Identification of a Novel N-Carboxamoyl Glucuronide: In Vitro, In Vivo, and Mechanistic Studies

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Received October 6, 2009; accepted December 11, 2009

ABSTRACT:

1-[4-Aminomethyl-4-(3-chlorophenyl)-cyclohexyl]-tetrahydro-pyrimidin-2-one, 1, was developed as an inhibitor of dipeptidyl peptidase-4 enzyme. Biotransformation studies with 1 revealed the presence of an N-carboxamoyl glucuronide metabolite (M1) in rat bile and urine. N-Carboxamoyl glucuronides are rarely observed, and little is understood regarding the mechanism of N-carboxamoyl glucuronidation. The objectives of the current investigation were to elucidate the structure of the novel N-carboxamoyl glucuronide, to investigate the mechanism of N-carboxamoyl glucuronidation in vitro using stable labeled CO2, UDP glucuronosyltransferase (UGT) reaction phenotyping, and to assess whether M1 was formed to the same extent in vitro across species—mouse, rat, hamster, dog, monkey, and human. Structure elucidation was performed on a mass spectrometer with accurate mass measurement and MSn capabilities. 13C-labeled carbon dioxide was used for identification of the mechanism of N-carboxamoyl glucuronidation. Mechanistic studies with 13C-labeled CO2 in rat liver microsomes revealed that CO2 from the bicarbonate buffer (in equilibrium with exogenous CO2) may be responsible for the formation of M1. M1 was formed in vitro in liver microsomes from multiple species, mainly rat and hamster, followed by similar formation in dog, monkey, mouse, and human. M1 could be detected in UGT1A1, UGT1A3, and UGT2B7 Super-somes in a CO2-rich environment. In conclusion, our study demonstrates that formation of M1 was observed in microsomal incubations across various species and strongly suggests incorporation of CO2 from the bicarbonate buffer, in equilibrium with exogenous CO2, into the carboxamoyl moiety of the formed N-carboxamoyl glucuronide.

Human UDP-glucuronosyltransferases (UGTs) catalyze the conjugation of glucuronic acid to a variety of xenobiotic and endobiotic molecules (Remmel et al., 2009). The UGTs are expressed in many tissues, including liver, kidney, intestine, colon, adrenal glands, spleen, lung, skin, testes, ovaries, olfactory glands, and brain (Tukey and Strassburg, 2000). Conjugation via glucuronidation is an important step in the elimination of many endogenous molecules from the body, including bilirubin, bile acids, steroid hormones, thyroid hormones, retinoic acids, and others (Burchell et al., 1998). The glucuronidation reaction proceeds by transfer of a glucuronic acid moiety from UDP-glucuronic acid (UDPGLA) to an acceptor molecule and occurs with a wide range of functional groups such as aromatic and aliphatic alcohols, carboxylic acids, thiols, primary, secondary, tertiary, and aromatic amino groups, and acidic carbon atoms (Remmel et al., 2007). One such conjugation reaction at a carboxamic acid functionality results in the formation of an N-carboxamoyl glucuronide. Carboxamic acids are formed when carbon dioxide reacts with primary or secondary amines, and subsequent glucuronidation of the carboxamic acid results in N-carboxamoyl glucuronide formation. Few reactions have been reported in the literature for N-carboxamoyl glucuronidation. N-Carboxamoyl glucuronides of amino acids and certain xenobiotics have been reviewed by Schaefer (2006). Other xenobiotics that are reported to form N-carboxamoyl glucuronide are tocainide (Elvin et al., 1980), rimantadine (Brown et al., 1990), carbendil (Schaefer, 1992), mofegiline (Dow et al., 1994), sertraline (Obach et al., 2005), sitagliptin (Vincent et al., 2007), and amosulalol (Suzuki and Kamimura, 2007). Little is known about the mechanism of this reaction and the human UGTs that catalyze it.

