrition due to undesirable ADME properties. However, during lead development or the optimization phase such quantitative information is often not available. The primary reason for this is the unavailability of radiolabeled substance during this stage of the drug discovery and development program. The focus of this study is to employ an alternate methodology or a tool to achieve quantitative mass balance information and determine elimination pathways of molecules during early discovery and lead optimization phase without employing a radiolabeled compound. We propose that nuclear magnetic resonance (NMR), specifically $^{19}$F NMR, can be used as a comprehensive tool to obtain quantitative mass balance information at any stage of drug development as long as the molecules contain one or more fluorine atoms in them. $^{19}$F NMR-based quantitation has several advantages over the most commonly used $^1$H NMR-based methods: simpler profiles (frequently only a single aromatic fluorine or CF$_3$ group is present in drug candidates), reduced chance for peak overlap due to larger fluorine chemical shift range and above all no interfering background signals from endogenous substances. Although not all approved drugs or drug candidates in development contain fluorine atoms; in recent years, their presence has become increasingly common. By one recent estimate, one-fourth of the recently approved drugs and strikingly three out of the five top-selling drugs contain one or more fluorine atoms (Wang et al., 2014). Selective installation of fluorine atoms into small molecule drug candidates not only reduces their in vivo...
metabolic turnover and increases lipophilicity but also in some instances enhances binding efficacy and selectivity to the target (Müller et al., 2007).

NMR is inherently quantitative, in that the signal intensity observed in the NMR spectrum is directly proportional to the number of nuclei that give rise to a specific resonance. Thus, quantitative NMR is routinely used in pharmaceutical analysis to estimate the purity and potency of active pharmaceutical ingredients (Holzgrabe et al., 2005). Also, the application of fluorine NMR to drug metabolism studies is not new and has been successfully used previously in the detection, identification, and quantitation of metabolites of marketed anticancer and antifungal drugs in human biofluids (Malet-Martino et al., 2005; Martino et al., 2006; Sylvia & Gerig, 1993). These earlier investigations mostly focused on drugs, for which disposition has been characterized by other conventional methods. Herein, we demonstrated that for potential drug candidates that contain one or more fluorine atoms, quantitative 19F NMR can be used as a tool to determine the mass balance and elimination pathways prior to entering clinical development. Further, we demonstrated that the 19F NMR-based quantitation can be reliably used to estimate the steady-state systemic exposure of circulating entities with the potential to identify differences, if any, in the metabolism between animals used in non-clinical safety assessments and humans as early as possible during the drug development process. Since, in addition to the parent compound, metabolites were observed in rat and dog plasma, structural characterization by MS and NMR for these metabolites was also presented.

Materials and methods

Materials

The fluoropyrimidine compound and its metabolite standards (A and B) were synthesized by Eli Lilly and Company (Indianapolis, IN). The vehicle for oral administration was 1% (w/v) hydroxyethylcellulose (Hercules Inc., Wilmington, DE), 0.25% (v/v) polysorbate 80 (Spectrum Chemical, New Brunswick, NJ) and 0.05% Xiameter AFE-1501 Antifoam (Dow Corning Corporation, Midland, MI) in purified water. All reagents used for the synthesis of metabolites A and B were purchased from Sigma-Aldrich (St. Louis, MO).

Animals

Male and female Sprague–Dawley rats (220–350 g) were purchased from Harlan Laboratories, Inc. (Scottsdale, PA) and the bile duct cannulation was performed by the supplier. Male and female beagle dogs were from Covance Research Products (Greenfield, IN). All procedures used in this study are in compliance with the United States Department of Agriculture (USDA) Animal Welfare Act (9 CFR Parts 1, 2, and 3); the guide for the Care and Use of Laboratory Animals: Eighth Edition, Institute for Laboratory Animal Research, The National Academies Press, Washington, DC; and the National Institutes of Health, Office of Laboratory Animal Welfare. Whenever possible, procedures in this study are designed to avoid or minimize discomfort, distress and pain to animals.

Pharmacokinetics and excretion in rats

Four rats/sex received an oral dose of 30 mg/kg. Blood samples were taken via femoral artery into tubes containing K3EDTA at 0.5, 2, 4, 8, 24 and 48 h post-dose. Samples were kept on wet ice and centrifuged to obtain plasma within 60 min of blood collection. Cumulative bile samples were collected in tubes over ice for periods 0–6, 6–12, 12–18 and 18–24 h post-dose, urine samples were collected for periods 0–12, 12–24, 24–36 and 36–48 h post-dose and feces samples were collected between 0–24 and 24–48 h post-dose.

