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The use of ^{18}O-exchange and base-catalyzed *N*-dealkylation with liquid chromatography/tandem mass spectrometry to identify carbinolamide metabolites

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Oxidation of *N*-alkyl-substituted amides is a common transformation observed in metabolism studies of drugs and other chemicals. Metabolism at the alpha carbon atom can produce stable carbinolamide compounds, which may be abundant enough to require complete confidence in structural assignments. In a drug discovery setting, rapid structural elucidation of test compounds is critical to inform the compound selection process. Traditional approaches to the analysis of carbinolamides have relied upon the time-consuming synthesis of authentic standards or purification of large enough quantities for characterization by nuclear magnetic resonance (NMR). We describe a simple technique used in conjunction with liquid chromatography/tandem mass spectrometry (LC/MS/MS) which demonstrates the chemical identity of a carbinolamide by its distinctive ability to reversibly exchange [^{18}O]water through an imine intermediate. A key advantage of the technique is that the chromatographic retention times of metabolites are preserved, allowing direct comparisons of mass chromatograms from non-treated and [^{18}O]water-treated samples. Metabolites susceptible to the treatment are clearly indicated by the addition of 2 mass units to their original mass. An additional test which can be used in conjunction with ^{18}O-exchange is base-catalyzed *N*-dealkylation of *N*-(α-hydroxy)alkyl compounds. The use of the technique is described for carbinolamide metabolites of dirlotapide, loperamide, and a proprietary compound. Copyright © 2010 John Wiley & Sons, Ltd.

The *N*-dealkylation of amines is a commonly encountered metabolic transformation of drugs and xenobiotics.[1] The reaction is effected by multiple forms of cytochrome P450 enzymes[2] and involves the hydroxylation of the alkyl carbon atom alpha to the nitrogen atom to generate an unstable carbinolamine intermediate. Dissociation of this intermediate yields the *N*-dealkylated amine and an aldehyde or ketone from the alkyl portion of the molecule.[3,4] Alkyl substituents on amide nitrogens can also undergo CYP-catalyzed hydroxylations adjacent to the amide nitrogen atom. For example, the enzyme peptidylglycine α-amidating mono-oxygenase catalyzes the formation of an α-hydroxyglycine intermediate as a first step in peptide amidation.[5] In contrast to carbinolamines, the carbinolamides thus formed are often stable and can be isolated.[6–15] The stability of such structures has been attributed to intramolecular hydrogen bonding between the carbonyl oxygen and the hydroxyl hydrogen atoms, possibly with the participation of a solvent water molecule.[16] Due to the isobaric relationship between carbinolamides and metabolites hydroxylated at other positions, the use of mass spectrometry to distinguish between the two species requires diagnostic ions to be present in the tandem mass (MS/MS) spectrum. Alternatively, structural characterization of a carbinolamide metabolite relies on matching the chromatographic retention time and mass spectrum to an authentic synthetic standard, or on nuclear magnetic resonance (NMR) analysis of the purified metabolite. Either of these approaches is resource- and time-intensive.

In the course of our studies on the metabolic profiles of dirlotapide (CP-742,033; Slentrol) in dogs[17] and rats, we identified a major metabolite that was determined by liquid chromatography/tandem mass spectrometry (LC/MS/MS) to be a debenzylated product with additional oxidation on either the amide nitrogen atom or *N*-methyl carbon atom. Due to the extreme lability of other bonds in the molecule, the characteristic loss of formaldehyde, which would be expected from an *N*-hydroxymethyl metabolite, could not be observed in the mass spectrum. Consequently, the structure could not be determined unambiguously from its mass spectral fragmentation pattern. The metabolite was then purified from dog feces and its structure was established by proton NMR to be the stable carbinolamide metabolite.

As a consequence of the intense effort required to determine the structure of the dirlotapide metabolite by traditional approaches, we have used this metabolite to

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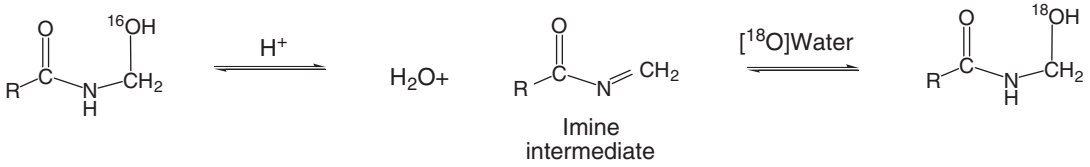

Scheme 1.

develop a method using two simple chemical reactions in conjunction with LC/MS/MS that enables an unambiguous distinction between a stable carbinolamide and other possible hydroxylated species. As shown in Scheme 1 we took advantage of the chemistry of the proposed metabolite, reasoning that an *N*-α-hydroxyalkyl structure should reversibly dehydrate under acidic conditions, thus allowing the incorporation of an atom of ^{18}O when the reaction is conducted in ^{18}O-enriched water. This species could then be observed as a peak in the mass spectral chromatogram at the same retention time as the original metabolite, but now with a mass addition of 2 u. We further reasoned that, under basic conditions, deprotonation of the hydroxymethyl group should promote the irreversible loss of the alkyl group to produce the *N*-dealkylated metabolite.

The method has been used to identify a proposed carbinolamide metabolite of loperamide, and to characterize metabolites from two proprietary compounds in discovery programs at Pfizer. We suggest the use of this technique as a rapid, unequivocal means to identify hydroxylations on alkyl groups alpha to the nitrogen atom of amides, which avoids the time-consuming purification of a metabolite as would be required for structural elucidation by NMR. An advantage of using [^{18}O]water for the derivatization procedure is that complex samples, such as crude plasma, urine, or fecal extracts, can be analyzed by comparison with control samples without the complication of shifted HPLC retention times.

EXPERIMENTAL

Chemicals

[^{14}C]Dirlotapide was prepared by Perkin Elmer Life Sciences (Boston, MA, USA). The material was diluted with non-radiolabeled dirlotapide to an appropriate specific activity (see below) by the Pfizer Radiochemical Synthesis Group (Groton, CT, USA). Unlabeled dirlotapide, *N*-desbenzyl-*N*-desmethyl dirlotapide (M4), and compound A were synthesized by Pfizer (Groton, CT, USA). Loperamide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, sodium hydroxide, and concentrated sulfuric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Water enriched in ^{18}O (97 atom %) was purchased from Isotech (Sigma-Aldrich). All other solvents and reagents were of the highest grade commercially available, and were used without further purification.

Animal studies

Feces, bile, urine, and blood for preparation of plasma were collected from male and female Sprague-Dawley rats administered approximately 50 μCi of [^{14}C]dirlotapide (specific activity 6.74 mCi/mmol, radiochemical purity >99% by HPLC).

Similar samples were collected from male and female beagle dogs given approximately 17 μCi/kg of [^{14}C]dirlotapide (specific activity 5.65 mCi/mmol, radiochemical purity >96% by HPLC).

In vitro metabolism studies

Loperamide and compounds C and D were incubated with rat and monkey liver microsomes at substrate concentrations of 20 μM and microsomal protein concentrations of 1 mg/mL. Compound A was incubated with human liver microsomes and human cryopreserved hepatocytes at substrate concentrations of 10 μM. Hepatocyte incubations with compound A were conducted in Williams' E media containing 750 000 viable cells/mL and were maintained under an atmosphere of 5:95 CO_2/O_2. Compound B was incubated with rat and human liver microsomes at 10 μM substrate concentrations and microsomal protein concentrations of 2 mg/mL.

