Short Communication

Synthesis and Identification of the Quaternary Ammonium-Linked Glucuronide of 1-Phenylimidazole in Human Liver Microsomes and Investigation of the Human UDP-Glucuronosyltransferases Involved

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ABSTRACT:

1-Phenylimidazole was investigated as a potential model substrate with respect to formation of a quaternary ammonium-linked glucuronide (N\textsuperscript{+}-glucuronide) at an aromatic type tertiary amine. A reference sample of the potential N\textsuperscript{+}-glucuronide metabolite of 1-phenylimidazole was obtained by organic synthesis. The structural identity of the metabolite formed by incubation of 1-phenylimidazole with human liver microsomes was proven to be the N\textsuperscript{+}-glucuronide by exhibiting the same HPLC retention time and electrospray ionization mass spectrum as the reference sample. The screening of 1-phenylimidazole against a panel of nine expressed human UDP-glucuronosyltransferases indicated the involvement of UGT1A3 and UGT1A4 in the formation of the N\textsuperscript{+}-glucuronide metabolite.

The glucuronidation of an aliphatic or aromatic tertiary amine functional group of a substrate leads to the formation of a polar quaternary ammonium-linked glucuronide metabolite (N\textsuperscript{+}-glucuronide) (Burchell et al., 1997). Such N\textsuperscript{+}-glucuronidation plays a significant role in the metabolic elimination of many aliphatic tertiary amine-containing therapeutic agents, including many H\textsubscript{1} antihistamine and tricyclic antidepressant drugs (Hawes, 1998). In contrast, although an aromatic tertiary amine is also a common structural feature of many xenobiotics, the N\textsuperscript{+}-glucuronide metabolite has been reported for only about ten substrates including major metabolites of anastrozole (McCann et al., 1997), lamotrigine (Sinz and Remmel, 1991), nicotine (Caldwell et al., 1992), and tioconazole (MacRae et al., 1990). In fact, there is a lack of knowledge regarding this metabolic pathway, including identification of the UDP-glucuronosyltransferase (UGT)\textsuperscript{1} enzymes involved (Green and Tephly, 1998) and substrate specificities. A monosubstituted imidazole was selected as a prototype substrate to study glucuronidation at an aromatic tertiary amine, since 5-membered polyaza ring systems are commonly encountered in drug structures, and imidazoles are a model for more complex systems in that they can be regarded to possess one aliphatic-like tertiary amine and one aromatic-type tertiary amine. Also, 1-phenylimidazole was studied because all the observed aromatic N\textsuperscript{+}-glucuronide metabolites of 5-membered polyaza ring systems have occurred in such N-substituted compounds, albeit with complex substituents, as with anastrozole and tioconazole (Midgley et al., 1981; Nakano et al., 1989; Takeuchi et al., 1989; MacRae et al., 1990; Rush et al., 1990, 1992; Sinz and Remmel, 1991; McCann et al., 1997).

Reported in the present work is the definitive identification of the N\textsuperscript{+}-glucuronide metabolite of 1-phenylimidazole and the delineation of the UGT enzymes involved after incubation of the substrate with human liver microsomes and nine expressed human UGT enzymes, respectively.

Materials and Methods

Chemicals. 1-Phenylimidazole (Transworld Chemicals, Rockville, MD), Tris base, alamethicin, ethyl 2-pyridylacetate, UDP-glucuronic acid (ammonium salt), magnesium chloride, β-saccharic acid 1,4-lactone (Sigma, St. Louis, MO), perchloric acid (BDH chemicals, Toronto), and lithium hydroxide (Aldrich, Milwaukee, WI) were of reagent grade. All organic solvents (EM Science, Gibbstown, NJ) were of HPLC grade.Double distilled water (18 ± 0.05 ohm cm), deionized and purified by a Milli-Q Water system, was used. HPLC mobile phase solvents were filtered through Millipore 0.45-μm filters before use.

Chemical Synthesis of β-1-Phenylimidazole N\textsuperscript{+}-Glucuronide. A mixture of 1-phenylimidazole (1) (0.72 g, 0.05 mol) and methyl (2,3,4-tri-O-acetyl-α-D-glucopyranosyl bromide) uronate (2) (2.8 g, 0.07 mol) (Fig. 1) was heated in a vial under nitrogen at 70°C for 24 h. The acetyl-protected α-bromo sugar was synthesized by a literature method (Aboul-Enein, 1977) and stored at −20°C until used. The reaction mixture melt was dissolved in methanol (15 ml) and filtered. The filtrate was made alkaline by adding lithium hydroxide (45 ml, 0.1 M), stirred for 15 min at room temperature and then extracted with ether (3 × 50 ml). The aqueous methanolic reaction mixture was made acidic to pH 6.0 with glacial acetic acid. The mixture was concentrated, and the aqueous layer was frozen and lyophilized. The solid material so obtained was dissolved in a minimum amount of water (5 ml) and then loaded on strong cation-exchange resin (Dowex 50W × 8, 50–100 mesh, 2.5 × 50 cm). The column was washed with four bed volumes of distilled water, and the quaternary ammonium salt was eluted with 2 M aqueous ammonia solution. The UV-absorbing fractions were collected and concentrated to give N\textsuperscript{+}-glucuronide as a colorless oil. A portion of the purified material was hydrolyzed by acidification of a portion of the product to give the free amine as a white solid.