I was developed as a small molecule inhibitor of dipeptidyl peptidase-4 (adenosine deaminase complexing protein-2) for the treatment of type 2 diabetes. In an effort to understand the mechanism of clearance of 1 in rats, metabolite identification studies were carried out in rat plasma, urine, and bile samples. Biotransformation studies revealed that an N-carboxamoyl glucuronide (M1) was present in rat bile and urine. The objectives of the current investigation were as follows: 1) to elucidate the structure of this novel N-carboxamoyl glucuronide in rat bile and urine, 2) to study the mechanism of N-carboxamoyl glucuronide formation in vitro in an appropriate model using 13C-labeled carbon dioxide, 3) to study whether M1 could be biosynthesized in vitro in rat liver microsomes, 4) to compare the formation of M1 in liver microsomes across various species, and 5) to identify the UGT isozymes involved in the formation of M1 in vitro.

ABBREVIATIONS: UGT, UDP-glucuronosyltransferases; 1, 1-[4-aminomethyl-4-(3-chlorophenyl)-cyclohexyl]-tetrahydro-pyrimidin-2-one; UDPGA, UDP-glucuronic acid; MS, mass spectrometry; LC, liquid chromatography; MeCN, acetonitrile; ESI, electrospray ionization; RF, radiofrequency; MS/MS, tandem mass spectrometry.
Materials and Methods

Chemicals and Reagents. 1 was synthesized at Novartis Institutes for Biomedical Research (Basel, Switzerland). Liver microsomes (Sprague-Dawley rat, beagle dog, cynomolgus monkey, Syrian hamster, and CD1 mouse; 20 mg/ml) were purchased from XenoTech, LLC (Lenexa, KS) and human liver microsomes (20 mg/ml, pooled lot 20) were purchased from CellzDirect (Austin, TX). β-Glucuronidase (isolated from Helix pomatia) and UDPGA were purchased from Sigma-Aldrich (St. Louis, MO). UGT Supersomes at a 5 mg/ml protein concentration (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) were obtained from BD Biosciences (Franklin Lakes, NJ). Solvents and reagents were MS-grade and were purchased from Mallinckrodt Biomedicine Research (Basel, Switzerland). Liver microsomes (Sprague-Dawley rats were purchased from Mallinckrodt Baker (Phillipsburg, NJ). NaH\(^{13}\)CO\(_3\) (99% pure) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA).

In Vivo Studies in Sprague-Dawley Rats. The in-life portion of the study was conducted at Novartis Institutes for Biomedical Research (Cambridge, MA) in accordance with institutional animal care and use committee protocols. Three male Sprague-Dawley rats with cannulated bile ducts were administered an intravenous 1 mg/kg dose of \(\text{I}\), formulated in 100 mM phosphate buffer, pH 7.4. Predose (0 h) and postdose bile and urine were collected up to 30 h (at intervals of 0–4, 4–7, and 7–30 h). Plasma was obtained from the blood samples collected at predose (0 h) and postdose (1 and 6 h) for metabolic profiling. Rat bile, urine, and plasma were stored at −80°C until analysis.

