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Application of Quantitative NMR in Pharmacological Evaluation of Biologically Generated Metabolites: Implications in Drug Discovery

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List of Abbreviations

ESI-LC/MS: electrospray ionization-liquid chromatography mass spectrometry
LC/UV/MS: liquid chromatography/ultraviolet spectroscopy/mass spectrometry
MIST: metabolites in safety testing
MRM: multiple reaction monitoring
MS/MS: mass spectrometry/mass spectrometry
¹H-NMR: proton nuclear magnetic resonance
SPE: solid phase extraction

ABSTRACT

It is important to gain an understanding of the pharmacological activities of metabolite(s) of compounds in development, especially if they are found in systemic circulation in humans. Pharmacological evaluation of metabolites is normally conducted with synthetic standards which become available during various stages of drug development. However, the synthesis of metabolite standards may be protracted, taking anywhere from several weeks to months to be completed. This often slows down early pharmacological evaluation of metabolites. Once a metabolite(s) is found to possess comparable (or greater) pharmacological activity than the parent compound, additional studies are carried out to better understand the implications of circulating pharmacologically active metabolite(s). To conduct some of these studies as early as possible without slowing the progression of a compound in development is important, especially if critical go or no-go decisions impinge on the outcomes from these studies. An early pharmacological evaluation of significant metabolites is hereby proposed to be conducted in the drug discovery stage so that all pertinent studies and information can be gathered in a timely manner for decision-making. It is suggested that these major metabolites be isolated, either from biological or chemical sources, and quantified appropriately. In the case of biologically generated metabolites, NMR is proposed as the tool of choice to quantitate these metabolites prior to their evaluation in pharmacological assays. For metabolites that have the same UV characteristics as the parent compound, quantitation can be conducted using UV spectroscopy instead of NMR. In this paper, we propose a strategy that could be used to determine the pharmacological activities of metabolites isolated in sub-milligram quantities.

INTRODUCTION

Pharmacological evaluation of new chemical entities is traditionally performed in early drug discovery. Enzyme and cell-based assays are often used to initially assess the potential pharmacological activities of compounds prior to further evaluation in animal models. Frequently, the relationship between the structure and pharmacological activity is further studied by chemically synthesizing close structural analogues with the intention of optimizing drug-receptor interaction for improved potency. This SAR work is critical during the lead optimization phase of new potential therapeutic agents as modifications of chemical templates can also lead to significant alterations in physico-chemical properties, which in turn can dramatically influence the ADME characteristics of compounds. Significant chemistry effort is made in the lead optimization stage to generate compounds with desired pharmacological and ADME properties. An alternative, yet less exploited way of creating close analogues from a pharmacologically active template is through biological means, such as enzyme-mediated metabolism or microbial conversion.

During early drug discovery studies, metabolic modifications of chemical templates by various sub-cellular and cellular systems from different animal species are routinely investigated. Metabolite profiles, which are often obtained by combination of liquid chromatography, mass spectrometry and ultraviolet spectroscopy (LC/MS/UV), identify new chemical entities produced by these biological enzymes. However, because of the low levels of these metabolites, further pharmacological evaluations of these compounds have not routinely been performed in the past.

Metabolic conversion has not traditionally been considered as a source of generating new chemical entities for further pharmacological evaluation. In addition to regarding metabolites merely as excretory products, there are several other reasons why medicinal chemists have not considered metabolites as potential drug candidates. In the past, elucidating the structures of these metabolites was a challenge. Providing a list of hypothetical structures based on inconclusive mass spectral data was one of the reasons for lack of interest in these metabolites by the medicinal chemists and biologists.

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However, with advancements in mass spectrometry and NMR techniques, it has become a routine practice to obtain structures of metabolites in early discovery (Mutlib and Shockcor, 2003). A significant hurdle in the past has been the inability to isolate sufficient quantities of metabolites in relatively pure form for further evaluation. Recently, it has become easier to produce sufficient amounts of metabolites using any of the various biological or biomimetic systems. It has been shown that these biological systems can be used reliably to generate metabolites in the microgram to milligram range. Despite the ability to generate and isolate sufficient quantities of pure samples of metabolites, the challenge has been to obtain an accurate weight of the compounds for proper evaluation in pharmacological assays.

A strategy advocating the use of NMR as a quantitative tool to determine the concentrations of biologically synthesized metabolites has been discussed in the literature and elsewhere (Espina et al., 2009). Traditionally, biologically isolated metabolites have not been considered as a source of generating reference standards for further quantitative work. However, because of the FDA guidance on safety testing of metabolites, some laboratories have contemplated using these biologically synthesized metabolites as “reference standards” for preliminary quantitative studies. ^1H NMR has been proposed as the method of choice in authenticating and quantitating these biologically synthesized metabolites prior to their further use as standards in more sensitive LC/MS based quantitative assays (Vishwanathan et al., 2009). We have successfully used this approach to obtain metabolite concentrations and exposure values using sample pooling methods (Vishwanathan et al., 2009). A secondary consequence of being able to obtain accurate weights of isolated metabolites is further evaluation of their pharmacological activities. Once the exact structure and concentration of the isolated metabolite is known, it can be submitted to pharmacologists for further testing in enzyme- and cell-based assays. In this paper, we describe a strategy that was used to effectively elucidate the structure and determine the concentrations of metabolites prior to evaluation of pharmacological activities of these biologically generated compounds. A compound which was found to be highly metabolized by human liver microsomes (see Figure 1), was studied extensively along with its major metabolite(s) in animals to better understand their

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pharmacokinetic behaviors in animals after it was shown that the metabolite was equipotent to the parent compound.

MATERIALS AND METHODS

Chemicals and Supplies

Compound **1** (see Figure 1) was obtained from Department of Chemical Sciences, Pfizer Global Research and Development, Pearl River, NY. The chemical purity of this compound was shown to be greater than 98% by HPLC/UV. NADPH was purchased from Sigma Chemical Co. (St. Louis, MO). Liver microsomes from rats, cynomolgus monkeys and humans were purchased from XenoTech LLC (Lenexa, KS). Discovery[®] DSC-18 (C₁₈) cartridges were purchased from Sigma Chemical Co. (St. Louis, MO).

Solvents used for chromatographic analysis were HPLC or ACS reagent grade and were purchased from EMD Chemicals (Gibbstown, NJ). All deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). All general solvents and reagents were the highest grade available commercially.

In Vitro Incubations and Isolation of Metabolites for NMR Characterization and Quantitation

A large-scale incubation of compound **1**, at a concentration of 0.02 mM, with human liver microsomes was conducted to generate metabolite **M1** (see Figure 1). A total of 50 x 2 mL incubations were conducted in 0.1 M potassium phosphate buffer (pH 7.4), containing 1 mg/mL microsomal protein, 5 mM NADPH, and 8 mM MgCl₂ at 37° C for 60 min. Protein precipitation was conducted using 4 mL of 2% formic acid in acetonitrile per incubation. The tubes were vortexed for 1 min and centrifuged at 2400 x g for 10 minutes at 4° C. The supernatants were transferred to clean culture tubes after which the organic phase was removed under a stream of nitrogen at 30 °C. The aqueous phase from each tube was pooled and subjected to a cleanup on solid phase C₁₈ extraction cartridges.

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Two Discovery[®] DSC-18 (C₁₈, 60 mL capacity) cartridges were used for sample cleanup prior to further purification on a HPLC system. The cartridges were washed with 60 mL of acetonitrile followed by the same volume of H₂O. The aqueous supernatant was loaded onto the cartridges (approximately 50 mL/cartridge) and allowed to flow through completely under gravity. The cartridges were then washed with one volume of H₂O. The wash fractions were analyzed by injecting 50 µL of samples on a Thermo LTQ ion trap mass spectrometer and were found to be void of both compound **1** and **M1**. Elution of **M1** was done using 25 mL aliquots of solvent varying in concentrations of acetonitrile in water (10%-100% acetonitrile; with 10% stepwise increase). Aliquots (50 µL) of each fraction were injected on the Thermo LTQ mass spectrometer; **M1** was found to elute completely in the 30%, 40% and 50% fractions. These fractions were pooled (75 mL total) and subsequently reduced in volume to ~ 4 mL which was subjected to further purification on an Agilent 1100 HPLC system equipped with a fraction collector. Aliquots (500 µL) of the sample were injected on a Varian Pursuit C₁₈ (250 x 10 mm, 5 µm) semi-preparative column coupled to the HPLC system. The mobile phase consisted of (**A**): 0.1% formic acid in H₂O and (**B**): acetonitrile which was delivered at 5 mL/min. The gradient consisted of increasing the % organic (**B**) from the initial value of 25% to 50% in 7 min followed by a linear ramp to 95% **B** in one minute before re-equilibrating the column. Compound-related components were monitored using a UV detector set at 300 nm. Fractions containing **M1** were pooled and lyophilized. Approximately 1 mg of **M1** was isolated as its formate salt in >95% purity as determined by LC/UV/MS and NMR analysis. The isolated metabolite was handled in a glove box to minimize contamination with water prior to NMR analysis.

*In Vitro Metabolism of Metabolites **M1a-1** and **M1a-2** (Enantiomers of **M1**)*

Metabolites **M1a-1** and **M1a-2** (see Figure 2), chemically synthesized enantiomers of **M1**, were incubated with male Sprague-Dawley rat, male cynomolgus monkey and male human liver microsomes (1 mg/mL) fortified with cytosol (2 mg/mL) in the presence of NADPH (5 mM), MgCl₂ (8 mM), GSH (2 mM) and UDPGA (2 mM) in a total volume of 1 mL (adjusted with 0.1 M phosphate buffer, pH 7.4) at 37° C for 60 minutes. Protein

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precipitation was conducted using 2 x volumes (2 mL) of 2% formic acid in acetonitrile and samples centrifuged (3400 rpm for 5 min) before transferring the supernatants to clean culture tubes. The solvents were completely removed under a stream of nitrogen at 30 °C. The dried samples were reconstituted in 200 µL of the HPLC mobile phase (1:9 v/v, acetonitrile:5 mM ammonium acetate). Aliquots of reconstituted samples were analyzed by LC/MS to assess the extent of metabolism of each enantiomer in each species.