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1 Abbreviations used are: UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; ESI, electrospray ionization; MS, mass spectrometry; CHAPS, (3-[3-cholamidopropyl)dimethylamino]-1-propane sulfonate.

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were collected and lyophilized under high vacuum. The solid residue so obtained contained a mixture of two compounds as indicated by two closely eluting peaks (peak 1, 
$R_t = 7.49$ min; peak 2, $R_t = 8.43$ min) in the HPLC chromatogram. These two compounds were separated by fractional crystallization from aqueous methanol and their structures were shown by $^1$H NMR and electrospray ionization mass spectrometry (ESI-MS) to be $\beta$-1-phenylimidazolide N$^\beta$-glucuronide (3) and 1-phenylimidazolide 4$^\prime$,5$^\prime$-ene N$^\gamma$-glucuronide (4), respectively.

$^1$H NMR Spectroscopy and Mass Spectrometry. $^1$H NMR spectra were recorded using a Bruker AMX 500 FT (500 MHz) spectrometer at ambient temperature. The mass spectrometer was a Micromass Quattro II Triple Quadrupole system operated in the positive ion mode under electrospray conditions.

1-Phenylimidazolide 4$^\prime$,5$^\prime$-ene N$^\gamma$-glucuronide (4). $^1$H NMR (D$_2$O): $\delta$ 4.10 (1H, m, sugar 3$^\prime$), 3.95 (1H, d, sugar 5$^\prime$, $J_{5,4} = 9.52$ Hz), 5.54 (1H, d, sugar 1$^\prime$, $J_{1,2} = 8.44$ Hz), 7.50–7.60 (5H, m, phenyl), 7.85 (1H, d, H-5 imidazole, $J_{4,5} = 2.16$ Hz) and 7.88 (1H, d, H-4 imidazole, $J_{4,5} = 2.14$ Hz). ESI-MS: $m/z$ (M$^+$) = 321.

Isolation and Rate of Formation of 1-Phenylimidazolide N$^\beta$-Glucuronide in Human Liver Microsomes. Microsomes were prepared from pooled human liver microsomes (equal weight taken from four livers) obtained from the International Institute for the Advancement of Medicine (Exton, PA) by differential centrifugation using a literature procedure (Huskey et al., 1993). The protein content of microsomal suspensions was determined by the method of Lowry et al. (1951) using human serum albumin as a reference standard. The standard reaction mixture (500 $\mu$L) for N-glucuronidation, consisting of MgCl$_2$ (10 mM), alamethicin (25 $\mu$g/mg of protein), UDPGA (5 mM), human liver microsomes (0.5 mg), Tris buffer (50 mM, pH 7.4), and 1-phenylimidazolide (1.25 mM) was incubated for 120 min at 37°C. The reaction was stopped by cooling on ice and adding aqueous perchloric acid (1%, 500 $\mu$L). The microsomal mixture was centrifuged at 9,000g for 10 min. The supernatant was loaded on an activated C-18 solid phase extraction cartridge (1 g, Varian Mega Bondelute) followed by washing with water (3 ml) and ether (2 ml), and dried by passing air. The N$^\beta$-glucuronide was eluted by methanol (1 ml) and subsequently analyzed by HPLC and ESI-MS.

N$^\beta$-Glucuronidation of 1-Phenylimidazolide by Expressed Human UDP-Glucuronosyltransferases. Microsomes from human B lymphoblastoid cells expressing UGT1A1, UGT1A4, UGT1A6, UGT1A9, and UGT2B15, and control microsomes (without vector) were purchased from Gentest (Woburn, MA). Microsomes from Sf9 insect cells infected with a baculovirus containing cDNA for human UGT1A3, UGT1A7, UGT1A10, and UGT2B7, and W1S9-WT control baculosomes were purchased from Panvera, Madison. The control products were used as negative controls for their respective UGT preparations. The microsomal mixture (200 $\mu$L) consisting of MgCl$_2$ (10 mM), saccharic acid lactone (3 mM), alamethicin (12.5 $\mu$g), UDPGA (5 mM), protein (0.2 mg), Tris buffer (50 mM, pH 7.4), and the substrate (1.25 mM) in 5 $\mu$L of methanol was incubated and subsequently analyzed as for the determination of the rate of glucuronidation in human liver microsomes.

