Biosynthesis of Drug Metabolites and Quantitation Using NMR Spectroscopy for Use in Pharmacologic and Drug Metabolism Studies

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Abstract

The contribution of drug metabolites to the pharmacologic and toxicologic activity of a drug can be important; however, for a variety of reasons metabolites can frequently be difficult to synthesize. To meet the need of having samples of drug metabolites for further study, we have developed biosynthetic methods coupled with quantitative NMR spectroscopy (qNMR) to generate solutions of metabolites of known structure and concentration. The specific quantified isolates were used as standards in the drug discovery setting as substrates in pharmacology assays, for bioanalytical assays to establish exposure, and/or as analytical standards for bioanalytical work to ascertain exposure, among others. We illustrate five examples of metabolite biosynthesis and qNMR. The types of metabolites include one glucuronide and four oxidative products. Concentrations of the isolated metabolite stock solutions ranged from 0.048 to 8.3 mM, with volumes from approximately 0.04 to 0.150 ml in hexadeutated dimethylsulfoxide. These specific quantified isolates were prepared dimethylsulfoxide; ERETIC 2, electronic reference to access in vivo concentrations 2; HMBC, heteronuclear multiple-bond correlation; HPLC, high-performance liquid chromatography; HPS, high-pressure liquid chromatography-mass spectrometry; HSQC, heteronuclear single-quantum coherence; PDA, photo diode array; PDE2, phosphodiesterase-2; qNMR, quantitative NMR.

Introduction

A majority of drugs undergo metabolism as their main mechanism of clearance. A variety of enzymes catalyze oxidation, reduction, hydrolysis, and conjugation reactions (Parkinson et al., 2013). Delineation of the metabolic pathways of new drug candidates is expected by regulatory agencies. An understanding of the metabolism of new chemical entities can aid in drug research to design compounds with improved pharmacokinetic properties (i.e., low clearance, low first-pass metabolism, and half-lives suitable for convenient dosing). The metabolites of drugs and new drug candidates can be important for several reasons. First, because metabolism can be a major clearance mechanism for a new drug, structural knowledge of the main metabolites permits an informed inquiry into what drug metabolizing enzymes may be responsible for clearance. Knowledge of enzymes responsible for clearance can permit an understanding of the potential for interpatient variability in pharmacokinetics, drug-drug interactions, and the impact of genetic polymorphisms on drug and metabolite exposure (Zhang et al., 2007). Second, drug metabolites can possess similar binding potency to the same pharmacologic target as the parent drug, and thus could contribute to the clinical effect (Obach, 2013). Knowledge of potency, pharmacokinetics, and target tissue distribution of active metabolites is important for establishing the pharmacokinetic-pharmacodynamic relationship and dosing regimen. It can also be possible that an active metabolite can be a drug itself; three common antihistamines—fenofenadine, desloratadine, and cetirizine—are all active metabolites of first-generation antihistamines (terfenadine, loratadine, and hydroxyzine, respectively) with improvements in clinical profiles. Third, drug metabolites have been subjected to increased focus over the past decade because of their potential to be responsible for toxicity (i.e., the metabolites in safety testing or “MIST” issue; Baillie et al., 2002; Smith and Obach, 2009; http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Multidisciplinary/M3_R2/Step4/M3_R2__Guideline.pdf). During the development of a new drug, an assurance is needed that metabolites to which humans are exposed have been acceptably tested for toxic potential. Usually this is assessed by virtue of metabolite exposure in laboratory animals after administration of the parent drug.

Because of these points, it is important to obtain authentic standards of human drug metabolites. These standards can be used to test for target activity or to construct calibration curves for measurement of drug concentrations. However, chemical synthesis of metabolites can be
challenging. A metabolite of interest can be one structure of many regiochemical and/or stereochemical possibilities, requiring the synthesis of several isomers to establish which possibility is correct. As an alternative to chemical synthesis, microbiological and biomimetic methods have been described for the generation of drug metabolites (Bernadou and Meunier, 2004; Li et al., 2009). However, use of these methods requires proof that the correct metabolite isomer is being made by the system employed.

When drug-metabolizing enzymes from human or laboratory animal species are employed as the biologic system to prepare an authentic standard of a metabolite, there is greater assurance that the truly desired metabolite is being made (Li et al., 2009). However, a challenge of such biologic reagents is the generation of enough product to permit isolation of enough material to weigh. These techniques can produce the desired metabolites but generally only in the tens of micrograms range (5–500 nmoles). It is not feasible to accurately weigh isolates of this magnitude. However, quantitative NMR (qNMR) can be used to provide concentrations of isolated metabolite in this weight range (Espina et al., 2009, Vishwanathan et al., 2009, Walker et al., 2011). Although NMR is typically thought of as a qualitative technique that yields structural information, the quantitative nature of NMR has long been used in the field of natural products (Pauli et al., 2012). What separates NMR from other spectroscopic techniques (e.g., mass spectrometry, UV/visible spectrophotometry) as a quantitative method is the uniform response to hydrogen atoms. This enables several different strategies using internal and external standards to quantitate unknown organic compounds without a qualified reference standard.

We describe methods for making and isolating metabolites in quantities from 5 to 500 nmoles. Metabolites are generated using liver microsomes in incubation volumes of tens of milliliters and isolated using high-pressure liquid chromatography (HPLC). The residues in fractions containing metabolites of interest are analyzed by qNMR to yield small volumes (~50–150 µl) of metabolite solutions of known concentrations (typically 0.1–5 mM) in hexadeuterrated dimethylsulfoxide (DMSO-6D). These solutions can then be used as stock solutions from which dilutions can be made to test for in vitro pharmacologic activity, to use as standards for calibration curves, or to use in other drug metabolism studies. The methods are illustrated using five examples (Fig. 1), and the uses of these biosynthesized standards are briefly described.

