N-GLUCURONIDATION OF NICOTINE AND COTININE BY HUMAN LIVER MICROSOMES AND HETEROLOGOUSLY EXPRESSED UDP-GLUCURONOSYLTRANSFERASES

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ABSTRACT:

Nicotine is considered the major addictive agent in tobacco. Tobacco users extensively metabolize nicotine to cotinine. Both nicotine and cotinine undergo N-glucuronidation. Human liver microsomes have been shown to catalyze the formation of these N-glucuronides. However, which UDP-glucuronosyltransferases contribute to this catalysis has not been identified. To identify these enzymes, we initially measured the rates of glucuronidation by 15 human liver microsome samples. Fourteen of the samples glucuronidated both nicotine and cotinine at rates ranging from 146 to 673 pmol/min/mg protein and 140 to 908 pmol/min/mg protein, respectively. The rates of nicotine glucuronidation and cotinine glucuronidation by these 14 samples were correlated, $r = 0.97$ ($p < 0.0001$). The glucuronidation of nicotine and cotinine by heterologously expressed UGT1A3, UGT1A4, and UGT1A9 was also determined. All three enzymes catalyzed the glucuronidation of nicotine. However, the rate of catalysis by UGT1A4 Supersomes was more than 30-fold greater than that by either UGT1A3 Supersomes or UGT1A9 Supersomes. Interestingly, when expressed per UGT1A protein, measured by a UGT1A specific antibody, cell lysate from V79-expressed UGT1A9 catalyzed nicotine glucuronidation at a rate 17-fold greater than did UGT1A9 Supersomes. UGT1A4 Supersomes also catalyzed cotinine N-glucuronidation, but at one-tenth the rate of nicotine glucuronidation. Cotinine glucuronidation by either UGT1A3 or UGT1A9 was not detected. Both propofol, a UGT1A9 substrate, and imipramine, a UGT1A4 substrate, inhibited the glucuronidation of nicotine and cotinine by human liver microsomes. Taken together, these data support a role for both UGT1A9 and UGT1A4 in the catalysis of nicotine and cotinine N-glucuronidation.

Nicotine is the addictive compound in tobacco products (Benowitz, 1988). Metabolism of nicotine varies among individuals, and this variation has been suggested to contribute to both the frequency and intensity of smoking (Oscarson, 2001; Tyndale and Sellers, 2002). Nicotine is extensively metabolized to cotinine. The first step in the metabolism to cotinine is P450[1] 2A6-catalyzed 5′-oxidation. It has been suggested that altered P450 2A6 activity and expression influences smoking behavior (Oscarson, 2001). Variations in the extent of nicotine conversion to cotinine occurs in smokers (Benowitz and Jacob, 1994; Benowitz et al., 1994, 2002; Perez-Stable et al., 1998), and recently, a relationship between nicotine clearance and the intake of nicotine from cigarettes was reported in an Asian-American population (Benowitz et al., 2002).

In most individuals, nicotine 5′-oxidation is the major pathway of nicotine metabolism. However, nicotine is also metabolized to its N-glucuronide conjugate. Cotinine is also N-glucuronidated. Glucuro-

nide conjugates account for from 0 to 50% of the nicotine excreted by tobacco users and from 0 to 70% of the excreted cotinine (Byrd et al., 1992; Benowitz et al., 1994; Davis and Curvall, 1999). The N-glucuronide of cotinine (Fig. 1) has been synthesized and the structure of the cotinine glucuronide excreted by smokers has been confirmed (Caldwell et al., 1992). Nicotine contains two sites for glucuronide conjugation: the nitrogen atoms of the pyridine or pyrrolidine ring. Nicotine N-glucuronidated at the pyridine ring (Fig. 1) was synthesized and the presence of this glucuronide in the urine of smokers confirmed by LC-MS/MS analysis and comparison to the standard (Byrd et al., 2000, Gorrod and Schepers, 1999). However, whether or not the pyrrolidine N-glucuronide may also be formed in tobacco users is unknown, since typically nicotine glucuronides in human samples have been measured as the nicotine released by β-glucuronidase treatment.

There is significant individual variability in nicotine and cotinine glucuronidation among smokers, and it has been suggested that the same enzyme catalyzes the N-glucuronidation of both compounds (Benowitz et al., 1994; Nakajima et al., 2002c). It has been reported that black smokers excrete lower levels of nicotine and cotinine N-glucuronides than do white smokers. The authors of that study (Benowitz et al., 1999) have hypothesized that a subset of smokers, mostly black, are slow metabolizers with regard to nicotine and cotinine N-glucuronidation, suggesting that the enzyme catalyzing N-glucuronidation is polymorphic. It has also been suggested that the same enzyme may glucuronidate NNAL, a carcinogenic metabolite of the tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Benowitz et al., 1999).

[1] Abbreviations used are: P450, cytochrome P450; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; UGT, UDP-glucuronosyltransferase; propofol, 2,6-diisopropylphenol; UDPGA, UDP-glucuronic acid; HLM, human liver microsome(s); ESI, electrospray ionization; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography.

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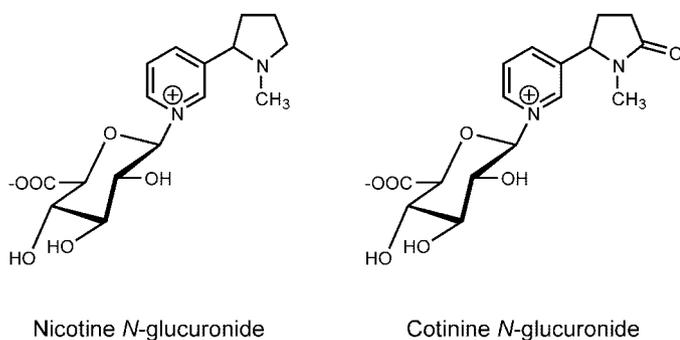
Fig. 1. *Nicotine and cotinine N-glucuronides.*

Glucuronidation reactions are catalyzed by UDP-glucuronosyltransferases (UGTs). Recently, the formation of nicotine and cotinine glucuronides by human liver microsomes was quantified by two laboratories (Ghosheh et al., 2001; Ghosheh and Hawes, 2002a,b; Nakajima et al., 2002a,c). However, the UGTs catalyzing the N-glucuronidation of nicotine and cotinine were not identified. The interindividual variation in the urinary excretion of nicotine and cotinine glucuronides that has been observed is most likely a result of differences in the protein expression of the UGTs catalyzing their formation. Among the UGTs in human liver, only UGT1A3 and UGT1A4, which are 93% identical in amino acid sequence, are reported to catalyze the formation of quaternary ammonium-linked glucuronides (Green et al., 1995; Green and Tephly, 1998; Vashishtha et al., 2001). However, UGT1A3 mRNA is expressed at much lower levels than is UGT1A4 in the liver (Mojarrabi et al., 1996). We previously reported that UGT1A9 catalyzed the N-glucuronidation of NNAL, a nicotine-derived nitrosamine (Nguyen et al., 2000). The product of this reaction is the N-glucuronide of the pyridine ring, which is analogous to the N-glucuronides of cotinine and nicotine. Therefore, the UGTs 1A3, 1A4, and 1A9 are all possible candidates as catalysts of nicotine and cotinine glucuronidation.