Toxicokinetics in dogs

One dog/sex received either twice daily doses of 1.5 and 3 mg/kg or once daily dose of 6 mg/kg for 7 days. Blood samples were collected from each animal via jugular vein at the following time points relative to dosing on Days 1 and 7 into tubes containing K3EDTA: 0 (pre-dose on Day 7 only), 0.5, 1, 2, 4, 8, 12, 16 and 24 h post-dose. Samples were kept on ice to centrifuge to obtain plasma within 60 min of blood collection. Only plasma samples collected with the 6 mg/kg dose group was used for the toxicokinetic analysis by LC-MS/MS and profiling and exposure determination by NMR.

Toxicokinetic analysis by LC-MS/MS

Concentrations of fluoropyrimidine test compound in plasma were determined using an LC-MS/MS assay. Briefly, an aliquot of 25 μL plasma sample was mixed with 180 μL of internal standard solution (100 ng/mL D6-metformin in 5% formic acid/acetoniitrile) and centrifuged to remove the precipitated proteins. The supernatant was diluted 10-fold with 5% formic acid/acetoniitrile and analyzed by LC-MS/MS using a Betasil Silica 50 mm × 2.1 mm × 5 μm column (Thermo Fisher Scientific Inc., Waltham, MA) with a gradient mobile phase (mobile phase A: 1000:2:2 water/formic acid/1 M ammonium bicarbonate and mobile phase B: 1000:25:2:2 acetoniitrile/water/formic acid/1 M ammonium bicarbonate) delivered at 1.5 mL/min. Selected reaction monitoring (SRM) transitions in positive ion mode with precursor and product ions for the analyte (310.1 → 212.1) and internal standard (326.1 → 265.1) were acquired with Sciex API4000 (Applied Biosystems/MDS Sciex, Foster City, CA) tuned to achieve unit resolution (0.7 DA at 50% FWHM) using Analyst software, version 1.4.2 (SCIEX Headquarters, Framingham, MA). The dynamic range of the assay was 1–5000 ng/mL. The plasma concentrations were then used to estimate the pharmacokinetic parameters using Watson Bioanalytical LIMS, Version 7.4 (Thermo Fisher Scientific Inc., Waltham, MA).

Sample preparation for NMR analysis

Plasma

Standards were prepared by spiking 100 μL of appropriate stock solutions of the fluoropyrimidine test compound in methanol into 900 μL of control rat plasma to give final amounts of 30, 10, 3 and 1 μg. The samples were mixed briefly by vortexing and four volumes of acetonitrile were added to precipitate the plasma proteins. The samples were then centrifuged at 1500 rpm for 10 min; supernatants
removed and dried using a stream of nitrogen at 45°C. To each dried plasma extract, 700 μL of 9:1 CH₃OH–CD₃OD was added, sonicated in a bath sonicator for 5 min and centrifuged at 14,000 rpm for 10 min. The supernatant was transferred to a 5 mm NMR tube for analysis. Plasma samples obtained from four rats post-dose were combined per time point and a 0–48 h AUC pool was generated for male and female rats separately using the plasma pooling method described by Hop et al. (1998). The pooled plasma (950 μL) was extracted, dried and reconstituted as described for the plasma standards.

Plasma standards were also prepared in dog control plasma as described for the rat plasma. Zero to sixteen hour AUC pooled samples were prepared from Day 1 (pooled plasma volume 1.55 mL) and Day 7 (pooled plasma volume 1.60 mL) from individual animals and were extracted, dried and reconstituted in the NMR solvent as described before.

**Urine**

Appropriate stock solutions of fluoropyrimidine test compound in CD₃OD (70 μL) were added to 630 μL of control rat urine to yield final amounts of 70, 21, 7 and 2.1 μg of standards. The samples were then centrifuged at 14,000 rpm for 10 min and the clear supernatant was transferred to 5 mm NMR tubes for analysis. Urine samples collected over 0–48 h post-dose were pooled across time points per animal by aliquoting 20% volume of the excreted urine at each collection interval. An aliquot (630 μL) of each pooled urine was mixed with 70 μL of CD₃OD, centrifuged at 14,000 rpm for 10 min and the supernatant was transferred to 5 mm NMR tubes for analysis.

**Bile**

Appropriate stock solutions of fluoropyrimidine test compound in CD₃OD (70 μL) were added to 630 μL of control rat bile to yield final amounts of 21, 7, 2.1 and 0.7 μg of standards. The samples were then centrifuged at 14,000 rpm for 10 min and the supernatants were transferred to 5 mm NMR tubes for analysis. Bile samples collected over 0–24 h after dosing the compound were pooled across time points per animal by aliquoting 10% volume of the excreted bile at each collection interval. An aliquot (630 μL) of each pooled bile was mixed with 70 μL of CD₃OD, centrifuged at 14,000 rpm for 10 min and the supernatant was transferred to 5 mm NMR tubes for analysis.