Materials and Methods

Compound A [(3S)-3-amino-1-hydroxy-6-phenoxo-3,4-dihydroquinolin-2(1H)-one], compound B [4-azetidin-1-yl]-3-(5-(4-ethylphényl)-1-methyl-1H-pyrazol-4-yl)-1-methyl-1H-pyrazol[3,4-d]pyrimidine, compound C, [N-[(2,4-di-tert-butyl-5-hydroxyphenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamido], and compound D [ethyl 1-{[(3-dimethylcarbamoyl)-4-{[(4-(trifluoromethyl) biphenyl-2-yl)carbonyl]amino}[phenyl]acetyl]oxy}methyl]-1,3-dihydro-2-benzofuran-1-carboxylate] were synthesized by Pfizer (Groton, CT). Azanavar and [methyl [(5S,10S,15S)-11-benzyl-5-tert-butyl-10-hydroxy-15,15-dimethyl-3,6,13-trioxo-1[5]-pyridin-2-yl]benzoyl] 2-oxa,4,7,8,11,12-tetraazaheptadecan-14-yl]carbamate] was obtained from Sequoia Research Products (Pangbourne, UK). Recombinant homologously expressed CYP3A5 (123 pmol/mg microsomal protein) was obtained from Panvera (Madison, WI). NADPH and uridine diphosphate glucuronic acid were from Sigma-Aldrich (St. Louis, MO). Human and female dog liver microsomes as well as dog intestinal microsomes were purchased from BD Gentest (Woburn, MA). The human liver microsomes consisted of a pool of 50 male and female donors. All deuterated solvents were purchased from Cambridge Isotope Laboratories (Tewksbury, MA).

Equipment

The following equipment was used in these preparations unless otherwise noted: Jouan Model CT422 centrifuge (Saint-Herblain, France); Beckmann-Coulter Spinchron DLX centrifuge (Brea, CA); Genevac Evaporator (Genevac, Valley Cottage, NY); Jasco PU-980 HPLC pump (Jasco EZ-2 Analytical Instruments, Easton, MD); AB Sciex Triple TOF 5600 mass spectrometer (Framingham, MA) with a Shimadzu CBM-20A controller (Shimadzu USA, Columbia, MD); LC-20ADXR pumps (Shimadzu USA); CTC PAL Autosampler (Leap Technologies, Carrboro, NC) and Agilent 1100 PDA (photo diode array) (Agilent Technologies, Santa Clara, CA); Thermo-Fisher high-pressure liquid chromatography–mass spectrometry (HPLC-MS) containing Surveyor quaternary HPLC, Surveyor PDA detector, and LTQ mass spectrometer (Thermo Fisher, Wilmington, DE); Gilson FC-204 fraction collector (Gilson, Middletown, WI); Shimadzu semi-preparative HPLC system containing a SIL-HTC autosampler, two LC-20AD solvent pumps, an SPD-M20A diode array detector, and a PRC-10 fraction collector (Shimadzu USA); Nexus II dry box (Vacuum Atmospheres, Hawthorne, CA); and Bruker Avance 600 MHz NMR spectrometer controlled by Topspin V3 with a TCI Cryo probe (1.7 or 5 mm) (Bruker BioSpin Corporation, Billerica, MA). The polypropylene microtiter plates were from Arctic White (Bethlehem, PA).

General Method 1

In general method 1, the parent compound (typically 10–100 µM) is incubated in an in vitro system containing a source of drug metabolizing enzyme and cofactors necessary to effect the biotransformation of interest. The reaction volume typically ranges between 10 and 100 ml and is performed in an Erlenmeyer flask in a shaking water bath at 37°C. At the end of the incubation, the reaction is terminated by addition of acetonitrile (CH3CN; 1–4 volumes), and the mixture is spun in a centrifuge to remove the precipitant. The supernatant is subject to vacuum centrifugation to remove organic solvent, and to the remaining supernatant is added aqueous formic acid to a total volume of 25–150 ml. This mixture is subject to centrifugation at 40,000g, and the resulting clear supernatant is applied to an HPLC column through an HPLC pump. After the material is applied, the column is moved to a gradient HPLC-MS system coupled to a fraction collector, and an HPLC solvent program is applied to elute the product(s) of interest. Fractions containing the metabolite(s) of interest are combined, and the solvent removed by vacuum centrifugation. Residues are dissolved in DMSO-d6 for NMR analysis.

General Method 2

In general method 2, incubations are performed as in method 1. After centrifugation of terminated incubation mixtures as previously, the supernatants are transferred to clean 50-ml polypropylene centrifuge tubes and concentrated to dryness in a Genevac vacuum centrifuge. The residues in each tube are reconstituted 10% CH3CN in water (<2 ml). The reconstituted samples are combined in a 2-ml HPLC vial and are injected onto a HPLC semipreparative system as multiple 300-µl injections collecting the postcolumn effluent on a fraction collector. Fractions containing the metabolite(s) of interest are combined, and the solvent removed by vacuum centrifugation. Residues are dissolved in DMSO-d6 for NMR analysis.

Example A: Biosynthesis of Compound A Glucuronide Using Method 1

Compound A (50 µM) was incubated with human liver microsomes (2 mg/ml) containing 25 µg/ml amelamin, 5 mM MgCl2, and 5 mM uridine diphosphate glucuronic acid in a total volume of 20 ml KH2PO4 (100 mM, pH 7.4). The incubation was conducted in a 250-ml Erlenmeyer flask in a shaking water bath maintained at 37°C for 1 hour. At the end of the incubation, 20 ml of CH3CN was added, and the mixture was transferred to two 50-ml polypropylene conical tubes and vigorously mixed on a vortex mixer. The tubes were spun in a Jouan centrifuge at 1700g for 5 minutes, and the supernatant was transferred to new 50-ml polypropylene conical tubes. The tubes were subjected to vacuum centrifugation in a Genevac evaporator set at the HPLC mixture setting for approximately 1 hour to remove the CH3CN. The remaining solution was added to water to a total volume of ~100 ml and formic acid (1 ml). This mixture was subjected to centrifugation in a Beckmann centrifuge at 40,000g for 30 minutes to clarify the supernatant. The supernatant was transferred to a 100-ml graduated cylinder and directly applied onto a Varian Polaris C18 column (4.6 × 250 mm; 5 µm particle size) through a Jasco HPLC pump at a flow rate of ~0.8 ml/min. After application of the 100 ml, another ~10 ml of 0.1% formic acid was pumped onto the column to ensure that the HPLC lines were cleared of the supernatant.