Cotinine levels are routinely used as a measure of tobacco exposure and nicotine metabolism in tobacco users. The extent of both cotinine and nicotine glucuronidation in individuals will clearly affect these levels. Therefore, knowing what enzymes contribute to the formation of these nicotine metabolites will increase our ability to interpret data on urinary cotinine and tobacco exposure. In addition, the levels of unmetabolized nicotine to which a smoker is exposed will depend on the rate of nicotine glucuronidation by that individual. Therefore, different levels of the enzymes that catalyze the N-glucuronidation of nicotine may contribute to any role nicotine metabolism plays in smoking behavior.

In the present study, we characterized the catalysis of nicotine and cotinine glucuronidation by 15 human liver microsome samples and by UGT1A3, UGT1A4, and UGT1A9. A role for UGT1A4 and UGT1A9 in the catalysis of nicotine and cotinine glucuronidation by human liver microsomes was further investigated by carrying out inhibition studies with imipramine and propofol, respectively.

Materials and Methods

Materials. Reagents for glucuronidation assays including UDPGA, saccharolactone, alamethicin, (−)-nicotine, (−)-cotinine, p-nitrophenol, imipramine-HCl, and propofol were purchased from Sigma-Aldrich (St. Louis, MO). The glucuronide conjugates of p-nitrophenol, nicotine, and cotinine were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Radiolabeled UDP-glucuronic acid [glucuronyl-^{14}C(U)] (specific activity >300 nCi/nmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). [5-^{3}H]-(S)-Nicotine (11 Ci/mmol) was synthesized from (S)-5′-bromonicotine by Moravek Biochemicals (Brea, CA). (S)-5′-Bromonornicotine was synthe-

sized as described previously (Jacob, 1982) and then methylated with formaldehyde and sodium borohydride to generate (S)-5′-bromonicotine, which was purified by distillation. HLM 2, HLM 3, HLM 5, HLM 6, HLM 10, and HLM 12 were a gift from Dr. Rory Remmel (University of Minnesota, Minneapolis, MN). Microsome samples not listed above were prepared from tissue samples provided by Dr. F. Peter Guengerich (Vanderbilt University, Nashville, TN) using previously published protocols (Fowler et al., 1994). UGT1A3, UGT1A4, and UGT1A9 Supersomes were purchased from BD Gentest (Woburn, MA). V79 (Chinese hamster fibroblast) cell lines overexpressing UGT1A9 were kindly donated by Dr. Brian Burchell (University of Dundee, Dundee, Scotland). Coomassie protein stain and standards were purchased from Pierce Endogen (Rockford, IL). Rabbit anti-UGT1A antibody (Strassburg et al., 1999) was a gift from Dr. Robert Tukey (University of California, San Diego, CA). The goat anti-rabbit IgG horseradish peroxidase-conjugated antibody was purchased from Amersham Biosciences Inc. (Piscataway, NJ).

Growth of V79 Cell Line. V79 cell lines expressing UGT1A9 were grown as described previously (Wooster et al., 1991) using Dulbecco's modified Eagle's medium containing Glutamax supplemented with 10% Nuserum I (New England Biolabs, Beverly, MA). Cells were screened with 0.2 mg/ml geneticin and harvested from 150×25 mm tissue culture dishes in phosphate-buffered saline. Cells were pelleted and resuspended in 10 mM Tris-acetate (pH 7.4), 1 mM EDTA, and 20% glycerol. Suspensions were subjected to three rounds of freeze-thawing before gentle homogenization. The cell lysate was stored at −80°C until analysis.

p-Nitrophenol Glucuronidation by Human Liver Microsomes. The glucuronidation of p-nitrophenol by 15 human liver microsome samples was assayed under the following conditions: 0.05 mg/ml protein, 0.025 mg/ml alamethicin (containing 0.01% ethanol), 500 μM p-nitrophenol, 5 mM UDPGA, 8.5 mM saccharolactone, and 10 mM MgCl$_2$ in 50 mM potassium phosphate (pH 7.1). The alamethicin concentrations and preincubation conditions used were as determined previously (Fisher et al., 2000) and confirmed with one HLM to result in the maximum rate. However, when a new solution of alamethicin was prepared at the same concentration, the rates varied, suggesting that the alamethicin obtained from Sigma-Aldrich varied between batches. Reactions were carried out in 100 μl at 37°C for 15 min before stopping with 1/10 volumes of 0.3 N ZnSO$_4$ and 0.3 N Ba(OH)$_2$ as previously described (Ren et al., 2000). p-Nitrophenol glucuronide was analyzed using HPLC System I and quantified by comparison to a standard curve. Reactions rates with HLM 134 were linear for 30 min with protein concentrations between 50 and 250 μg/ml protein.

Glucuronidation of Nicotine by Human Liver Microsomes. Glucuronidation of nicotine was assayed under the following conditions: 2 mg/ml protein, 0.1 mg/ml alamethicin, 5 mM [5-^{3}H]nicotine (3–6 nCi/nmol), 10 mM UDPGA, 8.5 mM saccharolactone, and 10 mM MgCl$_2$ in 50 mM potassium phosphate (pH 7.1) or 50 mM Tris-HCl (pH 8.9). As controls, samples were assayed in the absence of UDPGA. Nicotine glucuronidation was linear with time, from 20 to 80 min, and protein concentration, from 1 mg/ml to 4 mg/ml. Reaction rates catalyzed by HLM 109 were determined at 200 μM, 500 μM, 1 mM, 5 mM, 10 mM, and 20 mM nicotine. Reactions were carried out in 100-μl total volume for 1 h at 37°C in a shaking water bath and stopped with 5 μl of trifluoroacetic acid. Samples were mixed thoroughly and centrifuged, and the supernatant was passed through 0.2-μm nylon MicroSpin centrifuge filters (ChromTech, Inc., Apple Valley, MN) before analyzing by HPLC System II. Nicotine glucuronides produced by each sample coeluted with the standard. To confirm the identity of the nicotine glucuronides, ^{3}H-product and standard were collected from HPLC, hydrolyzed with 0.2 N NaOH at 80°C for 1 h, and then analyzed with HPLC System II. The percentage recovery of nicotine from the added standard was determined and the formation of [^{3}H] nicotine glucuronide was calculated. Some samples were assayed with 2 mM ^{14}C-labeled UDPGA (0.9 nCi/nmol) and unlabeled nicotine, then analyzed as outlined above.