Microsomal systems contained 10 mM $MgCl_2$ and 100 mM potassium phosphate buffer at pH 7.4 in final volumes of 2 to 4 mL, and were initiated by the addition of NADPH (1.3 mM final concentration). All reaction mixtures were maintained at 37°C and were gently shaken for the duration of the incubation period. At the end of the reaction timecourse incubations were terminated by the addition of four volumes of cold acetonitrile with thorough mixing. Following centrifugation for 5 min at 1100 *g*, supernatants were evaporated in a vacuum centrifuge, and residues were reconstituted in HPLC mobile phase for analysis.

HPLC, mass spectrometry, and radioactivity monitoring

For [^{14}C]dirlotapide analyses, feces or plasma samples were extracted with 5 mL of acetonitrile by mixing thoroughly for 10 min, followed by centrifugation for 5 min at 1100 *g*. This procedure was conducted three times, then for each sample the supernatants were combined and the solvent was evaporated under vacuum in a Genevac vacuum centrifuge. Samples were analyzed by LC/MS/MS with offline radioactivity profiling using an LC-ARC stopped-flow system (AIM Research, Hockessin, DE, USA). The HPLC column effluent was mixed with Stop-Flow AQ scintillation counting fluid, and radiolabeled sample components were measured in 12 s fractions with a counting time of 30 s. For both LC/MS/MS and LC-ARC analyses, the HPLC systems consisted of an Agilent 1100 pump, membrane degasser, and autosampler. Chromatography was performed using a 150 mm × 4.6 mm Zorbax Stable-bond CN column packed with 5 micron particles. The mobile phase consisted of 5 mM ammonium acetate, pH 6.8 (solvent A), and acetonitrile (solvent B) at a flow rate of 0.7 mL/min. The mobile phase composition was 20% B from 0–3 min, then increased to 40% B by 43 min, then to 90% B by 68 min, held isocratically at 90% B until 73 min, then decreased to 20% B by 74 min, after

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which the column was re-equilibrated at 20% B until 79 min. Dirlotapide eluted at approximately 58 min under these conditions, and all metabolites eluted earlier.

For *in vitro* metabolite studies of loperamide and compounds C and D, acetonitrile-quenched incubation mixtures were centrifuged for 5 min at 1100 *g*, then supernatants were evaporated to dryness under vacuum. Residues were reconstituted in 20:80 acetonitrile/water prior to analysis by LC/MS/MS. The HPLC system was as described above, but the HPLC column was an Xterra RP18 3.0 × 150 mm column (Waters, Milford, MA, USA) packed with 5 micron particles. The mobile phase consisted of ammonium acetate, pH 6.8 (solvent A), and acetonitrile (solvent B). The column was held at 20% B for 3 min, then ramped to 70% B over the next 25 min, washed at 90% B until 34 min, then was changed back to starting conditions at 35 min and re-equilibrated for an additional 5 min. Loperamide eluted at 24.7 min, and all metabolites eluted earlier.

Metabolites of [^{14}C]dirlotapide, loperamide, and compounds C and D were identified using a TSQ Quantum triple-quadrupole mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) operated in positive-ion mode. The electrospray ionization (ESI) source was operated at 4200 V with a capillary temperature setting of 275°C. Q1 scans, precursor-ion scans and product-ion scans were used for structural elucidation. For dirlotapide and its metabolites, precursor-ion scans for precursors of *m/z* 249 and 421 fragment ions at collision energies (CE) of 60 and 40 V, respectively, were used for metabolite monitoring. Product-ion scans were collected at collision energies ranging from 20–60 V, and utilized a collision gas pressure ranging from 0.7–1.5 mTorr of argon.

Accurate mass measurements of dirlotapide metabolites were obtained using a Q-TOF-2 quadrupole-time-of-flight mass spectrometer (Waters, Milford, MA, USA) equipped with an ESI source operated in positive-ion mode. An internal lock mass (Leu enkephalin, +*m/z* 556.2766) was used throughout the analysis via a lockspray, allowing the calibrant to be introduced into the mass spectrometer every 5 s. For loperamide metabolites, elemental formulae were generated by analysis on an LTQ-Orbitrap XL (ThermoFisher Scientific, San Jose, CA, USA) at a resolution setting of 30 000 (specified at *m/z* 400).

Metabolite determinations from incubations with compounds A and B were performed with an LTQ Orbitrap XL mass spectrometer using an ESI source in positive-ion mode. The HPLC system consisted of an Accela pump, autosampler, and diode-array detector. Full scan mass spectra were acquired at a resolution setting of 15 000 (specified at *m/z* 400) with data-dependent MS^n at 30% (compound A) or 65% (compound B) normalized collision energy for metabolite fragmentation studies. The mobile phase consisted of 5 mM ammonium formate adjusted to pH 3 with formic acid (solvent A) and acetonitrile (solvent B) flowing at 1 mL/min. For compound A, separation was achieved with a 4.6 × 150 mm Kromasil C18 column (Varian, Torrance, CA, USA) packed with 5 micron particles. The gradient profile was 5% B from 0–3 min, 50% B by 28 min and held isocratically for 1 min, then back to starting conditions by 30 min, followed by 5 min of column re-equilibration before the next injection. For compound B, a Synergi Hydro RP (Phenomenex, Torrance, CA, USA) 4.6 × 150 mm, 4 micron column was used for separation. The gradient program was 5% B from 0–3 min, 60% B by 25 min, 80% B by 35 min, 95% B by 38 min, then held at starting conditions for a further 10 min.

Nuclear magnetic resonance

For NMR studies, dirlotapide metabolite M2 was extracted with acetonitrile from approximately 200 g of dog feces and purified using semi-preparative conditions on a model 215 fraction collector (Gilson, Middletown, WI, USA). A Monochrom CN 10 × 150 mm column (MetaChem, Torrance, CA, USA) was used with the gradient described above at a flow rate of 2.5 mL/min. An approximately 4 min window of column eluent was isolated which contained several metabolites, including M2. After several injections of the crude fecal extract, the HPLC fractions were combined and the solvent was evaporated. The residue was reconstituted in 20:80 acetonitrile/H_2O, and a further purification step was performed on an analytical scale using an Ace 4.6 × 150 mm phenyl column (Advanced Chromatography Technologies, Aberdeen, UK) packed with 3 micron particles. The solvent system for this separation consisted of water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.6 mL/min. The mobile phase gradient began at 40% B and changed to 60% B by 40 min, then ramped to 90% B at 41 min and was held there until 51 min, then changed back to starting conditions at 52 min, with a 5 min re-equilibration period before the next injection. This purification step allowed for good separation of metabolite M2 from other metabolites in the fraction. Manual collection of the HPLC fractions containing M2 was performed, and these were combined and evaporated as before. Several cycles of reconstitution in acetonitrile-d_3 followed by evaporation under vacuum were performed to remove residual protonated solvents. Finally, samples were dissolved in 0.15 mL of acetonitrile-d_3 '100%' (Cambridge Isotope Laboratories, Andover, MA, USA). Based on liquid scintillation analysis of the purified metabolite, approximately 40 μg of M2 were isolated.