Liquid Chromatography/Mass Spectrometry

Analysis of SPE Fractions, Purity Check and Metabolite Characterization: The fractions from the C18 cartridges were analyzed by flow injection analysis, whereby aliquots (50 µL) from various fractions from SPE were injected directly onto the mass spectrometer using a 1:1 (v/v) mixture of acetonitrile : 0.1% formic acid with the solvent delivered at 0.2 mL/min. Upon the isolation of the metabolite **M1**, liquid chromatography/mass spectrometry (LC/MS) was conducted to obtain structural information as well as to ensure the purity of the isolated metabolite. HPLC was conducted on a Phenomenex C₁₈ Luna column (150 x 2.1 mm) using acetonitrile and 5 mM ammonium acetate as the mobile phase. The composition of the mobile phase was changed from 10% acetonitrile to 40% in the first 18 minutes after which the column was washed with 95% acetonitrile for 5 minutes. Subsequently the column was re-equilibrated with 10% acetonitrile prior to the next injection. Deuterated water (5 mM ammonium acetate) was substituted in LC/MS experiments designed to determine the number of exchangeable protons in metabolite **M1**. The flow rate was set at 200 µL/min. Mass spectral data was obtained with a LTQ Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) which was equipped with an electrospray ionization source and operated in the positive ionization mode with the electrospray needle kept at +4500 kV. Settings for the mass spectrometer were optimized to provide a structurally relevant range of product ions from MS/MS (under high energy collisional dissociated mode) and MSⁿ (ion trap mode) experiments. The capillary temperature and voltage were set at 370 °C and 29 V, respectively. Ultrapure nitrogen was used as the nebulizer gas and curtain gas. The relative collision

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energy was kept at 30 eV for generating product ions in both MS/MS and MSⁿ experiments. Other parameter settings for the analyses included arbitrary values of 55, 20 and 10 for sheath, auxiliary, and sweep gases (ultrapure nitrogen), respectively. To establish the structure of **M1** unequivocally, synthetic standards of various isomeric forms of this metabolite, **M1a** (endo-isomer, racemic), **M1a-1** (endo isomer, enantiomer 1, 1R,5R,6R), **M1a-2** (endo isomer, enantiomer 2, 1S,5S,6S), and **M1b** (exo-isomer, racemic) (see Figure 2), were injected onto the LC/MS to compare the retention times and mass spectral fragmentation patterns. Chiral separation of **M1a-1** and **M1a-2** was conducted as described previously (Chen et al., 2010). An aliquot of **M1** was injected on the same chiral column to compare the retention time of this metabolite with the HPLC separated enantiomers, **M1a-1** and **M1a-2**. It was found that **M1a-1** and **M1a-2** had retention times of 10.2 and 13.4 min, respectively. Metabolite **M1** showed identical retention time as **M1a-1** (see Chen et al, 2010).

LC/MS Analysis of Microsomal and Rat Plasma Extracts: The microsomal and plasma extracts were subjected to LC/MS analysis using the same experimental conditions as described above.

Quantitative LC/MS Analysis of Plasma Samples from Pharmacokinetic Studies:

Compound **1**, **M1a-1** and **M1a-2** were quantified in male rat plasma utilizing a non-validated LC/MS/MS assay. The assay limit of quantification was 1 ng/mL using 50 μ L aliquots of rat plasma. The standard curve ranged from 1 to 1000 ng/mL for all compounds. All samples, when needed, were appropriately diluted. The analysis was accepted if three-fourths or more of the back-calculated concentrations of the calibration standards and at least two-thirds of the quality control samples were within 20% of their theoretical values. All calibration standards and QC samples met these criteria. The coefficient of determination (r^2) was > 0.9991 . Experimental and calibration samples were prepared by adding 50 μ L aliquots of samples or standards into the appropriate well of a 96-well disposable plate. Control blanks were prepared by adding 50 μ L of control matrix to the appropriate well(s). Calibration samples were prepared by adding 10 μ L of the appropriate working calibration standards (prepared in acetonitrile) to the appropriate well

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containing 50 μ L of blank plasma. To experimental and control blank samples was added 10 μ L of acetonitrile. Compound **1**, **M1a-1** and **M1a-2** were extracted from plasma by protein precipitation using 200 μ L of acetonitrile containing 100 ng/mL of internal standard. Samples were vortexed for 3 minutes and then centrifuged at 3400 rpm for 5 minutes. A 150 μ L aliquot of the supernatant was transferred to a microplate using a Tomtec Quadra 96. Ten microliters of this sample was injected for analysis.

Chromatographic resolution was achieved on a Discovery C₁₈ HPLC column (50 \times 2.1 mm, 5 μ m) at ambient temperature with the same mobile phase gradient used for the metabolite characterization (see above) All analyses were carried out using the Cohesive Aria LX2 HPLC system with LEAP autosampler interfaced with a Sciex API 4000 mass spectrometer. The LC eluate was sprayed into the mass spectrometer at +5 kV and desolvation of droplets was aided by heated desolvation gas. Quantitative LC/MS analysis was conducted by operating the mass spectrometer in the multiple reaction (MRM) mode. The protonated molecule of the analytes (compound **1**, m/z 489.2; **M1**, **M1a-1** and **M1a-2**, m/z 505.2) and internal standard (m/z 475.2) were selected by Q1 set at a resolution of 0.7 Da (FWHM). Collision-activated dissociation was carried out in Q2 with nitrogen at collision energy of 45 eV. Fragment ions of the analytes (m/z 489.2 \rightarrow 395.2 and m/z 505.2 \rightarrow 411.2) and internal standard (m/z 475.2 \rightarrow 381.2) were mass analyzed by Q3 at a resolution of 0.7 Da (FWHM). Data acquisition and reduction including peak integration were carried out with Analyst software version 1.4.1. Drug plasma concentrations were quantified utilizing Watson LIMS version 7.3.1.

NMR Spectroscopy

NMR Instrument: NMR data were recorded at 30°C on a 600 MHz Bruker Avance III spectrometer with a 5 mm CPTCI CryoProbe™ (Bruker BioSpin Corporation, Billerica, MA) at the basic frequency of 600.13 MHz. The spectrometer was controlled using TopSpin® (Bruker BioSpin, v 2.1 pl 1). All data collection was performed without spinning the samples. The probe was tuned and matched to the specific frequency using

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the automatic tuning method (ATMA) from Bruker BioSpin. The samples were field frequency locked using the ^2H signal of the deuterated solvent. Shimming was performed on each sample prior to data acquisition using the TopShim automatic shimming method from Bruker BioSpin on the solvent peak. Each data set was collected under automation with the same parameter set using the ICON-NMR software (Bruker BioSpin, v 3.7) and a SampleJetTM sample changer (Bruker BioSpin Corporation, Billerica, MA) or manual sample changes.

Qualitative NMR Analysis of Metabolite MI: The isolated metabolite, **MI**, was evaporated to dryness under a nitrogen stream, and dissolved in 500 μL DMSO- d_6 (D, 99.96%) (Cambridge Isotope Laboratories, Inc., Andover, MA) and transferred into a 5 mm NMR tube. Proton, HSQC, and HMBC experiments were performed. All chemical shifts were referenced to the DMSO signal at ^1H δ 2.49 ppm. The proton spectra were acquired with 32,768 data points over a 6,602 Hz spectral window using a 30° pulse and standard pulse sequence. Data were Fourier transformed with a 0.3 Hz line broadening window function. The 2D ^1H - ^{13}C HSQC spectra were acquired with 1024 data points in F_2 , and 256 increments in F_1 , with 32 scans per increment, using a phase sensitive pulse sequence.

Quantitative NMR Analysis of Metabolite MI: The quantitative NMR technique used in this study was similar to what has been described previously (Espina et al., 2009). Briefly, each sample was dissolved in the same volume (500 μL) of the solvent and was placed in the magnet at the same depth. Each proton spectrum was acquired with 65,536 data points over a 12,019 Hz spectral window using a 90° (8.3 μs) pulse and the gradient 1D nuclear Overhauser effect spectroscopy (NOESY) presat pulse sequence (noesygprr1d), using a 5 mm CPTCI CryoProbeTM (Bruker BioSpin Corporation, Billerica, MA). Eight scans with an acquisition time of 2.7 sec. were collected for each spectrum. A pre-acquisition delay of 30 sec. was used during the data acquisition. The receiver gain was set automatically using the more concentrated solution and was kept constant throughout each of the data sets. Data were Fourier transformed with a 0.3 Hz line broadening window function. The spectra were transferred into ACD/SpecManager

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(ACD/Labs, Inc, v 11.0). The FID Reconstruction method was applied to the full spectrum during automatic baseline correction prior to integration. All the spectra in a series (absolute intensity) were combined using the SpecManager Series command. Integration of the spectra in a series was performed manually using group treatment within SpecManager. For quantitation purposes, integration of all aromatic protons (δ 8.37, 8.30, 7.55, and 7.44) of compound **1** was used to generate calibration curve as described previously (Espina et al., 2009). An average of the normalized integral value for compound **1** was plotted against the concentration values to create a calibration curve as described before (Espina et al., 2009). The table of common integrals for the series was transferred to Microsoft Excel for analysis. All samples were analyzed by NMR in triplicates (three separate determinations of the same sample on different occasions). The concentrations of quality control (QC) samples and metabolite **M1** were obtained using the calibration curve generated from compound **1**. All spectra were referenced to the residual solvent signal.

Pharmacological Evaluation of Metabolite M1

Using NMR, the exact concentration of the isolated metabolite was measured using the calibration curve constructed with the compound **1** as described previously by Espina et al (2009). Briefly, the NMR spectra of the parent compound were obtained at 5 different concentrations and a calibration curve created based on the integration of aromatic proton signals (average). The concentration of metabolite **M1** was calculated using the standard curve as described previously (Espina et al., 2009). Another metabolite of compound **1**, available as a synthetic standard, was used as a quality control to ensure that the NMR quantitation was verified by gravimetric determination. The results are presented in Table 1. It has been shown by Espina et al. during the validation of the quantitative NMR method, that the concentrations of metabolites measured using this approach is within 10% of the gravimetrically determined nominal values. Hence, the measured concentration of the isolated metabolite was used to obtain the absolute amount of metabolite that was isolated from the biological source. Subsequently, this sample (with the concentration of the metabolite known) was used to prepare a series of diluted

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samples that were used for measuring the binding and functional potency of the isolated metabolite on the target receptors. In the same study, a solution of compound **1** was prepared at a similar concentration as the isolated metabolite **M1**. The concentrations of both samples were confirmed by quantitative NMR before being simultaneously evaluated in the pharmacological assays. Upon the availability of the synthetic standards of metabolite **M1**, pharmacological evaluations were conducted using solutions prepared from compounds which were weighed accurately using analytical balances. The pharmacological activities of various compounds, including that of the isolated metabolite **M1**, are shown in Table 2.

Pharmacokinetics of Compound 1, M1a-1 and M1a-2

Single dose IV (2 mg/kg) studies were conducted in male Sprague-Dawley rats with compounds **1**, **M1a-1** and **M1a-2** (pure enantiomers).