The HPLC column was then transferred to a Thermo LTQ HPLC-UV-MS system with the mass spectrometer operated in the positive ion mode. The
Effluent was split between the mass spectrometer and a Gilson fraction collector at a ratio of approximately 1:15. The mobile phase used consisted of 0.1% formic acid (mobile phase A) and CH₃CN (mobile phase B) at a flow rate of 0.8 ml/min. The mobile phase composition commenced at 95% A/5% B, was held at that composition for 5 minutes, followed by a linear gradient to 50% A/50% B at 25 minutes, a second gradient to 20% A/80% B at 30 minutes, held at this composition for 5 minutes, then re-equilibrated at initial conditions for 10 minutes.

The mobile phase program and data collection were started by making a dummy injection of water from the autosampler. Fractions were collected every 20 seconds into a wide-welled polypropylene microtiter plate. Fractions collected in the region of interest (i.e., that where the mass spectrometer
showed the presence of mlz 447, the protonated molecular ion of the glucoronide metabolite) were injected (5 μl) onto a second Polaris C18 column using the same mobile phase gradient to test for purity. Those containing the product of interest were combined into a 15-ml conical glass tube, and the solvent was evaporated by vacuum centrifugation in the Genevac evaporator. Upon dryness, they were prepared for NMR analysis as described later.

**Example B: Activity-Gram and Biosynthesis of Two Hydroxy Metabolites of Compound B Using Method 1**

Compound B (10 μM) was incubated with dog liver microsomes (2 mg/ml) in KH2PO4 (0.1M, pH 7.4) containing MgCl2 (3.3 mM) and NADPH (1.3 mM) in a total volume of 1 ml at 37°C. After 45 minutes, the incubation was terminated by addition of CH3CN (5 ml), the precipitate was removed by centrifugation (5 minutes, 1700 g), and the supernatant was evaporated in vacuo. The residue was reconstituted in 0.1 ml 1% formic acid and injected onto HPLC-MS. The system contained a Varian Polaris C18 column (4.6 × 250 mm; 5 μm) and the mobile phase comprised 0.1% formic acid (solvent A) and CH3CN (solvent B) at a flow rate of 0.8 ml/min. The gradient program began with 95% A/5% B for 5 minutes followed by a linear gradient to 20% A/80% B at 50 minutes. The effluent was split between the mass spectrometer and a fraction collector at a ratio of about 1:15. The fractions were collected every 24 seconds in a 96-well microtiter plate. The plate was transferred to a vacuum centrifuge to dry the plate.

After drying, the plate was provided to the in vitro pharmacology laboratory (Pfizer), and each well was tested for inhibition of phosphodiesterase-2 (PDE2) activity. By plotting the PDE2 activity observed in each well against the time of collection for each well, a chromatographic representation of pharmacologic activity was made. This representation has been called an activity-gram, (Obach, 2013). This activity-gram showed that the two hydroxyl metabolites could have PDE2 inhibition activity, albeit the signal was weak. Nevertheless, observation of these signals in the activity-gram triggered a larger-scale biosynthesis of these two metabolites with the intent of more accurately evaluating their pharmacologic activity on the isolated materials.

Compound B (20 μM) was incubated with dog liver microsomes (2 mg/ml) containing 3.3 mM MgCl2, and 1.3 mM NADPH in a total volume of 50 ml KH2PO4 (100 mM, pH 7.4). The incubation was conducted in a 500-ml Erlenmeyer flask in a shaking water bath maintained at 37°C for 1 hour. At the end of the incubation, 50 ml of CH3CN was added, and the mixture was transferred to four 50-ml polypropylene conical tubes and vigorously mixed on a vortex mixer. The tubes were centrifuged at 1700g for 5 minutes, and the supernatant was transferred to four new 50-ml polypropylene conical tubes. The tubes were subjected to vacuum centrifugation in a Genevac Evaporator (set at HPLC mixture setting) for approximately 2 hours to remove the CH3CN. After 45 minutes, the incubation was terminated by addition of CH3CN (5 ml), the precipitate was removed by centrifugation (5 minutes, 1700g), and the supernatant was evaporated in vacuo. The residue was reconstituted in 0.1 ml 1% formic acid and injected onto HPLC-MS. The system contained a Varian Polaris C18 column (4.6 × 250 mm; 5 μm particle size) through a Jasco HPLC pump at a flow rate of 0.8 ml/min. After application of the 100 ml, another ~15 ml of 0.1% formic acid was pumped onto the column to ensure that the HPLC lines were cleared of the supernatant.

The HPLC column was then transferred to an HPLC-MS system as in example A. The effluent was split between the mass spectrometer and a Gilson fraction collector at a ratio of approximately 1:15. The mobile phase used consisted of 0.1% formic acid (mobile phase A) and CH3CN (mobile phase B) at a flow rate of 0.5 ml/min. The mobile phase composition commenced at 90% A/10% B, was held at that composition for 5 minutes, followed by a linear gradient to 5% A/95% B at 25 minutes, held at this composition for 5 minutes, then re-equilibrated at initial conditions for 15 minutes.

The mobile phase program and data collection were started by making a dummy injection of water from the autosampler. Fractions were collected every 20 seconds into a wide-welled polypropylene microtiter plate. Fraction purity was assessed using a Halo C18 column (3.0 × 50 mm; 2.7 μm particle size) on an HPLC-MS system consisting of a Shimadzu CBM-20A controller, LC-20ADXR pumps, CTC PAL Autosampler, and Agilent 1100 PDA in line with an AB Sciex Triple TOF 5600 mass spectrometer operated in the positive ion mode.

The mobile phase used consisted of 0.1% formic acid (mobile phase A) and CH3CN (mobile phase B) at a flow rate of 0.45 ml/min. The mobile phase composition commenced at 95%/5%, was held at that composition for 1 minute, followed by a linear gradient to 0%/A/10% B at 7.9 minutes, held at this composition for 1 minute, then re-equilibrated at initial conditions for 2 minutes. Those fractions containing the product of interest were combined into 15-ml glass conical tubes, and the solvent was evaporated by vacuum centrifugation. Upon dryness, they were prepared for NMR analysis as described later.