All NMR spectra were recorded on a Bruker Avance 600 MHz (Bruker BioSpin Corporation, Billerica, MA, USA) controlled by XWIN-NMR V3.5 and equipped with a 2.5 mm BBI probe. 1D spectra were recorded using a sweep width of 12 000 Hz and a total recycle time of 3.6 s. The resulting time-averaged free induction decays were transformed using an exponential line broadening of 0.3 Hz to enhance signal to noise. All spectra were referenced using residual acetonitrile-d_3 ($\delta = 1.94$ ppm relative to TMS, $\delta = 0.00$). The multiplicity edited HSQC data were recorded using the standard pulse sequence provided by Bruker. A 1K × 128 data matrix was acquired using a minimum of 128 scans and 16 dummy scans with a spectral width of 8000 Hz in the f2 dimension. The data was zero-filled to a size of 1K × 1K. A relaxation delay of 1.5 s was used between transients.

Substitution with ^{18}O-enriched water

Carbinolamide metabolites, either as purified HPLC fractions or as crude sample extracts, were dried under vacuum or a stream of N_2. Residues were then redissolved in 15 μL of

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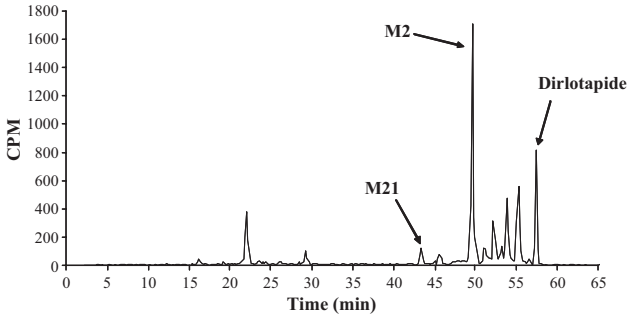

Figure 1. Representative HPLC radiochromatogram of an extracted rat fecal sample following a single oral dose of [^{14}C]dirlotapide.

acetonitrile and 30 μL of ^{18}O-enriched water was added. Concentrated H_2SO_4 (5 μL) was added to catalyze water exchange, and after mixing well the tubes were capped and left at room temperature for 1 h. The ^{18}O reaction mixture was centrifuged and then analyzed by LC/MS/MS without any pH modification.

N-Dealkylation by base treatment

Aqueous mixtures (100 μL) containing carbinolamide metabolites were alkalinized with the addition of 10 μL of 10 N NaOH and the solutions were allowed to stand at room temperature for 1 h. The sample was neutralized by the addition of 10 μL of 10 N H_2SO_4 and centrifuged before analysis by LC/MS/MS.

RESULTS AND DISCUSSION

Dirlotapide and numerous metabolites were identified in feces, bile, and plasma of Sprague-Dawley rats and beagle dogs after oral administration of [^{14}C]dirlotapide (Fig. 1). The proposed carbinolamide metabolite M2 was observed in significant quantities in all rat and dog matrices examined, and was the most abundant metabolite present in rat fecal samples. M21, a ring-hydroxylated analogue of M2, was also observed in rat and dog feces. Metabolites M4 and M24, the products of amide *N*-dealkylation from M2 and M21, respectively, were observed in plasma and/or feces as well. A scheme depicting the relevant metabolism of dirlotapide is shown in Fig. 2.

[^{14}C]Dirlotapide had an HPLC retention time of approximately 57 min. Full scan MS spectra consisted of an $[M+H]^+$ ion at *m/z* 675, with some unavoidable source fragmentation which produced a less intense ion at *m/z* 554. The product-ion mass spectrum of *m/z* 675 at 20 V CE contained ions at *m/z* 91, 122, 421, 483, 526, 554, and 585 (Fig. 3(A)). Loss of the benzyl group produced the *m/z* 91 and 585 ions, while loss of *N*-methyl-*N*-benzylamine produced the acylium ion at *m/z* 554,

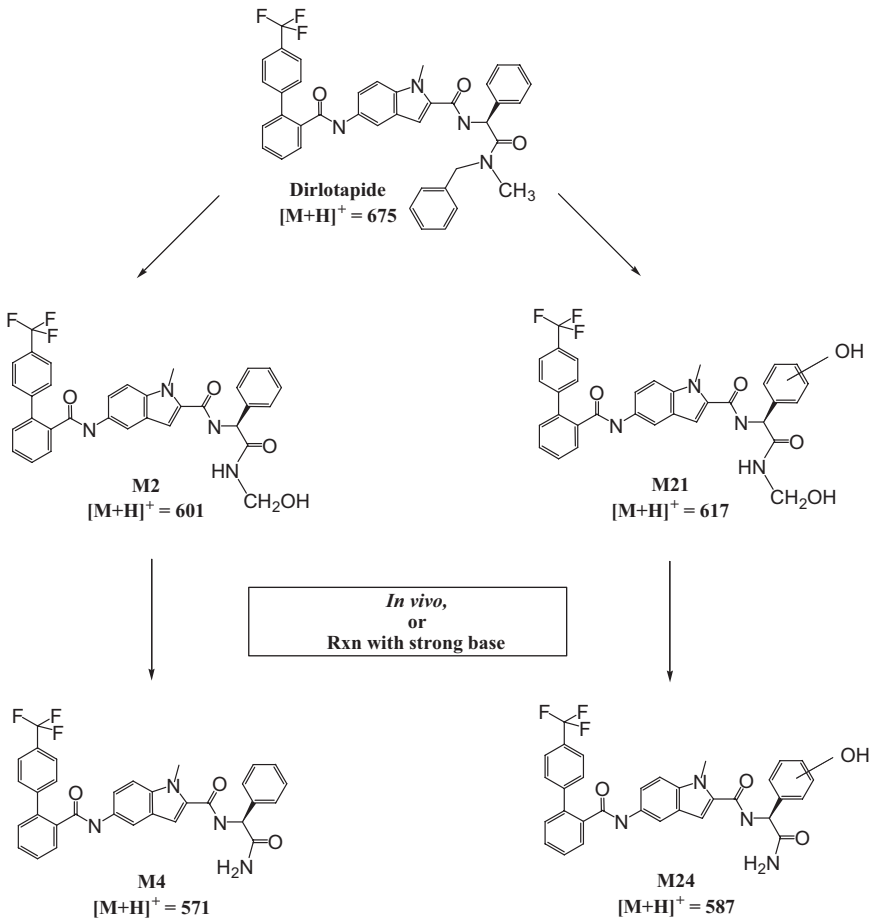

Figure 2. Schematic diagram of the relevant dirlotapide metabolic pathway. Some intermediate metabolites have been omitted for clarity.

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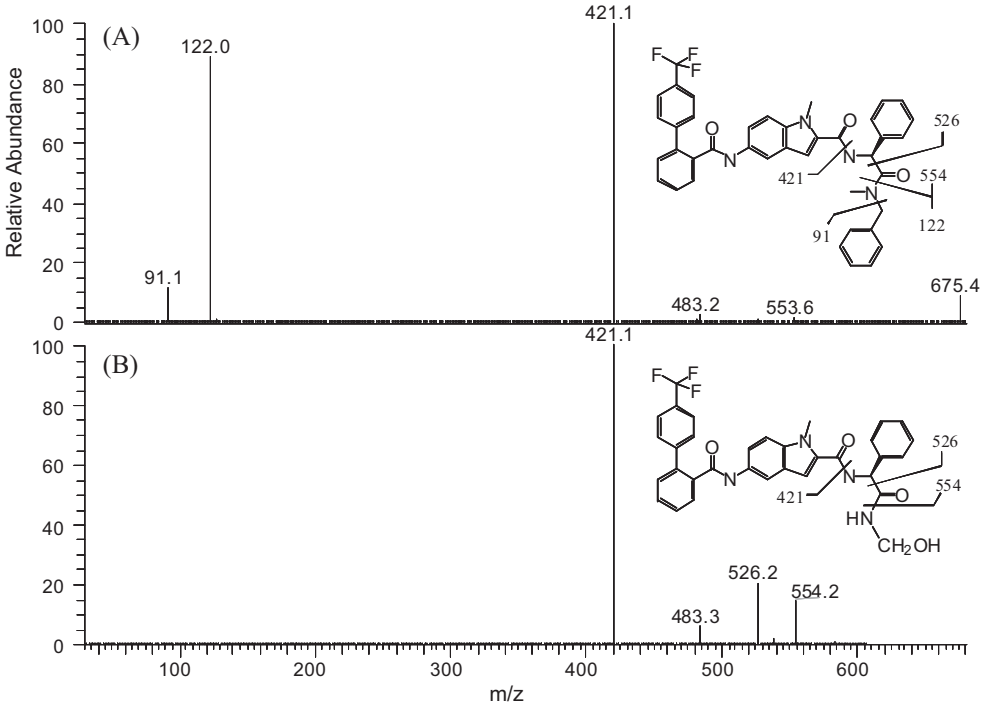