Male Sprague-Dawley rats (n=3 per dose group) were purchased from Charles River (Raleigh, NC) and acclimated in an IACUC accredited facility for a few days prior to dosing. Animals used in the study were surgically cannulated for jugular and femoral veins by the vendor. The compounds were formulated in 5% dextrose and 0.5% lactic acid, pH 3.5 for intravenous administration. The IV dose volume was 1 mL/kg. The compound was administered to animals that had access to food and water ad libitum. Serial samples (N = 10) of blood (300 µL) were taken at the following time points: 0, 2, 10, 15, 30, 60, 120, 240, 360 and 1440 min post-dose *via* the indwelling jugular vein catheter into EDTA tubes and stored at -70°C before HPLC/MS analysis of compound **1**, **M1a-1** and **M1a-2** (see above). An aliquot (100 µL) of plasma samples from selected time points (30 min, 2 h and 6 h) were treated with acetonitrile (200 µL) and samples centrifuged prior to transferring the supernatant to another set of clean tubes. The solvents were removed under a stream of nitrogen and the dried residues reconstituted in 100 µL of the HPLC mobile phase before qualitative LC/MS analysis (see above) was conducted to determine the plasma metabolite profiles.

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Pharmacokinetic Analysis

The pharmacokinetic parameters for individual animals were determined using a non-compartmental analysis module (Model 201) of the pharmacokinetic software package WinNonlin, ver. 5.1 (Pharsight, CA). The program applies a model-independent approach and the standard methods described previously (Gibaldi and Perrier, 1975). The systemic clearance (CL_T) was calculated as $Dose/AUC_{0-\infty}$, and volume of distribution at steady-state (Vd_{ss}) was calculated as $MRT \cdot CL_T$, where MRT is $AUMC_{0-\infty}/AUC_{0-\infty}$. The area under the plasma concentration versus time curve (AUC) was calculated using the linear trapezoidal method. Where warranted, the slope of the apparent terminal phase was estimated by log-linear regression using at least 3 data points and the terminal rate constant (λ) was derived from the slope. $AUC_{0-\infty}$ was estimated as the sum of the AUC_{0-t} (where t is the time of the last measurable concentration) and C_t/λ . The apparent terminal half-life ($t_{1/2}$) was calculated as $0.693/\lambda$. For pharmacokinetic calculations, all drug concentration values below the assay limit of quantification (BLQ) were treated as zero.

Statistics

A least-squares linear regression analysis of the UV peak areas versus concentrations of Compounds **1**, **M1a-1** and **M1a-2** were performed using GraFit 5[®] from Erithacus Software. The concentrations of Compounds **1**, **M1a-1** and **M1a-2** in the dosing solutions were determined from the standard curve linear regression equations by inverse prediction using Microsoft Excel 2003. Calculations of means and standard deviations were also performed using Microsoft Excel 2003.

RESULTS

Identification and Characterization of Metabolite M1

The in vitro metabolism studies conducted with compound **1** showed that it was primarily converted to a single metabolite (**M1**) in the presence of human and cynomolgus monkey liver microsomes (Figure 1). Mass spectral analysis of **M1** showed that it was a hydroxylated metabolite with $[M+H]^+$ at m/z 505, an increment of 16 amu from the parent molecular weight. LC/MS analysis conducted using deuterated water showed that there were three exchangeable protons, consistent with the presence of an additional hydroxyl group in metabolite **M1** (Figure 3). Initial NMR analysis of this metabolite showed that hydroxylation had taken place on one of the pro-chiral carbons forming part of the bridge of the bicyclic ring system. ^1H NMR of metabolite **M1** (d^6 -DMSO, 600 MHz) δ 9.22 (s, 1H), 9.20 (s, 1H), 8.37 (d, 2H, $J = 7.0$ Hz), 8.30 (t, 2H, $J = 8.4$ Hz), 7.55 (d, 2H, $J = 6.3$ Hz), 7.44 (d, 2H, $J = 8.8$ Hz), 4.73 (d, 1/2H, $J = 7.4$ Hz), 4.55 (d, 1/2H, $J = 7.3$ Hz), 4.51 (d, 1/2H, $J = 5.8$ Hz), 4.34 (d, 1/2H, $J = 5.9$ Hz), 4.27 (m, 1H), 3.99 (dd, 1H, $J = 16.8, 11.0$ Hz), 3.92-3.55 (m, 11H), 2.37 (m, 1H), 1.71 (m, 1H). The hydroxylation of compound **1** produced a chiral center; hence leading to the possibility of enantiomers. The enantiomeric composition of the isolated metabolite **M1** could not be determined by NMR analyses. Subsequently, the hydroxylation was shown to have occurred in a very stereo-specific manner (see structures in Figure 2). The stereochemistry of the hydroxylated metabolite was confirmed by chemical synthesis and subsequent chiral separation of resulting enantiomers (Chen et al., 2010). The partial ^1H -NMR of **M1** and two synthetically prepared racemic isomers, **M1a** (endo) and **M1b** (exo) are shown in Figure 3. As shown, the ^1H -NMR of the isolated **M1** matched that of the racemic endo-isomer, **M1a** (Figure 3). The ^1H -NMR of the exo-isomer (**M1b**) was found to be distinctly different than that of the metabolite **M1**. For the rest of NMR data for the endo and exo isomers of **M1** please refer to Cheng et.al. (2010). Furthermore, it was found that **M1** and **M1b** had different HPLC retention times (though same MS/MS spectra) when analyzed by LC/MS (data not shown). However, it was found that **M1** and **M1a** showed identical retention times on HPLC as well as displaying identical MS/MS

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fragmentation pattern (Figure 4). However, **M1a** was racemic in nature and it was important to demonstrate if the isolated metabolite was racemic or present as a pure enantiomer. Hence additional studies were conducted to separate the two enantiomers of **M1a** into **M1a-1** and **M1a-2** by chiral HPLC (see Chen et al., 2010). It was demonstrated clearly by Chen et al that the biologically produced metabolite **M1** corresponded to **M1a-1**; hence the formation of metabolite **M1** by human liver microsomes appear to have taken place in a very stereo-specific manner. Interestingly enough, each of the enantiomers, **M1a-1** and **M1a-2**, displayed two sets of signals for the bridgehead protons believed to be due to the chair-boat conformations of the bicyclic ring system (Figure 5). The HSQC data shows that the methylene carbon of the bridgehead had two chemical shifts (δ 53.0 and 57.5), and each was correlated to two sets of proton signals

In Vitro Metabolism of M1a-1 and M1a-2

Further in vitro metabolism studies were conducted with synthetic standards of enantiomers **M1a-1** and **M1a-2**. LC/MS analysis of microsomal extracts showed that **M1a-1** and **M1a-2** were both relatively stable in rat liver microsomes, producing only a single hydroxylated metabolite (Figure 6a). However, interestingly, **M1a-2**, was found to be extensively metabolized by monkey and human liver microsomes to a single hydroxylated metabolite (complete structure not determined). In contrast **M1a-1** was found to be relatively stable in the presence of monkey and human liver microsomes (Figure 6b).

Quantitation of Biologically Generated Metabolite M1

Metabolite **M1** was produced in sufficient quantities by human liver microsomes to be characterized and quantitated by NMR analysis. The quantitation of **M1** was conducted using the method as described previously (Espina et al. 2009). The concentration of **M1** was determined to be 1.72 mM (see Table 1). A quality control (QC) sample (another synthetic metabolite standard of compound **1**) was used during the NMR analysis to

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ensure that reliable concentration of **M1** was obtained. Three concentrations of the QC (0.2, 2.0 and 3.5 mM) were evaluated by quantitative NMR against gravimetrically determined values. The % difference between NMR and gravimetrically determined concentrations were found to be less than 15% at all three concentrations.

*Pharmacological Activities of the Isolated Metabolite **M1** and Chemically Synthesized Metabolites **M1a-1** and **M1a-2**.*

For **M1**, the isolated material showed low nM binding and affinity to the receptor, that was equivalent to that of the parent compound. This initial evaluation using the isolated material was later confirmed by testing the corresponding correct enantiomer which had similar in vitro binding as was previously obtained using the isolated material (see Table 2). Interestingly, the epimeric form (**M1a-2**) of this metabolite was approximately two times less active than **M1a-1** in three out of four pharmacological assays (Table 2). However, it appears that an introduction of a hydroxyl group did not alter the pharmacological activity significantly as compared to the parent compound.

*In Vivo Pharmacokinetics of Compound **1**, **M1a-1** and **M1a-2***

Figures 7a-c illustrates semilogarithmic plasma concentration-time profiles for compounds **1**, **M1a-1** and **M1a-2** after 2 mg/kg intravenous bolus doses. The concentration-time profiles were best described by one-compartment models with first-order elimination rate constants. Pharmacokinetic parameters were characterized by high clearance compared to liver blood flow, moderate volume of distribution compared to plasma volume and short elimination half-lives (see Table 3). Both metabolites had similar clearance and volume of distribution. Oral administration of the compounds (10 mg/kg) produced non-detectable levels of the compound and hence the data are not shown in this report.

DISCUSSION

NMR has traditionally been used to elucidate structures of small and large molecules (Prestgard, 2001; Mutlib and Shockcor, 2003). NMR, especially in combination with mass spectrometry and other hyphenated techniques, is customarily used to fully elucidate structures of previously uncharacterized metabolites (Mutlib and Shockcor, 2003). While this qualitative aspect of NMR has played a significant role in drug metabolism studies, the quantitative capabilities of NMR in metabolism studies have not been fully realized until recently. With recent improvements in the NMR technology, it is now considered as an important quantitative tool despite its lower sensitivity as compared to more widely used mass spectrometers. Recently, in response to the demands from the regulatory agencies to better define the exposures of metabolites in humans and in preclinical species (US FDA, 2008), strategies based on the application of NMR as a quantitative tool for metabolite quantitation have been proposed (Espina et al., 2009; Dear et al., 2008). NMR has been used in the past as an analytical tool to determine concentrations of synthetic and biosynthetic products (Warren et al., 1976; Vinson et al., 1978; Weiner and Eisenreich, 1997; Groen et al., 1998; Silvestre et al., 2001; Holzgrabe et al., 2005; Pauli et al., 2005, 2007, 2008; Rizzo and Pinciroli, 2005; Diehl et al., 2007; and Shao et al., 2007), metabolites, catabolites and endogenous compounds in biological fluids (Malet-Martino et al., 1986; Monte et al., 1994; Desmoulin et al., 2002; Lutz et al., 2004; Orhan et al., 2004; Skordi et al., 2004; Moazzami et al., 2007), or impurities present in products (Hays, 2005; Malz and Jancke, 2006). The theoretical aspects and validation of quantitative NMR have been discussed and reviewed in the literature (Mackenzie IS, 1984; Evilia RF, 2001; Burton et al., 2005; and Malz and Jancke, 2005).

