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Comparison between Radioanalysis and ^{19}F Nuclear Magnetic Resonance Spectroscopy in the Determination of Mass Balance, Metabolism, and Distribution of Pefloxacin

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ABSTRACT

Mass balance and metabolism studies using radiolabeled substances are well recognized as an important part of the drug development process. In this study, we directly assessed the use of fluorine nuclear magnetic resonance (^{19}F NMR) to achieve quantitative mass balance, metabolism, and distribution information for fluorinated compounds, without the need for radiolabeled synthesis or study. As a test case, the disposition of pefloxacin, a fluoroquinolone antibiotic, was evaluated in rats using quantitative ^{19}F NMR in parallel with a radiolabeled study. Urine, bile, and feces samples were collected over specific periods after oral administration of either 25 mg/kg [^{14}C]pefloxacin or 25 mg/kg pefloxacin and were subsequently profiled by radioactivity or ^{19}F NMR, respectively. The percentage of dose excreted in each matrix was comparable between the two methods, with the total dose recovered by radioactivity and ^{19}F NMR determined to be 86.8% and 81.8%, respectively. In addition, plasma samples were collected to determine the exposure of pefloxacin and its circulating metabolites. The plasma exposure of pefloxacin determined by ^{19}F NMR was within 5% to that calculated by a validated liquid chromatography-tandem mass spectrometry bioanalytical method. By both methods, pefloxacin was identified as the major circulating entity, with pefloxacin glucuronide as the major circulating metabolite. Quantitative analysis of metabolites in excreta was generally comparable between the two methods. In selected tissues, both methods indicated that the parent drug accounted for most of the drug-related material. In summary, we have demonstrated that ^{19}F NMR can be used as an alternative method to conventional radiolabeled studies for compounds containing fluorine without the need for radiolabeled synthesis/study.

Introduction

One of the most outstanding features of nuclear magnetic resonance (NMR) spectroscopy that sets it apart from other analytical technologies is that it 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. Therefore, quantitation is independent of chemical structures. As a consequence, NMR spectroscopy has been widely used as a quantitative tool in several areas of pharmaceutical research (Holzgrabe et al., 2005; Bradley et al., 2010; Do et al., 2011; Barding et al., 2012; Pauli et al., 2012; Lindon and Wilson, 2015). Quantitative NMR has also found applications in drug metabolism studies and has been used in the quantitation of metabolites of anticancer, antifungal, and antipsychotic drugs (Sylvia and Gerig, 1993; Malet-Martino et al., 2005; Martino et al., 2006; Shamsipur et al., 2007). Mutlib et al. (2012) demonstrated that mass balance of fluorinated compounds can be obtained using quantitative fluorine nuclear magnetic resonance (^{19}F NMR). Studies conducted in rats with two fluorinated compounds that were also radiolabeled with carbon-14 showed that the two methods produced comparable results. To demonstrate the value of ^{19}F NMR in a discovery setting, the investigators also studied a fluorinated compound after i.v. administration to dogs and showed that mass balance can be estimated using ^{19}F NMR; this finding was later confirmed by additional quantitative studies using mass spectrometry (MS/MS). Based on this work, the authors outlined a strategy to understand the routes of excretion or mass balance in animals for fluorinated compounds in conjunction with ^{19}F NMR; however, these investigations were limited to mostly mass balance information, and no comparative quantitative data in each matrix were provided for parent/metabolites. More importantly, the investigators did not profile plasma, which is a distinct advantage of using ^{19}F NMR compared with single-dose radiolabeled studies. This is because steady-state exposure of major or significant metabolites can be obtained to address the human metabolite coverage without the need for metabolite standards or radiolabeled substance using ^{19}F NMR. Thus, to our knowledge, these earlier investigations are relatively narrow in scope and did not encompass comprehensive mass balance, metabolism, and distribution studies. Recently, our laboratory has demonstrated the utility of ^{19}F NMR early in drug discovery by investigating the disposition of a fluoropyrimidine compound; however, in that investigation, NMR results were not directly compared with the widely used gold standard radiolabeled study (Hu et al., 2015). The objectives of the present investigation were to evaluate by ^{19}F NMR the mass balance and metabolism of pefloxacin, a fluoroquinolone antibiotic, in rats in parallel with a radiolabeled study and critically compare the results between the two methods. Additionally, we also conducted an exploratory assessment of the distribution of pefloxacin-related material in selected rat tissues, including brain, heart, kidney, liver, lung, and spleen,

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ABBREVIATIONS: AUC, area under the plasma concentration-time curve; ^{19}F NMR, fluorine nuclear magnetic resonance; LC-MS/MS, liquid chromatography-mass spectrometry; LSC, liquid scintillation counting; S/N, signal-to-noise ratio.

by both methods. The absorption, distribution, metabolism, and elimination of pefloxacin in nonclinical species and humans have been previously described in a nonradiolabeled study (Montay et al., 1984).

Materials and Methods

Materials

Pefloxacin as mesylate dihydrate and norfloxacin was purchased from Sigma-Aldrich (St. Louis, MO). [^{14}C]pefloxacin was synthesized at Quotient Bioresearch (Cardiff, UK). The vehicle used for oral administration of [^{14}C]pefloxacin or pefloxacin was deionized water.

Animals

Male Sprague-Dawley rats, intact and bile duct–cannulated, were purchased from Envigo RMS, Inc. (Indianapolis, IN). The animals were acclimated for 3 days before dose administration. At dosing, the animals weighed 228 to 320 g and were 9 to 11 weeks of age. All procedures used in this study are in accordance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare. Whenever possible, procedures in this study have been designed to avoid or minimize discomfort, distress, and pain to animals.

Synthesis of [^{14}C]Pefloxacin

Synthesis of radiolabeled pefloxacin (Supplemental Scheme 1) was accomplished in six steps, starting from diethyl 2-[^{14}C]malonate **1**. Initial reaction with triethyl orthoformate led to ethoxymethylenemalonate **2**, which was coupled with chrorofluoroaniline **3** to provide N-substituted compound **4**. The cyclization of **4** in refluxing diphenyl ether gave hydroxyquinoline derivative **5**, which, after N-ethylation, followed by hydrolysis of ester **6,** gave oxoquinolone **7**. Finally, coupling of chloride **7** with N-methylpiperazine provided target [^{14}C]pefloxacin with radiochemical purity of 99.7% and specific activity (after dilution with the unlabeled material) of 39.6 μCi/mg.

Study Design and Sample Collection

The overall study design is summarized in Table 1. Rats in all groups received an oral dose of 25 mg/kg of either [^{14}C]pefloxacin (100 μCi/kg) or pefloxacin. From group 1 rats, blood samples of approximately 1 ml were collected from three rats/time point via jugular vein into tubes containing K_2EDTA at 0.5, 1, 2, 4, 8, 12, 24, and 48 hours postdose for pharmacokinetic analysis. Blood samples were kept on wet ice and centrifuged (1300*g*) to obtain plasma within 60 minutes of blood collection. The plasma sample collected at each time point was divided into approximately two aliquots, one for the pefloxacin concentration measurement by liquid chromatography with tandem mass spectrometry (LC-MS/MS) method and the other by ^{19}F NMR. From group 2 rats, additional blood samples of approximately 1.8 ml were collected from three rats per time point via jugular vein into tubes containing K_2EDTA at 0.5, 1, 2, and 4 hours postdose for metabolite profiling by radioactivity. From groups 3 and 4 rats, cumulative excreta samples were collected postdose into plastic containers over ice for periods 0–6, 6–12, 12–24, 24–48, and 48–72 hours for bile; 0–12, 12–24, 24–48, and 48–72 hours for urine and at 24-hour intervals through 72 hours for feces; group 3 samples were used to determine the mass balance and metabolite profiling by ^{19}F NMR; and group 4 samples were used to determine the mass balance and metabolite profiling by radioactivity. Blood and selected tissue samples, including brain, heart, kidney, liver, lung, and spleen, were collected from one animal/time point at 0.5, 1, 2, and 24 hours postdose from groups 5 and 6 to evaluate the distribution of pefloxacin- or [^{14}C]pefloxacin-related material in various tissues by ^{19}F NMR or radioactivity, respectively.