LC-MS\(^{\text{a}}\) Method for Metabolite Identification. Urine and bile samples were pooled from each time interval (equal volumes) and centrifuged at 6440 g for 5 min before analysis. An aliquot (0.2 ml) of each plasma sample was transferred to a 2-ml Eppendorf tube containing acetonitrile (0.2 ml), and the samples were vortexed and centrifuged (3200 g) for 5 min to precipitate the proteins. The respective supernatants were removed and transferred to 1-ml conical glass inserts before analysis. Predose samples (0 h) were used as control samples (background ion spectra) for LC-MS\(^{\text{a}}\) analyses. Samples were analyzed for the presence of metabolites with the help of a Thermofisher Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) capable of MS\(^{\text{a}}\) scanning and accurate mass measurement interfaced with a 3X Ti HPLC pump and CTC PAL autosampler (Leap Technologies, Carrboro, NC). The analytes (injected as 20-μl aliquots) were separated on a Symmetry C18 analytical column (5 μm, 2.1 × 150 mm; Waters, Milford, MA) with a 35-min gradient elution method. Mobile phase A consisted of 10 mM ammonium formate in MS-grade water with 0.1% formic acid. Mobile phase B consisted of MS-grade acetonitrile with 0.1% formic acid. The sample aliquots were eluted at a flow rate of 0.25 ml/min with 5% B over 5 min. Thereafter, the percentage of mobile phase B was gradually increased to 40% B over 20 min and to 95% over 5 min. After the elution of \(\text{I}\) and its metabolite, the column was returned over 1 min to 5% B, where it was held for 3 min before the next injection. ESI in positive ion mode was performed at a capillary temperature of 375°C, a sheath gas flow rate of 35 ml/min, source voltage of 4.20 kV, source current of 100 μA, capillary voltage of 35 V, and tube lens voltage of 100 V. A multipole RF amplifier was set at 400 Vp-p, along with multipole 0 offsets at −1.25 and −4.75 V, lens 0 voltage at −6.50 V, lens 1 voltage at −13 V, gate lens offset at −62 V, multipole 1 offset at −7 V, and front lens at −5 V. The instrument calibration was set at a multiple RF frequency of 2824 Hz, main RF frequency of 11875.5 Hz, and pulsed Q dissociation collision energy factor of 10. Fourier transformation mass spectrometry-generated accurate mass measurement was carried out at a mass resolution of 60,000. An ion trap mass spectrometry-based data-dependent scan after collision-induced dissociation was performed at a normalized collision energy of 35. Activation Q was set at 0.25, and activation time was 30 ms.

In Vitro Generation of N-Carbamoyl Glucuronide M1. Glucuronidation experiments were conducted by modification of previously reported protocols (Shaffer et al., 2005; Argikar and Remmel, 2009a,b). Incubations (0.5 ml) were conducted in 2-ml DW-96-well plates (Analytical Sales and Products, Inc., Pompton Plains, NJ) under a CO\(_2\)-rich atmosphere at 37°C in a shaking water bath. A constant environment of CO\(_2\) was maintained by slow, but continuous, purging with CO\(_2\) gas. The incubations contained liver microsomes (either rat, human, monkey, hamster, mouse, or dog) at a protein concentration of 1 mg/ml or human UGT isoforms at a protein concentration of 0.2 mg/ml in 0.1 M NaHCO\(_3\) buffer (pH 7.5), substrate \(\text{I}\) (20 μM), alamethicin (50 μg), MgCl\(_2\) (5 mM), and \(\delta\)-saccarolactone (1 mg/ml). Each reaction was started by the addition of UDPGA (3.2 mg/ml). After 2 h of incubation, the reaction was quenched with 0.5 ml of MeCN and centrifuged for 5 min at 4630 g. The resulting supernatants were transferred to clean DW-96-well plates with glass inserts (1 ml) for LC-MS\(^{\text{a}}\) analysis.

In Vitro Generation of N-Carbamoyl Glucuronide M1 using \(\text{CO}_2\) buffer. Incubations (0.5 ml) were conducted in a DW-96-well plate (2 ml) under a CO\(_2\)-rich atmosphere at 37°C in a shaking water bath. Each incubation contained rat liver microsomes (protein concentration of 1 mg/ml) in 0.1 M KH\(_2\)PO\(_4\) buffer (pH 7.5), substrate \(\text{I}\) (20 μM), alamethicin (50 μg/mg protein), MgCl\(_2\) (5 mM), and \(\delta\)-saccarolactone (1 mg/ml). The reaction was started by the addition of UDPGA (3.2 mg/ml). After 2 h of incubation, each reaction was quenched with 0.1 ml of MeCN and centrifuged for 5 min at 4630 g. The resulting supernatants were transferred to a clean DW-96-well plate with glass inserts (1 ml) for LC-MS\(^{\text{a}}\) analysis.