We have demonstrated that NMR could be utilized to quantitate metabolites isolated from biological sources (Espina et al., 2009). In that study it was shown that the compounds, from which the metabolites are derived, could be used to create calibration curves for quantitative analysis. This approach was successfully used to obtain exposure values of metabolites present in plasma of preclinical species, hence circumventing the need for synthetic standards of metabolites or radiolabeled studies. Recently, we showed the

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value of conducting quantitative NMR studies in addressing metabolites in safety testing (MIST) (Vishwanathan et al., 2009). The application of quantitative ^{19}F -NMR in obtaining mass balance of fluorinated compounds was demonstrated by us and others (Graffe, 1998, 1999; Lenz et al., 2002; Duckett et al., 2006; and Mutlib et al., 2009). The ability to quantitate microgram quantities of metabolites led us to further investigate the possibility of using quantitative NMR in accelerating the pharmacological evaluation of metabolites earlier in drug discovery. In the past, pharmacological testing of metabolites was conducted primarily with synthesized metabolite standards. However, frequently, the synthesis of metabolites is protracted taking several weeks to months before the authentic standards are available for further evaluation. Knowledge of the pharmacological activities of major metabolites earlier can lead to the design of appropriate studies to better understand the implications of such metabolites, especially if they are postulated to have biological activity similar to the parent compounds. Once it is known that a metabolite possesses pharmacological activity similar to the parent compound (or in some cases more potent), its chemical synthesis can be accelerated to confirm its pharmacological activity. Furthermore, since pharmacologically active metabolites need to be quantitated in clinical samples, the availability of synthetic standards of metabolites in a timely manner can facilitate the assay of this metabolite present in clinical samples. An initial pharmacological evaluation of biologically generated metabolites can be used in the decision making steps whether chemistry resources are needed for its synthesis. Obviously synthesis of pharmacologically inactive metabolite can be either delayed or not conducted depending upon the fate of the compound as it progresses into development. The other advantage of knowing the pharmacological activity of a metabolite earlier is that it allows one to file a patent on it, hence protecting the intellectual property of a company. Pharmacologically active metabolites can contribute significantly to the overall therapeutic or adverse effects of drugs. Many active metabolites, such as atorvastatin (Liptor) (Lennernas H, 2003), simvastatin (Zocor) (Williams and Feely, 2002), fluoxetine (Prozac) (Cheer and Goa, 2001), fexofenadine (Allegra) (Meeves and Appajosyula, 2003) and cetirizine (Zyrtec) (Golightly and Greos, 2005) have been developed as drugs in their own right. In an attempt to demonstrate the value of early pharmacological evaluation of biologically-

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derived metabolites, a series of studies based on initial results, were conducted with compound **1** and its synthesized metabolite standards. The purpose of these was to demonstrate some of the outcomes and impact of conducting early metabolite identification and quantitation in discovery. The cascade of studies that may occur as a result of confirmatory metabolite identification and quantitation are described in this paper (see Scheme 1).

Compound **1** was found to be metabolized to a major single hydroxylated metabolite by human liver microsomes. As shown in Figure 1, the hydroxylation took place on a pro-chiral center leading to the possibility of several isomers. While the position of hydroxylation was easily determined by initial NMR analysis of the isolated metabolite, the exact stereochemistry could only be resolved by conducting synthesis and chiral HPLC separation of enantiomers (Chen et al., 2010). After the identity of the metabolite was unequivocally confirmed by HPLC and mass spectral comparisons with synthetic standards, additional studies such as pharmacological and pharmacokinetic evaluations were conducted. It is important to note that it was close to 12 months (from the time the position of hydroxylation on **M1** was determined) before synthetic standards of this metabolite were available for pharmacological evaluations. In contrast, it took 3-4 weeks from the time the major hydroxylated metabolite was initially identified in human liver microsomes before pharmacological evaluation of the isolated metabolite was conducted. The protracted time required to obtain the synthetic standard of this metabolite was due to a number of reasons including complex synthesis needed to achieve the desired stereospecificity. While the initial pharmacological tests on the isolated metabolite were considered reliable and accurate, it was important to demonstrate (as part of the verification process) that the synthetic standard produced similar or identical results as the isolated metabolite in pharmacological evaluations (see Table 2). Indeed, the results were very similar and hence confirmed the value of conducting quantitative NMR on isolated metabolites so that pharmacological assessments could be made earlier with such isolated metabolites rather than waiting for synthetic standards of metabolites. Interestingly, the introduction of hydroxyl group on the bridged morpholine ring system did not diminish the pharmacological activity of the metabolite **M1**. This information

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was useful to chemists as this led to design of other molecules with modified bicyclic ring system (Chen et al., 2010). Fluorinated analogues were also considered so that the formation of the hydroxylated metabolite could be mitigated. By evaluating the pharmacological activities of metabolites, one may also gain an insight into structure-activity (SAR) relationship; hence potentially influencing the design of newer molecules (see Scheme 1). For example, in the case of compound **1**, fluorine and methyl groups were substituted in the metabolic soft spot, realizing that an introduction of a small group at that position was not going to compromise the pharmacological activity. Further studies with these analogues are in progress. Often the chemical synthesis of metabolite(s) is accelerated once it is realized that it possesses pharmacological activity (see Scheme 1). This in turn allows additional testing of the metabolite in a pharmacology model. Other studies such as in vitro metabolic disposition or in vivo pharmacokinetic evaluation of these synthetic metabolite standards could then easily be conducted. The information derived from all these studies can lead to better understanding of the disposition of the parent compound as well its pharmacologically active metabolite in preclinical species. In some cases, the properties of metabolite (or other synthesized) analogues may be more favorable than the parent compound; hence potentially creating a new drug candidate. With compound **1**, it was found that the major metabolite **M1** was produced in a very stereospecific manner, existing as the enantiomer **M1a-1**. Furthermore, it was shown that this enantiomer was more metabolically stable than **M1a-2**, which was shown not to be produced as a metabolite. The racemic mixture (**M1a**) also displayed liver microsomal instability due to the rapid metabolism of enantiomer, **M1a-2**. If this hydroxylated metabolite was further pursued as a potential drug candidate, it would be desirable to synthesize the more stable enantiomer, **M1a-1**. The significant difference in the metabolic disposition of the two enantiomers is consistent with what has been reported before for other racemic compounds (Mutlib and Nelson, 1997 and literature cited therein).

Pharmacokinetic studies showed that metabolite **M1** (**M1a-1** enantiomer) had higher clearance and lower exposure values as compared to the parent compound **1**. The overall pharmacokinetic behavior of two enantiomers, **M1a-1** and **M1a-2** were similar. This

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observation was consistent with in vitro metabolism data that showed similar metabolic stability in the presence of rat liver microsomes. Furthermore, the in vitro metabolism data suggested that these hydroxylated metabolites (**M1a-1** and **M1a-2**) were not predisposed to glucuronidation as evidenced by lack of glucuronide conjugates in the metabolite profile (Figure 6a). Furthermore, the LC/MS analysis of plasma extracts obtained from the pharmacokinetic studies showed an absence of any glucuronide conjugates, consistent with in vitro metabolism data (data not shown). It is postulated that the high clearance of **M1a-1** and **M1a-2** could be due to their rapid elimination in bile, though further studies will be needed to confirm this. The high clearance of this metabolite (**M1a-1**) observed in rats led to termination of this compound as a viable candidate for further development.

A strategy is proposed to ensure that reliable pharmacology results are obtained from metabolites that are isolated from biological sources (see Scheme 2). Once a metabolite of interest is selected, it is proposed that a scale-up and subsequent isolation and characterization take place to confirm the structure of the metabolite. The NMR analysis of the isolated metabolite is used to confirm the identity (and also purity) of the isolated metabolite. Subsequently, the metabolite can be quantitated by HPLC/UV if the absorption characteristics of the metabolite and the parent compound are considered to be similar. Usually, if the site of metabolism is remote from the UV chromophore, the absorption characteristics are relatively same for the parent compound and its metabolite(s). If the metabolite UV spectrum is not the same as the parent compound, ¹H-NMR can be used to generate the calibration curve for quantitation purposes (Espina et al., 2009). The parent compound, in this case Compound **1**, can be used to generate the standard calibration curve which could be used to quantitate the isolated metabolite (Espina et al., 2009). A standard solution of the parent compound similar in concentration to that of isolated metabolite is made and both preparations are subjected to quantitative analysis by NMR. Once the concentrations are verified, both samples are submitted for side by side comparison of pharmacological activities (Scheme 2). The preparation of a separate standard solution of the parent compound (such as compound **1**) acts as a quality control for both NMR analysis and also for pharmacological evaluation.

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Alternatively, to ensure the integrity of the results, an analogue of the parent compound or synthetically available metabolite standard, could be weighed and its concentration verified by NMR so that pharmacological evaluations could be performed (Table 1). In the case of compound **1**, a synthetic standard of metabolite that was used as the QC for quantitative NMR was found to be inactive (data not shown).

The value of performing quantitative NMR on isolated metabolites for the purpose of further pharmacological tests was demonstrated. A better understanding of the disposition of compound **1** and its metabolite **M1** was achieved once it was demonstrated that **M1** was pharmacologically active. Because sufficient quantities of **M1** were readily available for quantitation as well as for method development, the synthesis and identification of correct enantiomer was greatly facilitated. Subsequent pharmacokinetic evaluations of the pharmacologically active chemically synthesized metabolite showed that it had the propensity to be rapidly cleared *in vivo*, making it an unlikely candidate for further development. Overall, as illustrated with this example, design of experiments and critical resource-sparing decisions could be facilitated if pharmacological activities of metabolites are known much earlier in the drug discovery and development process.

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Authorship Contributions

Participated in research design: Mutlib, Espina, Venkatesan, Dehnhardt, Chaudhary

Conducted experiments: Espina, Vishwanathan, Chen, Babalola, Chaudhary

Contributed new reagents or analytical tools: Espina

Performed data analysis: Mutlib, Espina, Venkatesan, Chaudhary

Wrote or contributed to the writing of the manuscript: Mutlib, Espina, Chen, Dehnhardt, Venkatesan, Chaudhary

Other: Talaat, Scatina, Mansour acquired funding for the research and reviewed manuscript

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List of Schemes

Scheme 1: Potential studies and outcomes as a result of evaluating pharmacological activities of metabolites earlier in the drug discovery process.

Scheme 2: Workflow demonstrating the application of quantitative NMR in evaluating pharmacological activities of isolated metabolites for comparison with parent compound.

List of Figures

Figure 1: Metabolism of compound **1** to metabolite **M1** in the presence of monkey and human liver microsomes.

Figure 2: Chemical structures of synthetic isomeric forms of metabolite **M1**. The correct stereochemistry of metabolite **M1** was found to correspond to the endo-isomer, **M1a-1**.