Analysis of Total Radioactivity

Radioactivity concentrations in plasma (groups 2 and 6) and in bile and urine (group 4) were determined directly by liquid scintillation counting (LSC) using model 2900TR liquid scintillation counter (Packard Instrument Company, Meriden, CT) after adding Ultima Gold XR scintillation cocktail to a known amount of the sample. Radioactivity concentrations in fecal homogenates was determined by combustion of a known amount of fecal homogenate in a model 307 Sample Oxidizer (Packard Instrument Company, Meriden, CT) and trapping the resulting $^{14}CO_2$ in a mixture of Perma Fluor and Carbo-Sorb. Radioactivity concentrations in tissue samples collected from group 6 rats were determined by LSC after digestion with 1 N sodium hydroxide.

Bioanalysis and Pharmacokinetics of Pefloxacin

Plasma samples collected from group 1 rats were analyzed for pefloxacin using a validated LC-MS/MS method. Briefly, 20 μl of plasma sample was extracted by protein precipitation by mixing with 50 μl of internal standard solution (5 ng/ml pefloxacin-d3 in 1:1 CH_3OH-H_2O) and 300 μl CH_3CN. The mixture was centrifuged at 3200 rpm for 2 minutes. An aliquot of the supernatant was diluted 4 × with 1:9 CH_3OH-H_2O and analyzed on a X-Bridge C18, 5 μm, 2 × 30-mm column (Waters Corporation, Milford, MA) using a gradient mobile phase (mobile phase A: 50 mM NH_4HCO_3 in H_2O and mobile phase B: 20 mM NH_4HCO_3 in MeOH) delivered at a flow rate of 1.5 ml/min). Selected reaction monitoring transitions in positive ion mode with precursor and product ions for pefloxacin (*m/z* 334.2 → *m/z* 290.2) and pefloxacin-d3 (*m/z* 337.2 → *m/z* 293.2) were acquired with Sciex API 6500 mass spectrometer using Analyst software version 1.6.2 (SCIEX Headquarters, Farmingham, MA). The dynamic range of the assay was 1–5000 ng/ml.

Pharmacokinetic parameters were calculated using noncompartmental analysis (NCA) in Phoenix WinNonlin Professional Edition, Version 6.2.1 (Pharsight Corporation A Certara Company, Princeton, NJ).

Extraction of Matrices for Metabolite Profiling by Radioactivity

Plasma. A known aliquot of plasma samples obtained from three rats in group 2 at 0.5, 1, 2, and 4 hours postdose were combined across animals by time point. The samples were then pooled along with control rat plasma to make a 0- to 4-hour plasma area under the plasma concentration-time curve (AUC) pool (Hop et al., 1998). Similarly, plasma samples from a single rat at each time point in group 6 were also pooled to generate a 0.5- to 2-hour AUC pool. Approximately 1 ml of the AUC pooled plasma sample (groups 2 and 6) was combined with 3 ml of 0.1% formic acid in CH_3CN, vortex-mixed for 1 minute, shaken for 10 minutes, centrifuged, and the supernatants were removed. The pellets were re-extracted twice with 1 ml of water and 2 ml of 0.1% formic acid in CH_3CN. All supernatants were combined, and an aliquot in duplicate was analyzed by LSC. The extraction

TABLE 1

Study design

Group[a]	No. of Animals		Compound	Samples Collected	Purpose
	BDC	Intact			
1	0	12	Pefloxacin	Blood	Plasma exposure by LC-MS/MS and ^{19}F NMR
2	0	6	[^{14}C]Pefloxacin	Blood	Metabolite profiling by radioactivity
3	4	0	Pefloxacin	Bile, urine, feces	Excretion and metabolite profiling by ^{19}F NMR
4	4	0	[^{14}C]Pefloxacin	Bile, urine, feces	Excretion and metabolite profiling by radioactivity
5	0	4	Pefloxacin	Blood and tissues	Distribution by ^{19}F NMR
6	0	4	[^{14}C]Pefloxacin	Blood and tissues	Distribution by radioactivity

BDC, bile duct–cannulated.
[a]Administered pefloxacin dose was 25 mg/kg in all groups.

recoveries were determined to be 91.1% and 94.3% for groups 2 and 6, respectively. The remaining supernatants were concentrated by evaporation under a stream of nitrogen to a final volume of approximately 0.5 ml before analysis.

Urine. An aliquot (1 ml) of urine samples collected from four rats in group 4 at 0–12 and 12–24 hours were pooled across animals by time interval to generate a single 0–12 and 12–24 hours pooled samples. The pooled urine samples were analyzed directly without any pretreatment.

Bile. An aliquot (1 ml) of bile samples collected from four rats in group 4 at 0–6 and 6–12 hours were pooled across animals by time interval to generate a single 0- to 6- and 6- to 12-hour pooled samples. The pooled samples were diluted 1:5 v/v with water before analysis.

Feces. Feces samples collected from four rats in group 4 at 0–24 and 24–48 hours were homogenized with a known amount of EtOH:H_2O (1:1, v:v). An aliquot (2.8 g) of the homogenized samples were pooled by time interval to generate a single 0–24 and 24–48 hours pooled samples. Approximately 1 g of each feces pool was mixed with 3 ml of 0.1% HCOOH in CH_3CN, vortex mixed for 1 minute, shaken for 10 minutes, centrifuged, and the supernatant was removed. The pellets were further extracted twice with 1 ml of H_2O and 2 ml of 0.1% HCOOH in CH_3CN, with the exception that the 24–48 hours pool was extracted one more time with the same solvent mixture. The combined supernatants were evaporated to near dryness under a stream of nitrogen and reconstituted in water before analysis. The extraction recoveries for the 0-to 24- and 24- to 48-hour fecal homogenates were 90.5% and 78.7%, respectively.

Tissues. Tissue samples, brain, heart, kidney, liver, lung, and spleen were excised, rinsed with saline, and blotted dry, weighed, and placed on wet ice for processing from a single rat at 0.5, 1, and 2 hours postdose in group 6. The tissues were then homogenized by adding a known amount of H_2O (approximately 0.5 to 2 times depending on the tissue) using a probe type homogenizer at room temperature. The homogenized tissue samples were then pooled to generate a single 0.5- to 2-hour AUC pooled sample (Hop et al., 1998). A similar pool was made from plasma collected from a single rat at 0.5, 1, and 2 hours in group 6. The radioactivity in each pooled sample was determined by LSC. Approximately 1 g of each AUC pooled sample (plasma and tissues) was combined with 3 ml of 0.1% HCOOH in CH_3CN, vortex-mixed for 1 minute, shaken for 20 minutes, and centrifuged, and the supernatants were removed. The pellets were re-extracted with 1 ml of water and 2 ml of 0.1% HCOOH in CH_3CN as described. The supernatants were combined, and duplicate aliquots were analyzed by LSC to determine the extraction recovery. The extraction recovery ranged from 91.5% to 98% for all tissues and 90.2% for plasma. The remaining combined supernatants then were concentrated by evaporation under a stream of nitrogen to near dryness and reconstituted in H_2O before analysis.