Results and Discussion

The synthesis of $\beta$-1-phenylimidazolide N$^\beta$-glucuronide (3) was accomplished in two steps as depicted in Fig. 1. Initially, various commonly used approaches to the synthesis of glucuronide metabolites (Kaspersen and Van Boeckel, 1987; Stachulski and Jenkins, 1998) were investigated without success. For example, no evidence of reaction could be found when 1-phenylimidazole (I) and the acetyl-protected-α-sugar (2) were treated under either reflux in toluene (Stachulski and Jenkins, 1998) or phase transfer reaction conditions (aqueous sodium bicarbonate/benzene, room temperature) (Luo et al., 1992). However, the reported approach to the formation of N-glucuronides by treatment of the aglycone with the α-bromo sugar at mild temperature and without a catalyst (Caldwell et al., 1992) was successfully applied. Thus, reversed phase HPLC-UV analysis (cyano column) of the reaction product mixture obtained by keeping the melt of I and 2 under nitrogen for 24 h at 70°C indicated the presence of two major reaction products. These two products were identified by ESI-MS analysis of the residue obtained by collecting the appropriate fraction of mobile phase as the triacetyl ester derivatives of 1-phenylimidazolide N$^\beta$-glucuronide and 1-phenylimidazolide 4$^\prime$,5$^\prime$-ene N$^\gamma$-glucuronide (retention times: 16.25 and 17.45 min in the peak area ratio of 1:5.5). Hydrolysis of the reaction mixture containing these two intermediates occurred in highest yield with lithium hydroxide than with sodium hydroxide, sodium bicarbonate, or sodium carbonate. HPLC monitoring of the hydrolytic reaction with lithium hydroxide indicated the gradual disappearance of the esterified compounds.

![Fig. 1. Reaction scheme for the synthesis of 1-phenylimidazolide N$^\beta$-glucuronide.](http://image-url.com)
and the appearance of two products with much shorter retention times (7.49 and 8.43 min). The two products represented by these new peaks were separated and purified via strong cation-exchange resin chromatography and fractional crystallization. The identity and purity of the 1-phenylimidazole N\textsuperscript{1}-glucuronide (retention time 7.49 min, 5% yield) was proven by \textsuperscript{1}H NMR (D\textsubscript{2}O) and ESI-MS (M\textsuperscript{+} = 321, Fig. 3A). A doublet at δ 5.5 in the \textsuperscript{1}H NMR spectrum was assigned to the β-anomeric proton (C\textsubscript{1}-H) of the glucuronic acid moiety and the coupling constant of 8.44 Hz confirmed the β-anomeric assignment (J values for α and β anomers of glucuronides are in the range 2 to 4 Hz and 7 to 10 Hz, respectively) (Kaspersen and Van Boeckel, 1987). The other product was identified using similar instrumental techniques as the 1-phenylimidazole 4',5'-ene N\textsuperscript{1}-glucuronide (4, retention time 8.43 min, 10% yield). For the \textsuperscript{1}H NMR spectra of both 3 and 4, as expected (MacRae et al., 1990), no signal corresponding to the 2H proton of imidazole was observed due to deuterium exchange. It is noteworthy that there have been only a few other reports to the formation of 4',5'-ene by-products, despite the numerous reports of the synthesis of glucuronide metabolites (Stachulski and Jenkins, 1998). It is likely that due to the similarity of the chromatographic properties of these by-products and the desired glucuronide compounds they may be common, but infrequently recognized, impurities. The anomeric configuration of these synthetic by-products requires investigation.

Incubation of 1-phenylimidazole with activated human liver microsomes led to the formation of its N\textsuperscript{1}-glucuronide metabolite, which was isolated from the microsomal mixture by solid phase extraction. The structure of the metabolite was confirmed by comparing the ESI mass spectrum (M\textsuperscript{+} = 321, Fig. 3B) and HPLC retention time with that of the synthetic standard. The identity of the molecular ion peak

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**Fig. 2.** A HPLC chromatogram of extracts from incubation of 1-phenylimidazole with human liver microsomes and UDP-glucuronic acid in the presence of alamethicin.

**Fig. 3.** Positive ion electrospray mass spectrum of 1-phenylimidazole N\textsuperscript{1}-glucuronide obtained from synthesis (A) and human liver microsomal mixture (B).
Only human UGT1A3 and UGT1A4 have been shown to catalyze the glucuronidation of an aliphatic tertiary amine determined in human liver microsomes (Le Bigot et al., 1983; Dahl-Puustinen and Tephly, 1997), which were found to be optimal at pH 7.4 and 25 μg of alamethicin/mg of protein, respectively. The channel forming peptide alamethicin was found to be more effective than four examined detergents (emulgen 911, Triton X-100, lubrol PX, and CHAPS) in the activation of UGT catalytic activity (unpublished work). Saccharic acid lactone was added to inhibit any inherent glucuronidase activity present in the microsomes. Under optimum conditions the rate of glucuronidation of 1-phenylimidazole was found to be 270 ± 22 pmol/min/mg at a substrate concentration of 1.25 mM.