Figure 3. MS/MS spectra of dirlotapide (A) and dirlotapide metabolite M2 (B).

which upon loss of CO produced the *m/z* 526 ion. The *m/z* 483 ion appears to be formed by elimination of HNCO from *m/z* 526 with recombination of the resulting tropyllium ion and the fragment containing the biphenyl and indole ring systems. The *m/z* 421 fragment is the acylium ion containing the biphenyl and indole ring systems. At higher collision energy (CE), a *m/z* 249 fragment was observed due to the acylium ion containing the biphenyl ring system, which yielded *m/z* 221 and 201 ions after successive neutral losses of CO and HF.

[^{14}C]Dirlotapide metabolite M2 was observed at a retention time of 48.5 min, and had an $[M+H]^+$ ion at *m/z* 601. The collision-induced dissociation (CID) spectrum of M2 had ions at *m/z* 249, 421, 483, 526, and 554 (Fig. 3(B)) in common with dirlotapide, suggesting debenzylation of dirlotapide and hydroxylation on either the *N* atom or *N*-methyl group. Accurate mass measurements on M2 yielded *m/z* 601.2083, differing from the mass calculated for $C_{33}H_{28}N_4O_4F_3$ (*m/z* 601.2063) by 3.4 ppm. These data supported the assignment of oxidation on either the amide *N* atom or methyl group. No distinction could be made between the two by mass spectrometry. The *m/z* 554 ion resulting from in-source fragmentation occurred so readily that the diagnostic loss of formaldehyde expected from the CID of an *N*-hydroxymethyl structure, which in the case of M2 would produce an *m/z* 571 ion, could not be observed. Incubation of an isolated sample of M2 with 20% $TiCl_3$ for 1 h produced no change in the spectrum or retention time, suggesting that M2 was not an *N*-oxide metabolite.[18]

Due to the abundance of M2 in the circulation of the rat and dog, it was deemed necessary to better characterize its structure. NMR spectroscopy could provide an unambiguous determination of the site of oxidation; however, this would require the laborious purification of a concentrated sample of M2 from dog or rat fecal extracts. Therefore, we attempted derivatizations designed to take advantage of the pH-dependent dehydration/rehydration equilibrium of the proposed *N*-hydroxymethyl compound. An HPLC fraction containing M2 was collected and the solvent was evaporated under a stream of N_2. The residue was reconstituted in ACN, and [^{18}O]water was added with a small amount of concentrated sulfuric acid as a catalyst to promote production of the imine. After 1 h, an HPLC peak at the retention time of M2 (Figs. 4(A) and 4(B)) was observed at *m/z* 603 (2 mass units higher than before ^{18}O exchange), by Q1 scans and precursor-ion scans for precursors of the *m/z* 421 fragment ion. The product-ion spectrum of *m/z* 603 was identical to that of M2 (*m/z* 601), suggesting that a single ^{18}O atom was incorporated into the molecule at the position

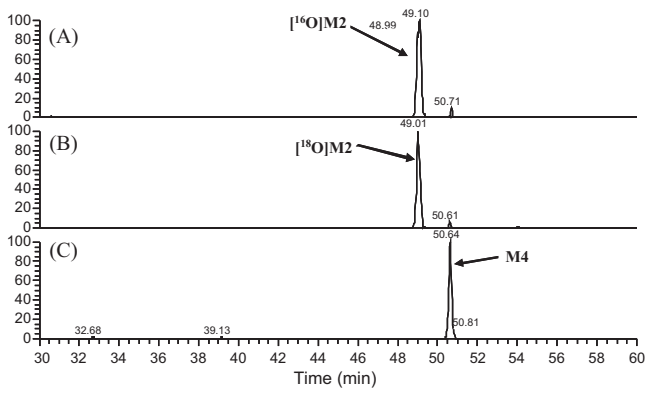

Figure 4. Precursor-ion (*m/z* 421 fragment) chromatograms of an isolated sample of dirlotapide metabolite M2: (A) untreated control, (B) after treatment with [^{18}O]water and H_2SO_4, and (C) after treatment with 2 N NaOH.

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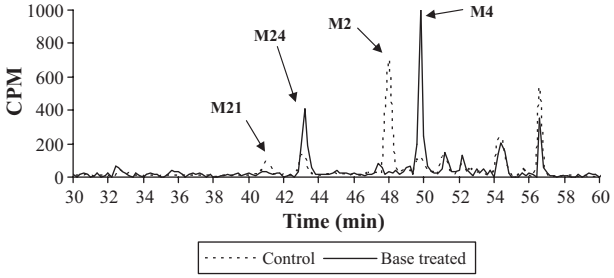

Figure 5. Radiochromatograms of a crude rat fecal extract before (Control) and after (Base treated) treatment with 10 N NaOH. The conversion of M21 into M24 and of M2 into M4 can be observed.

of the amide *N*-methyl group, and demonstrating the ability of M2 to form an imine intermediate. Additionally, we surmised that in strong base solutions, the deprotonation of the alcoholic *O* atom would drive M2 to *N*-dealkylate by the loss of formaldehyde. In fact, incubation of an HPLC fraction containing M2 with 2 N NaOH for 1 h at room temperature produced *N*-desbenzyl-*N*-desmethyl-dirlotapide (M4, Fig. 4(C)), which was verified by comparison of LC/MS/MS retention times and CID spectra with an authentic analytical standard of M4.