Metabolite Profiling and Identification by Radioactivity and LC-MS/MS

Pefloxacin and its metabolites in various matrices (plasma, urine, bile, feces, and tissues) were separated on an Atlantis T3 C18 150 × 4.6-mm, 3-μm column (Waters UK, Elstree, Hertfordshire, UK) with a flow rate of 1 ml/min. The mobile phase consisted of 10 mM aqueous ammonium formate (mobile phase A) and CH_3CN (mobile phase B). Analytes were eluted with the following gradient [time (min)/% B]: 0/5, 5/5, 10/15, 15/15, 22/20, 26/30, 31/95, 36/95, and 36.1/5. The column effluent was split either between radiodetector (Beta-RAM model 4, LabLogic, Sheffield, South Yorkshire, UK) and MS (10:1) during urine and bile sample analyses or between fraction collector and MS (4:1) during plasma, feces, and tissue sample analyses. When using a fraction collector, fractions were collected at 10-second intervals into 96-well plates containing solid scintillant, and radioactivity in each well was determined using TopCount (PerkinElmer, Beaconsfield, Buckinghamshire, UK) analysis. Subsequently, radiochemical profiles were generated based on radioactivity counts. Metabolite identification was performed by accurate mass MS and MS/MS using a Thermo Fisher LTQ XL Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific Inc., Hemel Hempstead, Hertfordshire, UK) with a full scan resolution of 30,000.

Extraction of Matrices and Sample Preparation for ^{19}F NMR Analysis

Plasma. Plasma standards were prepared by spiking 50 μl of appropriate stock solutions of pefloxacin in methanol into 1 ml of control rat plasma yielding final concentrations of 30, 10, 3, and 1 μM pefloxacin. The samples were extracted and dried as described for the radioactivity analysis. The dried extracts were reconstituted in 630 μl of 1:1 H_2O-CH_3OH plus 70 μl of CD_3OD, sonicated in a bath sonicator for 5 minutes, and centrifuged at 14,000 rpm for 10 minutes. The clear supernatant was transferred to a 5-mm NMR tube for analysis. A known aliquot of plasma samples collected from group 1 rats ($n = 3$) was combined across animals per time point and then pooled along with a control rat plasma to generate a 0- to 24-hour plasma AUC pool (Hop et al., 1998). The pooled sample (440 μl) was extracted and reconstituted as described already before NMR analysis.

Urine. Urine standards, yielding final concentrations of 300, 100, 30, 10, and 3 μM pefloxacin, were prepared by mixing 630 μl of the rat control urine with 70 μl of CD_3OD and 5% CH_3COOH and appropriate concentration of pefloxacin stock solutions. The mixture was centrifuged at 14,000 rpm for 10 minutes, and the clear supernatant was transferred for NMR analysis. Urine samples collected from four rats in group 3 at 0–12 and 12–24 hours were pooled by 10% volume across animals by time interval to generate a single 0- to 12- and 12- to 24-hour pooled samples. An aliquot of the pooled urine sample (630 μl) was mixed with 70 μl of CD_3OD and 5% CH_3COOH, centrifuged, and transferred for NMR analysis. Acetic acid (5%) was added to urine and other matrix samples, including bile and feces (see following sections) to obtain acceptable NMR linewidth.

Bile. Bile standards, yielding final concentrations of 300, 100, 30, 10, and 3 μM pefloxacin, were prepared by mixing 630 μl of the rat control bile with 70 μl of CD_3OD and 5% CH_3COOH and appropriate concentration of pefloxacin stock solutions. The mixed sample was centrifuged as before, and the clear supernatant was transferred for NMR analysis. Bile samples collected from four rats in group 3 over 0–6 and 6–12 hours were pooled by 10% volume across animals by time interval to generate single 0- to 6- and 6- to 12-hour pooled samples. The pooled samples, 630 μl each, were processed as before before NMR analysis.

Feces. Feces standards, yielding final concentrations of 150, 100, 50, 10, and 3 μM pefloxacin, were prepared by mixing appropriate concentrations of pefloxacin with approximately 0.5 g of rat control fecal homogenate. The homogenate was then extracted and dried as detailed for the radioactivity analysis. To the dried extract, 630 μl of 1:1 H_2O-CH_3OH plus 70 μl of CD_3OD and 5% CH_3COOH was added, sonicated for 5 minutes in a bath sonicator, centrifuged at 14,000 rpm for 10 minutes, and transferred for NMR analysis. Fecal samples collected from four rats in group 3 over 0–24 and 24–48 hours after homogenization were pooled by 2% weight across animals by time interval to generate a single 0–24 and 24-48 hours pooled sample. The pooled samples, approximately 0.5 g each, were extracted and reconstituted before analysis as described already.

Tissues. Tissue samples, brain, heart, kidney, liver, lung, and spleen, were collected and processed as described already—for radioactivity analysis from control and group 5 rats. A single tissue standard, yielding a final concentration of 100 μM pefloxacin, was prepared for each control tissue by mixing appropriate concentration of pefloxacin with approximately 0.5 g of each rat control tissue homogenate. The homogenate was extracted as described for radioactivity analysis and reconstituted as described before for NMR analysis. Each tissue sample collected from group 5 rats after homogenization was pooled to generate a 0- to 24-hour AUC pool, extracted, and reconstituted as before. In addition, the tissue and plasma samples collected at the 1 hour time point (approximate C_{max} based on radioactivity for all tissues and plasma) from group 5 rats were also processed as before for NMR analysis.

Metabolite Profiling by ^{19}F NMR

All ^{19}F NMR experiments were performed on a Bruker Avance III 600 MHz spectrometer equipped with a QCI HFCN quadruple resonance cryoprobe (Billerica, MA). Fluorine NMR spectra were recorded with a total of 32,000 complex points and a spectral width of 100 ppm (56,446 Hz) centered at δ -120 ppm.

Measurement of Longitudinal Relaxation Time. The relaxation time (T_1) for pefloxacin in matrices or matrix extracts was measured using a typical inversion recovery method by incrementing the duration of the interpulse delay time (τ) in 0.1-second steps. The signal was nulled with an average delay of 0.2 second, based on which T_1 was calculated to be approximately 0.3 second ($T_1 = \tau_{null}/\ln 2$).

A 5-second preacquisition delay, together with an acquisition time of 0.6 second, resulting in a total of 5.6-second repetition delay (well over the 5 × T_1 recommended for quantitative NMR experiments) was used for all experiments. Proton decoupling during acquisition was achieved using a standard Waltz-16 decoupling sequence centered at 8 ppm. Raw data were zero-filled to 64,000 complex points and a 2-Hz line-broadening window function was applied before

Fourier transformation. ^{19}F chemical shifts were referenced with respect to $CFCl_3$ at 0 ppm. The spectra were phase and baseline corrected before integration was carried out. NMR run times ranged from 6.5 minutes to 24 hours, depending on the sample concentration for plasma, urine, bile, feces, and tissue standards and study samples. Signal averaging with an experimental run time beyond 24 hours to improve the signal-to-noise ratio (S/N) was not conducted because longer analysis times are not expected to provide significant S/N improvements (in NMR, gains in S/N are proportional to the square root of the number of scans). The data acquisition time can be estimated a priori based mostly on the number of fluorine atoms/groups present in the molecule (CF versus. CF_2 versus CF_3 or