**Feces**

Appropriate stock solutions of fluoropyrimidine test compound in methanol (100 μL) were added to 900 μL of rat control fecal homogenate (fecal homogenate was prepared by adding three volumes of 1:1 methanol–water to fecal pellets) to yield final amounts of 70, 21, 7, 2.1 and 0.7 μg of standards. The samples were mixed by vortexing and extracted with equal volume of methanol three times. The combined supernatants were dried and to each dried extract 700 μL of 9:1 CH₃OH–CD₃OD was added. The samples were then sonicated for 5 min in a bath sonicator, centrifuged at 14,000 rpm for 10 minutes and transferred to 5 mm NMR tubes for analysis. Fecal homogenates after dosing the compound were prepared as described above. An aliquot (20%) of fecal homogenates thus obtained were pooled across time points (0–48 h) per animal, extracted, dried and reconstituted as described before for the fecal standards.

**Confirmation of parent compound in bile and feces samples**

After completion of profiling experiments by ¹⁹F NMR, a known amount of parent compound was spiked into selected bile and feces study samples in order to confirm the presence of the parent compound in these matrices. Such spiking was not performed for plasma and urine study samples, since identification of parent resonance was straightforward and no signal overlap is suspected in these matrices.

**Isolation of metabolite B from rat urine**

Urine collected from male and female rats (n = 4/sex) over the 0–48 h post-dose were combined (~80 mL) and applied directly on a Waters XBridge C18 column (10 × 250 mm). The column was eluted using 10 mM ammonium acetate (mobile phase A) and acetonitrile (mobile phase B) following the gradient (min, %B): 0–2, 10%; 2–20, 30%; 20.1–25, 90% with a flow rate of 7 mL/min. Fractions were collected every 15 s and fractions containing the metabolite B were pooled and re-purified over a Waters XBridge C18 (4.6 × 150 mm) column using 0.2% formic acid and acetonitrile as mobile phases. Fractions containing metabolite B were pooled, dried and analyzed by NMR.

**Synthesis of metabolites A and B**

**Metabolite A**

To a solution of fluoropyrimidine test compound (315 mg) in trifluoroacetic acid (10 mL) was added 10 equivalents of 30% hydrogen peroxide and magnetically stirred for 6.5 h at 50°C. At the end of this period, the reaction mixture was cooled to room temperature, diluted with water (3 mL) and evaporated under reduced pressure to remove trifluoroacetic acid. The remaining aqueous solution was filtered through a SCX (Silicycle, 10 g) cartridge and subsequently purified on a C18 column with ammonium bicarbonate–acetonitrile as the mobile phase to yield metabolite A (230 mg). The mass spectrum of metabolite A showed a MH⁺ ion at m/z 326, 16 mass units higher than the corresponding MH⁺ ion of the parent compound.

**Metabolite B**

The synthesis of metabolite B was initiated starting from a 4-chloro intermediate and accomplished in three steps. First, to a solution of 4-chloro substituted fluoropyrimidine intermediate (325 mg) in dimethylsulfoxide was added 7.5 equivalent of 1 N aqueous sodium hydroxide. The mixture was heated at 100°C for 90 min, cooled to room temperature, diluted with 10 equivalents of 1 N aqueous hydrochloric acid and the precipitated product was filtered (291 mg). The 4-hydroxy fluoropyrimidine (or fluoropyrimidone) intermediate thus obtained (185 mg) in dioxane was mixed with three equivalents of boronic acid, 0.5 equivalent of
RuPhos(Pd)Gen2 and three equivalents of aqueous sodium carbonate and heated at 100 °C for 90 min under atmospheric nitrogen. Aqueous work up followed by purification over normal phase silica gel yielded the Suzuki coupled 4-hydroxy fluoropyrimidine intermediate (136 mg), which upon deprotection with trifluoroacetic acid/anisole (10:1 v/v) at 100 °C for 3 h followed by silica gel chromatography afforded metabolite B (108 mg). The mass spectrum of metabolite B showed a MH⁺ ion at m/z 326, 16 mass units higher than the corresponding MH⁺ ion of the parent compound.