**Example C: Biosynthesis of Compound C Hydroxy Metabolite Using Method 1**

The hydroxy metabolite of compound C, which had been detected in dog and human intestinal microsomal incubations, was scaled up for isolation. Two separate incubations of compound C were performed in 50-ml propyloxypropene centrifuge tubes at 37°C for 40 minutes in a shaking water bath. The incubation volume was 10 ml and consisted of 0.1 M KH2PO4 buffer (pH 7.4), MgCl2 (3.3 mM), dog intestinal microsomes (1.0 mg/ml), compound C (100 μM), and NADPH (1.3 mM). Reactions were terminated by the addition of CH3CN (20 ml). The solutions were centrifuged (1800g, 5 minutes), and the supernatants were transferred to clean 50-ml polypropylene centrifuge tubes and concentrated to dryness. The residue in each tube was reconstituted with 600 μl of 10% CH3CN in water. The reconstituted samples were combined in a 2-ml HPLC vial and were placed onto a Shimadzu semi-preparative HPLC-UV system. The sample was injected as four 300 μl injections collecting the postcolumn effluent on a fraction collector. Separation was performed on a Zorbax RX C8, 9.4 × 250 mm, 5 μm semi-preparative HPLC column (Agilent Technologies).

The mobile phase consisted of 0.1% formic acid (solvent A) and CH3CN (solvent B) at a flow rate of 4.0 ml/min. The gradient started at 15% B for 5 minutes, ramped linearly to 90% B over 35 minutes, held isocratically at 90% B for 10 minutes, returned to the initial condition over 1.0 minute, and allowed to equilibrate for 4.0 minutes. The HPLC fractions were collected throughout the run at 1.0-minute intervals. Aliquots (100 μl) of fractions at the retention time of the UV peak corresponding to the hydroxy metabolite were analyzed by liquid chromatography–mass spectrometry. Fractions containing the metabolite of interest were combined into a single 15-ml glass centrifuge tube and concentrated to dryness. The residues were reconstituted in 200-μl DMSO-d6 for NMR analysis.

**Example D: Biosynthesis of Compound D Hydroxy Metabolite Using Method 2**

The hydroxy metabolite of compound D, which had been detected in dog and human intestinal microsomal incubations, was scaled up for isolation. Two separate incubations of compound D were performed in 50-ml polypropylene centrifuge tubes at 37°C for 40 minutes in a shaking water bath. The incubation volume was 10 ml and consisted of 0.1 M KH2PO4 buffer (pH 7.4), MgCl2 (3.3 mM), dog intestinal microsomes (1.0 mg/ml), compound D (100 μM), and NADPH (1.3 mM). Reactions were terminated by the addition of CH3CN (20 ml). The solutions were centrifuged (1800g, 5 minutes), and the supernatants were transferred to clean 50-ml propyloxypropene centrifuge tubes and concentrated to dryness. The residue in each tube was reconstituted with 600 μl of 10% CH3CN in water. The reconstituted samples were combined in a 2-ml HPLC vial and were placed onto a Shimadzu semi-preparative HPLC-UV system. The sample was injected as four 300 μl injections collecting the postcolumn effluent on a fraction collector. Separation was performed on a Zorbax RX C8, 9.4 × 250 mm, 5 μm semi-preparative HPLC column (Agilent Technologies).

The mobile phase consisted of 0.1% formic acid (solvent A) and CH3CN (solvent B) at a flow rate of 4.0 ml/min. The gradient started at 15% B for 5 minutes, ramped linearly to 90% B over 35 minutes, held isocratically at 90% B for 10 minutes, returned to the initial condition over 1.0 minute, and allowed to equilibrate for 4.0 minutes. The HPLC fractions were collected throughout the run at 1.0-minute intervals. Aliquots (100 μl) of fractions at the retention time of the UV peak corresponding to the hydroxy metabolite were analyzed by liquid chromatography–mass spectrometry. Fractions containing the metabolite of interest were combined into a single 15-ml glass centrifuge tube and concentrated to dryness. The residues were reconstituted in 200-μl DMSO-d6 for NMR analysis.
pH 7.4) containing MgCl₂ (3.3 mM) and an NADPH regeneration system (BD Gentest) at 37°C in a shaking water bath. After 100 minutes, CH₃CN was added (30 ml), and the mixture was spun at 1700g for 5 minutes. The supernatant was transferred to 50-ml polypropylene conical tubes and subjected to vacuum centrifugation in a Genevac Evaporator (set at HPLC Mixture setting) for approximately 1 hour to remove the CH₃CN. To the remaining solution was added 0.5 ml of formic acid and water to a total volume of 50 ml. This solution was spun at 40,000g for 30 minutes, and the resulting supernatant was transferred to a 50-ml graduated cylinder and directly applied onto a Varian Polaris C18 column (4.6 × 250 mm; 5 µm particle size) through a Jasco HPLC pump at a flow rate of 0.8 ml/min. After application of the 50 ml, another ~ 10 ml of 0.1% formic acid was pumped onto the column to ensure that the HPLC lines were cleared of the supernatant. This column was transferred to the aforementioned HPLC-MS system (as in example A). The products were eluted using a mobile phase gradient consisting of 0.1% formic acid in water (solvent A) and CH₃CN (solvent B) at 0.8 ml/min.

The mobile phase started at 5% B for 5 minutes, an instant increase to 25% B at 5.1 minutes, a linear gradient to 65% B at 50 minutes, a 10-minute wash at 95% B, and re-equilibration to initial conditions over 10 minutes. Each eluent was passed through a photodiode array UV detector (200–400 nm) and then split (approximately 1:6) between the LTQ mass spectrometer and a fraction collector. Fractions were collected every 20 seconds. Each fraction collected in the retention time region of interest where hydroxyatazanavir metabolites eluted (~22–24 minutes) was tested for purity by HPLC. Those fractions containing products of interest were combined as appropriate into 15-ml conical glass tubes, and the solvent removed in vacuo. This material was analyzed by NMR.