Results

Structural Rationalization of \(\text{I}\). Upon ESI in positive ion mode at a collision energy of 35, \(\text{I}\) showed a protonated molecular ion [M + H] of \(m/z\) 322. The LC retention time was approximately 15.5 min (Fig. 1). The collision-induced dissociation product ion spectrum (Fig. 2) afforded fragment ions with \(m/z\) values of 305, 222, 205, and 101. Fragment ion \(m/z\) 305 was formed by the loss of the ammonium moiety. Fragment ion \(m/z\) 222 was formed by the loss of the tetrahydropyrimidin-2(1H)-one moiety. Subsequent loss of ammonia from fragment ion \(m/z\) 222 resulted in an ion of \(m/z\) value 205. Fragment ion \(m/z\) 101 was confirmed as the tetrahydropyrimidin-2(1H)-one moiety of \(\text{I}\) by accurate mass measurement.

Structural Rationalization of \(\text{M1}, N\)-Carbamoyl Glucuronide of \(\text{I}\). ESI of \(\text{M1}\) in positive ion mode at a collision energy of 35 resulted in a protonated molecular ion of \(m/z\) value 542, higher than \(\text{I}\) by 220 atomic mass units (Fig. 3). This was detected at a retention time of approximately 19.1 min (Fig. 1). The collision-induced dissociation product ion spectrum of \(\text{M1}\) afforded fragment ions with \(m/z\) values of 305, 222, 205, and 101. Fragment ion \(m/z\) 305 was formed by the loss of the ammonium moiety. Fragment ion \(m/z\) 222 was formed by the loss of the tetrahydropyrimidin-2(1H)-one moiety. Subsequent loss of ammonia from fragment ion \(m/z\) 222 resulted in an ion of \(m/z\) value 205. Fragment ion \(m/z\) 101 was confirmed as the tetrahydropyrimidin-2(1H)-one moiety of \(\text{I}\) by accurate mass measurement.

Results from β-Glucuronidase Treatment. As described under Materials and Methods, the rat bile containing \(\text{M1}\) was treated with β-glucuronidase for 2 h. The enzyme-treated bile sample showed almost complete loss of \(\text{M1}\) and a marked generation of the parent, \(\text{I}\), in comparison with the control sample containing buffer instead of the enzyme β-glucuronidase. No parent was observed in the pretreatment
control (0–4 h bile sample, with no treatment with β-glucuronidase). The corresponding carbamic acid of 1 is presumably unstable and was not observed after β-glucuronidase-mediated hydrolysis. These data support the fact that m/z 542 was indeed a glucuronide that reverted back to the parent, as illustrated in Fig. 4.

Results from Experiments with 13C-Labeled CO2. In experiments performed under an environment of labeled 13CO2, m/z corresponding to 543 was observed (Fig. 5). This matched with the expected stable labeled N-carbamoyl glucuronide of 1 containing 13CO2. Furthermore, MS/MS of ion 543 revealed m/z 367 along with other fragments that accurately matched the fragment ions from MS/MS of m/z 542 (the N-carbamoyl glucuronide containing CO2). The fragment m/z 367 corresponded to a stable labeled carbamic acid of 1 (Fig. 5). Because of the incorporation of labeled 13CO2, not only was m/z 367 1 atomic mass unit higher than the previously identified m/z 366, but also was in disagreement with the nitrogen rule, as expected. This finding shows that incorporation of 13CO2 to yield 13C-labeled M1 was successful in an environment of 13CO2.

Species Selectivity and Phenotyping. To compare the formation of M1 across species, incubations were performed with 1 in liver microsomes from multiple species. Among the species tested, M1 was formed readily in rat and hamster, followed by comparable formation in dog, monkey, mouse, and human (Fig. 6). Because of the lack of a synthetic standard of M1, formation of M1 relative to 1 was based on MS response and was considered a measure of the extent of M1 formation in incubations with liver microsomes and cloned expressed UGTs. UGT phenotyping experiments in UGT Supersomes under an environment of CO2 revealed that formation of M1 was catalyzed by UGT1A3, UGT1A1, and UGT2B7, as shown in Fig. 7.