19F NMR analysis

One dimensional (1D) 19F NMR experiments were performed on a Bruker Avance III 600 MHz Spectrometer equipped with a QCI HFCN quadruple resonance Cryo probe (Billerica, MA). Spectra were recorded with a spectral width of 100 ppm centered at –130 ppm and a total of 32 k complex points.

Longitudinal relaxation time (T₁) measurement

The T₁ for the parent compound in CD₃OD was measured using a simple inversion recovery method by incrementing the duration of the inter-pulse delay in 0.1 s steps. The signal was nulled with a delay of 0.5 s, based on which T₁ was calculated to be approximately 0.7 s (T₁ = τ null/ln2).

For all quantitative fluorine NMR experiments, a 5-s pre-acquisition delay (>5 × T₁) was used together with an acquisition time of 0.6 s, resulting in a total of 5.6-s repetition delay. Proton decoupling during acquisition was achieved using a standard WALTZ-16 decoupling sequence centered at 8 ppm. Raw data were zero-filled to 64 k complex points and a 2 Hz line broadening window function was applied before Fourier transformation. 19F chemical shifts were referenced with respect to CFCl₃ at 0 ppm. NMR run times ranged from approximately 6.5 min to 13 h, depending on the sample amount for plasma, urine, bile and feces standards and study samples. For matrix standards with lowest amount in the calibration range, 8 k scans with a total NMR run time of approximately 13 h were used, except for plasma where only 4 k scans were used. To gain maximum possible sensitivity within reasonable time, 8 k scans with a total NMR run time of approximately 13 h were used for all study samples.

NMR-based quantitation

For each matrix, 19F spectra were obtained using a range of amounts (see above for the calibration range for each matrix). The 19F spectrum obtained with the highest amount was usually assigned the nominal value and used as reference spectrum to calibrate the remainder of the amounts in the calibration range using the built-in ERETIC2 tool (TopSpin version 3.2, Bruker Corporation, Billerica, MA) based on the PULCON method (Wider & Dreier, 2006). From the these values, standard curves were generated for each matrix and the highest amount was back calculated for each matrix based on the linear fit of the respective standard curve. Quantitation of parent and metabolites observed as peaks in the 19F NMR spectra of study samples for each matrix was determined using the back calculated highest amount for each matrix. With few exceptions, most peaks in the study samples were quantitated when their S/N ratios were at or above the respective standard S/N ratios. When necessary, deconvolution was performed to quantitate overlapping peaks using the Mnova NMR software version 9.0.0 obtained from Mestrelab Research, Escondido, CA (Cobas & Sykora, 2009).

Structural characterization of metabolites A and B by NMR

Standard 1D and 2D NMR experiments were performed on a Varian Inova 600 MHz spectrometer equipped with an HCN triple resonance Cold probe (Agilent Technologies, Palo Alto, CA) to support the characterization of metabolites. Standard 1D 13C NMR experiments were performed on a Varian VNMRS spectrometer equipped with a 13C Cold probe. Chemical shifts were referenced with respect to TMS at 0 ppm for 1H and 13C.

Results

Standard curves

In order to quantitate peaks observed in the 19F NMR spectrum of plasma, urine, bile and feces, standard curves using the parent compound were generated for each matrix by measuring 19F NMR spectra over a range of amount as detailed in the “Methods” section. Overall, the correlations by linear fit observed between nominal and NMR-measured amounts data were excellent with r² = >0.99 for all matrices (Supplemental Figures S1 and S2).

Limit of quantitation

Limit of quantitation (LOQ) was determined based on the S/N ratio observed for the standard solutions in each matrix. The S/N ratio for the lowest amount in the calibration range was calculated to be 48 for plasma (lowest amount 1 μg), 62 for urine (lowest amount 2.1 μg), 15 for bile (lowest amount 0.7 μg) and 59 for feces (lowest amount 0.7 μg).

Plasma exposure, excretion and mass balance in rats

The male and female rat plasma profile by 19F NMR is shown in Figure 1 and the systemic exposure (AUCₐₖ₋₄₈) of parent compound and metabolite B is summarized in Table 1. In 0–48 h AUC pooled male and female plasma, the parent compound accounted for the majority of the compound-related exposure (AUC), approximately 64 and 76% in male and female rats, respectively. Plasma exposure for the parent compound was similar between male (152 μg h/mL) and female (131 μg h/mL) rats (Table 1). In addition, systemic exposure determined for the parent compound using conventional LC-MS/MS method is also listed for comparison in Table 1. In spite of the fact that the exposure results were from two different studies that used different doses, there seems to be a good concordance between the two methods. The percent dose excreted in urine, bile and feces and the mass balance are summarized in Table 2. In male rats, the mean dose recovered in excreta over 0–48 h was approximately 40%, with approximately 28% in urine, 9% in bile and 3% in feces. In female rats, the mean dose recovered in excreta over the same period was 55%, with approximately 40% in urine, 8% in bile and 7% in feces.
Metabolite profiling of plasma, urine, bile and feces