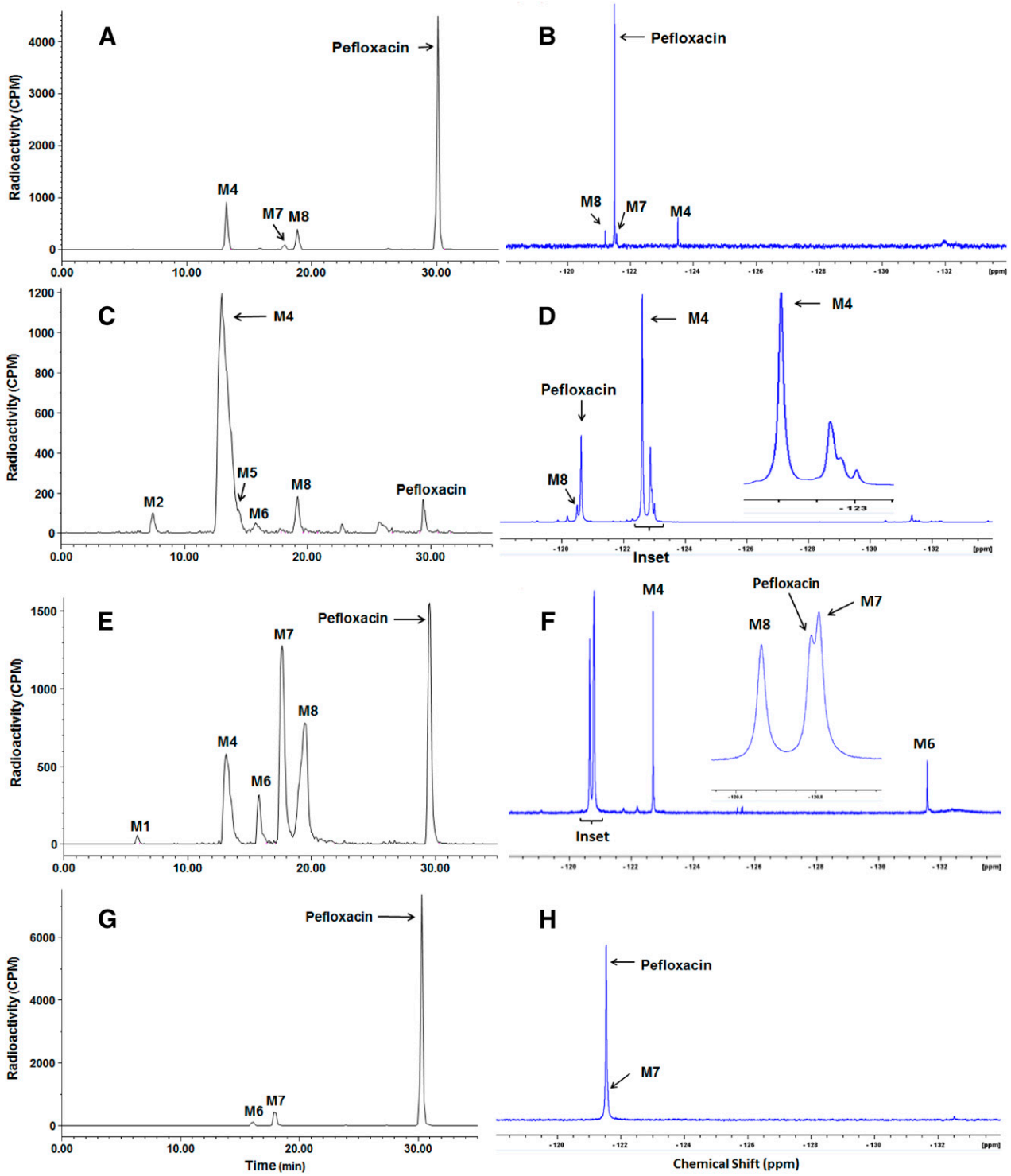

Fig. 1. Radioactivity and ^{19} NMR profiles of AUC pooled plasma (A and B), bile (C and D), urine (E and F), and feces (G and H) after a single 25-mg/kg oral dose of [^{14}C]pefloxacin or pefloxacin to male Sprague-Dawley rats. AUC plasma pools were generated using plasma samples collected from 0.5 to 2 hours for radioactivity analysis and 0 to 24 hours for ^{19}F NMR analysis. Identity of pefloxacin and norfloxacin (M7) in ^{19}F NMR profiles was confirmed by spiking respective standards in plasma, urine, bile, and feces study samples. The assignment of other metabolites in the ^{19}F NMR spectra was based on comparison with radioactivity profile and consistency of ^{19}F chemical shifts across matrices.

TABLE 2

Plasma exposure of pefloxacin and metabolites as percentage of total drug-related exposure after a single 25-mg/kg oral dose of [^{14}C]pefloxacin or pefloxacin to male Sprague-Dawley rats

Compound	Percent Exposure Relative to Total Drug-Related Exposure		
	Radioactivity		^{19}F NMR
	0–4 h AUC	0.5–2 h AUC[a]	0–24 h AUC
Parent (pefloxacin)	64.8	70.3	80.7
M7 (norfloxacin)	4.3	2.4	3.9
M4 (pefloxacin glucuronide)	16.9	16.2	10.5
M8 (pefloxacin + O)	10.0	8.7	4.9

[a]Plasma samples were collected at 0.5, 2, and 24 hours postdose. Because of an oversight, a 24-hour sample was not used in the AUC pool.

multiple chemically equivalent CF_3), magnetic-field strength (lower versus higher), and probe type (room temperature versus cryogenically cooled).

Standard Curves and NMR-based Quantitation Standard curves for pefloxacin were generated in each matrix by measuring ^{19}F NMR spectra over a range of concentrations, as described before (Hu et al., 2015). Briefly, the ^{19}F spectrum obtained with the highest concentration was assigned the nominal concentration value and used as reference spectrum to calibrate the remainder of the spectra in the concentration range using the built-in ERETIC2 tool (TopSpin version 3.2) based on the PULCON method (Wider and Dreier, 2006). For all matrices, the correlation by linear fit observed between nominal and NMR-measured concentrations was excellent, with $r^2 \geq 0.99$ (Supplemental Fig. 1). The limit of quantitation was determined based on the S/N observed for the standards of pefloxacin in various matrices. The S/N for the lowest concentration in the calibration range was calculated to be 14 for plasma (1 μM pefloxacin), 9 for urine (3 μM pefloxacin), 14 for bile (3 μM pefloxacin), and 16 for feces (3 μM pefloxacin). For each matrix, quantitation of pefloxacin and its metabolites observed as peaks in the ^{19}F NMR spectra of study samples was also accomplished using the ERETIC2 tool. As before, the ^{19}F peak obtained with the highest concentration for each matrix was assigned the nominal value and used as reference spectrum to determine the concentrations of pefloxacin and metabolites, with subsequent correction for the linear fit, with the exception of tissues where correction for the linear fit was not made since only a single standard was used (see preceding). Spiking experiments with available standards, pefloxacin and norfloxacin, were performed to confirm their identity in the ^{19}F NMR spectrum of selected matrices. Where necessary, deconvolution was performed using Mnova NMR version 9.0.0 (Mestrelab Research, Escondido, CA) before quantitation of overlapping peaks http://www.ebyte.it/library/downloads/Poster_GSD_ENC09.pdf. Finally, the percentage of dose eliminated in each matrix was calculated by adding individual peak or selected region concentrations observed in the NMR spectrum for that matrix, and mass balance was calculated by adding the dose eliminated in urine, bile, and feces over specific periods.

Qualitative Profiling of Bile Samples and Peak Area Determination of Pefloxacin and Pefloxacin Acyl Glucuronide (M4) by LC-MS/MS with and without 5% Acetic Acid

Since 5% acetic acid was added to bile samples during ^{19}F NMR analysis to obtain acceptable NMR linewidth (see preceding discussion), additional LC-MS/MS experiments were conducted to evaluate the impact of acetic acid on the stability of the pefloxacin acyl glucuronide metabolite (M4) in bile. An aliquot of 0- to 6- and 6- to 12-hour bile study samples from group 3 rats was reconstituted with 10% CH_3OH and 5% acetic acid (similar to ^{19}F NMR sample preparation) and analyzed immediately and after 24 hours at room temperature after reconstitution, along with bile samples without the addition of 5% acetic acid. Pefloxacin and its metabolites were separated on an ACQUITY UPLC BEH C18, 2.1 × 100 mm, 1.7-μM column (Waters Corporation, Milford, MA) with a flow rate of 0.5 ml/min. The mobile phase consisted of water with 1% HCOOH (mobile phase A) and CH_3CN with 1% HCOOH (mobile phase B). Analytes were eluted with the following gradient [time (min)/%B]: 0/2, 0.5/2, 0.6/11, 3.8/11, 3.9/90, 4.25/90, 4.5/2 and 6/2. Analyte identification and peak area determinations for pefloxacin and M4 were performed by accurate mass MS and MS/MS using a Thermo Fisher Q Extractive mass spectrometer (Thermo Fisher Scientific, San Jose, CA) with a full scan resolution of 30,000.