Cotinine Glucuronidation by Human Liver Microsomes. Reactions were carried out according to the protocol for nicotine except that 10 mM [^{14}C]UDPGA (0.3 nCi/nmol) and 5 mM cotinine were used. Reactions were carried out in the presence and absence of cotinine. Glucuronidation rates were linear with time and protein content. Glucuronidation rates by HLM 109 were determined at 200 μM, 500 μM, 1 mM, 5 mM, 10 mM, and 20 mM cotinine. Cotinine

glucuronides were analyzed on HPLC System II, confirmed by coelution with standard and sensitivity to base hydrolysis.

MS/MS Analysis of Human Liver Microsome-Catalyzed Nicotine and Cotinine Glucuronidation. The identifications of nicotine and cotinine glucuronides were confirmed by collection from HPLC System II followed by analysis by mass spectrometry. Collected fractions were analyzed on a Thermo Finnigan (San Jose, CA) LCQ Deca instrument using electrospray ionization (ESI) with positive ion detection. Electrospray conditions used to generate MS data were as follows; voltage, 3 kV; current, 1.4 μA; and capillary temperature, 350°C. MS/MS data were obtained with source set as follows: capillary voltage, 2.85; activation amplitude, 30%; isolation width, 1.5 atomic mass units; activation Q, 0.25; and activation time, 30 ms. A continuous flow of 0.2 ml/min of 50% water/50% methanol with 1% acetic acid was passed through the ESI source, and into the flow was injected 10 μl of collected fraction with 10 μl of TFA (pH 1.56) collection buffer.

Propofol and Imipramine Inhibition of Nicotine and Cotinine Glucuronidation. Rates of nicotine and cotinine glucuronidation by human liver microsomes were measured in the presence of inhibitor, either 100 μM propofol (containing 0.05% methanol) or imipramine (100 μM or 1 mM) and compared with rates in the absence of inhibitor. Reaction conditions and analyses were as described above.

Imipramine and Propofol Glucuronidation Catalyzed by Human Liver Microsomes. The glucuronidation of imipramine catalyzed by human liver microsomes was determined in the presence and absence of 100 μM propofol. Microsomes (2 mg/ml) were incubated with 0.1 mg/ml alamethicin, 1 mM imipramine, 2 mM UDPGA, and 8.5 mM saccharolactone in 50 mM Tris-HCl (pH 8.9) containing 10 mM MgCl$_2$ for 60 min. Reactions were carried out in the presence and absence of both UDPGA and protein and terminated by the addition of 1/10 volumes of 0.3 N ZnSO$_4$ and 0.3 N Ba(OH)$_2$. Identification of the imipramine glucuronide was confirmed by the release of imipramine upon treatment with 0.2 N NaOH. Imipramine glucuronide formation was analyzed using HPLC System III (Nakajima et al., 2002b). Glucuronides were quantified by comparison of peak areas to a standard curve. Propofol glucuronidation rates were determined as for imipramine except that [^{14}C]UDPGA (0.8–2.0 mCi/mmol) and 1 mM propofol were used and reactions were terminated with an equal volume of methanol. Propofol glucuronide was quantified by radioflow HPLC using System IV.

Glucuronidation by Expressed UGTs. Nicotine glucuronidation by UGT1A3, UGT1A4, and UGT1A9 Supersomes and V79-expressed UGT1A9 cell lysate were quantified under the following conditions: 1 to 2 mg/ml protein, 0.05 to 0.1 mg/ml alamethicin, 5 mM [5-^{3}H]nicotine (10–20 nCi/nmol), 2 mM UDPGA, 3 mM saccharolactone, and 10 mM MgCl$_2$ in 50 mM potassium phosphate (pH 7.1) or 50 mM Tris-HCl (pH 8.9). Cotinine glucuronidation rates were determined using 2 mM [^{14}C]UDPGA (20 nCi/nmol) and 5 mM cotinine under the same conditions outlined above. The reaction volume was 100 μl and samples were terminated with 5 μl of 100% TFA after 2 h at 37°C in a shaking water bath. Samples were centrifuged at 4°C, and the supernatant was removed and centrifuged through MicroSpin filters before analyzing by HPLC System II.

Propofol glucuronidation activity with UGT1A4 and UGT1A9 Supersomes and V79-expressed UGT1A9 were determined with conditions as described above for nicotine except that 100 μM propofol and 2 mM [^{14}C]UDPGA (4.8 nCi/nmol) were used as substrates and the incubation time was 1 h. Reactions were stopped by the addition of equal volumes of methanol. Propofol glucuronide quantification was done with HPLC System IV. The detection of propofol glucuronide peak was dependent on both enzyme and propofol.

LC-MS/MS Analysis of Nicotine and Cotinine Glucuronidation by UGTs. UGT reactions were carried out as described above with nonradioactive substrates. HPLC analysis was with System II except that the mobile phase was 0.025% TFA (pH 2.5). Detection was by reaction ion monitoring on a Thermo Finnigan TSQ 7000 instrument using electrospray ionization, m/z 339 for nicotine and m/z 353 for cotinine, each with loss of 176. The ESI source was set as follows: voltage, 2 kV; capillary temperature, 280°C; collisional gas, 2 mTorr; collisional voltage, −20 V; auxiliary gas, 30; and sheath gas, 100. Standard curves were generated with 1.5 to 120 pmol of nicotine and cotinine glucuronide standards. Samples were analyzed twice, once without added glucuronide standard and once with the addition of standard at a concentration similar to that of the glucuronide detected in the sample.

Western Blot Analysis of UGT1A in Overexpressed Cell Lines. Western blots were carried out according to the protocol outlined by Nguyen and Tukey (1997) with minor modifications. Fifty nanograms to 5 μg of total protein from Supersomes, UGT1A3, UGT1A4, UGT1A9, and V79-expressed UGT1A9 cell lysate were analyzed. A 1:1,000 dilution of rabbit anti-UGT1A antibody (Strassburg et al., 1999) with a 1:100,000 dilution of goat anti-rabbit IgG with horseradish peroxidase conjugate was used. Membrane probing was carried out for 1 h with each antibody at 37°C on an orbital shaker. UGT1A protein was visualized by chemiluminescence with the ECL Plus Kit (Amersham Biosciences) according to the instructions outlined by the manufacturer. The relative levels of UGT1A protein in each sample were determined by volume analysis on a Storm 840 scanning densitometer under conditions in which the densitometry response was linear with protein for each sample.