The 0–48 h AUC pooled male and female rat plasma profiles are shown in Figure 1. In both male and female rat plasma only two components, parent and a single metabolite, were detected by 

\[ ^{19}F\text{NMR} \]

The metabolite, designated as metabolite B, was identified as 4-hydroxyfluoropyrimidine or fluoro-4-pyrimidone (see below for structure identification) and it represented approximately 36 and 24% of the total compound-related exposure in male and female rats, respectively (Table 1).

Figure 1. 

\[ ^{19}F\text{NMR profiles of 0–48 h AUC pooled, female Sprague–Dawley rat plasma following a single oral dose of fluoropyrimidine compound (30 mg/kg) [Panel A] and 0–16 h AUC pooled female beagle dog plasma following daily oral doses of fluoropyrimidine compound (6 mg/kg) for seven days [Panel B]. The broad peak observed near –150 ppm in both rat and dog plasma profiles was also observed in respective plasma standards and may be attributed to an unknown fluoro polymer impurity likely associated with the plasma collection tubes.} \]

Table 1. Systemic exposure (AUC) of parent compound and metabolite B in male and female Sprague–Dawley rats following a single oral dose of fluoropyrimidine compound (30 mg/kg).

| Methods | Dose (mg/kg) | Mean AUC\(_{0–48}\) (µg h/mL) | Mean AUC\(_{0–48}/\text{dose}\) (µg h/mL/mg) |
|---------|-------------|-----------------|-----------------|
|         | Male | Female | Male | Female | Male | Female | Male | Female |
| 
^{19}F-NMR | 30 | 152 | 87 | 131 | 41 | 5.06 | 2.90 | 4.37 | 1.38 |
| LC-MS/MS\(\text{a}\) | 20 | 107 | NC | 75 | NC | 5.36 | NC | 3.75 | NC |

AUC\(_{0–48}\) = area under the plasma concentration–time curve from time 0–48 h. NC = not calculated.

\(\text{a}\)Internal data from a different study. Concentrations of fluoropyrimidine test compound in plasma were determined with a LC-MS/MS assay. Briefly, an aliquot of 25 µL plasma sample was mixed with 180 µL of internal standard solution (100 ng/mL D6-metformin in 5% formic acid/acetoneitrile), and centrifuged to remove the precipitated proteins. The supernatant was diluted 10-fold with 5% formic acid/acetoneitrile and analyzed by LC-MS/MS using a Betsai Silica 50 mm x 2.1 mm x 5 µm column (Thermo Fisher Scientific, Inc., Waltham, MA) with a gradient mobile phase (mobile phase A: 1000:2 water/formic acid/1 M ammonium bicarbonate and mobile phase B: 1000:25:2:2 acetonitrile/water/formic acid/1 M ammonium bicarbonate delivered at 1.5 mL/min. Selected reaction monitoring (SRM) transitions in positive ion mode with precursor and product ions for the analyte (310.1 → 212.1) and internal standard (326.1 → 265.1) were acquired with an Applied Biosystems/MDS Sciex API4000 tuned to achieve unit resolution (0.7 DA at 50% FWHM) using Analyst software (version 1.4.2). The dynamic range of the assay was 1–5000 ng/mL. Pharmacokinetic parameters were calculated using Watson Bioanalytical LIMS, Version 7.4 (Thermo Fisher Scientific Inc., Waltham, MA).

Metabolite profiling of plasma, urine, bile and feces

The 0–48 h AUC pooled male and female rat plasma profiles are shown in Figure 1. In both male and female rat plasma only two components, parent and a single metabolite, were detected by 

\[ ^{19}F\text{NMR} \]

A total of 3–5 quantifiable components were detected in male and female rat urine. Parent compound was the major component detected in urine, constituting approximately 14 and 32% of the dose in male and female rats, respectively. Metabolite B was the major metabolite and accounted for 11% of the dose in male urine and 7% of the dose in female urine. Other metabolites detected in urine individually constituted <2% of the dose.

Figures 4 and 5 show the 

\[ ^{19}F\text{NMR profiles of representative male and female bile and feces, respectively. A total of 3–4 and 3–5 quantifiable components were detected in male and female rat bile and feces, respectively. Parent compound was observed as a minor component and metabolites} \]

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constituted the majority of dose excreted in bile; whereas, in feces, parent compound accounted for 3 and 7% of the dose in male and female, respectively. Other metabolites detected in feces each were 51% of the dose.