NMR Sample Preparation and Analysis

All samples, including parent compounds, were prepared for NMR analysis as follows. Tubes containing the dried products (isolates are typically not visible to the naked eye) were transferred to a vacuum chamber which was attached to the glove box. Transfer pipettes, pipette tips, and NMR tubes were stored in the glove box to remove adsorbed water from their surfaces to minimize the potential for sample contamination. All transfer pipettes, pipette tips, and NMR tubes were stored in the glove box to remove adsorbed water from their surfaces to minimize the potential for sample contamination.

NMR spectra were recorded on a Bruker Avance 600 MHz instrument controlled by Topspin V3 and equipped with a 5 mm or 1.7 mm TCI Cryo probe. The one-dimensional spectra were recorded using an approximate sweep width of 8400 Hz and a total recycle time of ~7 seconds. The resulting time-averaged free induction decays were transformed using an exponential line broadening of 1.0 Hz to enhance signal to noise. The two-dimensional data were recorded using the standard pulse sequences provided by Bruker. At a minimum a 1K × 128 data matrix was acquired using a minimum of 2 scans and 16 dummy scans, with a spectral width of 10,000 Hz in the f2 dimension. The data were zero-filled to at least 1K data point. ¹H and ¹³C spectra were referenced using residual DMSO-d₆ (¹H δ = 2.50 relative to tetramethylsilane, δ = 0.00. ¹³C δ = 39.50 relative to tetramethylsilane, δ = 0.00).

qNMR was performed in three ways: the artificial signal insertion for calculation of concentration observed (aSICCO) method (Walker et al., 2011), the electronic reference to access in vivo concentrations 2 (ERETIC 2) (Bruker 2012 method), or an internal standard of maleic acid (Malz and Jancke, 2005). For aSICCO and ERETIC 2, an authentic 10 mM standard of maleic acid was used as a calibrant.

Results

Compound A. Compound A is a kynurenine aminotransferase inhibitor with a hydroxamic acid motif (Tuttle et al., 2013). The O-glucuronide conjugate of compound A was successfully prepared from compound A incubated with human liver microsomes and conditions and cofactors to support glucuronidation reactions. In the HPLC trace of the isolation of compound A glucuronide (Fig. 2, A and B), it can be observed that two glucuronide metabolites were formed. Compound A can undergo glucuronidation at the hydroxamic acid oxygen and the amine nitrogen. The N-glucuronide was the minor product and not further characterized. The major glucuronide of interest was the O-glucuronide, eluting around 17.4 minutes. The fractions containing the O-glucuronide appeared pure by HPLC/UV-MS analysis (Fig. 2, C and D). NMR spectral data confirmed the overall purity of the sample and the site of glucuronidation as the hydroxamic acid oxygen. The structure was confirmed with three critical pieces of NMR data: 1) the presence of a ¹H resonance (δ 5.04, doublet, ¹H, J = 7.9 Hz) not observed in the parent; 2) the ¹H-¹³C heteronuclear single-quantum coherence (HMQC) data that linked this new ¹H resonance to a ¹³C resonance at δ 109.6; and 3) a positive nuclear Overhauser effect response in H7 when the new resonance at δ 5.04 is irradiated (Fig. 3, A).

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A–C). These data can only be satisfied by glucuronidation of the hydroxamic acid substituent. The concentration of the sample, determined by qNMR (aSICCO), was 0.053 mM.

This material was used as a standard for bioanalysis of in vivo matrices. The O-glucuronides arising from direct glucuronidation have the potential to undergo in-source fragmentation, and if not chromatographically resolved from their parent drugs, their presence can confound quantitation of the parent drug. This material was used to show that this phenomenon was not operative in this case. The material was also used in a rat pharmacokinetic study to address the potential for enterohepatic recirculation. Rats showed high oral bioavailability and low clearance despite rat in vitro metabolism data demonstrating a high rate of glucuronidation. Analysis of bile from bile duct cannulated rats after intravenous and oral administration of compound A showed that the glucuronide was present at 26% (intravenous) and 39% (oral) of the dose in bile.

**Compound B.** In the example of the phosphodiesterase inhibitor compound B (Helal et al., 2012), an observation had been made that the pharmacologic effect observed in dogs was greater than what would be expected from the known potency of compound B and its concentration in plasma. Thus, the potential existed for an active metabolite(s). Compound B was incubated with dog liver microsomes, and the metabolites observed (Fig. 4A) included two hydroxyl metabolites (m/z 390), one doubly hydroxylated metabolite (m/z 406, retention time approximately 29 minutes), and two metabolites arising from oxidative opening of the azetidine ring (m/z 406, retention time = 18.77 minutes and m/z 392, retention time = 30.40 minutes). The fractions were tested for in vitro pharmacologic activity to yield an activity-gram (Fig. 4B). In addition to the unconsumed parent drug, there were two other peaks of activity that correlated with the two singly hydroxylated metabolites.