HPLC Systems. The HPLC systems consisted of either Shimadzu LC-10ADvp pumps and a Shimadzu SPD-10ADvp spectrophotometer (Shimadzu, Kyoto, Japan) or Waters 510 solvent delivery pumps and a Waters λ-Max model 480 LC spectrophotometer with a Waters model 710B autoinjector (Waters, Milford, MA). For radioactive detection, a β-RAM radioflow detector (IN/US Systems, Tampa, FL) was used. System I used a Phenomenex (Torrance, CA) 10-μm C18 Bondclone column (3.9 × 300 mm); p-nitrophenol glucuronide was eluted with 90% [25 mM triethylamine (pH 2.1)]:10% acetonitrile (Hanioka et al., 2001) at a flow rate of 1 ml/min. Absorbance was measured at 304 nm. System II was that used previously to analyze cotinine and nicotine (Hecht et al., 2000). Nicotine and cotinine N-glucuronide standards eluted at 7 and 15 min, respectively. Detection of cotinine and nicotine glucuronide metabolites was by radioactivity. System III used the C18 Bondclone column of System I and a mobile phase similar to that used by Nakajima et al. (2002). Imipramine glucuronide was eluted with 70% [50 mM potassium phosphate (pH 5)] and 30% acetonitrile. The flow rate was 1 ml/min. Imipramine and its glucuronide eluted at 36 and 12 min, respectively. System IV used a C18 Bondclone column. The elution gradient used, with a flow rate of 1 ml/min, was from 50% 25 mM triethylamine (pH 2.1) and 50% methanol to 100% methanol in 20 min, and then held at 100% methanol for 10 min. Propofol glucuronide eluted at 20 min and propofol at 23 min. Detection was by radioactivity.

NMR Analysis of Nicotine N-Glucuronide. Reactions with either HLM 129 or UGT1A4 Supersomes (2.5 mg/500 μl) were carried out as described previously in the absence of radiolabel. Nicotine glucuronide metabolites were purified by HPLC with a 5-μm Hypersil Hypercarb column (Thermo Finnigan; 100 × 3 mm) eluted with water/methanol as follows: 100% water for 10 min, then a linear gradient to 50% methanol in 40 min. Nicotine N-glucuronide standard eluted at 29 min, and glucuronides formed by UGT1A4 and human liver microsomes were collected at this retention time. Samples were evaporated to dryness and resuspended in CD$_3$OD. ^{1}H NMR spectra were acquired at 25°C on a Varian 800 MHz NMR spectrometer (NMR facility, University of Minnesota) δ 9.2–9.0 (d, 2H, pyridinium 2 and 6), 8.7 (m, 1H, pyridinium 4), 8.1 (m, 1H, pyridinium 5), 5.7 (s, 1H, anomeric proton 1′), 4.02 (m, 1H, glucuronide 5′), 3.7–3.6 (m, 2H, glucuronide 3′, 4′), 3.55 (m, 1H, pyrrolidine 2), 3.45 (m, 1H, 2′), 3.3 (m, 1H, pyrrolidinium 5b), 2.5 (m, 1H, pyrrolidine 5a), 2.4 (m, 1H, pyrrolidine 3b), 2.3 (s, 3H, N-CH$_3$), 2.0 (m, 1H, pyrrolidinium 3a), 1.9 (m, 1H, pyrrolidinium 4a), and 1.8 (m, 1H, pyrrolidinium 4b). This spectrum is identical to that obtained for the pyridine N-glucuronide standard.

Results

The rates of p-nitrophenol glucuronidation by 15 human liver microsome samples were determined. This planar phenol is primarily glucuronidated by UGT1A6 and UGT1A9 (Ethell et al., 2002) and, to a lesser extent, by other UGTs. This lipophilic aglycone was used to 1) determine that all human liver microsome preparations had glucuronidation activity and 2) to establish conditions at which to measure glucuronidation activity.

The rate of p-nitrophenol glucuronidation catalyzed by HLM 129 was determined in the presence of a 5:1 ratio by weight of protein to lysophosphatidylcholine or a 20:1 ratio of protein to alamethicin. The rate was 2-fold higher in the presence of alamethicin when compared with lysophosphatidylcholine. When the UDPGA concentration was

TABLE 1

Rates of glucuronidation by human liver microsomes

Rates for each substrate were determined as described under *Materials and Methods* and are the average of duplicate determinations, except in the case of nicotine and cotinine, where rates are the average of two independent experiments each carried out in duplicate. All duplicate values varied by less than 10%.

Sample	Substrates				
	PNP[a]	Nicotine[b]	Cotinine[b]	Imipramine[c]	Propofol[c]
109	34[d]	406	558	–[e]	–
123	32[d]	N.D.[f]	N.D.	115	N.D.
126	75	146	140	109	25
127	42[d]	326	467	338	77
129	59,35[d]	212	325	260	160
130	53	154	191	218	75
132	89	184	231	273	170
133	77	348	388	209	48
134	31[d]	347	545	420	173
2	53	673	869	–	–
3	48	616	908	–	–
5	48	207	239	–	–
6	47	324	469	–	–
10	95	432	748	–	–
12	48	367	523	–	–

[a] Rates (nmol/min/mg protein) were determined with 0.5 mM PNP and 5 mM UDPGA.

[b] Rates (pmol/min/ml) were determined at pH 7.1 with 5 mM [^{3}H]nicotine (3 nCi/nmol) and 10 mM UDPGA or 5 mM cotinine with 10 mM [^{14}C]UDPGA (1 nCi/nmol).

[c] Imipramine, 1 mM, and propofol, 1 mM, glucuronidation rates (pmol/min/mg) were determined with 2 mM UDPGA or 2 mM [^{14}C]UDPGA (4 nCi/nmol), respectively.

[d] Rates were determined with a different preparation of alamethicin from that used for other determinations.

[e] –, not determined.

[f] N.D., not detected; limit of detection: nicotine, 50 pmol/min/mg protein; cotinine, 100 pmol/min/mg protein; imipramine, 50 pmol/min/mg protein; and propofol, 20 pmol/min/mg protein.