Results

Pharmacokinetics of Pefloxacin. After oral administration to male rats, pefloxacin was rapidly absorbed with a T_{max} of 1 hour. Pefloxacin concentrations in plasma declined to below the limit of quantitation by 48 hours postdose. The terminal half-life of pefloxacin in plasma was estimated to be 2.5 hours.

The systemic exposure (the AUC from 0 to 24 hours [AUC_{0-24}]) of pefloxacin calculated by ^{19}F NMR using AUC pool with adequate sampling from group 1 rats (18.57 μg · h/ml) was within 5% of the plasma exposure calculated by NCA (19.50 μg · h/ml) or estimated by AUC pooling from the plasma concentrations determined by a validated LC-MS/MS method (19.46 μg · h/ml).

Profiling of plasma AUC pool with sparse sampling (0.5, 1, and 2 hours) from group 6 rats by radioactivity showed pefloxacin as the predominant component (70.3% of the total radioactivity exposure) and the presence of eight metabolites, of which only pefloxacin glucuronide (M4, 16.2% of the total radioactivity exposure), norfloxacin (M7, 2.4% of the total radioactivity exposure), and pefloxacin + O (M8, 8.7% of the total radioactivity exposure) had exposures >2% of the total radioactivity exposure (Fig. 1A and Table 3). Profiling of plasma AUC pool with sparse sampling (0.5, 1, 2, and 24 hours) from group 5 rats by ^{19}F NMR also revealed pefloxacin as the major circulating component (80.7% of the total drug-related exposure) and the presence of three quantifiable metabolites (M4, M7, and M8) with exposures 10.5%, 3.9%, and 4.9%, respectively, of the total drug-related exposure (Fig. 1B and Table 2).

Metabolite Profiling and Excretion of Pefloxacin by Radioactivity and ^{19}F NMR. The excretion of radioactivity in urine, bile, and feces over specific intervals is summarized in Table 4. After oral administration,

TABLE 3

Percent dose eliminated in urine, bile, and feces after a single 25-mg/kg oral dose of [^{14}C]pefloxacin or pefloxacin to male bile-cannulated Sprague-Dawley rats

Matrix	Collection Interval	Percent Dose Eliminated	
		Radioactivity[a]	^{19}F NMR[b]
Urine	0–24 h	22.81	26.57
Bile	0–12 h	44.76	39.41
Feces	0–48 h	19.27	15.81
Total	NA	86.84	81.79

NA, not applicable.
[a]Values represent mean (n = 4).
[b]Values represent single determination (a known amount of sample from n = 4 animals were pooled for each matrix before analysis).

TABLE 4

Recovery of dose as pefloxacin and metabolites in urine, bile, and feces after a single 25-mg/kg oral dose of [^{14}C]pefloxacin or pefloxacin to male bile-cannulated Sprague-Dawley rats

Method	Parent/Metabolite	% Dose Eliminated			
		Urine (0–24)	Bile (0–12 h)	Feces (0–48 h)	Total
Radioactivity profiling	Parent (pefloxacin)	4.94	1.87	12.86	19.67
	M4	3.32	32.94	BQL	36.26
	M6	1.18	0.52	0.31	2.01
	M7 (norfloxacin)	6.09	0.19	1.30	7.58
	M8	5.70	1.80	0.13	7.63
^{19}F NMR profiling	Parent (pefloxacin)	5.18	9.50	15.81[b]	30.49
	M4	3.93	16.88	BQL	20.81
	M6	1.64	[a]	BQL	1.64
	M7 (norfloxacin)	6.60	BQL	[c]	6.60
	M8	6.21	1.50	BQL	7.71

[a]Could not be positively identified. Three unidentified peaks detected accounted for 4.24%, 2.51%, and 1.74% of dose.
[b]Value includes M7 (norfloxacin).
[c]Overlapped with pefloxacin and could not be resolved in data analysis by Mnova MNR.

the overall mean recovery of radioactivity over specified time intervals was 86.8%, with 44.8%, 22.8%, and 19.3% of pefloxacin-related radioactivity recovered in bile (0–12 hours), urine (0–24 hours), and feces (0–48 hours), respectively. Profiling by ^{19}F NMR of bile, urine, and feces over the same specified time intervals as radioactivity resulted in a comparable recovery of 81.8%, with 39.4%, 26.6%, and 15.8% of the administered dose recovered in bile, urine, and feces, respectively (Table 3). Of the total radioactivity dose recovered in bile, urine, and feces, a substantial portion of the dose (40.1%, 21.6%, and 14.7%, respectively) was accounted for by pefloxacin and metabolites. Figure 1, C, E, and G shows the metabolic profile of bile, urine, and feces by radioactivity, and Table 4 summarizes the major metabolites identified and the amounts recovered in urine, bile, and feces by radioactivity (see Table 5 for a complete list of metabolites identified and amounts recovered). By comparison, Fig. 1, D, F, and H shows the metabolic profile of bile, urine, and feces, respectively, by ^{19}F NMR; Table 4 also summarizes the amount of pefloxacin and major metabolites eliminated in various matrices as quantitated by ^{19}F NMR. By radioactivity, unchanged pefloxacin accounted for approximately 20% of the administered dose (Table 4). Metabolites that accounted for >5% of the dose included M4 (36.2%), M7 (7.6%), and M8 (7.6%), with formation of M4 representing the major biotransformation pathway of pefloxacin (Fig. 2). As shown in Table 4, the amount of unchanged pefloxacin dose recovered in excreta was 30.5% by NMR; consistent with radioactivity analysis, the major metabolite identified by ^{19}F NMR was also M4.

Peak Area Determination of Pefloxacin and Pefloxacin Acyl Glucuronide by LC-MS/MS with and without 5% Acetic Acid. The peak area ratios of pefloxacin acyl glucuronide to pefloxacin as determined by LC-MS/MS with and without 5% acetic acid for the 0- to 6-hour bile sample were nearly identical (0.109 and 0.105, respectively). Similarly, the peak area ratios of pefloxacin acyl glucuronide to pefloxacin with and without 5% acetic acid for the 6- to 12-hour bile sample were identical (0.089). Furthermore, no differences in the ratios were observed when the samples were analyzed immediately or after 24 hours after the addition of 5% acetic acid. Thus, these results indicate that pefloxacin acyl glucuronide is stable under the conditions of ^{19}F NMR analysis.

Tissue Distribution of Pefloxacin by Radioactivity and ^{19}F NMR. Distribution of pefloxacin-related material in selected tissues, including brain, heart, kidney, liver, lung, and spleen, is shown in Fig. 3A by radioactivity and in Fig. 3B by ^{19}F NMR. Pefloxacin tissue-to-plasma ratios by both methods are summarized in Supplemental Table 1. By both methods, the parent drug was the predominant compound in all tissues, and, with the exception of the brain, total drug-related concentrations were higher in all tissues relative to plasma. The brain-to-plasma ratio was determined to be approximately 0.4 by both radioactivity and ^{19}F NMR, indicating that pefloxacin did cross the blood-brain barrier. Metabolite concentrations in tissues were generally low as evaluated by both methods.