Although complete mass balance was not achieved in this exploratory study, it is reasonable to conclude that urinary excretion of the parent compound and its metabolites was the major route of elimination. In urine and feces, unchanged parent compound was recovered to the extent of approximately 17% (male) and 39% (female) of the administered dose. Metabolite B was the major metabolite accounting for approximately 15% (male) and 9% (female) of the dose and profiling by 19F NMR indicated that metabolism is mostly oxidative.

**Toxicokinetics in dog**

The systemic exposure (AUC) of the parent compound and metabolite A (N-oxide) in dogs after a single dose and after daily doses for 7 days as determined by 19F NMR is summarized in Table 3 and compared with those determined by conventional LC-MS/MS method. Parent drug was the predominant component in male and female dog plasma and only a single metabolite (metabolite A), other than that observed in rat plasma (only metabolite B was observed in rat plasma), was observed (Figure 1). After daily doses for 7 days exposure to parent compound increased noticeably in female dogs (approximately 53% when compared to single dose). No increase in metabolite exposure, however, was observed after single dose versus multiple doses. Overall, the calculated exposure for the parent compound and metabolite A was similar between the NMR and LC-MS/MS methods.

**Isolation of metabolite B**

Metabolite B is the major metabolite in rat urine. In order to identify its structure, microgram quantities of metabolite B

| Sex    | Urine   | Bile    | Feces   | Total   |
|--------|---------|---------|---------|---------|
| Male   | 28.0 ± 11.8 | 8.2 ± 4.6 | 3.2 ± 3.4 | 39.4 ± 9.5 |
| Female | 39.5 ± 11.9 | 8.1 ± 4.2 | 7.1 ± 1.9 | 54.6 ± 15.1 |

BQL = below quantitation limit.
were isolated by repeated HPLC purification from the pooled male and female urine samples.

**Structural characterization of metabolites A and B**

The mass spectrum of metabolite B showed a protonated molecular ion consistent with addition of an oxygen atom to the parent compound. Further mass spectral fragments observed in the MS/MS spectrum suggested that the oxygen was added to the pyrimidine ring. Although profiling by $^{19}$F NMR does not always provide structural information, the large chemical shift difference ($>10$ ppm) observed for the fluorine between the parent compound and metabolite B clearly indicated that the site of oxidation is in the vicinity of fluorine substitution, namely, the pyrimidine ring (Table 4). In order to identify the specific site of oxidation, conventional NMR investigation of metabolite B was undertaken. A detailed analysis of 1D and 2D $^1$H and $^{13}$C NMR data of

![Figure 3](image-url)  
**Figure 3.** $^{19}$F NMR profiles of 0–48 h pooled individual male Sprague–Dawley rat urine (Panels A, B, C and D) and all male pooled urine (Panel E) following a single oral dose (30 mg/kg) of fluoropyrimidine compound. The fluorine chemical shift for metabolite B was variable in individual rat urine samples (Panels A, B, C and D), but coalesced into one when pooled together (Panel E) indicating that metabolite B is pH sensitive and may exist in different isomeric forms. Similar profiles were observed with female rat urine samples (data not shown).

![Figure 4](image-url)  
**Figure 4.** $^{19}$F NMR profiles of pooled male (Panel A) and female (Panel B) Sprague–Dawley rat bile (0–24 h) following a single oral dose (30 mg/kg) pyrimidine compound. Inset: Deconvolution of overlapping peaks by the MNova NMR software (Pink: experimental spectrum and Blue: deconvoluted spectrum). Spiking with the parent compound indicated parent as a minor component in the overlapped region of the spectrum.
metabolite B together with the parent compound as shown in Table 4 (only partial assignments are shown) led to the structural assignment of metabolite B.