FIG. 2. *Radioflow HPLC analysis of nicotine glucuronidation by human liver microsomes.*

[5-^{3}H]Nicotine (5 mM, 3 nCi/nmol) was incubated with UDPGA and human liver microsomes for 60 min. Panel A, analysis of reaction mixture. Arrows indicate retention time of coinjected standards. Panel B, analysis of the peak collected at 7 min (A) following treatment with 1 M NaOH. Details of reactions and analyses are as described under *Materials and Methods*.

increased from 2 mM to 5 mM, the rate of *p*-nitrophenol glucuronidation with alamethicin increased 8%. All 15 samples had quantifiable rates of *p*-nitrophenol glucuronidation (Table 1). The rates varied about 3-fold among samples, ranging from 31 to 95 nmol/min/mg. Some of this variability may be due to differences in alamethicin solutions. The rate of *p*-nitrophenol glucuronidation by human liver microsomes 129 was either 59 or 35 nmol/min/mg, depending on the preparation of alamethicin used. A single alamethicin solution was used for all subsequent experiments.

Catalysis of [5-^{3}H]nicotine glucuronidation by human liver microsomes was quantified by radioflow HPLC (Fig. 2). One radioactive product coeluting with nicotine *N*-glucuronide standard was detected (7 min, Fig. 2A). The peak eluting at 4 min was formed in the absence of UDPGA and was not analyzed further. When the metabolite at 7 min was collected and treated with base, it was quantitatively converted to [5-^{3}H]nicotine (Fig. 2B). When [^{14}C]UDPGA and unlabeled nicotine were used in the analysis of human liver microsome-catalyzed nicotine glucuronidation, the results were quantitatively identical (data not shown).

The 7-min metabolite isolated by HPLC was further analyzed by electrospray ionization MS. A single ion was detected, *m/z* 339, protonated nicotine glucuronide. Product ion spectrum of *m/z* 339 contained a single ion, *m/z* 163, consistent with the neutral loss of glucuronic acid, which is characteristic of glucuronide conjugates. Identical results were obtained from MS and MS/MS analysis of the nicotine glucuronide standard.

Nicotine glucuronidation was determined for all 15 samples at pH 7.1. The rate of nicotine glucuronidation by HLM 109 increased approximately 8-fold, from 43 to 329 pmol/min/mg, when the concentration of nicotine was increased from 200 μM to 5 mM (data not shown). An increase in nicotine concentration from 5 to 20 mM increased glucuronidation rates another 2-fold. Nicotine glucuronidation rates by the remaining samples were determined at 5 mM nico-

tine. Fourteen of the 15 samples catalyzed nicotine glucuronidation at rates ranging from 146 to 673 pmol/min/mg (Table 1). HLM 123 failed to produce detectable nicotine glucuronide. However, this sample had *p*-nitrophenol glucuronidation activity similar to that of other samples. HLM 123 also catalyzed imipramine glucuronidation but not propofol glucuronidation (Table 1).

When cotinine glucuronidation by human liver microsome samples was quantified by radioflow HPLC using [^{14}C]UDPGA, a single product peak was detected. It coeluted with cotinine glucuronide standard and, when collected and treated with base, quantitatively released [^{14}C]glucuronic acid. Identification of this metabolite as cotinine glucuronide was confirmed by mass spectrometry. The metabolite was collected from HPLC and analyzed by electrospray ionization MS. A single ion was detected, protonated cotinine glucuronide, *m/z* 353. Subsequent MS/MS analysis of *m/z* 353 ion generated a single ion, *m/z* 177, protonated cotinine. This fragment is due to neutral loss of the glucuronic acid moiety. Analysis of cotinine *N*-glucuronide standard gave the same results.

The rate of cotinine glucuronidation by HLM 109 was determined over a range of concentrations. The glucuronidation rate increased more than 20-fold when the concentration of cotinine was increased from 200 μM to 5 mM. An increase in cotinine concentrations from 5 mM to 20 mM increased glucuronidation rates by 1.6-fold. Glucuronidation rates by all human liver microsome samples were determined at 5 mM cotinine. Cotinine was glucuronidated by 14 of 15 samples at rates ranging from 140 to 908 pmol/min/mg protein (Table 1). HLM 123 did not produce any detectable amount of cotinine glucuronide. Cotinine glucuronidation rates were equal to or higher than nicotine glucuronidation rates. The rates of nicotine glucuronida-
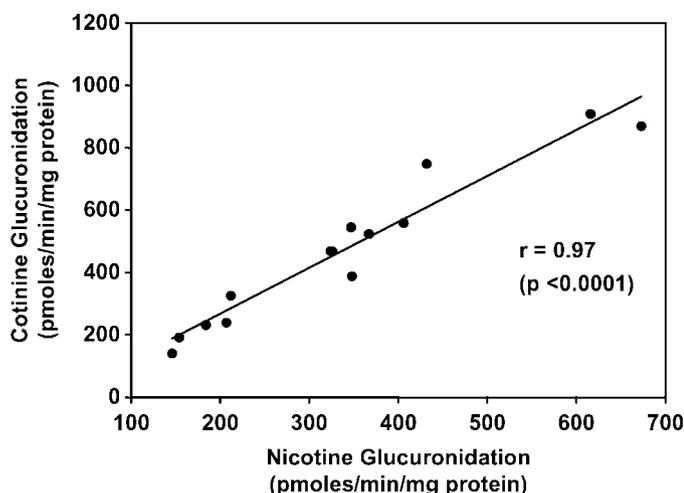
FIG. 3. *Correlation of nicotine and cotinine glucuronidation by human liver microsomes.*

tion at pH 7.1 correlated well with cotinine glucuronidation rates for these 14 samples (Fig. 3), $r = 0.97$ ($p < 0.0001$). Neither the nicotine nor the cotinine glucuronidation rate correlated with the rates of p-nitrophenol glucuronidation ($r = 0.20$ and 0.24, respectively, and $p > 0.4$).

The effect of increased pH on nicotine and cotinine glucuronidation by six human liver microsome samples was determined. The pK_a of the pyrrolidine ring nitrogen is 7.8; therefore, at pH 7.1, 83% of the nicotine is protonated, but at pH 8.9, this decreases to 8%; hence, a 10-fold increase in the more lipophilic free base nicotine occurs. Nicotine glucuronidation rates increased between 1.2- and 2-fold when the pH was increased from 7.1 to 8.9. The pH effect was greater at lower nicotine concentrations (data not shown). A change in pH between 7.1 and 8.9 will have no effect on cotinine protonation. However, the cotinine glucuronidation rates measured at pH 8.9 increased between 1.7- and 2.1-fold when compared with the rates at pH 7.1. Increases varied among samples. For the six human liver microsome samples analyzed at pH 8.9, the rates of nicotine and cotinine glucuronidation were not correlated ($r = 0.67$, $p = 0.15$). The correlation at pH 7.1 for these same six samples was 0.90 ($p = 0.015$).