Discussion

N-Carbamoyl glucuronidation is a rarely observed disposition process. To date N-carbamoyl glucuronidation has been reported for a small number of drugs containing primary and secondary amino functionalities, for example, sitagliptin (Vincent et al., 2007) and sertraline (Obach et al., 2005). Of the earlier reports for N-carbamoyl glucuronidation few were for tocainide (Elvin et al., 1980; Ronfeld et al., 1982). Since then, such a metabolic pathway has been reported for rimantadine (Brown et al., 1990), carvedilol (Schaefer, 1992), mofegiline (Dow et al., 1994), and most recently for amosulalol (Suzuki and Kamimura, 2007), in addition to sitagliptin and sertraline. Carbamic acids are known to be intrinsically unstable and are likely to form without enzymatic catalysis under physiological conditions. However, there has been some evidence for characterization of carbamic acids of xenobiotics in solution phase by NMR analysis. Carbamic acid derivatives of piperazine (Kirsch et al., 2000) and des-methyl org1377 (Delbressine et al., 1990) were identified in this manner, generated after reaction of NaHCO3 with respective parent molecules. However, stable carbamic acids have also been reported by Shaffer et al. (2005) and Hayakawa et al. (2003). A trans-esterification type reaction has been used as a method of trapping an unstable
carbamic acid. Straub et al. (1988) were successful in isolation of the ethyl ester of benzazepine after ethanolysis of benzazepine N-carbamoyl glucuronide in the presence of sodium metal and anhydrous ethanol. Liu et al. (2001) used a similar ethanolysis reaction to form an ethyl ester of a carbamate of “compound I.”

N-Carbamoyl glucuronides of unstable carbamic acids can convert back to parent moieties upon hydrolysis (Tremaine et al., 1989) or even during sample preparation. Rimantadine-N-carbamoyl glucuronide identified by post-trimethylsilyl or pentafluorobenzyl derivatization gas chromatography-MS, was documented to revert back to the parent after treatment with glusulase (Brown et al., 1990). For N-carbamoyl glucuronides of unstable carbamic acids, complete hydrolysis back to the parent compound has been reported after enzymatic hydrolysis. In the present study, we were unable to isolate and identify the corresponding carbamic acid of I upon treatment with β-glucuronidase (Fig. 4). However, we were able to observe complete conversion of the glucuronide to the parent, compared with the control. Furthermore, the fragment ion m/z 322 corresponding to I was formed by loss of both CO₂ and glucuronide moieties. This diagnostic fragmentation pattern for N-carbamoyl glucuronides (Shaffer et al., 2005), coupled with the accurate mass-enabled LC-MSⁿ characterization of the intact N-carbamoyl glucuronide, provides firm evidence for the novel N-carbamoyl glucuronide formation.