Figure 3. Partial ^1H NMR spectra of the aliphatic region of **M1** (bottom) and synthetic standards of the endo isomer **M1a** (middle) and exo isomer **M1b** (top).

Figure 4. High-resolution MS/MS spectra of **M1**, and the endo (**M1a**) and the exo (**M1b**) isomers.

Figure 5. ^1H - ^{13}C HSQC spectrum of **M1** showing two different conformations for the bridgehead protons (signals shown within the rectangle) of the bicyclic ring. The same pattern was observed with individual enantiomers **M1a-1** and **M1a-2** as well as the racemic mixture, **M1a**. For the corresponding proton NMR signals, please see Figure 3.

Figure 6a. LC/MS total ion chromatograms (TIC) showing that both enantiomers, **M1a-1** (bottom) and **M1a-2** (top) were metabolized in a similar manner by rat liver microsomes.

Figure 6b. LC/MS total ion chromatograms (TIC) showing in vitro metabolite profiles of enantiomers, **M1a-1** (bottom) and **M1a-2** (top) in the presence of human liver microsomes. **M1a-2** was highly unstable as compared to **M1a-1**. Similar results were obtained with cynomolgus monkey liver microsomes (data not shown).

Figure 7: Plasma concentrations of compound **1** (a), **M1a-1** (b) and **M1a-2** (c) following a single 2 mg/kg intravenous dose in male SD Rats. Compounds **M1a-1** and **M1a-2** were below limit of quantitation 2 hrs post-dose.

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Table 1. NMR determination of the concentration of **M1** (isolated from microsomal incubations) produced from compound **1**

Compound	Nominal (weighed) concentration (mM)	Mean observed concentration by NMR (mM)	% RSD	% Difference from nominal value
compound 1	3.80	3.80	0.15	0.02
	2.50	2.48	0.09	0.80
	1.00	1.05	0.37	4.69
	0.50	0.50	0.08	0.11
	0.10	0.07	0.65	42.45
QC ^a	3.50	3.41	1.68	2.73
	2.00	2.12	0.33	5.77
	0.20	0.18	0.25	11.28
M1	----	1.72	0.16	----

^a Another metabolite of compound **1**, available as synthetic standard, was used as a quality control to ensure that the NMR quantitation was verified by gravimetric determination. NMR analysis was conducted in triplicates

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Table 2: Pharmacological evaluation of compound **1**, isolated metabolite **M1** and the synthetic enantiomers, **M1a-1** and **M1a-2**. Metabolite **M1** and **M1a-1** were shown to be identical by chiral HPLC (Chen Z et al., 2010).

Pharmacology Tests*	Compounds			
	M1 (isolated metabolite)	M1a-1 (R-enantiomer)	M1a-2 (S-enantiomer)	Compound 1
PI3K- α (nM)	4	4	9	8
PI3K- γ (nM)	33	50	93	51
mTOR (nM)	0.80	N/A	N/A	0.50
PC3 (nM)	0.08	0.04	0.06	0.02
MDA361 (nM)	0.03	0.02	0.05	0.04

* PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; PC3, prostate cancer cell line; MDA361, human breast adenocarcinoma cell line.
For description of the pharmacological assays, please see Venkatesan et al (2010).

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Table 3: Pharmacokinetic parameters of Compound **1**, **M1a-1** and **M1a-2** in Sprague Dawley rats following a single 2 mg/kg intravenous dose

Parameters	Compound 1	M1a-1	M1a-2
Cl _s (L/hr/kg)	2.3 ± 0.2	6.4 ± 0.8	7.4 ± 1.9
V _{dss} (L/kg)	2.4 ± 0.3	1.5 ± 0.4	0.9 ± 0.3
AUC _{0-inf} (ng*hr/mL)	867 ± 73	315 ± 36	283 ± 64
Half-Life (hrs)	0.9 ± 0.1	0.7 ± 0.1	0.2 ± 0.1