The relative rates of nicotine and cotinine glucuronidation by UGT1A3, UGT1A4, and UGT1A9 Supersomes were determined. Analyses were first carried out using [5-^{3}H]nicotine or [^{14}C]UDPGA and radioflow HPLC. A radioactive product that coeluted with nicotine N-glucuronide standard was detected. However, due to the relatively large amount of [^{3}H]nicotine used, a significant amount of radioactivity was also detected in this region in control reactions containing no UDPGA. Therefore, it was not possible to quantify the glucuronide product directly. To quantify the formation of nicotine N-glucuronide, the metabolite peak was treated with base and reanalyzed by radioflow HPLC, and the radioactivity coeluting with nicotine was determined. After a 2-h incubation, the extent of UGT1A4-catalyzed nicotine glucuronidation was 2 nmol/mg protein. [5-^{3}H]Nicotine glucuronide was detected as a product of both UGT1A3 Supersomes and V79-expressed UGT1A9 metabolism. The extent of glucuronidation by these two enzymes (per milligram of total protein) was less than 10% of that by UG1A4; therefore, quantitation was difficult.

LC-MS/MS analysis was used to further characterize and quantify the extent of nicotine glucuronidation by UGT1A3, UGT1A4, and UGT1A9. The glucuronide products were detected by monitoring the neutral loss of 176 from m/z 339 (Fig. 4). To increase the sensitivity
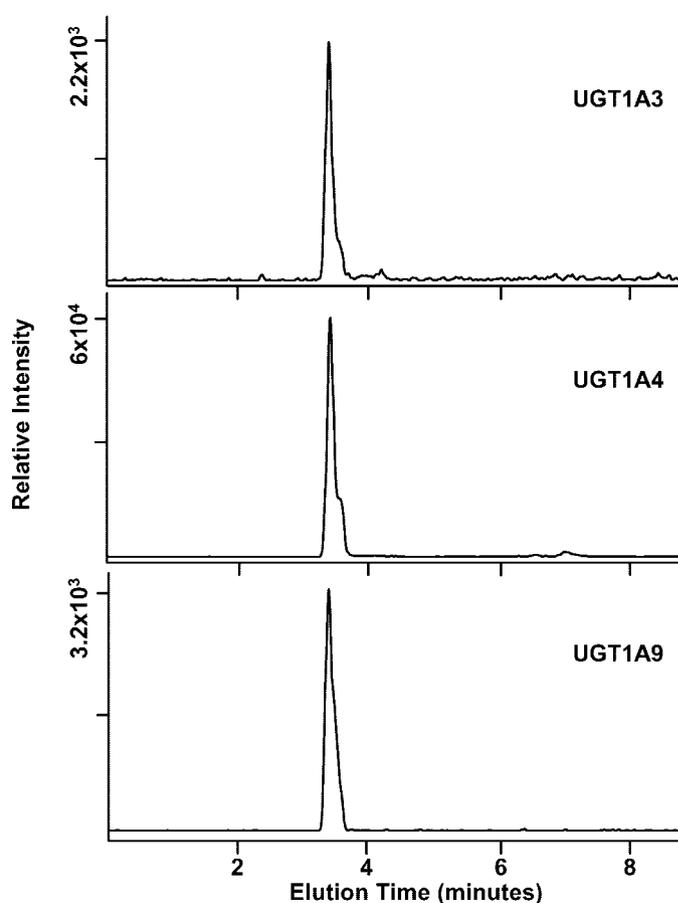
FIG. 4. *LC-MS/MS analysis of nicotine glucuronidation catalyzed by expressed UGTs.*

Nicotine (5 mM) was incubated with either UGT1A3 or UGT1A4 Supersomes (1 mg/ml) or V79-expressed UGT1A9 cell-lysate (2 mg/ml) and 2 mM UDPGA, pH 8.9. Detection was by reaction ion monitoring for a loss of 176 from m/z 363.

of glucuronide detection by LC-MS/MS, the TFA concentration of the mobile phase was decreased relative to that used for radioflow HPLC analysis. The lower concentration of TFA resulted in a decrease in the retention time of the nicotine N-glucuronide to 3.5 min (Fig. 4) from 7 min (Fig. 2). Analysis by LC-MS/MS confirmed that all three UGTs catalyzed the glucuronidation of nicotine at pH 8.9 (Fig. 4). The extent of glucuronidation by UGT1A4 Supersomes was 1,550 pmol/mg after a 2-h incubation, whereas the rates with UGT1A3 and UGT1A9 Supersomes were 50 and 20, respectively (Table 2). Interestingly, the extent of glucuronidation (expressed per milligram of total protein) by V79-expressed UGT1A9 cell lysates was about 2-fold greater than that by UGT1A9 Supersomes. In contrast, the rate of propofol glucuronidation by V79-expressed UGT1A9 cell lysates was one half the rate by UGT1A9 Supersomes, 98 versus 200 pmol/min/mg.

As noted above, the nicotine glucuronide product of both UGT1A4 and human liver microsomal metabolism coeluted with the pyridine N-glucuronide standard of nicotine. However, no standard for the pyrrolidine N-glucuronide was available, and these two glucuronides may coelute in the HPLC system used. Therefore, to confirm that UGT1A4 and human liver microsomes are generating the same N-glucuronide, the nicotine glucuronide metabolite formed by each human liver microsome was purified by HPLC and the NMR spectrum was obtained. The spectra obtained were identical to that of the pyridine N-glucuronide standard. There was no evidence for the formation of the pyrrolidine glucuronide metabolite with either UGT1A4 or human liver microsomes.

TABLE 2

LC/MS-MS determination of nicotine glucuronidation by UGT1A3, UGT1A4, and UGT1A9

Nicotine glucuronide was detected by LC/MS-MS after a 2-h incubation of enzyme preparations with 5 mM nicotine and 2 mM UDPGA. Quantification was by the addition of known quantities of nicotine glucuronide. Details are described under *Materials and Methods*.

UGT	Rate	Relative Expression[a]	Relative Rate[b]
	pmol/mg		
1A3 Supersomes	52	13	4
1A4 Supersomes	1550	13	119
1A9 Supersomes	20	13	2
V79 1A9	35	1	35

[a] Relative protein expression was determined by Western blot analysis with an antibody directed against UGT1A enzymes as described under *Materials and Methods*.
[b] Rates are adjusted for the relative levels of UGT1A protein expressed per milligram of total protein.

TABLE 3

Propofol inhibition of HLM-catalyzed N-glucuronidation

Rates were determined for each substrate in duplicate in the presence or absence of 100 μM propofol. Duplicates agreed within 10%.