The human UGT isoform(s) involved in the aromatc N-glucuronidation of 1-phenylimidazole in human liver microsomes were optimized, including regarding pH and latency disrupting agents (Burchell et al., 1997), which were found to be optimal at pH 7.4 and 25 μg of alamethicin/mg of protein, respectively. The channel forming peptide alamethicin was found to be more effective than four examined detergents (emulgen 911, Triton X-100, lubrol PX, and CHAPS) in the activation of UGT catalytic activity (unpublished work). Saccharic acid lactone was added to inhibit any inherent glucuronidase activity present in the microsomes. Under optimum conditions the rate of glucuronidation of 1-phenylimidazole was found to be 270 ± 22 pmol/min/mg at a substrate concentration of 1.25 mM.

The human UGT isoform(s) involved in the aromatic N-glucuronidation of 1-phenylimidazole was investigated under the same conditions that were optimized with respect to human microsomes. Of the human UGT isoforms examined, UGT1A3 and UGT1A4 were the only glucuronidase that were screened, only UGT1A3 and UGT1A4 were shown to catalyze the formation of the N-glucuronide metabolite. The rates of formation of the metabolite under these nonoptimized screening conditions at 1.25 mM substrate concentration were 41.9 ± 4.1 and 27.3 ± 2.2 pmol/min/mg for UGT1A3 and UGT1A4, respectively (Fig. 4).

There are various reports to the enzymatic activities and kinetics for the glucuronidation of an aliphatic tertiary amine determined in human liver microsomes (Le Bigot et al., 1983; Dahl-Puustinen and Bertilsson, 1987; Coughtrie and Sharp, 1991; Styczynski et al., 1992; Breyer-Pfaff et al., 1997; Mey et al., 1999) and expressed UGT isoforms (Green et al., 1995, 1998; Green and Tephly, 1996, 1998). Only human UGT1A3 and UGT1A4 have been shown to catalyze aliphatic type N-glucuronidation, although it has been pointed out that UGT1A3 likely does not make a significant contribution to hepatic metabolism in vivo due, in part, to the low expression in human liver (Green and Tephly, 1998). However, the contribution of extrahepatic tissue to such metabolism needs clarification, since, for example, both UGT1A3 and UGT1A4 are expressed in the gastrointestinal tract (Strassburg et al., 1998). Regarding glucuronidation at an aromatic tertiary amine, comparative data regarding enzymatic activities and the UGT isoforms involved are lacking, in that the only reports involve lamotrigine (Magdalou et al., 1992; Green et al., 1995; Furan et al., 1999). Also that lamotrigine has both primary amine and aromatic heterocyclic aza atoms has been noted (Green et al., 1995). The present screening data for UGT1A3 and UGT1A4 catalysis of 1-phenylimidazole glucuronidation are in the range of activities reported for the glucuronidation of various aliphatic tertiary amine substrates (Green and Tephly, 1998). Therefore, the present data indicate that of the UGTs so far examined, glucuronidation at both aliphatic and aromatic tertiary amine functional groups is selectively catalyzed by the same two UGT isoforms. This gives further evidence to previous observation that whereas glucuronidation at a primary or secondary amine is catalyzed by many UGT isoforms, including UGT1A3, UGT1A4, UGT1A6, and UGT1A9, glucuronidation at a tertiary amine is catalyzed only by UGT1A3 and UGT1A4 (Green and Tephly, 1998).

This preliminary investigation demonstrated that 1-substituted imidazoles are appropriate substrates to undertake investigation of substrate specificities involving the N-glucuronidation of tertiary aromatic amines. With the availability of a synthetic sample, the N-glucuronide was definitively identified as a metabolite of 1-phenylimidazole in human liver microsomes. Of the human UGT isoforms examined, UGT1A3 and UGT1A4 were the only glucuronosyltransferases that demonstrated a catalytic activity in the formation of β-1-phenylimidazole N-glucuronide.

**Fig. 4. Rate of formation of 1-phenylimidazole N-glucuronide in microsomes derived from human expressed UGTs.**

Assays were conducted using 5 mM UDP-glucuronic acid and 1.25 mM 1-phenylimidazole. Glucuronidation rates are given as mean ± S.D. of three determinations. No 1-phenylimidazole N-glucuronide was detected with UGT1A1, UGT1A6, UGT1A7, UGT1A9, UGT1A10, UGT2B7, and UGT2B15.

![Graph showing rate of formation of 1-phenylimidazole N-glucuronide for different UGT isoforms](image-url)