A crude rat fecal extract was then dried and mixed with [^{18}O]water and H_2SO_4 as described above, and the incorporation of ^{18}O into M2 and M21, a proposed *N*-hydroxymethyl metabolite with hydroxylation on the benzyl ring adjacent to the indole system, was easily observed. A previously identified carboxylic acid metabolite in this sample did not incorporate ^{18}O into its structure. In the crude extract, however, in contrast to the purified sample of M2, the addition of 5 μL of 2 N NaOH was insufficient to raise the pH high enough to promote *N*-dealkylation. A check of the pH of the sample after this treatment indicated a pH of approximately 9, which after 1 h produced no change in the radiochromatogram. However, after the addition of 10 μL of 10 N NaOH to a 100 μL aliquot of the crude extract, the pH was approximately 13. After 1 h the disappearance of M2 and M21 peaks in the radiochromatogram was observed, with a proportionate increase in the size of peaks corresponding to the *N*-dealkylated analogs M4 and M24, respectively (Fig. 5). These results are consistent with observations of reduced *N*-dealkylation rates for *N*-(hydroxymethyl)benzamide compounds at pH values below 10.5.[19]

The isotopic distribution of $[M+H]^+$ and $[M+NH_4]^+$ ions of M2, and $[M+H]^+$ ions of M21, both before and after treatment with [^{18}O]water and acid (Fig. 6) demonstrated the increase in abundance of the respective M+2 isotopes after ^{18}O-exchange. The mass peaks due to the ^{16}O isotopes which remained after treatment (*m/z* 601 and 617, respectively, for M2 and M21, Figs. 6(B) and 6(D)) were due either to residual [^{16}O]water in the sample at the time of the exchange experiment, or to the back-exchange of ^{16}O during liquid chromatography. The M+2 peaks in the untreated control sample (*m/z* 603 and 619, respectively, for M2 and M21, Figs. 6(A) and 6(C)) were due primarily to the ^{14}C label (specific activity of 6.74 mCi/mmol) at the carbonyl carbon atom nearest the biphenyl ring system.

To investigate the possibility of re-incorporation of ^{16}O into metabolites from the aqueous mobile phase component during

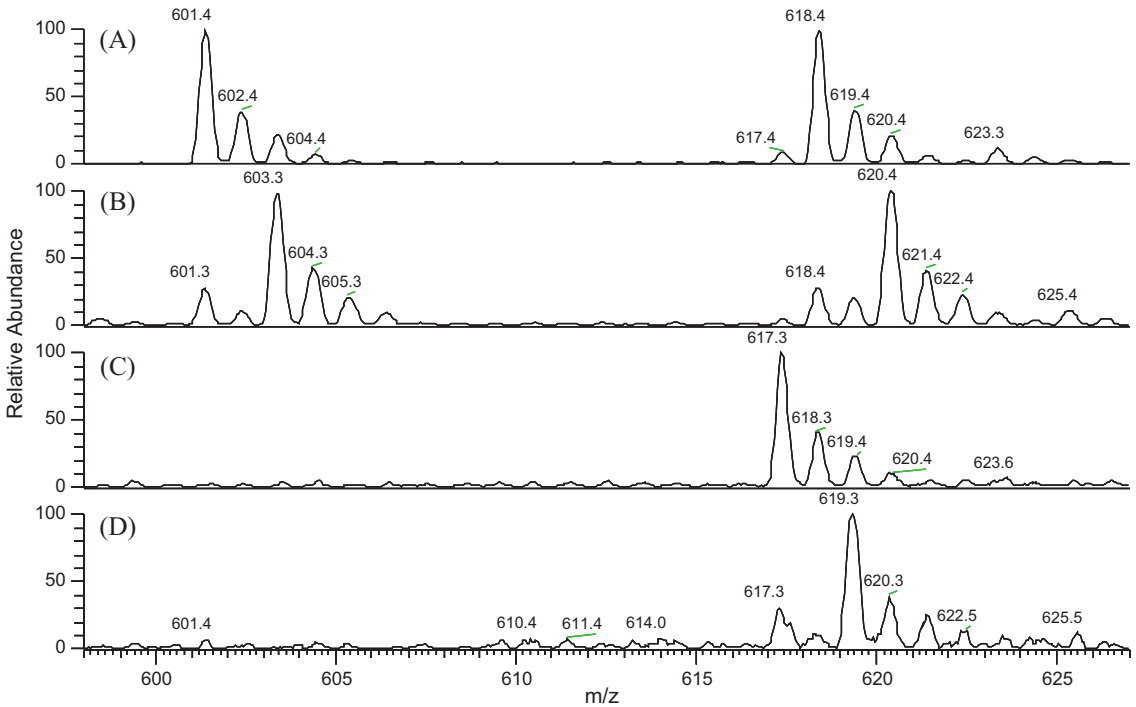

Figure 6. Isotopic distribution of dirlotapide metabolites M2 and M21 before and after treatment with [^{18}O]water and acid: (A) $[M+H]^+$ and $[M+NH_4]^+$ ions of M2 before treatment; (B) $[M+H]^+$ and $[M+NH_4]^+$ ions of M2 after treatment; (C) $[M+H]^+$ ions of M21 before treatment; and (D) $[M+H]^+$ ions of M21 after treatment.

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the course of liquid chromatography, especially with commonly used acidic mobile phases as low as pH 3, MS spectra of M2 were compared after ^{18}O-exchange using HPLC separations at both pH 3 and pH 6.8 conditions. The retention time ($t_R = 48.5$ min) of dirlotapide metabolite M2 was unchanged by varying the pH, and no changes in the isotopic distributions of the M and M+2 mass peaks at *m/z* 601 and 603 (due to ^{16}O and ^{18}O incorporation, respectively) could be discerned by inspection (Fig. 7). Additionally, by monitoring and integrating the multiple reaction monitoring (MRM) transitions for non-radiolabeled M2 of *m/z* 601 > 421 and 603 > 421, corresponding to [^{16}O]M2 and [^{18}O]M2, respectively, a quantitative measure of the relative amount of ^{16}O incorporation due to back-exchange during chromatography was established. The MRM peak area ratios ([^{18}O]M2/[^{16}O]M2) after use of the ^{18}O-exchange technique described above were 5.35 and 5.96, respectively, at pH 3.0 and pH 6.8, reflecting a 10.2% reduction in peak area for [^{18}O]M2 after chromatography at pH 3. By comparison, the [^{18}O]M2/[^{16}O]M2 ratio obtained for a control sample not treated with [^{18}O]water was 0.015 with chromatography performed at pH 6.8. These results suggested that re-equilibration of an ^{18}O-exchanged product with [^{16}O]water in the mobile phase during the course of liquid chromatography was minimal using either pH 3 or pH 6.8 analytical conditions, and did not preclude the use of the technique even for metabolites with HPLC retention times approaching 1 h.

Although we believed these observations to give unambiguous evidence for the *N*-hydroxymethyl structures of M2 and M21, we performed the isolation and purification of a sufficient amount of M2 for NMR analysis to firmly establish the validity of the technique. The spectrum of the M2 isolate contained three fewer downfield resonances when compared to similar data on dirlotapide. The singlet assigned as the methyl group of the benzylmethylamino moiety in dirlotapide was absent in the ^1H spectrum of the isolate. Furthermore, the inequivalent methylene pair at δ 4.49 and 4.76 which were assigned as the terminal benzyl methylene moiety of dirlotapide appeared as a broad singlet at δ 4.64 in the ^1H spectrum of the M2 isolate (Fig. 8). In addition, the multiplicity edited HSQC data contained a cross peak from the δ 4.64 resonance with a carbon chemical shift of 63.1 ppm and a phase that indicated a methylene. All of the NMR data was consistent with the proposed *N*-hydroxymethyl structure of M2.

In an effort to demonstrate the universality of the ^{18}O-exchange technique, we applied it to a reported carbinolamide metabolite of loperamide, and to metabolites of four proprietary compounds (compounds A–D). Loperamide contained an *N*,*N*-dimethylamide structure, while compound A was a cyclic *N*-alkylamide compound with multiple possible oxidation sites on the aliphatic moiety. Compound B contained an *N*,*N*-dimethyl amide moiety, while compounds C and D contained *N*-benzylamide moieties.