Structure Determination of Pefloxacin Metabolites. The structures of metabolites were proposed by accurate mass measurement, characteristic product ions observed in the MS^n spectra, and in comparison with a previously published report (Montay et al., 1984). Data summarizing the observed protonated molecular ions, elemental compositions, and characteristic product ions with proposed structures for all quantitated metabolites are included in the supplementary material (Supplemental Table 2). Detailed description of the structure determination for all metabolites is beyond the scope of this manuscript; however, a brief discussion on the structure of metabolite M4 is warranted because of the observed differences in its percentage of dose recovered between radioanalysis and ^{19}F NMR analysis. The protonated molecular ion of M4 was observed at *m/z* 510 (elemental composition $C_{23}H_{29}FN_3O_9$), 176 Da more than pefloxacin, indicating that it is a direct glucuronide of pefloxacin. A facile loss of the glucuronosyl residue in MS/MS did not permit its definitive structural assignment, acyl- versus N-glucuronide; however, the presence of carboxylic acid in pefloxacin and detection of more than one glucuronide with same mass suggested the likely structure of M4 as the acyl glucuronide. Furthermore, formation of acyl

TABLE 5

Pefloxacin and metabolites identified and amounts eliminated as percent dose in bile, urine, and feces after a single 25-mg/kg oral dose administration of [^{14}C]pefloxacin to male Sprague-Dawley rats

Pefloxacin/ Metabolite	Proposed Structure	Percent Dose Eliminated			
		Bile	Urine	Feces	Total
Pefloxacin	Pefloxacin	1.87	4.95	12.86	19.68
M1	Norfloxacin glucuronide	BQL	0.13	BQL	0.13
M2	Norfloxacin glucuronide	1.18	BQL	BQL	1.18
M3	Pefloxacin + O glucuronide	0.08	BQL	BQL	0.08
M4	Pefloxacin glucuronide	32.94	3.32	BQL	36.26
M5	Pefloxacin glucuronide	1.55	0.19	BQL	1.74
M6	Desethyl-pefloxacin + 2H	0.53	1.18	0.31	2.02
M7	Norfloxacin	0.19	6.09	1.30	7.58
M8	Pefloxacin + O	1.80	5.71	0.13	7.64
M9	Norfloxacin + O −2H	BQL	BQL	0.06	0.06
Total eliminated					86.84
Total identified					76.31

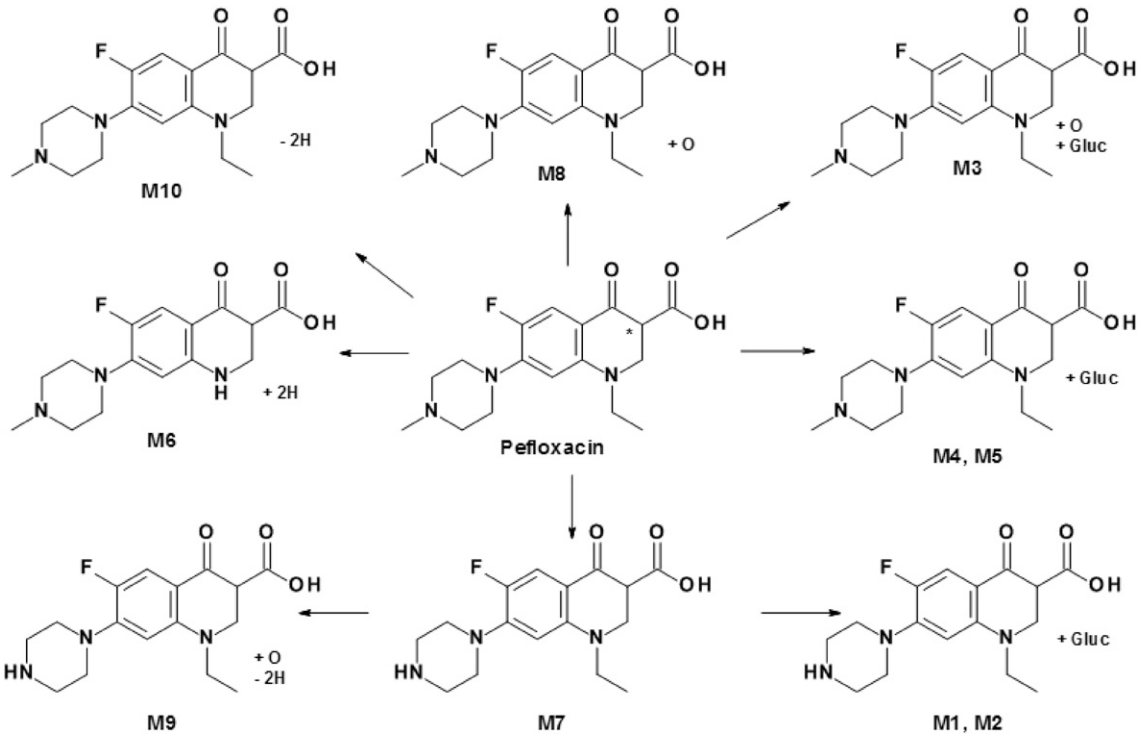

Fig. 2. Proposed metabolic pathways of pefloxacin in male Sprague-Dawley rats after a single oral 25-mg/kg dose of [^{14}C]pefloxacin. *Denotes the position of ^{14}C label.

glucuronide metabolites has been frequently reported for other members of this class of fluoroquinolone antibiotics (Sharma et al., 2008).

Discussion

Mass balance and metabolism studies in animals and humans are integral parts of the drug development process, and radiolabeled substances are generally used in these studies (Penner et al., 2012). Herein, we describe an alternative method to obtain mass balance, metabolism, and distribution information of pefloxacin using quantitative ^{19}F NMR and compare the results with a study conducted in parallel using a radiochemical analysis. Although the use of ^{19}F NMR or ^{1}H NMR in metabolism studies is not new, earlier applications are relatively narrow in scope and do not encompass comprehensive mass balance, metabolism, and distribution studies (Malet-Martino et al., 2006; Martino et al., 2005; Mutlib et al., 2012). Unlike ^{1}H NMR, in metabolism studies, quantitation based on ^{19}F NMR offers several advantages (Martino et al., 2005; Hu et al., 2015; Lindon and Wilson, 2015), among which the most important is the absence of interfering background signals from biologic matrices. The inclusion of fluorine atoms in small-molecule drug candidates or drugs has become increasingly popular, as this often leads to improved intrinsic potency; absorption, distribution, metabolism, and excretion and toxicologic properties (Müller et al., 2007; Gillis et al., 2015; Murphy and Sandford, 2015). As much as one third of the approved drugs, and notably three (atorvastatin, fluticasone, and lansoprazole) of the five top-selling drugs, contain fluorine atoms (Wang et al., 2014). Given the rising number of drugs and drug candidates containing fluorine atoms (Zhou et al., 2016), quantitative ^{19}F NMR appears to be an attractive alternative tool to radiolabeled studies to evaluate the disposition of at least one third of the drug candidates.