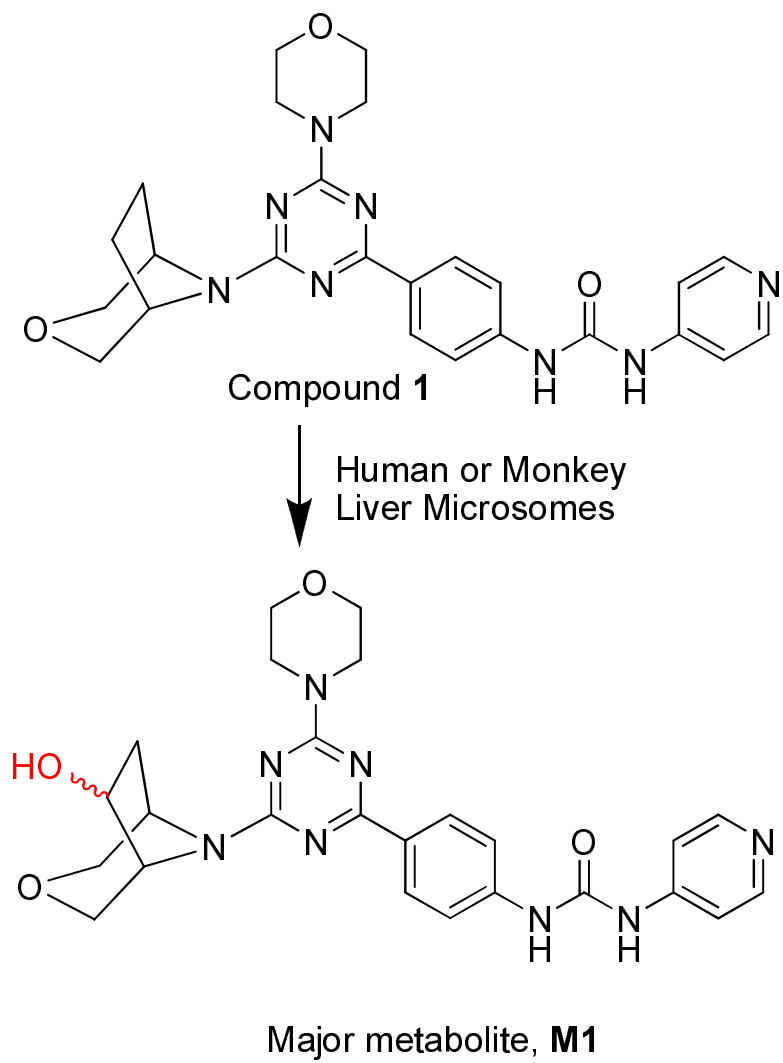


Figure 1

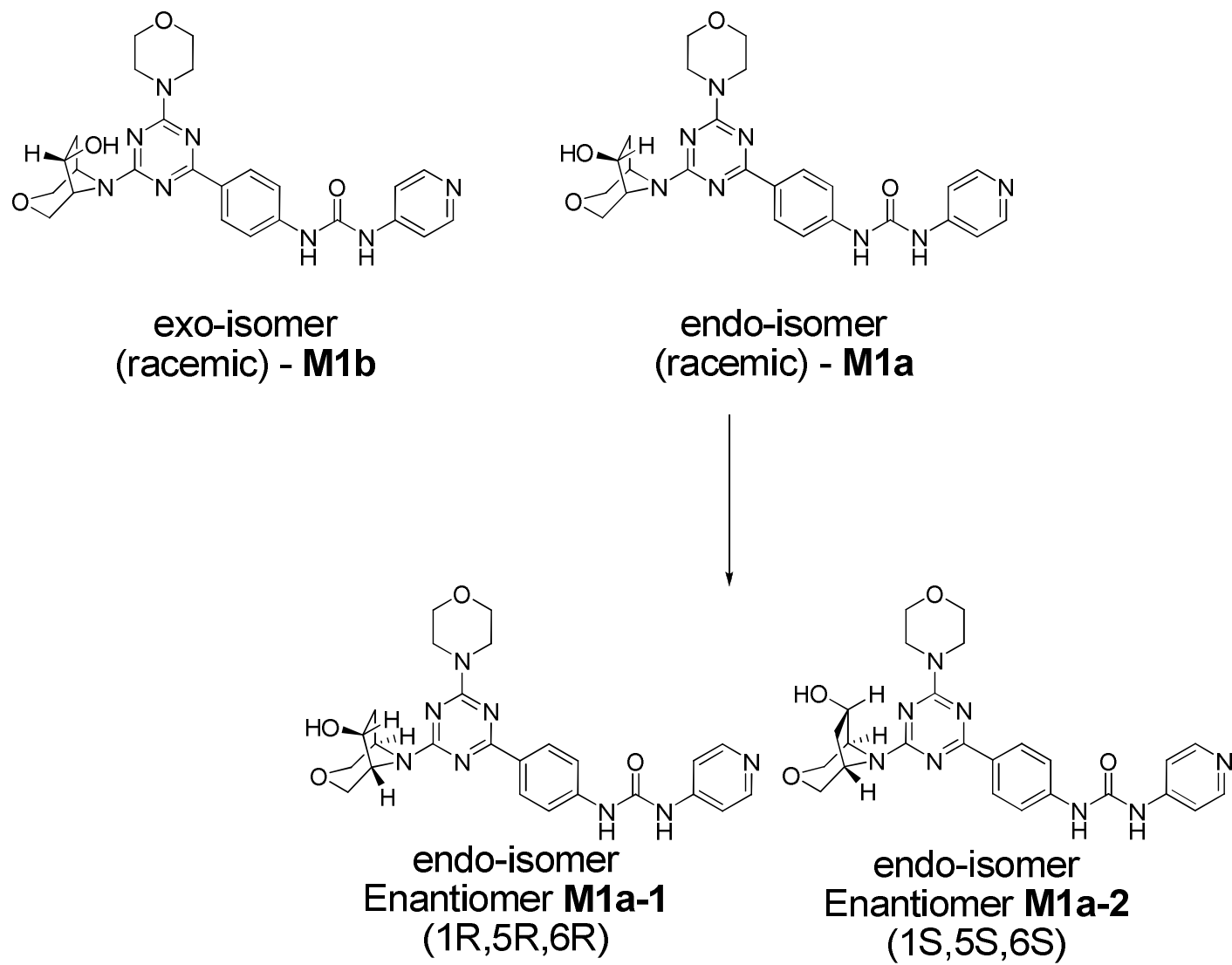


Figure 2

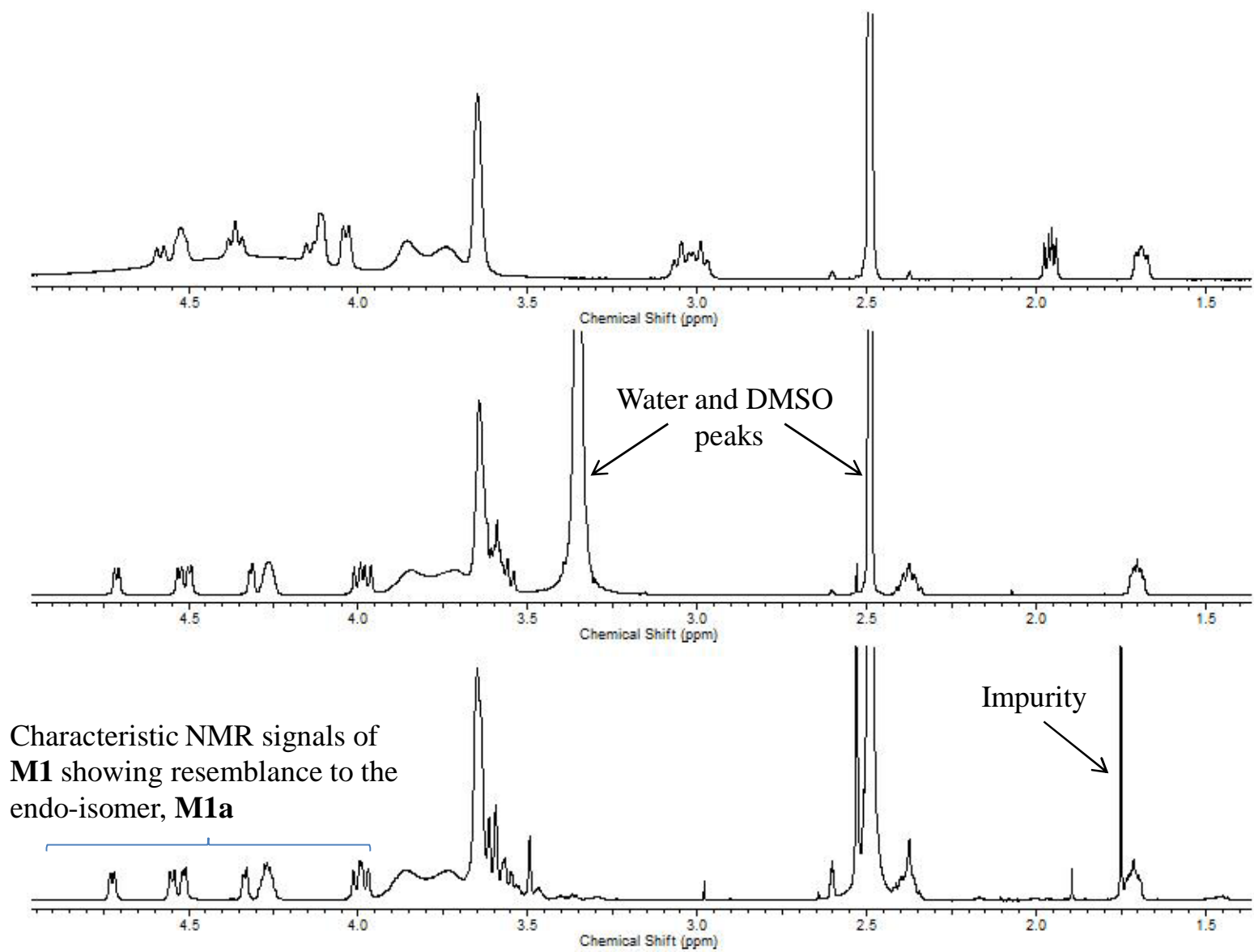


Figure 3

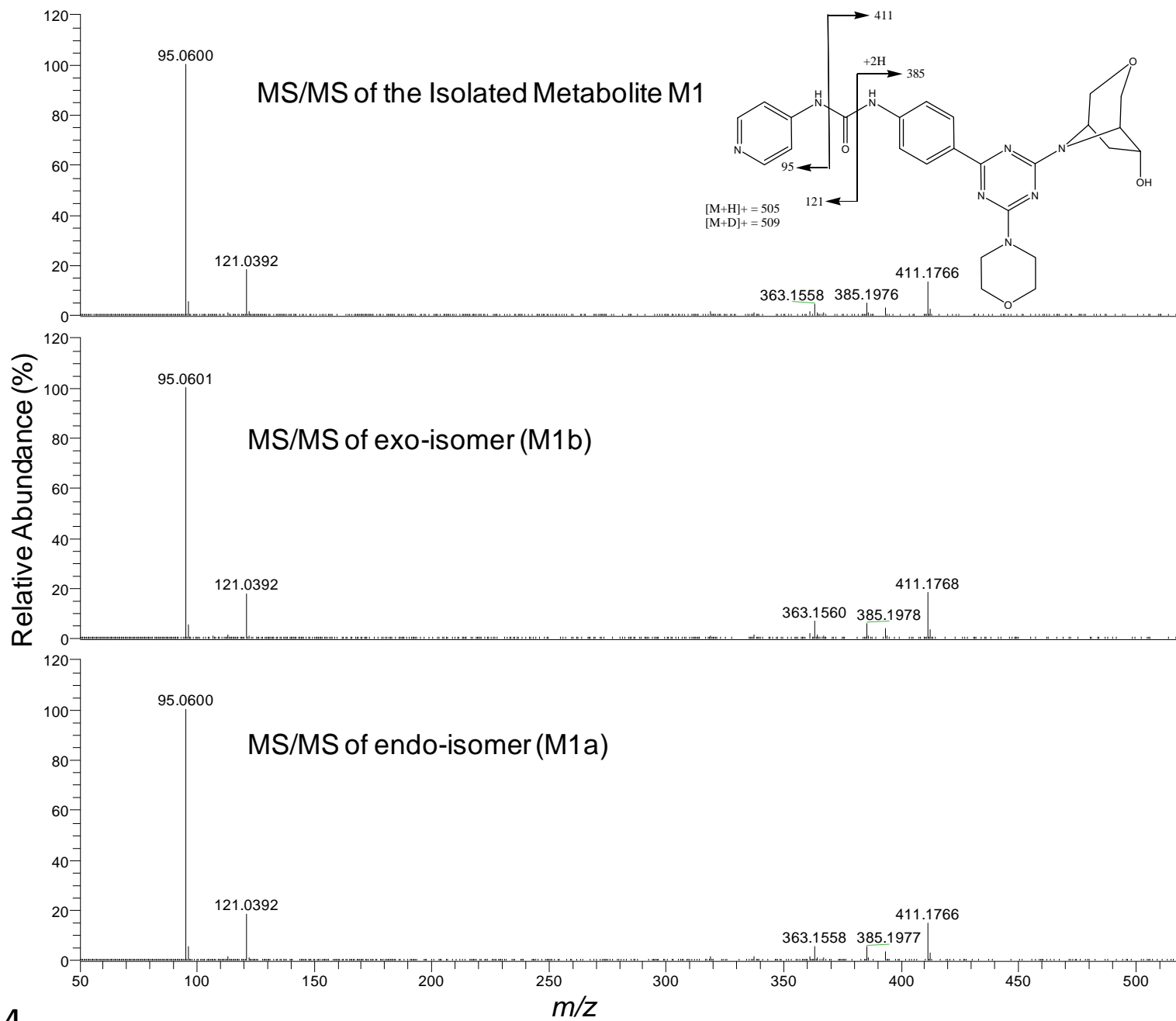


Figure 4

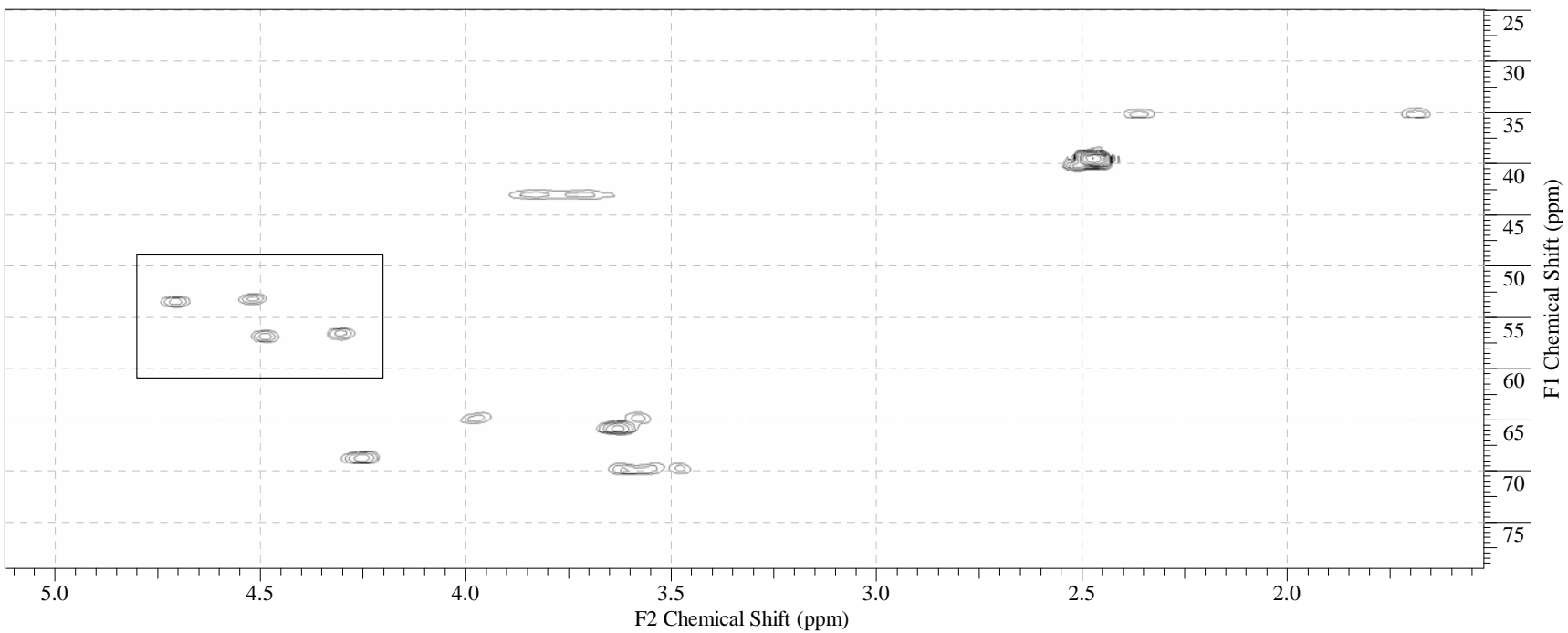


Figure 5

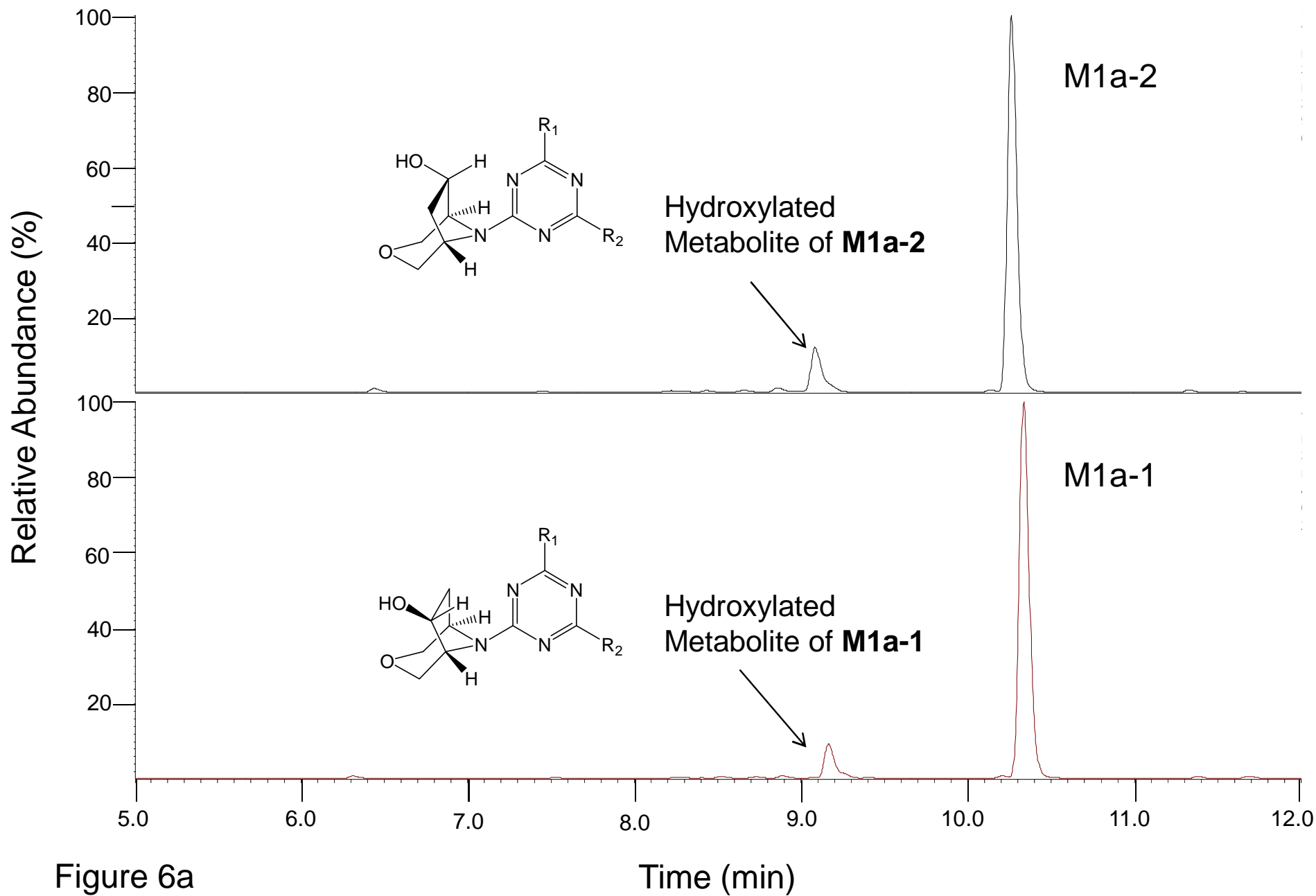


Figure 6a