As described previously,[20] the metabolism of loperamide in rat liver microsomes yielded *N*-desmethyl loperamide and *N*-desmethyl-*N*-hydroxymethyl loperamide as the major reaction products (Fig. 9). The MS/MS spectrum of loperamide consisted of a single prominent fragment ion at *m/z* 266 due to loss of the *N*,*N*-dimethyl-α,α-diphenylbutyramide moiety, and had minor ions at *m/z* 432 and 238 (Fig. 10(A)). The *m/z* 432 ion was due to loss of dimethylamine from loperamide, while the *m/z* 238 ion was due to the loss of ethylene from the *m/z* 266 ion. In contrast to *N*-desbenzyl-*N*-hydroxymethyl dirlotapide (M2), the identity of the carbinolamide metabolite of *N*-desmethyl loperamide (*m/z* 479) could be convincingly determined by analysis of CID mass spectra (Fig. 10) by virtue of a

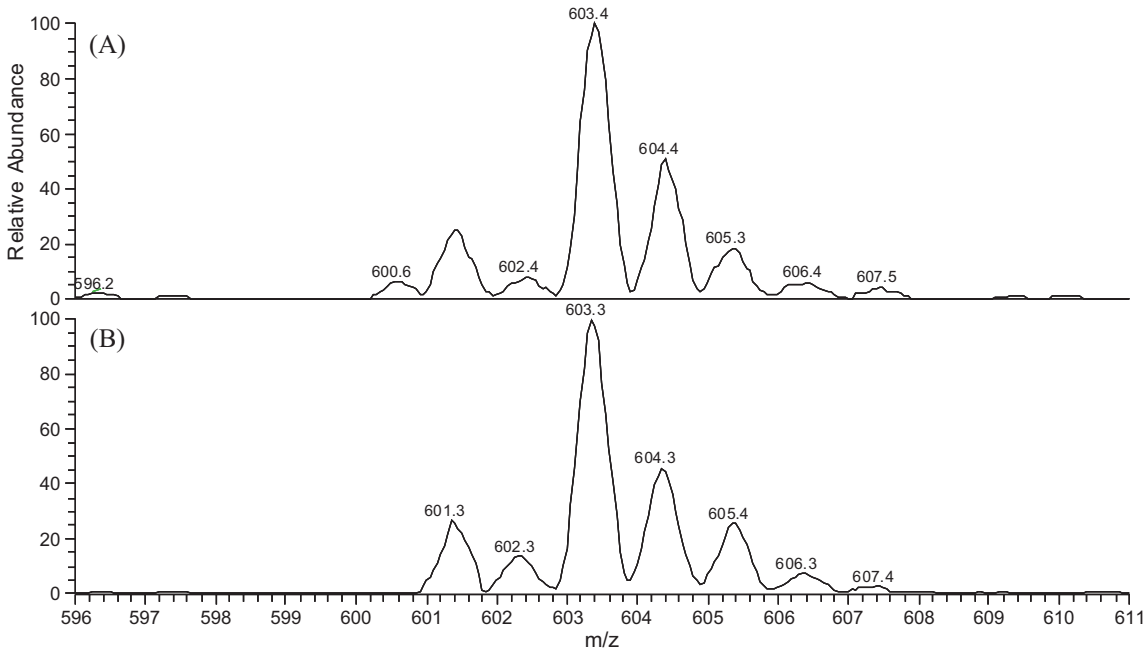

Figure 7. Isotopic envelopes of the $[M+H]^+$ ions of M2 at (A) pH 6.8 and (B) pH 3.0, demonstrating that back-exchange of ^{16}O from the aqueous mobile phase during the course of liquid chromatography did not occur to a significant extent. The HPLC retention time of M2 under both conditions was approximately 49 min.

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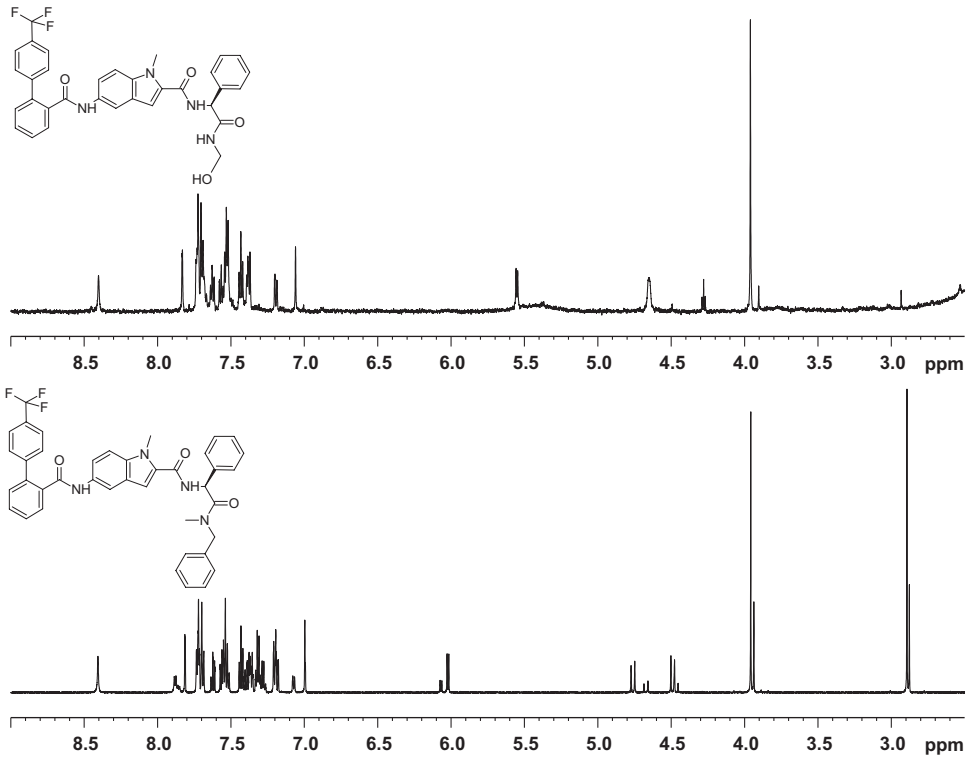

Figure 8. ^{1}H NMR spectra of dirlotapide metabolite M2 (top) and of dirlotapide (bottom) in ACN-d_3.

prominent ion at *m/z* 449 due to the neutral loss of formaldehyde.

To illustrate the ^{18}O-exchange technique with loperamide, an aliquot of the crude microsomal reaction mixture was dried and treated with [^{18}O]water and acid as described above. Analysis of the carbinolamide peak eluting at 18.2 min had [M]/[M+2]/[M+4] isotope ratios consistent with the addition of ^{18}O to a chlorine-containing compound. The CID spectra of *m/z* 479, 481, and 483 at $t_R = 18.2$ min, corresponding to [^{35}Cl^{16}O]*N*-desmethyl-*N*-hydroxymethyl loperamide; [^{35}Cl^{18}O]- (CID spectrum not shown) and [^{37}Cl^{16}O]*N*-desmethyl-*N*-hydroxymethyl loperamide; and [^{37}Cl^{18}O]*N*-desmethyl-*N*-hydroxymethyl loperamide following ^{18}O-exchange (Figs. 10(B)–10(D)) were indicative of ^{18}O substitution at the amide *N*-hydroxymethyl position, as shown by the loss of 32 u due to [^{18}O]formaldehyde in the sample incubated with [^{18}O]water, and by loss of unlabeled formaldehyde (30 u) in the untreated sample.