Inspection of the aromatic region of the proton spectrum revealed that the two protons situated ortho ($\delta$ 8.58 d, $J = 3.6$ Hz) and para ($\delta$ 9.01 d, $J = 2.6$ Hz) to the carbon bearing the fluorine substitution of the pyrimidine ring in the parent compound were conspicuously absent in metabolite B and was replaced by a single proton at 7.99 ppm (Table 4). In the parent compound, both protons showed a coupling to the fluorine but surprisingly no mutual coupling; however, in metabolite B the lone proton appeared as a singlet and showed no coupling to the fluorine. The absence of a coupling between the fluorine and hydrogen suggested that the site of oxidation was ortho to the fluorine substitution. Since the magnitude of $^1$H–$^{19}$F coupling cannot be reliably used to distinguish the site of oxidation, a $^1$H–$^{19}$F heteronuclear Overhauser effect spectroscopy (HOESY) experiment was performed both on the parent compound and metabolite B. The parent compound showed a strong HOESY correlation to proton at 8.58 ppm (ortho to fluorine substitution) and weak to moderate correlations to several other protons from the rest of the molecule indicating proximity of these protons to the fluorine atom. Metabolite B also showed weak to moderate HOESY correlations to the same protons from the rest of the molecule as the parent compound but did not show a correlation to the pyrimidine ring proton at 7.99 ppm, confirming the regiochemistry of oxidation as ortho to the fluorine substitution.

During profiling by $^{19}$F NMR, the fluorine resonance assigned to metabolite B showed significant shifts between samples (more prevalent in urine), but they coalesced when mixed into one sample (Figure 3). This indicates that metabolite B may be pH sensitive and could exist in keto-enol tautomeric forms as shown and the different chemical shifts for fluorine represent different pH-dependent populations of the two isomeric forms. Influence of pH on the chemical shift variation is well known in NMR spectroscopy (Kenwright et al., 2008; Miyataka et al., 2007).

Metabolite A is the only circulating metabolite observed in dog plasma after single or multiple doses. The $^{19}$F resonance for metabolite A, like metabolite B, is quite different than the parent compound but the shift is in the opposite direction suggesting the fluoropyrimidine ring as the site of metabolism and that metabolite A could be an N-oxide (LC/MS indicated addition of oxygen to the parent compound). This led to the synthesis of metabolite A for structure confirmation. The $^{19}$F NMR and standard 1 and 2D NMR analyses were also performed on the chemically synthesized metabolite A in order to confirm its structure. Partial assignments of metabolite A are also summarized in Table 4. When compared to the parent compound, significant up-field shifts (~10 ppm)

Table 3. Systemic exposure (AUC) of parent compound and metabolite A in male and female beagle dogs following daily oral doses of fluoropyrimidine compound (6 mg/kg) for 7 days.

| Day | Sex   | AUC$_{0–16}$ (µg h/mL) | LC-MS/MS | $^{19}$F NMR |
|-----|-------|------------------------|----------|--------------|
|     |       | Parent | Metabolite A | Parent | Metabolite A |
| 1   | Male  | 52.4  | 1.50         | 60.1   | 0.81         |
|    | Female | 33.0  | 1.74         | 42.8   | 2.28         |
| 7   | Male  | 44.6  | 1.60         | 64.7   | 0.78         |
|    | Female | 40.3  | 2.16         | 65.4   | 2.06         |
were observed for C-2, C-4 and C-6 (ortho or para to the N-oxide functionality) as a result of diminished electron density of the pyrimidine ring due to N-oxide formation, confirming the regiochemistry of N-oxide formation as N-3. The lack of $^1$H-$^{19}$F coupling between the fluorine and H-2 (para to fluorine substitution) suggests that the confirmation of the pyrimidine ring may be different in metabolite A than in the parent compound in which such coupling was observed. As with the parent compound, chemical shift assignments of H-2 (para to fluorine substitution) and H-4 (ortho to fluorine substitution) were confirmed by the $^1$H–$^{19}$F HOESY experiment.

### Discussion

NMR, mostly $^1$H NMR, has been used as a quantitative tool in several areas of research including process chemistry (Do et al., 2011; Holzgrabe et al., 2005), metabolomics (Lindon et al., 2000), fermentanomics (Bradley et al., 2010) and natural products (Pauli et al., 2012), to mention a few. Its application in drug disposition studies has also been emerging. In several recent investigations, $^1$H-NMR has been used to quantify small drug metabolites (Pauli et al., 2011; Holzgrabe et al., 2005), metabolomics (Lindon et al., 2011; Holzgrabe et al., 2005), and natural products (Pauli et al., 2012), to mention a few. Its application in drug disposition studies has also been emerging. In several recent investigations, $^1$H-NMR has been used to quantify small drug metabolites (Pauli et al., 2011; Holzgrabe et al., 2005), metabolomics (Lindon et al., 2011; Holzgrabe et al., 2005), and natural products (Pauli et al., 2012), to mention a few. Its application in drug disposition studies has also been emerging.