The mechanism of N-carbamoyl glucuronidation has not been experimentally proven until now. Spontaneous formation of carbamic acids has been documented for amino acids in the literature (Morrow et al., 1974). Formation of carbamic acids is proposed to be pH-dependent. Because amines may be protonated at lower pH values and the concentration of dissolved carbon dioxide decreases with an increase in pH, formation of carbamate is optimal in the pH range of 6 to 9 (Schaefer, 2006). In addition, because of their nature of being relatively un-ionized at physiological pH of 7.4, amino groups with lower pKₐ may form corresponding carbamic acids more readily. We attempted to identify the source of carbon dioxide that results in formation of an N-carbamoyl glucuronide. In vitro incubations with I were performed in rat liver microsomes under a saturated labeled...
$\text{CO}_2$ environment at atmospheric pressure in NaHCO$_3$ buffer. Control incubations were performed under a saturated environment of CO$_2$ at atmospheric pressure, with NaHCO$_3$ buffer. MS/MS analysis revealed that $N$-[13C]carbamoyl glucuronide was formed in the earlier incubations in proportions equal to $N$-[12C]carbamoyl glucuronide (Fig. 5). $N$-[12C]Carbamoyl glucuronide alone was formed in control incubations. This finding of incorporation of $^{13}\text{CO}_2$ in the product in equal proportions to CO$_2$ strongly indicates the prevalence of an equilibrium between CO$_2$ (gaseous) and dissolved CO$_2$. At the incubation pH of 7.4, the equilibrium extends further between the dis-
solved CO2 and the various anionic forms of bicarbonate. Whether the formation of the corresponding glucuronide occurs as a sequential reaction after formation of carbamic acid of I or whether the reaction proceeds through a concerted mechanism with a transition state involving the substrate, CO2, and UDPGA in the active site of UGTs cannot be deciphered at this time.

In the process of studying N-carbamoyl glucuronidation of carvedilol in dog and rat liver microsomes, Schaefer (1992) calculated the dissolved CO2 in the in vitro incubation medium. Bicarbonate buffer was reported to have 200-fold higher dissolved CO2 than phosphate buffer at atmospheric pressure. Furthermore, incubations containing bicarbonate buffer under a saturated CO2 environment resulted in 10-fold higher CO2 concentrations than those containing bicarbonate buffer devoid of a CO2 environment. We wanted to further elucidate the role of bicarbonate buffer in formation of 1-N-carbamoyl glucuronide. Therefore, the formation of M1 was evaluated in incubations containing NaHCO3 buffer at pH 7.4 and potassium phosphate buffer at pH 7.4. As measured by LC-MS/MS, formation of M1 was approximately 16-fold higher in the incubation containing NaHCO3 buffer at pH 7.4. As measured by LC-MS/MS, formation of M1 was reported to have 200-fold higher dissolved CO2 than phosphate buffer at atmospheric pressure. Furthermore, incubations containing bicarbonate buffer under a saturated CO2 environment resulted in 10-fold higher CO2 concentrations than those containing bicarbonate buffer devoid of a CO2 environment. We wanted to further elucidate the role of bicarbonate buffer in formation of 1-N-carbamoyl glucuronide. Therefore, the formation of M1 was evaluated in incubations containing NaHCO3 buffer at pH 7.4 and potassium phosphate buffer at pH 7.4. As measured by LC-MS/MS, formation of M1 was approximately 16-fold higher in the incubation containing NaHCO3 buffer than in the corresponding incubation in phosphate buffer, under an environment of CO2. This result supplements the equilibrium hypothesis and suggests that bicarbonate buffer may directly facilitate carbamic acid formation by this equilibrium mechanism. Hence, N-carbamoyl glucuronide formation because of higher amounts of dissolved CO2.

The preceding information reveals the lack of species specificity for formation of N-carbamoyl glucuronides. In vitro, N-carbamoyl glucuronides have been reported in a wide range of species, for example, for carvedilol in rat and dog liver microsomes (Schaefer, 1992), for BVT-2938 in rat, monkey, and human liver microsomal incubations (Edlund and Baranczewski, 2004), and for di-[25,35S]-2-amino-3-methyl-pentanoic-1,3-thiazolidine fumarate and its allo stereoisomer (Beconi et al., 2003). N-Carbamoyl glucuronides have been reported not only in in vitro incubations but also in in vivo tissues or body fluids. For example, the N-carbamoyl glucuronide conjugate of mefloquine was identified in dog urine (Dow et al., 1994), that of amosulalol in mouse bile (Suzuki and Kamimura, 2007), that of varenicline in rat, monkey, and human plasma and urine (Obach et al., 2006), that of ropinirole in monkey and human urine (Rami et al., 1999), and that of tocainide in urine of guinea pig, dog, cat, rabbit, and monkey (Gipple et al., 1982). We attempted to examine whether M1 was formed across multiple species and to measure the ratio of relative intensities of the N-carbamoyl glucuronide metabolite (M1/parent) across a variety of species. Among the species tested, the M1/parent ratio was maximal in liver microsomal incubations from rat and hamster, followed by comparable ratios in dog, human, monkey, and mouse (Fig. 6). The extent of M1 formation in vivo in the species mentioned earlier was beyond the scope of this investigation and remains unknown.