Initially, several attempts to demonstrate the *N*-dealkylation of *N*-desmethyl-*N*-hydroxymethyl loperamide using triethylamine (TEA) as the base were unsuccessful, as no change in the mass chromatogram of a TEA-treated rat

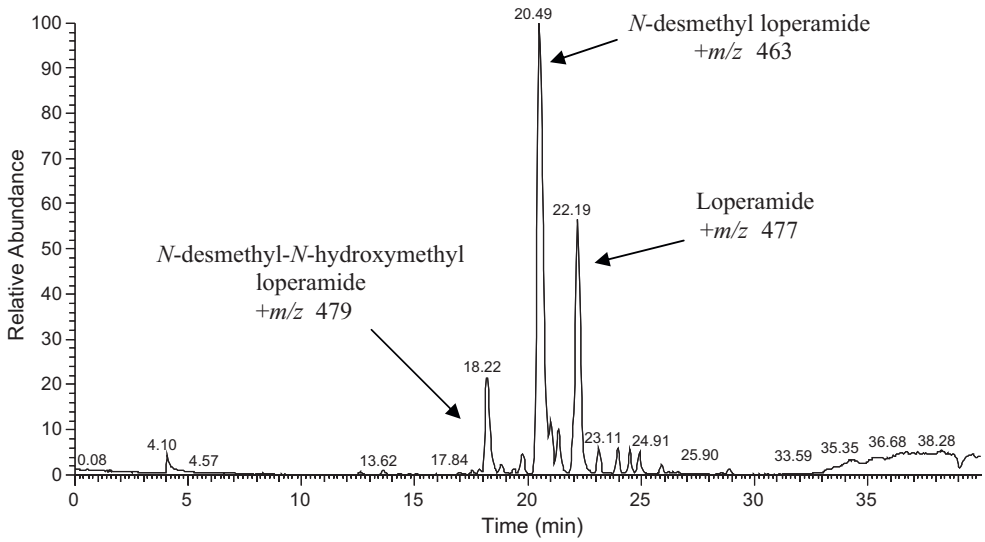

Figure 9. Base peak chromatogram of an incubation mixture of loperamide in rat liver microsomes after 30 min.

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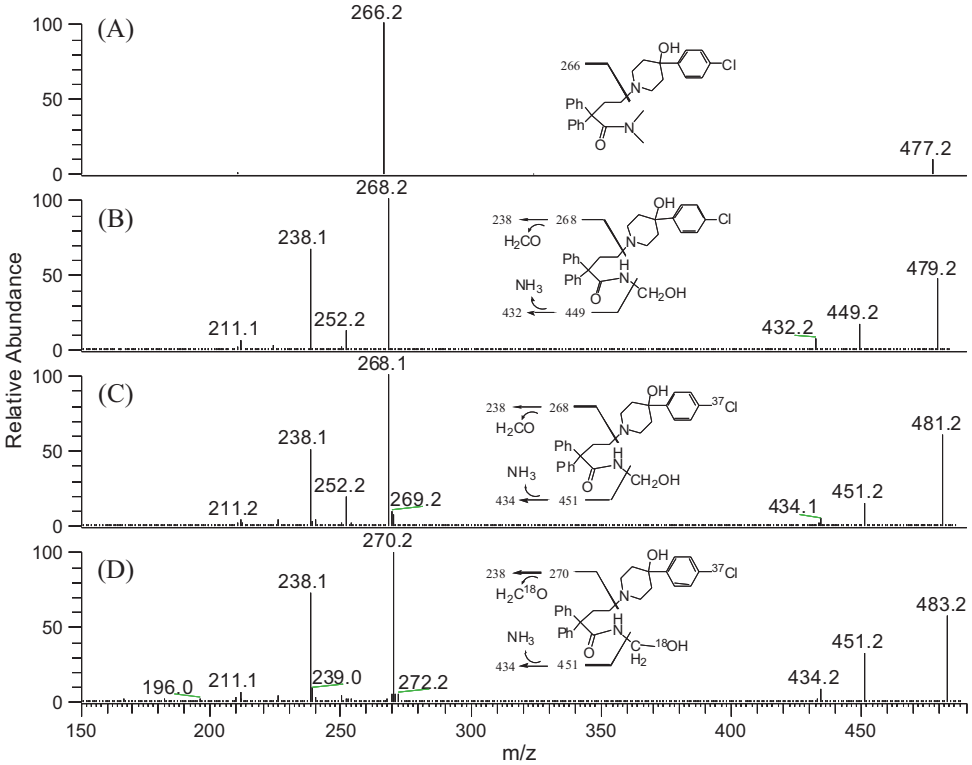

Figure 10. MS/MS spectra of (A) loperamide; (B) [$^{35}Cl^{16}O$]*N*-desmethyl, *N*-hydroxymethyl loperamide from an untreated sample; (C) [$^{37}Cl^{16}O$]*N*-desmethyl, *N*-hydroxymethyl loperamide from an untreated sample; and (D) [$^{37}Cl^{18}O$]*N*-desmethyl, *N*-hydroxymethyl loperamide from a sample treated with [^{18}O]water.

liver microsomal sample could be observed. In subsequent experiments, however, 10 μL of 10 N NaOH was substituted for TEA in the mixture. After 1 h at room temperature, the mixture was neutralized with an equimolar amount of H_2SO_4 prior to analysis by LC/MS/MS. The loss of the *m/z* 479 peak (*N*-desmethyl-*N*-hydroxymethyl loperamide) was easily observed by comparison with a control sample. Other metabolites were unchanged by base treatment, with the exception of a quaternary pyridinium metabolite resulting from oxidation of the piperidine ring. The latter metabolite was distinct chromatographically as well as by virtue of its mass and fragmentation properties, so its loss due to the base treatment did not complicate the analysis. The inability of TEA to drive the *N*-dealkylation of *N*-desmethyl-*N*-hydroxymethyl loperamide demonstrated the necessity for high pH to accomplish the reaction. Compound A is a proprietary molecule containing a cyclic amide with multiple possible sites of oxidation by drug-metabolizing enzymes. The MS^2 spectrum of compound A was sparse, containing only ions at *m/z* 195, 275, and 321 (Fig. 11(A)). The *m/z* 275 ion contained the piperidine amide moiety and residue R_1, while the *m/z* 321 ion resulted from cleavage of the amide C−N bond, producing a fragment which contained the piperidine moiety and residues R_2 and R_3.

Incubations of compound A with human liver microsomes or hepatocytes produced five singly oxygenated metabolites, in addition to several secondary metabolites. Of these, three primary singly oxidized metabolites (*m/z* 485, + 16 u relative to the parent compound) were shown by MS/MS spectra to be modified on the piperidine portion of the molecule. A careful analysis of the CID fragment ions contained in the MS/MS spectra of these three metabolites suggested that one was an *N*-oxide compound (losing oxygen, not water, during CID) on the piperidine moiety; and two were hydroxylations on the aliphatic ring system (due to facile dehydration during CID).[21] The ^{18}O-exchange technique was employed to distinguish hydroxylations α to the amide *N* atom from oxidations at other positions. A sample of compound A incubated with human hepatocytes was evaporated to dryness under vacuum, then reconstituted in ACN to which [^{18}O]water and H_2SO_4 were added. After approximately 1 h at room temperature, the sample was analyzed by LC/MS^n without further processing. Of the five separate singly oxidized metabolites observed, only one (retention time 20.8 min) was found to have incorporated an atom of ^{18}O after treatment by virtue of the presence of an enhanced M + 2 isotope peak at *m/z* 487. The MS/MS spectrum of this metabolite contained the *m/z* 195 ion both before and after treatment (Figs. 11(B) and 11(C)), in common with the parent compound, indicating that the metabolic modification was not on the R_3 portion of the molecule. Incorporation of ^{18}O was clearly indicated by dehydration of the *m/z* 293 ion (2 u higher than the corresponding ions before treatment), which, upon loss of $H_2^{18}O$, produced an *m/z* 273 ion, common to both pre- and post-treatment MS/MS spectra of this metabolite. Thus, of five isobaric singly oxidized metabolites, the one containing a hydroxyl modification alpha to the amide *N* atom was distinctly identified using the ^{18}O-exchange technique described here. Due to the cyclic nature of compound A and its carbinolamide metabolites, treatment with strong