Our work demonstrated that NMR-based quantitation of parent compound and its metabolites from biological samples such as plasma, urine, bile and feces does not require any special sample preparation compared to those associated with radioactivity profiling. In the present investigation, as in the case of radioactivity profiling, only plasma and feces were subjected to extraction prior to analysis while bile and urine samples were analyzed directly. In these instances, impact of matrix (urine or bile) on the NMR line width is minimal as can be seen in the urine or bile $^{19}$F NMR profile (Figures 2 and 4). However, matrix-induced $^{19}$F chemical shift is more apparent for both parent and metabolites (urine or bile versus plasma or feces). Overall, the chemical shifts of parent and metabolites are consistent for plasma and feces where direct matrix effects were eliminated through extraction. If desired, such extraction can be performed for all matrices prior to NMR analysis and our experience is that this may be necessary in some cases. NMR is often criticized for lack of sensitivity for ADME-related studies. However, mass balance studies are usually conducted at doses high enough for sensitivity not to be an issue. Current data were generated using a 30-mg/kg oral dose, but in our experience mass balance can be determined in rats when administered as low as 1 mg/kg (~1 μmol) intravenous dose (data not shown).
In addition, matrix samples can be concentrated 2–5 times either by evaporation or extraction if higher sensitivity is desirable and this process has worked well in our hands. Although full mass balance was not achieved in this pilot excretion study, the information gained from the analysis is still valuable in understanding the overall disposition of the molecule because such information cannot be easily obtained during early phase of the drug development by currently available methods. Potential reasons for the inability to obtain full mass balance in this study could be due to early termination of sample collection (collection only up to 48 h post-dose) and the rigor that was used in the conduct of in-life portion of the study (discovery ADME studies generally are not conducted with the same rigor as radiolabeled studies).

Quantitation of plasma components in toxicological species and human is critical to determine if separate safety assessment of circulating drug metabolites in humans is needed. The FDA (2008 and 2012) and ICH (2009) guidance recommends further safety assessment of metabolites if their exposure in human is >10% of the total drug-related exposure and at significantly greater levels in humans than the maximum exposure observed in the species used for non-clinical safety assessment. Furthermore, the guidance recommends that the human metabolite coverage should be demonstrated at steady-state exposures rather than after a single dose. Radiolabel studies are typically conducted after single dose administration for practical reasons and the plasma exposures for parent drug and metabolites after a single dose often do not represent steady-state exposures. However, this issue can be easily circumvented when using 19F NMR because no radiolabeled compound is needed for profiling by NMR. For example, plasma samples collected during repeat dose toxicology and multiple ascending dose (MAD) clinical studies, respectively, can be effectively used to determine the exposures of parent drug and metabolites at steady-state in animals and humans. One such example is illustrated in the present investigation by profiling plasma samples from a 7-day repeat dose toxicology study in dogs (Table 3 and Figure 1). In both male and female dogs, parent compound was detected as the major component and metabolite A was detected as the sole circulating metabolite. Although plasma samples were unavailable from a similar repeat dose toxicology study in rats for NMR analysis, 19F NMR profiling of rat plasma after a single dose revealed the presence of a different metabolite (metabolite B) than that observed in dog plasma. Thus, in the absence of metabolite standards, quantitation by NMR provides absolute exposure for circulating metabolites either after a single dose or multiple doses. In the future, NMR analysis can be extended to human plasma once MAD study is completed, and profiling by NMR could help to ascertain adequate exposures for human circulating metabolites in animals used for safety assessment.

One other advantage that 19F NMR offers is it does not require fractionation by HPLC prior to analysis. However, this could also be a disadvantage since without HPLC fractionation structural information for metabolites is lost. Nonetheless, the extent of chemical shift difference observed for fluorine between the parent drug and metabolites often can inform the region of biotransformation. For example, large 19F chemical shift differences observed between the parent compound and metabolites A (>5 ppm) and B (>10 ppm) suggest that metabolism has likely occurred in the proximity of the fluorine substitution or in the pyrimidine ring (Figure 6). This has been confirmed by qualitative LC/MS profiling and later comparison with synthetic standards.

**Conclusions**

We have demonstrated that 19F NMR-based quantitation is a useful tool to obtain mass balance and determine elimination pathways for leads or optimized drug candidates that contain one or more fluorine atoms. If accepted by the scientific community and regulatory agencies, 19F NMR-based quantitation may be routinely used to determine the steady-state...
exposure of parent drug and metabolites and determine the need for safety assessment of unique or disproportionate human metabolites during drug development. At the very least, the tool offers an opportunity to essentially define the human clearance and metabolism of a novel drug candidate, potentially from the very first clinical study without the need for a radiolabel synthesis/study.

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Declaration of interest
Eli Lilly and Company supported this study and all authors are employees of Eli Lilly and Company. The authors report no declarations of interest.

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