Literature reports on human UGT reaction phenotyping studies for N-carbamoylation reaction are even fewer. The varenicline-N-carbamoyl metabolite was reported to be formed by UGT2B7 only (Obach et al., 2006). Obach et al. (2005) reported that sertraline-N-carbamoyl glucuronidation was formed principally by UGT2B7 in vitro. Measurable activities were also observed with UGT1A3, UGT1A6, and UGT2B4. In the current investigation, we performed in vitro incubations with cloned expressed UGT Supersomes to identify the isozymes responsible for N-carbamoyl glucuronidation of I. We identified UGT1A1, UGT1A3, and UGT2B7 as being responsible for N-carbamoyl glucuronidation of I in vitro. Based on the metabolite/parent mass spectrometric responses (M1/parent) in incubations performed with recombinant UGT enzymes, UGT1A3 showed the maximal contribution in vitro (Fig. 7). It must be noted however, that these results are based on the relative activity of each UGT isoform in the Supersomes and may not represent the relative contribution of each UGT isoform in vivo.

N-Carbamoyl glucuronides, being rare, have not been well studied from a bioanalytical perspective. Liu and Pereira (2002) reported identification of an N-carbamoyl glucuronide of a thiazolidine-containing compound I, which was initially believed to be a mass spectroscopic interference. In addition, in-source fragmentation may lead to a dramatic decrease in signal or even lack of detection of N-carbamoyl glucuronides, especially if these glucuronides coelute with the parent. It is worth mentioning that more carbamoyl glucuronides have been identified in the past decade than before. Whether this identification can be attributed to newer mass spectrometers, advanced soft ionization techniques such as electrospray ionization, and advanced data acquisition techniques such as data-dependent scanning methodology or whether it is due to an increasing number of biotransformation studies on compounds containing primary and secondary amino groups remains to be ascertained.

Mechanistic studies with 13C-labeled CO2 suggest that formation of M1 was driven by exogenous CO2 (in equilibrium with CO2 dissolved in the buffer) in rat liver microsomes. This, to our knowledge, is the
first report documenting the formation of N-carbamoyl glucuronidation by use of $^{13}$CO$_2$ gas. Treatment of M1 with β-glucuronidase resulted in the formation of I and not the corresponding carbonate. It cannot be ascertained at this point whether I forms a carbamate before glucuronidation or whether the formation of M1 is a concerted process. Our data suggest that the N-carbamoyl glucuronide metabolite (M1) of I was observed in vitro in liver microsomes, across all the species tested, namely, rat, mouse, hamster, dog, monkey, and human. Based on the ratio of relative ion intensities (M1/parent), the N-carbamoyl glucuronide was formed in rat and hamster liver microsomes to a greater extent than in other species. UGT1A1, UGT1A3, and UGT2B7 (Supersomes) were observed to catalyze the N-carbamoyl glucuronidation of I. It is noteworthy that human UGTs other than human UGT2B7 may be involved in formation of carbamoyl glucuronides, as demonstrated by the novel N-carbamoyl glucuronide metabolite of I. In conclusion, our study demonstrates the MS*-based characterization, identification of mechanism of formation, and cross-species comparison of a novel N-carbamoyl glucuronide metabolite (M1), in addition to UGT phenotyping.

Acknowledgments. We thank Dr. Richard Sedrani and Sebastian Kopeck for providing compound I and George Marsh for supporting the in vivo experiments.

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