Upon observing that the two hydroxyl metabolites appeared to have some activity at the target enzyme, we undertook a larger scale bio-synthesis using dog liver microsomes. The HPLC purification chromatogram (Fig. 5A) shows a complex array of metabolite peaks, of which two major peaks were the two metabolites of interest (shown in the extracted ion chromatogram trace m/z 390 in Fig. 5B). Analytical HPLC analysis of the individual samples indicated the isolates were pure (Fig. 5, C and D). The fractions containing the two metabolites of interest were assessed by NMR, and the sites of oxidation and the concentrations of metabolites B1 and B2 were determined (Fig. 6). In the 1H spectrum of compound B, the resonance at δ 2.19 is assigned as the methylene C25 (Fig. 6A) (for a complete numbering scheme of all compounds, see Supplemental Fig. 4). The 1H of C24 and C26 were only observed as shallow broad peaks at δ 3.75. The absence of the 1Hs of C24 and C26 is most likely due to ring inversions of the azetidine. This is important in the characterization of metabolite B1. The 1H resonance of the methylene C25 observed in compound B was not observed in the
Compound C. The hydroxyl metabolite of compound C was successfully prepared from incubations of compound C with human liver microsomes and conditions and cofactors to support cytochrome P450 oxidation. In the HPLC trace of the isolation of metabolite C1, it can be observed that one major metabolite was formed, retention time 24.32 minutes (Figs. 7A and B). The collision-induced dissociation spectra of compound C and metabolite C1 indicate the presence of a single hydroxylation on one of the tert-butyl groups. The fractions containing the hydroxyl metabolite were pure, and a stock concentration in DMSO-d6 was 8.2 mM as determined by qNMR (ERETIC 2). The definitive NMR data that enabled the structural assignment of metabolite C1 were the 1H and 1H-13C heteronuclear multiple-bond correlation (HMBC) data sets (Fig. 8). The one-dimensional 1H data of the isolated metabolite C1 contain two singlet peaks at δ 1.30 and δ 1.39 integrating to six and nine hydrogens, respectively. Additionally there is a new singlet resonance not observed in the parent compound at δ 3.65 integrating as two hydrogens. These data are consistent with the oxidation of one of the two tert-butyl groups found on compound C. However, this cannot delineate which tert-butyl moiety was oxidized. To identify the site of oxidation, HMBC data must be used (Fig. 8). In the HMBC data set of metabolite C1 the tert-butyl resonance (δ 1.39) correlates to a quaternary carbon atom with a chemical shift of δ 132.4 (Fig. 8B). The resonance at δ 11.81 (assigned as the amide NH) also has a correlation to the same carbon at δ 132.4 (Fig. 8A). The only way to resolve these data is to assign the oxidation to the tert-butyl para to the amide of compound C. This material was used as a substrate for blood-to-plasma ratio determination (compound C = 0.780, metabolite C1 = 0.615), human plasma protein binding (fraction unbound, compound C = 0.00013, metabolite C1 = 0.0028), and in vitro efficacy determinations (EC50 compound C = 7 nM and metabolite C1 = 19 nM) to aid understanding the contribution of metabolite C1 to preclinical in vivo efficacy models.

Compound D. Compound D is a microsomal triglyceride transfer protein inhibitor that contains a metabolically labile ester linkage (Ryder et al., 2012). The primary metabolite of compound D was prepared from an incubation of compound D with dog intestinal microsomes. The HPLC analysis of the final incubate revealed a single dominant metabolite, metabolite D1, eluting before compound D (Fig. 9A). Semipreparative HPLC isolation of this metabolite yielded a chromatographically pure isolate containing a single peak (Fig. 9B). HPLC-MS data indicated metabolite D1 to have a mass of m/z 691, 16 Da greater than compound D, indicating a single-site oxidation. The tandem mass spectrometry data demonstrated that the oxidation was on the isobenzofuranone portion of the molecule. The NMR analysis indicated the isolated metabolite was mixture of two diastereomers resulting from the oxidation of C2 in the isobenzofuranone.

The critical NMR data that determined the site of oxidation are the 1H-13C HSQC data sets for compound D and metabolite D1 (Fig. 10). In the 1H-13C HSQC data of compound D, the cross peak at 1H 5.08/13C 73.3 is assigned as C2 of the benzofuran (Fig. 10A) (for a complete numbering scheme of all compounds, see Supplemental Fig. 4). In a similarly acquired data set of the isolated metabolite, these resonances are absent, and two new 1H resonances appear at δ 6.37 and 6.48. In the multiplicity edited 1H-13C HSQC, these resonances are correlated to a 13C chemical shift of δ 101.4 and indicate both arise from methines (Fig. 10B). This is consistent with the isole being a mixture of diastereomeric alcohols resulting from oxidation of the C2 carbon of compound D. Using an internal standard of 10 mM maleic acid, the concentration of the sum of both diastereomers together was determined to be 0.18 mM. The isolated metabolite was also used as substrate to determine its gut microsomal triglyceride transfer protein (gMTP) inhibitory IC50 value and was shown to be 30-fold less active than compound D and is therefore no concern.
Atazanavir. Two hydroxyatazanavir metabolites were prepared from an incubation of atazanavir and human CYP3A5. In the HPLC trace of the incubation extract (Fig. 11A), there are two very closely eluting metabolites of $m/z$ 720 (Fig. 11B). The earlier eluting metabolite was present in a fraction that contained a contamination of the later eluting metabolite (Fig. 11C) whereas the later eluting metabolite had only a trace of the earlier metabolite (Fig. 11D).

The $^1$H spectrum of atazanavir contains $^1$H resonances at $\delta$ 7.42 and $\delta$ 7.97 that are both doublets with coupling constants of 8.2 Hz each integrating to two $^1$H (Fig. 12A). The correlation spectroscopy data set indicates these resonances are strongly coupled to each other and to no other protons. These resonances are easily assignable to the four resonances of the single para substituted ring in atazanavir: H8, H9, H11, and H12 (for a complete numbering scheme of all compounds, see Supplemental Fig. 4). In the $^1$H spectrum of metabolite E1, the resonances of H8, H9, H11, and H12 are also present and unmodified from those observed in atazanavir. The resonances for H35, H36, H37, H38, and H39 are either absent or significantly shifted. Additionally, there is a new set of resonances that is consistent with a para substituted phenyl ring at $\delta$ 6.58 and $\delta$ 6.95, which are both doublets with coupling constants of 7.9 Hz and each integrate to two protons (Fig. 12B). The correlation spectroscopy data of the isolate of metabolite E1 also indicates these resonances are strongly coupled to each other and no to other protons. The resonances are most easily explained by the oxidation of C37 to form a para substituted phenol. Using qNMR (ERETIC 2), the concentration of the E1 metabolite was determined to be 0.05 mM and contained approximately 30% contamination of the E2 metabolite.

In the $^1$H spectrum of atazanavir, the resonances $\delta$ 0.63 and $\delta$ 0.77 (each singlet integrating to 9 Hs) are assigned as the tert-butyl groups (Fig. 12C). Because of the size and near symmetry of atazanavir, these two groups cannot be readily distinguished by NMR. In the $^1$H spectrum of the metabolite E2, the resonance at $\delta$ 0.63 has changed neither in chemical shift nor in integration. However, the $\delta$ 0.77 resonance has shifted slightly to $\delta$ 0.70 and now appears as a two singlets of equal intensity, with an integration equaling six protons. Additionally observed in the $^1$H spectrum of the isolate are two new resonances at $\delta$ 3.03 and $\delta$ 3.14 (Fig. 12D).