	Percentage Inhibition of *N*-Glucuronidation				
Sample		Nicotine[b]		Cotinine[c]	
	Imipramine[a] (pH 8.9)	pH 7.1	pH 8.9	pH 7.1	pH 8.9
109	9	16	4	70	35
127	6	45	3	58	18
130	0	32	6	64	41
132	5	30	17	61	32
133	0	16	27	64	42
134	3	25	24	66	42

[a] 1 mM imipramine and 2 mM UDPGA.
[b] 5 mM nicotine and 2 mM [^{14}C]UDPGA (1–2 nCi/nmol).
[c] 5 mM cotinine and 2 mM [^{14}C]UDPGA (0.5 nCi/nmol).

Western blot analysis using a UGT1A specific antibody was carried out to determine the relative amount of UGT1A protein in each of the enzyme preparations. All three UGT Supersomes preparations had similar amounts of UGT1A protein. However, the V79-expressed UGT1A9 cell lysate compared with UGT1A9 Supersomes had 1/13th the amount of UGT1A-reactive protein (Table 2). Therefore, the relative rates of UGT1A9 Supersomes and V79 UGT1A9 lysate per UGT1A protein were 2 and 35, respectively. The relative rate of UGT1A4 Supersomes was 119 (Table 2).

The ability of UGT1A3, UGT1A4, and UGT1A9 to catalyze the *N*-glucuronidation of cotinine was also determined. Analysis was by LC-MS/MS. A detectable level of cotinine glucuronide was formed by UGT1A4, but the amount was about 10% of the nicotine glucuronide formed under the same conditions (data not shown). This is in contrast to what was observed with human liver microsomes, which catalyzed cotinine glucuronidation to a greater extent than nicotine glucuronidation. Unlike nicotine glucuronidation, which was detected at pH 8.9, but not pH 7.1, cotinine glucuronidation was detected only at pH 7.1. Neither UGT1A3 nor UGT1A9 catalyzed cotinine glucuronidation at detectable levels.

To determine the relative contribution of UGT1A9 to both nicotine and cotinine glucuronidation by human liver microsomes, propofol was used as a competitive inhibitor. Propofol is proposed to be a selective substrate for UGT1A9 glucuronidation (Ebner and Burchell, 1993; Lockley et al., 2002). UGT1A9 Supersomes glucuronidated propofol at a rate of 200 pmol/min/mg protein, and as previously reported, propofol glucuronidation was not catalyzed by UGT1A4. The rates of propofol glucuronidation by human liver microsomes ranged from 25 to 173 pmol/min/mg protein (Table 1).

The possibility that propofol might inhibit UGT1A4 activity by human liver microsomes was investigated. Glucuronidation of imipramine is reported to be carried out by UGT1A4 (Nakajima et al., 2002b). The rate of imipramine glucuronidation catalyzed by human liver microsomes was measured in the absence and presence of 100 μM propofol. Among the six samples analyzed, the rates of imipramine glucuronidation varied 4-fold between 109 and 420 pmol/min/mg protein (Table 1). The addition of propofol had no effect on these rates (Table 3). These data suggest that 100 μM propofol is not inhibiting UGT1A4 activity in human liver microsomes.

The effect of propofol on human liver microsome-catalyzed nicotine and cotinine glucuronidation was determined at both pH 7.1 and 8.9 (Table 3). At pH 7.1, the addition of propofol decreased nicotine glucuronidation rates between 16 and 42%, but at pH 8.9, the rates were inhibited as little as 3%. We hypothesized that this difference in inhibition may simply be due to the increase in nicotine lipophilicity at pH 8.9, effectively resulting in a higher nicotine concentration at

pH 8.9 versus pH 7.1. As expected at lower nicotine concentrations, 200 μM and 20 μM, propofol is a more effective inhibitor of nicotine glucuronidation than it is at 5 mM nicotine (Fig. 5). At pH 7.1, propofol inhibited cotinine glucuronidation by human liver microsome 58% to 70%. When the reactions were run at pH 8.9, the extent of inhibition was less, ranging from 18 to 42% (Table 3). With either cotinine or nicotine as a substrate, the samples that were inhibited to the greatest extent at pH 7.l were not the samples in which we saw the highest inhibition at pH 8.9.

Imipramine inhibition of human liver microsome-catalyzed nicotine and cotinine glucuronidation was also determined. Investigators have used this UGT1A4 substrate to determine the extent to which UGT1A4 contributes to the glucuronidation of amines (Stevens et al., 2001) and recently to investigate a role for UGT1A4 in nicotine metabolism (Ghosheh and Hawes 2002b; Nakajima et al., 2002b). Addition of 1 mM imipramine to incubations containing 5 mM nicotine or cotinine completely inhibited nicotine and cotinine *N*-glucuronidation. At this concentration of imipramine, the glucuronidation of propofol was also significantly inhibited, from 41 to 53% (data not shown). Interestingly, the rates of *p*-nitrophenol glucuronidation by human liver microsome samples increased slightly when these reactions were carried out in the presence of 1 mM imipramine. Similarly, a more than 2-fold enhancement in activity was observed for expressed UGT1A4- and UGT1A9-catalyzed *p*-nitrophenol glucuronidation when imipramine was present. When the concentration of imipramine was decreased to 100 μM, human liver microsome-catalyzed propofol glucuronidation was no longer inhibited and nicotine glucuronidation was inhibited between 27 and 38% ($n = 5$, data not shown). The imipramine inhibition experiments were repeated for two samples, HLM 127 and HLM 134, with a lower nicotine concentration of 200 μM. The extent of imipramine inhibition increased slightly for each sample, from 32% to 42% for HLM 127 (Fig. 5) and from 25% to 40% for HLM 134.

Discussion

We report here the characterization of nicotine and cotinine glucuronidation by human liver microsomes and provide evidence that both UGT1A4 and UGT1A9 are catalysts of these reactions. Fourteen of the 15 samples that were analyzed catalyzed detectable rates of both nicotine and cotinine *N*-glucuronidation. By NMR analysis we confirmed that the product of UGT1A4-catalyzed nicotine metabolism was the pyridine *N*-glucuronide. As reported by Nakajima et al. (2002c), the rate of cotinine glucuronidation was equal to or greater than that of nicotine glucuronidation, and the rates were significantly correlated with each other. However, it is important to note that this
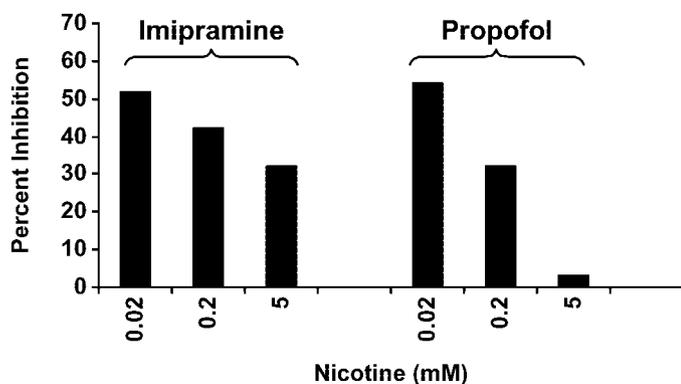
Fig. 5. *Inhibition of human liver microsome-catalyzed nicotine glucuronidation by imipramine and propofol.*