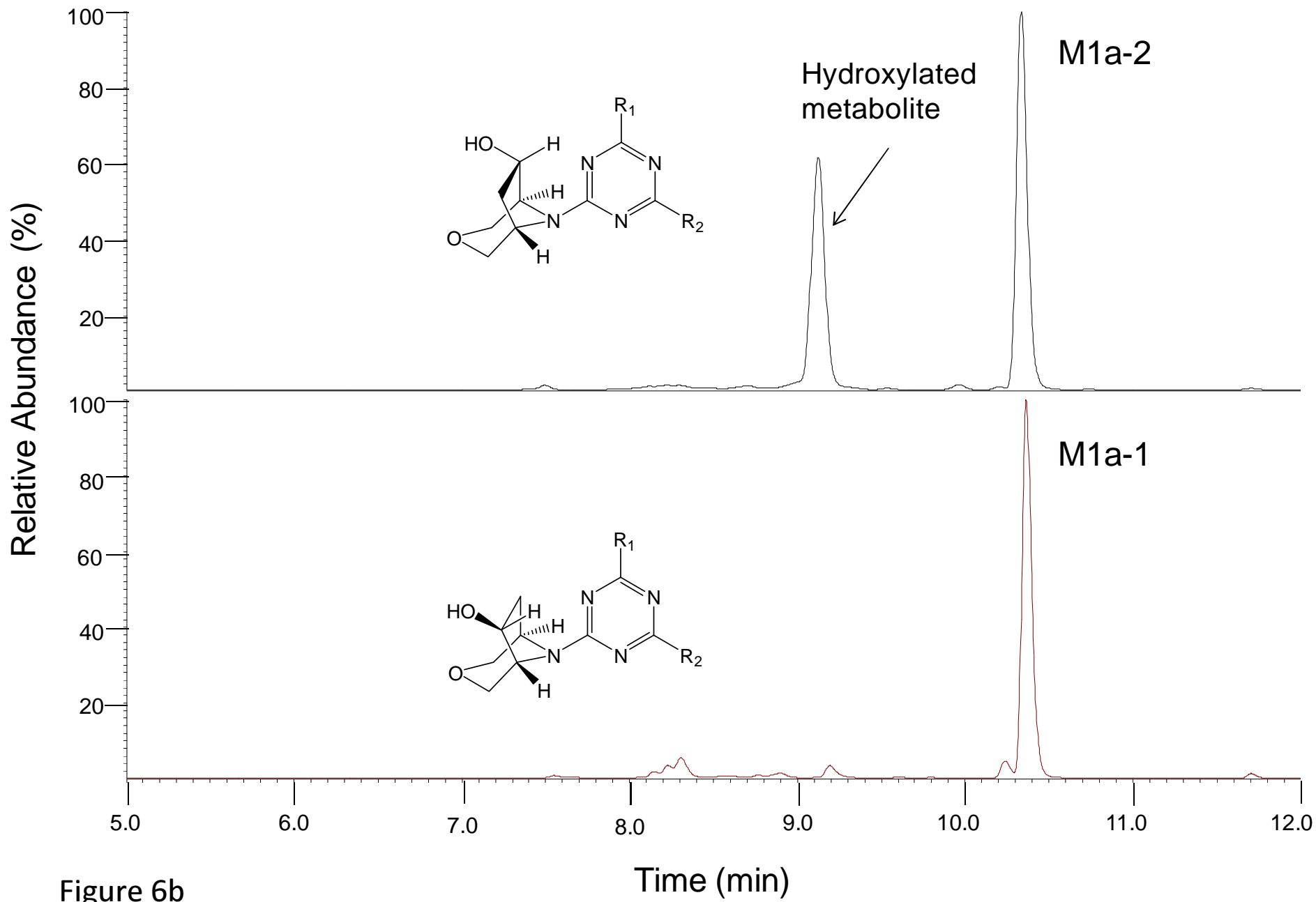


Figure 6b

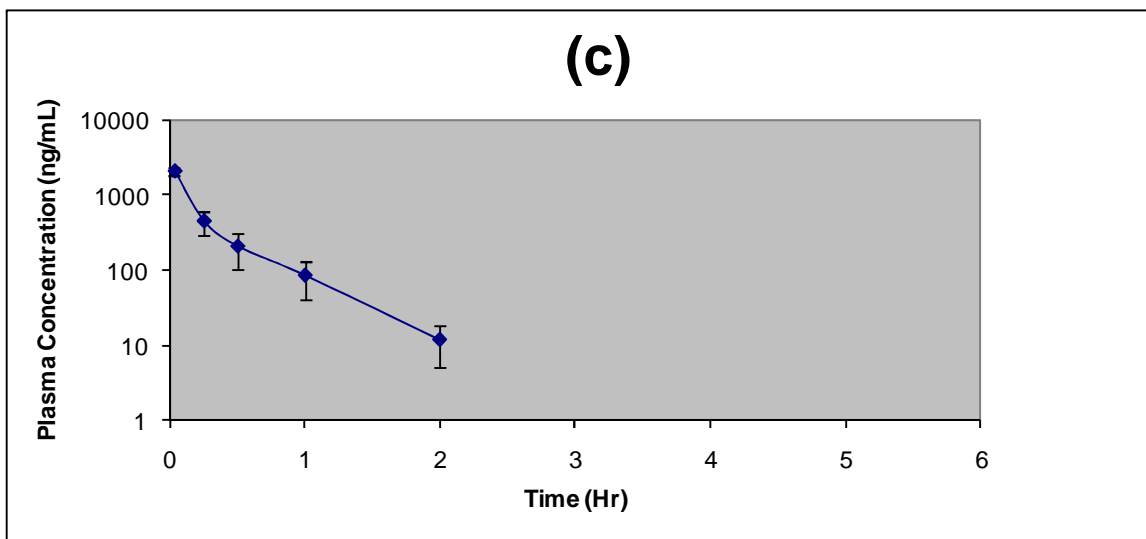
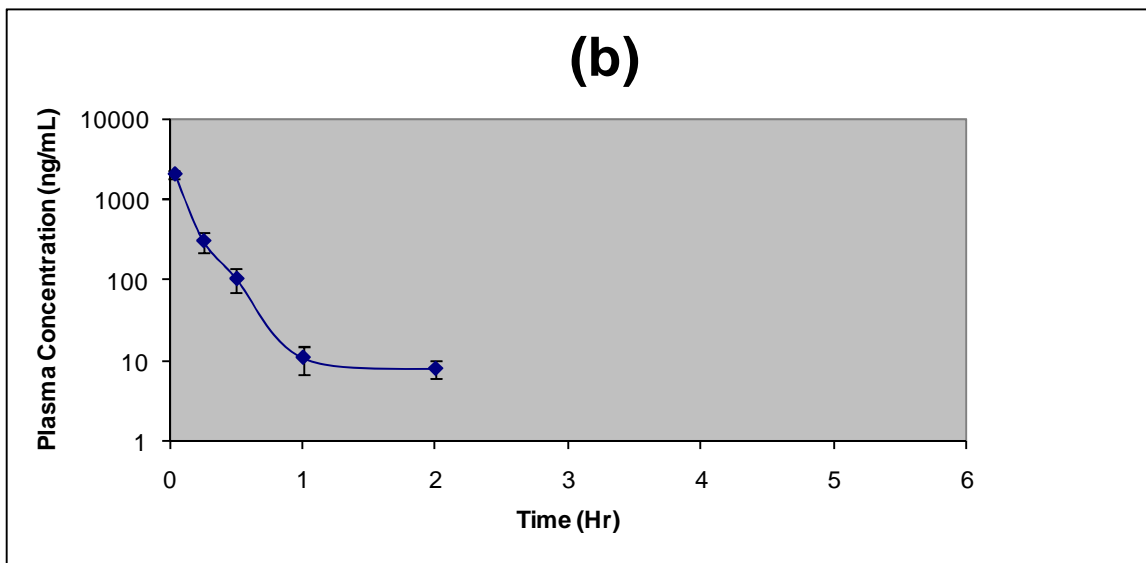
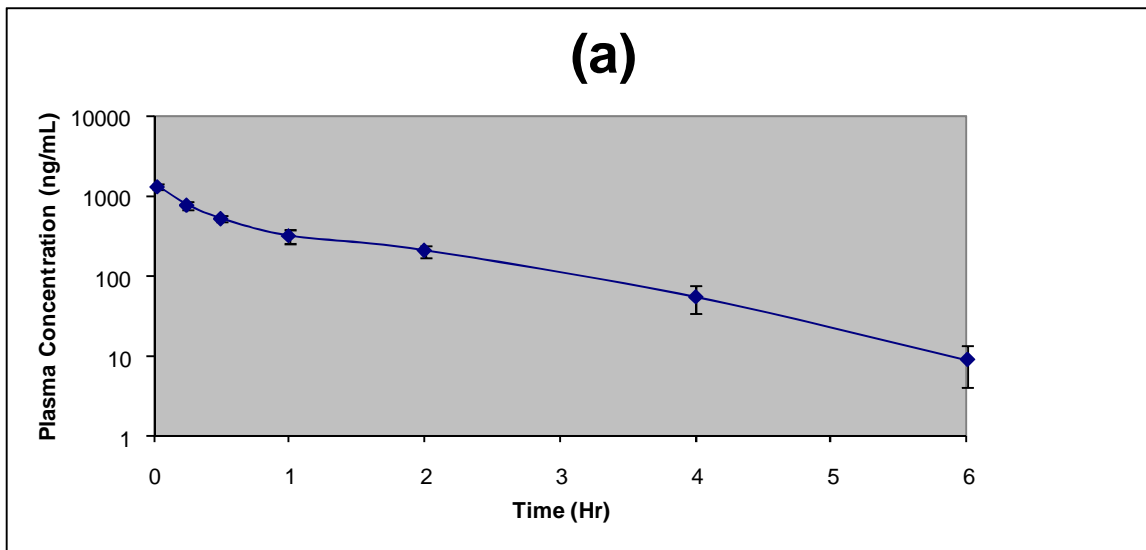
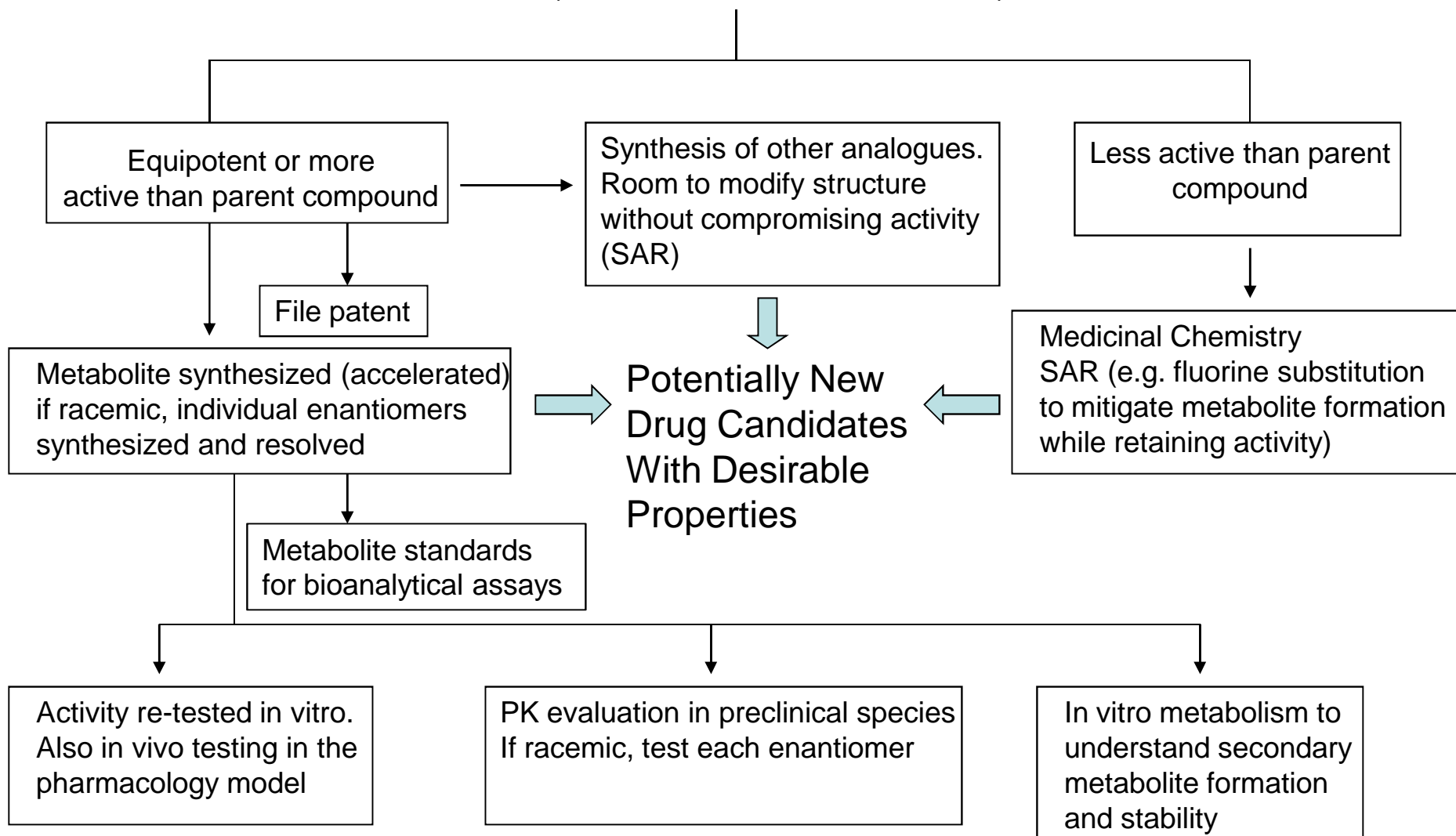
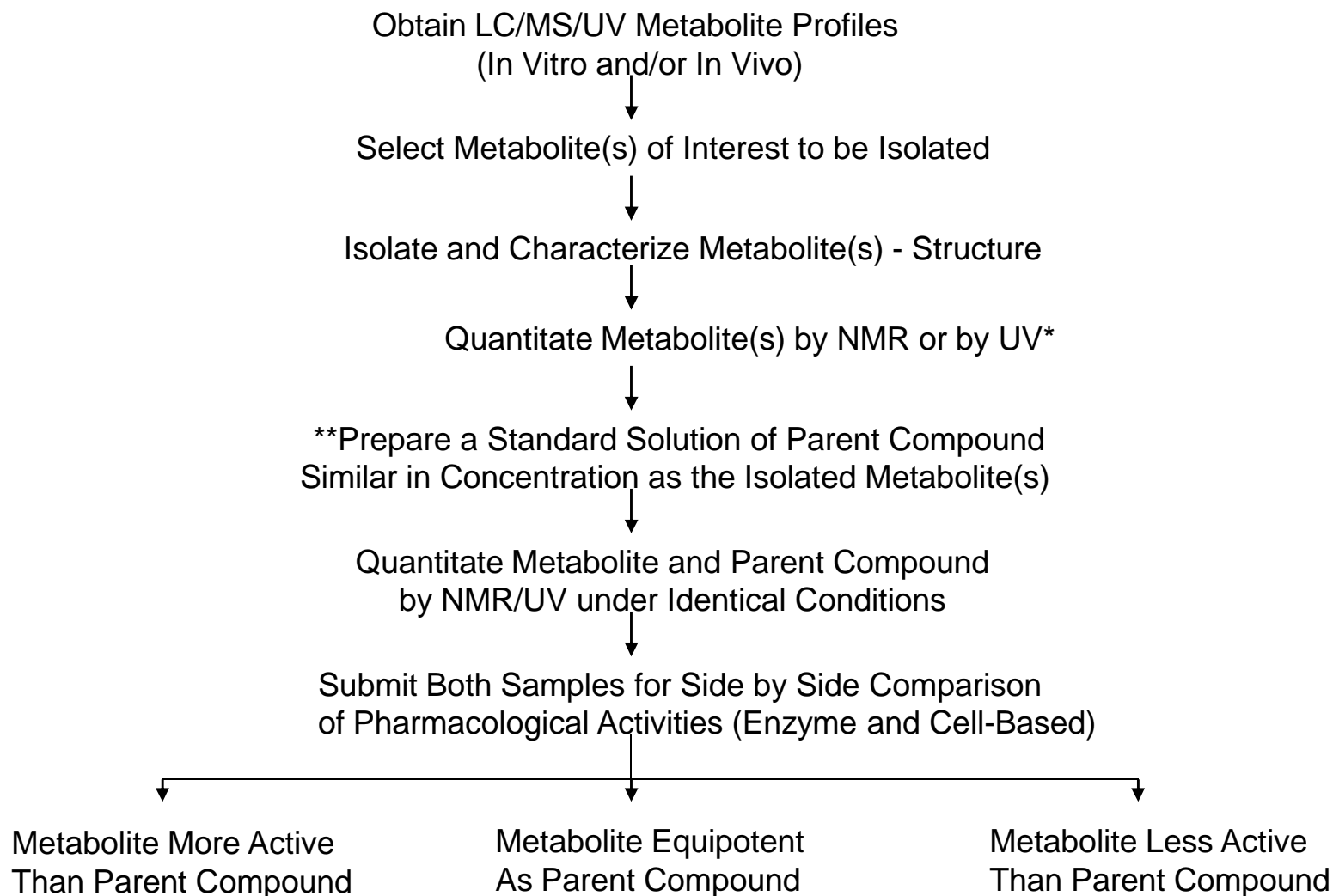


Figure 7

Pharmacological Evaluation of Metabolites (after NMR or UV Quantitation)



Scheme 1



*Metabolites can be quantitated by UV if the absorption characteristics of metabolites are considered to be the same as the parent compound.

** A standard solution of an analogue or chemically synthesized metabolite of the parent compound, the pharmacological activities of which were established previously, could be used instead.

Scheme 2