The multiplicity edited $^1$H-$^13$C HSQC data set indicates these resonances are inequivalent methylenes with a correlation to a carbon resonance at $\delta$ 67.5, whereas the $\delta$ 0.63 and $\delta$ 0.77 are methyls or methines correlating to carbon resonances at $\delta$ 26.2 and $\delta$ 21.3, respectively. Based on the $^1$H and $^13$C chemical shifts, the resonances...
at δ 0.70 are assigned as a pair of methyl groups with two separate chemical shifts.

All the NMR data from the E2 metabolite strongly suggest an oxidation of one of the tert-butyl groups. To define which tert-butyl has been oxidized, tandem mass spectrometry data were necessary. Atazanavir and the isolated metabolite E2 were analyzed on an Orbitrap mass spectrometer to develop a fragmentation pattern (Supplemental Figs. 1–3). Among the array of mass spectral fragments, metabolite E2 yielded a very minor ion at \( m/z \) 351.1915, indicating that the hydroxylation was on the portion indicated by carbons 18–51 (see Supplemental Fig. 4) and this fragmented further to \( m/z \) 164.1065 (unmodified 1-hydroxy-2-amino-4-phenylbutane). These were the only fragment ions that yielded the information needed to show which of the two tert-butyl groups was hydroxylated in metabolite E2. Using qNMR, the concentration of the E2 metabolite was determined to be 0.60 mM (ERETIC 2).

These two materials were used in the construction of standard curves for an HPLC-MS assay that was used to measure the enzyme kinetics of atazanavir metabolism in human liver microsomes, CYP3A4, and CYP3A5 (Tseng et al., 2014). This example demonstrates that in some cases metabolites do not even have to be pure for them to be of use.

**Discussion**

In this report, we have described methods useful for the generation of standards of metabolites via biosynthesis and qNMR. Metabolite standards generated in this fashion can be used for determination of in
vitro pharmacologic activity, for the creation of standard curves needed for HPLC-MS bioanalysis, and for in vitro drug metabolism studies. Both oxidized and conjugated metabolites have been generated and used in these types of experiments.

In general, the focus is on the generation of metabolites of relevance to humans and ideally a human in vitro system would ensure the production of the metabolite of interest, including the relevant stereochemistry. However, in selecting a biologic system for metabolite generation, it is best to select the system that will offer the greatest conversion to the metabolite of interest. In many instances, the best system to select may be liver microsomes from laboratory animal species and not humans. Also, employment of heterologously expressed human enzymes for the biosynthesis of a metabolite can offer advantages. Others have demonstrated the usefulness of microbes or mutated cytochrome P450 enzymes (Li et al., 2009). When using an alternative system, it is critical to ascertain whether the metabolite generated matches the desired product (in regio- and stereochemistry). In each example described in this report, a preliminary investigation of

Fig. 9. Preparation of metabolite D. (A) HPLC PDA trace (200–400 nm) of the compound D biosynthesis mixture applied to a Zorbax RX C8 (9.4 × 250 mm) HPLC column. (B) Extracted ion chromatogram (XIC) of m/z 690 indicating the elution time of the product of interest (~31 minutes) under analytical HPLC conditions.

Fig. 10. NMR of compound D and its metabolite D1. (A) $^1$H-$^{13}$C HSQC of compound D. (B) $^1$H-$^{13}$C HSQC of compound D1. (For a complete numbering scheme of all compounds, see Supplemental Fig. 4.)
various in vitro systems was performed to identify the system that would yield the metabolite(s) of interest in the best yield and with anticipated ease of isolation.

In our experiences generating metabolites in this manner, we have observed some subtle but critical technical aspects. 1) When loading an HPLC column by pumping a large volume of dilute aqueous extract through an HPLC pump, it is critical that the solution be clear and devoid of particles. Thus, spinning the solution at 40,000 g is an essential step and monitoring the column back pressure is recommended. Organic modifier concentration must be low enough to permit “packing” of the metabolite on the head of the column. 2) Upon obtaining an HPLC eluent fraction that possesses the metabolite of interest, we have found that removing the solvent in a vacuum centrifuge is superior to evaporation under inert gas. The material concentrates into the bottom of the tube for easier recovery into minimal (e.g., 0.05 ml) NMR solvent. We use conical glass tubes for this step. Drying under a flow of inert gas tends to spread the material over a larger surface area of the tube, which can lead to higher losses during reconstitution and introduction of possible contaminants from the drying gas and lines. 3) The volume of HPLC solvent in which the isolate is collected should be minimized. HPLC solvents are not 100% pure, thus the smaller the volume used in the collection, the lower the concentration of the nondrug-related impurities in the final isolate. 4) In almost all cases, the two largest impurities in the 1H NMR spectrum of an isolated metabolite are the residual nondeuterated solvent peak and water. The former can only be minimized through using the highest purity solvents available. Deuterated solvent manufacturers offer several levels of deuteration; obviously, the more isotopically pure the solvent, the greater the cost. However, when considering the total cost of the sample preparation (medicinal chemist time to synthesize the parent, the cost of hepatocytes/microsomes, the cost of isolation time, etc.), the relative cost of the highest quality deuterated solvent is minimal. The minimization of water as a contaminant in NMR samples is also controllable. After removing solvent from the final isolate, the sample is transferred and prepared in a dry box so that the residual water in the sample will be minimal.