Using pefloxacin as an example, our work demonstrated that the percent drug-related material recovered in urine, bile and feces, and quantitative mass balance can be readily estimated by ^{19}F NMR and that the results are comparable to a radiolabeled study conducted in parallel. There were some apparent differences in the percentage of recovery of pefloxacin and its major metabolite pefloxacin glucuronide (M4) between the two methods; ^{19}F NMR estimated greater recovery of pefloxacin (approximately 30% by ^{19}F NMR versus 20% by radioactivity) and conversely lower recovery of M4 (approximately 21% by ^{19}F NMR versus 39% by radioactivity). These differences are likely due to a combination of two factors: 1) the addition of 5% acetic acid during NMR analysis may have induced partial hydrolysis of the acyl glucuronide to generate pefloxacin [consistent with this, the recovery of pefloxacin in bile by ^{19}F NMR was slightly higher (approximately 10% of the dose) than the recovery by radioactivity (approximately 2% of the dose)]; however, acidic conditions are generally believed to stabilize acyl glucuronides and prevent from hydrolysis (Wang et al., 2006) with the exception of clopidogrel acid acyl glucuronide, which is known to be susceptible to hydrolysis under acidic conditions (Regan et al., 2010); and 2) during radioactivity analysis, it is possible that the primary acyl glucuronide and potential isomeric glucuronides as a result of acyl migration may have coeluted (note that the M4 peak is quite broad in the radiochromatogram) (Fig. 1C), but in NMR, they may have appeared as separate peaks. In fact, a cluster of unassigned ^{19}F peaks near the major glucuronide peak (range, δ –122.8 to −123.0 ppm) together accounted for approximately 8% of the dose (Fig. 1F, inset). It is not surprising to observe separate peaks for isomeric compounds in ^{19}F NMR as a result of the extreme sensitivity of fluorine resonances to structural and steric changes. To address factor 1, aliquots of study bile samples were treated with and without 5% acetic acid and subsequently analyzed by LC-MS (sample treated with 5% acetic acid likely to mimic conditions used during NMR analysis). The LC-MS results showed no differences in the peak intensity ratios of acyl glucuronide to unchanged parent drug between the samples treated with and without 5% acidic acid, suggesting that acid treatment did not induce hydrolysis of

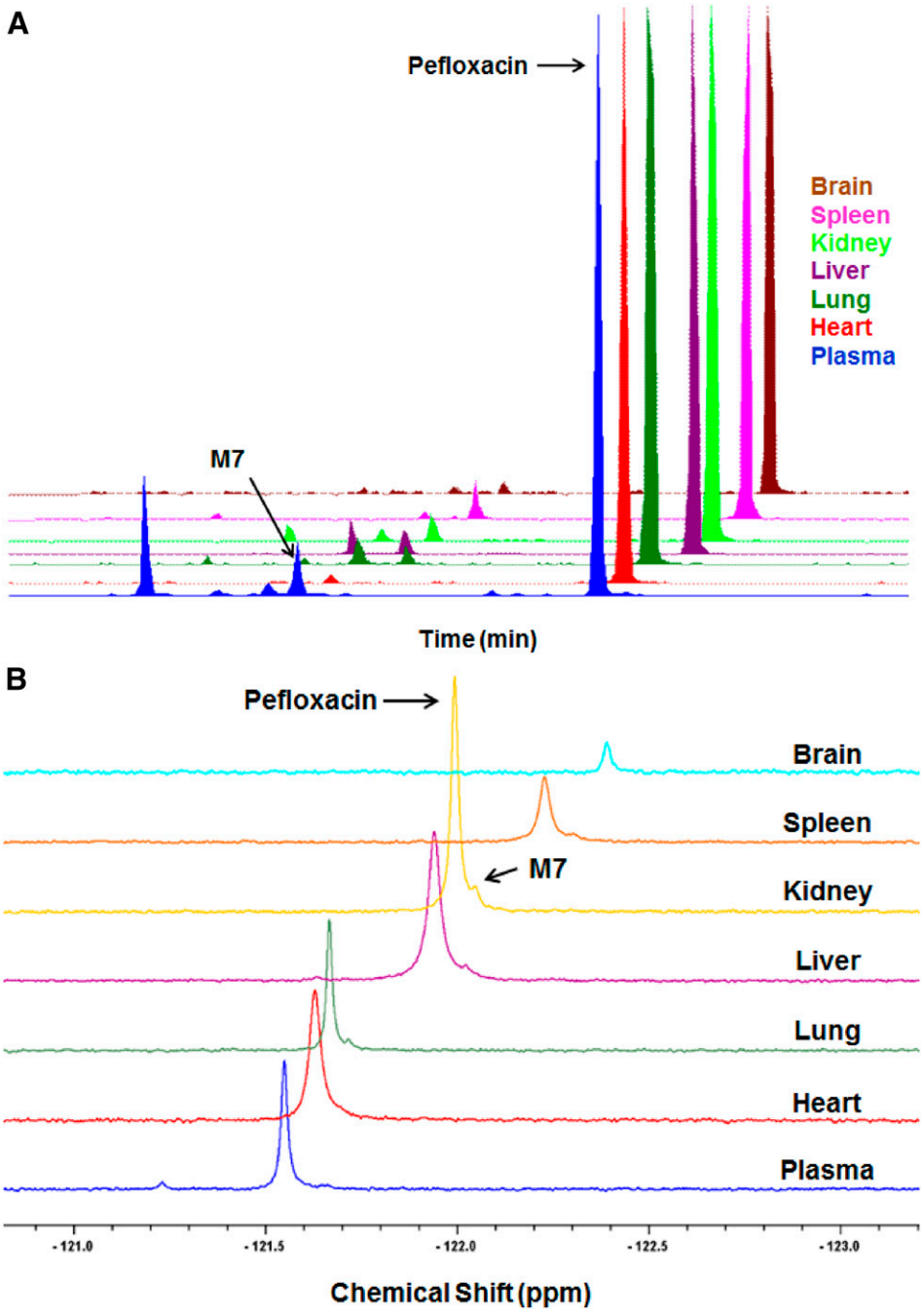

Fig. 3. Radioactivity (A) and ^{19}F NMR (B) profiles of selected tissues following a single 25- mg/kg oral dose of [^{14}C]pefloxacin or pefloxacin to male Sprague-Dawley rats. Radioactivity analysis was performed using a 0.5- to 2-hour AUC pool, and ^{19}F NMR analysis was performed using a 1-hour sample (C_{max} for most tissues). The ^{19}F NMR profile obtained using the 0- to 24-hour AUC pool is similar to the 1-hour sample but with less S/N.

pefloxacin acyl glucuronide. Assuming that the unassigned peaks accounting for 8% of the dose in the bile NMR profile are isomeric acyl glucuronides, the overall recovery of M4 is approximately 25%. Thus, the actual difference in the recovery of M4 and pefloxacin in bile between the two methods is approximately 8%, with higher recovery for M4 by radiochemical analysis and conversely higher recovery for pefloxacin by NMR analysis.

Regulatory guidance recommends further safety assessment of metabolites if their circulating exposure in humans at steady state is >10% of the total drug-related exposure and at significantly greater levels in humans than the maximum exposure observed in the animal species used for nonclinical safety assessment (http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/General/UCM292164.pdf; http://www.fda.gov/OHRMS/DOCKETS/98fr/FDA-2008-D-0065-GDL.pdf); and http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Multidisciplinary/M3_R2/Step4/M3_R2__Guideline.pdf). Therefore, determination of circulating levels of metabolites in toxicologic species and humans at steady state is critical to determine if separate safety assessment of circulating drug metabolites in humans is needed. For practical reasons, radiolabel animal studies are often conducted after the start of human trials (Penner et al., 2012). Furthermore, steady-state plasma exposure for parent drug and metabolites may show departure from that after a single dose. Because administration of radiolabeled substance is not required for the ^{19}F NMR-based method, these are not limitations. For example, steady-state exposures of parent drug and metabolites can be determined in animals and humans from plasma samples collected during repeat-dose toxicology and multiple ascending dose clinical studies, respectively. In the present investigation, pefloxacin exposure determined by ^{19}F NMR

was in good agreement with that derived from a validated LC-MS/MS method. Additionally, plasma profiling by ^{19}F NMR revealed presence of three quantifiable metabolites (M4, M7, and M8) with exposure M4 > M8 > M7. Profiling by radioactivity revealed the presence of eight metabolites; however, only NMR-quantifiable metabolites M4, M7, and M8 had exposures >2% of total drug-related exposure, with a similar order of relative abundance M4 > M8 > M7 as ^{19}F NMR profiling (Table 2). In humans, two circulating metabolites, M7 and pefloxacin N-oxide (presumably M8), have been reported (Montay et al., 1984; Jin, 2014). In the present study, ^{19}F NMR profiling of plasma enabled us to obtain quantitative information on these two human metabolites in rats. Thus, a distinct advantage of ^{19}F NMR is its ability to obtain quantitative information of major or significant metabolites to address the human metabolite coverage question without the need for metabolite standard(s) or radiolabeled substance.