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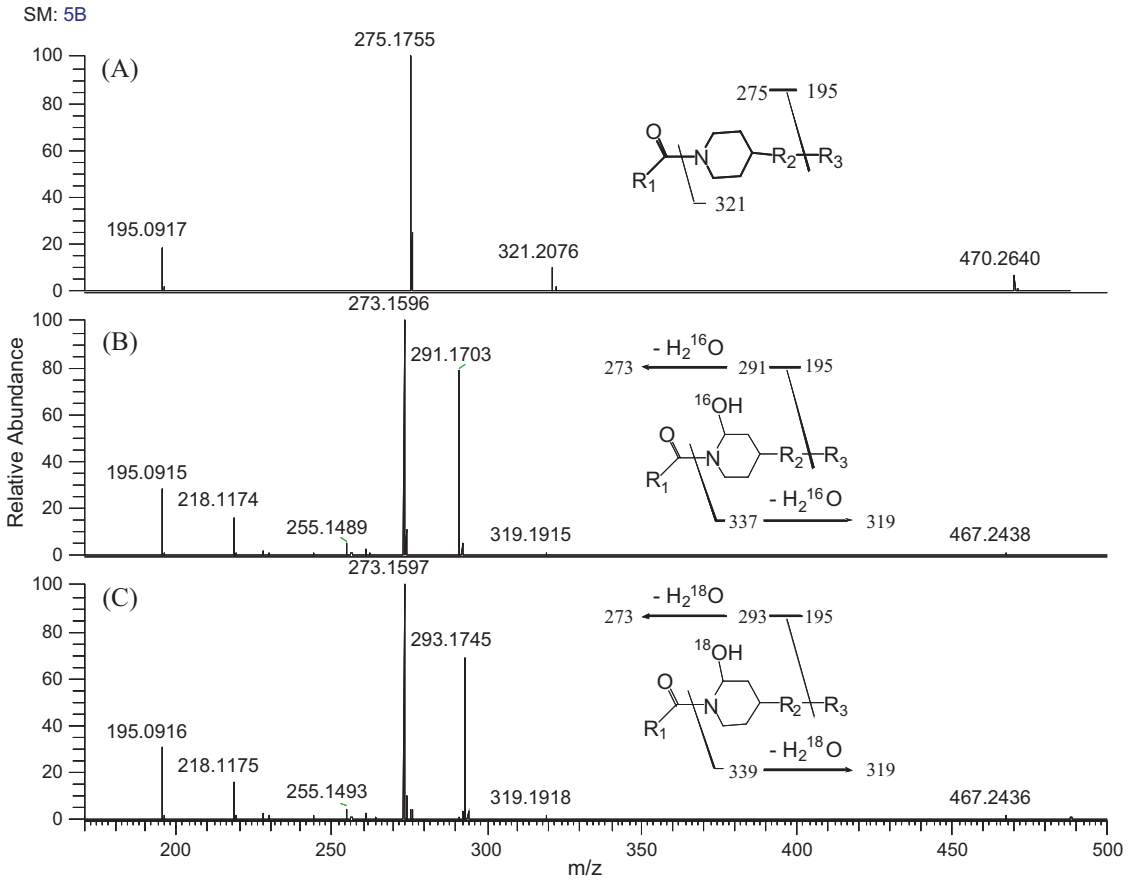

Figure 11. MS/MS spectra of compound A (A), and of a carbinolamide metabolite of compound A before (B) and after (C) treatment with [^{18}O]water and acid.

base would not be expected to produce *N*-dealkylation as was the case with dirlotapide and loperamide carbinolamide metabolites, and thus these reactions were not performed.

A glucuronide conjugate was also observed at 18.2 min at *m/z* 647 in the same hepatocyte incubation with compound A. Partial in-source fragmentation of this conjugate produced the aglycone at *m/z* 471 (+2 u relative to the parent compound), and MS/MS spectra of each of these ions were consistent with demethylation of the R_1 residue and oxidation on the piperidine portion of the molecule. After treatment with [^{18}O]water and H_2SO_4, M+2 peaks were observed for both the aglycone and glucuronide conjugate at *m/z* 473 and 649, respectively, each with an elemental formula consistent with the incorporation of ^{18}O into the structure. Thus, the glucuronide metabolite corresponded to a demethylated analogue of the carbinolamide metabolite just described, with conjugation on the R_1 moiety.

Compound B, containing an *N,N*-dimethylamide moiety, was observed by high-resolution MS to have produced a hydroxylated metabolite after incubation with rat and human liver microsomes, presumably on one of the amide *N*-methyl groups. Upon treatment with [^{18}O]water and H_2SO_4 as described, this metabolite *N*-dealkylated completely to the monomethyl amide derivative (data not shown), underscoring the high lability of some carbinolamides. This observation is consistent with a previous report,[6] in which the stability of *N*-hydroxymethyl-*N*-methylbenzamide was much less than that of *N*-hydroxymethylbenzamide.

Compounds C and D, structural analogs containing an *N*-benzyl amide moiety, each produced an abundant metabolite in rat and monkey liver microsomes for which mass spectrometry indicated a net loss of 2 u relative to the parent. Analysis of the metabolite of compound C by ^{1}H NMR spectroscopy (data not shown) demonstrated that under neutral conditions the metabolite existed as a hydroxylated structure (+16 u relative to parent) at the benzyl *C* atom α to the amide *N* atom. Dehydration reactions for compounds C and D occurred in the ESI source of the mass spectrometer to such an extent that no trace of the benzyl-hydroxylated metabolites could be observed. For compounds such as these, irreversible dehydration of metabolites under acid conditions, or loss of the label in the ion source of the mass spectrometer, would preclude use of the ^{18}O-exchange technique presented here.

CONCLUSIONS

A simple and rapid technique has been developed for conclusively distinguishing carbinolamide compounds from other possible isobaric *N*-oxygenated species. A key advantage of the technique as compared to other derivatizations which could be employed for the same purpose is the fact that chromatographic retention times are not altered as a result of chemical modification, thus allowing a direct comparison of mass chromatograms before and after treatment. The technique has been used with multiple

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isobaric metabolites of compound A to distinguish between those in which hydroxylation occurred α to the amide *N* atom, and those at other positions on the same aliphatic ring in which dehydration-rehydration equilibrium does not occur. In particular, the technique has shown utility for cases in which mass spectrometry alone was insufficient for complete refinement of the oxidation site in compounds of this type, or for which NMR spectroscopy was not feasible due to its relatively high sample mass requirements. The method is limited to structures in which the reversible formation of an imine intermediate can produce a stable ^{18}O-exchanged hydration product. The use of the technique with benzyl-hydroxylated amides may not be feasible due to the high rate of dealkylation or dehydration under the conditions used for analysis; however, this should be evaluated on a case-by-case basis.

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