Once the highest quality sample is prepared, the acquisition of the NMR data becomes the critical step in the process. NMR analysis is inherently less sensitive than MS analysis. However, since the introduction of reduced-temperature probes (Bruker cryo probes, Varian cold probes) in the late 1990s, the sample mass requirements for NMR have been consistently dropping. The reduction of temperature in the acquisition coils of an NMR probe can increase the signal to noise as much as a factor of 4 in a single pulse experiment over comparable room temperature probes. In multipulse experiments, this results in a practical increase in signal to noise of approximately a factor of 10. Concomitant with the development of cryoprobes was an effort to reduce the probe diameter and hence decrease the overall volume requirements for a sample. In terms directly relatable to a drug metabolism

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Fig. 11. Purification of atazanavir metabolites. (A) HPLC PDA trace (200–400 nm) of the atazanavir biosynthesis mixture applied to a Polaris C18 HPLC column. (B) Concurrent extracted ion chromatogram (XIC) of m/z 720 indicating the elution time of the products of interest (~23 minutes). (C and D) HPLC-PDA (200–400 nm) traces of the fractions containing metabolites E1 and E2.
scientist, if 20 nmol of a metabolite is isolated and reconstituted in a volume for a 5 mm probe (0.6 ml), the resulting sample concentration is 0.03 mM. This same quantity reconstituted for a 1.7 mm probe (0.040 ml) will result in a final concentration of 0.5 mM. It has been our experience that isolated samples with concentrations of 1.0 mM are usually sufficient for structural characterization and quantitation. Obviously this value is dependent on the complexity of the molecule. Sometimes other more sample-intensive experiments, direct observed carbon, or 1H-15N HMBC, for example, may be required. Conversely, much less sample may be required if the molecular modification is on a spectroscopically isolated portion of a molecule.

As an alternative to using 1H as the nuclei for quantitation, 19F could also be used. There are two distinct advantages to 19F qNMR over 1H qNMR. First, the inherent background of contaminating resonances from the isolation process is negligible with 19F NMR; second, the spectral width of 19F NMR is much larger that of 1H (250 ppm for 19F versus 12 ppm for 1H), allowing much greater selectivity. However, there is the obvious disadvantage that the molecule of interest must contain a 19F atom.

The examples we have described illustrate some uses of the biosynthesized metabolite solutions of known concentration in drug research. Metabolite stock solutions generated using these methods can frequently range between 0.05 and 5 mM. In most cases, these concentrations are high enough to permit use of these solutions as parent stock solutions that can be diluted to make standard curves for HPLC-MS bioanalytical methods. The glucuronide of compound A was used to quantitate rat bile samples by HPLC-MS, to determine the fraction of compound A excreted as its glucuronide. Stock solutions of metabolites B1 and B2 were used to test target potency and to make standard curves for the determination of metabolite concentrations in dog plasma samples by HPLC-MS. These plasma exposure values were important for understanding the concentration-effect relationship. The isolated metabolite C1 was used to establish absorption, distribution, metabolism, and excretion parameters (blood-to-plasma ratio and human plasma protein binding) as well as potency, all of which helped to provide a better understanding of the efficacy of compound C. The stock solution of compound D was used in potency assays to help establish a structure-activity relationship around this chemical series. The hydroxyatazanavir metabolites were used as standards for making enzyme kinetic measurements for CYP3A4 and CYP3A5 catalyzed metabolism and aided in understanding the relative importance of these two enzymes in atazanavir metabolism in humans (Tseng et al., 2014).

Other uses of the solutions of biosynthesized metabolites included the measurement of target receptor affinity. In the example of compound B, the activity-gram approach was used first to show that metabolites B1 and B2 could potentially have pharmacologic activity while the other metabolites would not. This triggered the biosynthesis and isolation of metabolites B1 and B2 and preparation of stock solutions of known concentration by qNMR, which were diluted into assay buffer for determination of intrinsic potency. Ultimately, if a metabolite is deemed to be important for the activity of a new drug (either efficacy and/or safety), a large quantity (i.e., grams) will need to be prepared for in-depth study. The methods described in this work can be used up front to determine whether such an investment in synthesis is warranted. Or if synthesis of the metabolite is
intractable, then biosynthesis and use of stock solutions of concentration established with qNMR may be the only avenue to have material for other investigations.

In conclusion, we have described in detail techniques to generate drug metabolites in stock solutions of known concentration. For this procedure, qNMR is critical, and the close collaboration between the drug metabolism scientist and NMR spectroscopist is essential. This is even more effective if the NMR instrumentation is established in the drug metabolism laboratory because the needs for this technique differ from the use of NMR in medicinal and process chemistry. These techniques can be essential for gathering important information on the activity of metabolites, the quantitative exposure to metabolites, and the absorption, distribution, metabolism, and excretion properties of metabolites.

Authorship Contributions

**Participated in research design:** Walker, Ryder, Bauman, Obach, Smith.

**Conducted experiments:** Walker, Ryder, Bauman, Obach.

**Performed data analysis:** Walker, Ryder, Bauman, Obach.

**Wrote or contributed to the writing of the manuscript:** Walker, Ryder, Bauman, Obach, Smith, Spracklin.

References


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Drug Metabolism and Disposition

BIOSYNTHESIS OF DRUG METABOLITES AND QUANTITATION USING NMR SPECTROSCOPY FOR USE IN PHARMACOLOGICAL AND DRUG METABOLISM STUDIES
Gregory S. Walker, Jonathan N. Bauman, Tim F. Ryder, Evan B. Smith, Douglas K. Spracklin, and R. Scott Obach

Supplemental Data

Atazanavir M$^n$ Data

Supplemental Figure 1
High Resolution Mass Spectrum of Atazanavir

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Supplemental Figure 1 (continued)

Note: m/z 194.0 and 273.8 are instrument interferences
Supplemental Figure 2
High Resolution Mass Spectrum of t-Butyl Hydroxyatazanavir

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Note: m/z 194.0 and 273.8 are instrument interferences
Note: m/z 194.0 and 273.8 are instrument interferences
Supplemental Figure 3

Schematic representation of the transformation of atazanavir and t-butyl hydroxyatazanavir into their respective metabolites. Atazanavir (m/z 705.3955) forms m/z 335.1967, and t-butyl hydroxyatazanavir (m/z 721.3905) forms m/z 351.1915 and m/z 164.1065. The diagram illustrates the chemical processes involved in these transformations.
Figure 4A

**Compound A Glucuronide**

**Compound A**

**Metabolite B1**

**Metabolite B2**

**Compound C**

**Metabolite C1**
Figure 4B

Compound D  Metabolite D1
Figure 4C