There are some limitations when evaluating metabolite profiling by ^{19}F NMR. As discussed previously (Hu et al., 2015; Lindon and Wilson, 2015), ^{19}F NMR chemical shift alone is far from providing complete structural information of metabolites unless suitable metabolite standards are available or used with other profiling methods, such as LC-MS. In spite of significant advances in the hardware and experimental methods, NMR sensitivity will continue to be an issue, particularly when disposition studies are needed for drugs that are administered at very low doses. In this instance, a CF_3 group or multiple chemically equivalent CF_3 groups, often found in drug candidates, may offer the necessary enhanced sensitivity compared with a single fluorine substituent (3 × or more); however, metabolite profiling of compounds in which fluorine atoms are attached to a sp^3 hybridized carbon (such as CF_3) may show very small chemical shift differences compared with substituent effects (perhaps owing to a lack of or weak resonance effects) relative to sp^2 hybridized carbon (such as substituted aromatic fluorine), in spite of known high sensitivity experienced by ^{19}F chemical shifts to subtle changes in the magnetic environment for organic compounds, illustrated by using celecoxib (Fig. 4) bearing a CF_3 group. Significant peak overlap was observed in the ^{19}F NMR profile of a mixture of celecoxib and three of its human metabolites (Fig. 5, A and B). Such a lack of resolution may prevent absolute quantitation of individual peaks (parent drug and metabolites). Nevertheless, it will still be useful in obtaining quantitative mass balance and excretion pathway information in such instances.

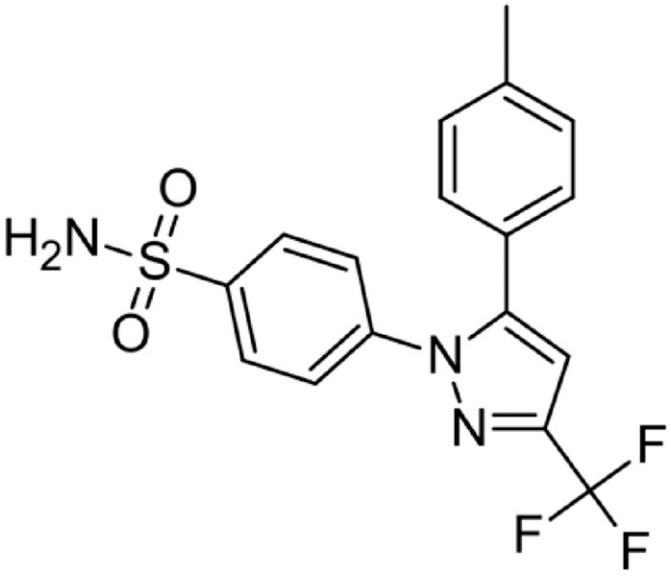

Fig. 4. Structure of celecoxib.

Most of the NMR-based quantitative analyses reported in the literature, including our analysis, use peak integrals post-Fourier transformation of the time-domain NMR data for quantitation. Quantitation based on peak integrals may introduce user bias because of the necessary manual phase and baseline corrections before analysis. Analysis based on the raw data, namely, the time-domain data, would provide uniform and even improved absolute quantitation of individual components in a mixture analysis. Such analysis has been recently reported for the quantitation of spent media components of mammalian cell cultures (Bradley et al., 2015) and may be adopted in the future in metabolism studies.

In summary, the results from this investigation demonstrated directly that quantitative mass balance, metabolism, and distribution information can be reliably obtained using ^{19}F NMR and that the results overall are in good concordance with a radiolabeled study performed in parallel. Furthermore, although the current analysis was performed after a single

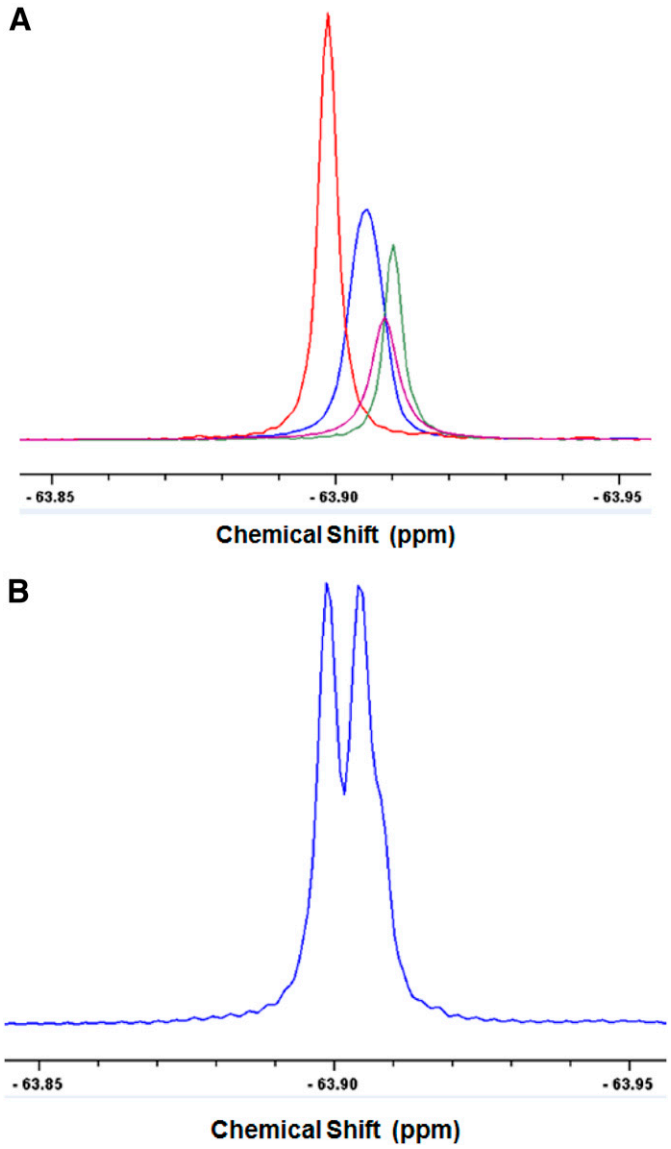

Fig. 5. Qualitative ^{19}F NMR profile of celecoxib and its main metabolites, hydroxycelecoxib, celecoxib carboxylic acid, and its corresponding acyl glucuronide. (A) Individual spectrum overlay. (B) Spectrum of mixture. Spectra were measured in 9:1 CH_3OH-CD_3OD with NMR conditions described in *Materials and Methods*. All resonances appeared in a small window of approximately 0.04 ppm (<25 Hz).

dose, unlike radiolabeled studies, no special effort is needed to perform ^{19}F NMR analysis at steady state to meet the stringent regulatory requirement of human metabolite coverage. Taken together, our work supports the broader use of ^{19}F NMR in absorption, distribution, metabolism, and excretion studies for drug discovery and development. Although ^{19}F NMR studies are unlikely to replace fully the need for standard LC-MS and radioactive experiments, they represent a powerful complementary tool to the more mainstream approaches.

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Authorship Contributions

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Conducted experiments: Hu, Katyayan, Kulanthaivel.

Performed data analysis: Hu, Katyayan, Kulanthaivel.

Wrote or contributed to the writing of the manuscript: Hu, Katyayan, Czeskis, Perkins, Kulanthaivel.

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