HLM 127 was incubated with the indicated concentrations of nicotine in the presence or absence of inhibitor (100 μM imipramine or 100 μM propofol), and the rate of nicotine glucuronidation was determined at pH 8.9. Details of the analysis are described under *Materials and Methods*. All nine reactions were carried out in duplicate, and rates agreed within 5% for 0.2 mM and 5 mM nicotine and within 15% for 0.02 mM. Percentage of inhibition was calculated from the means of each duplicate determination.

correlation was no longer significant when the rates of glucuronidation were determined at pH 8.9.

The influence of pH on the correlation between these two reactions may be due to the contribution of more than one enzyme and the result of two pH effects. One effect would be an increase in lipophilicity of nicotine at pH 8.9 versus 7.1, resulting in an effective increase in nicotine concentration at the active site of the enzyme. The second effect of increasing pH may be due to different pH optima for enzymes catalyzing nicotine and/or cotinine glucuronidation. Our data do not distinguish between these two possibilities.

A 1.2- to 2-fold increase in activity was observed for both cotinine and nicotine glucuronidation when the pH was changed from 7.1 to 8.9. However, the increased rates of glucuronidation for each substrate varied with sample. Ghosheh et al. (2002b) previously reported a 5-fold increase in the rate of nicotine glucuronidation by human liver microsomes when the pH was increased from 7.4 to 8.4, and a smaller, about 2.5-fold, increase in cotinine glucuronidation for this same increase in pH. It is unclear from the literature if the same 5-fold increase in nicotine glucuronidation rate was observed over a range of nicotine concentrations. The ratio of protonated to unprotonated nicotine would decrease from 2.5 to 0.25 when the pH is increased from 7.4 to 8.4. Therefore, the concentration of unprotonated nicotine, a lipophilic compound, would increase 3.5-fold over this range. This change in concentration may have a greater effect on the rate of glucuronidation at nicotine concentrations lower than the millimolar concentrations used in the present study.

It was reported by Nakajima et al. (2002c) that both imipramine and propofol were inhibitors of nicotine and cotinine glucuronidation by human liver microsomes. Imipramine is a substrate for UGT1A4 and propofol for UGT1A9 (Ebner and Burchell, 1993; Green et al., 1995; Nakajima et al., 2002b). Therefore, it was suggested that both these UGTs may play a role in nicotine and cotinine glucuronidation (Nakajima et al., 2002c). Our data support this conclusion. Imipramine and propofol inhibited both nicotine and cotinine *N*-glucuronidation by human liver microsomes (Table 3), and propofol inhibited nicotine glucuronidation to a greater extent at lower nicotine concentrations (Fig. 5). The latter data lead us to suggest that UGT1A9 may contribute to a greater percentage of glucuronidation at lower nicotine concentrations.

With the hypothesis that both UGT1A9 and UGT1A4 contribute to nicotine and cotinine glucuronidation, it is interesting to interpret some of the data for individual samples. For example, with HLM 123, we did not detect any nicotine or cotinine glucuronidation activity. However, this sample did catalyze the glucuronidation of imipramine at rates similar to those of other samples (Table 1). Is low or no UGT1A9 activity in HLM 123 the reason nicotine or cotinine glucuronidation was not detected? Or is a UGT other than UGT1A4 catalyzing imipramine glucuronidation in this sample? Analogously, is the nicotine glucuronidation activity in HLM 126, which has the same rate of imipramine glucuronidation as HLM 123, catalyzed by UGT1A9? (Propofol activity was detected in this sample.) Likewise, does the generally greater inhibition of cotinine glucuronidation by propofol (Table 3) suggest a greater contribution of UGT1A9 to cotinine glucuronidation compared with nicotine glucuronidation? Or is this difference due to a difference in concentration of nicotine and cotinine at the active site as a result of differences in lipophilicity?

To begin to answer some of the above questions, the relative rates of both nicotine and cotinine glucuronidation were determined with three heterologously expressed UGTs, 1A3, 1A4, and 1A9. Nicotine glucuronidation by UGT1A4 was readily detected when the reactions were carried out at pH 8.9. Much lower levels of activity were detected for UGT1A3 and UGT1A9. No nicotine glucuronidation was detected by any of these enzymes when the reaction was carried out at pH 7.1. Cotinine glucuronidation by UGT1A4 was detected when reactions were carried out at pH 7.1 but not at pH 8.9. The rate of UGT1A4-catalyzed cotinine glucuronidation was more than 10-fold lower than nicotine glucuronidation by this enzyme. No cotinine glucuronidation activity was detected with either UGT1A3 or UGT1A9. It seems likely that the greatly increased lipophilicity of nicotine at pH 8.9 (83% unprotonated) is why it was possible to detect nicotine glucuronidation but not cotinine glucuronidation.

Two other laboratories were unable to detect either cotinine or nicotine glucuronidation with any UGTs (Ghosheh and Hawes, 2002b; Nakajima et al., 2002c). The conditions under which Ghosheh et al. (2002b) ran their analyses were essentially the same as ours, except that the [^{14}C]nicotine specific activity was less than one-tenth that of the [5-^{3}H]nicotine used in the present study. This would decrease the limit of detection to the point where any nicotine glucuronide product would not have been detected. The more than 20-fold lower limit of detection we obtained using LC-MS/MS with reaction ion monitoring allowed us to also quantify the rates of UGT1A9- and UGT1A3-catalyzed nicotine glucuronidation and UGT1A4-catalyzed cotinine glucuronidation. Nakajima et al. (2002c) analyzed UGT-catalyzed nicotine and cotinine glucuronidation at pH 7.4 and at lower concentrations of substrates which, based on our results, would not result in a detectable level of glucuronidation by UGT Supersomes.

We previously reported that V79-expressed UGT1A9 cell lysate catalyzed the *O*-glucuronidation of NNAL (Ren et al., 2000) and the *N*-glucuronidation of NNAL (Nguyen et al., 2000). The latter reaction is analogous to nicotine glucuronidation. Therefore, we determined the activity of nicotine glucuronidation by V79-expressed UGT1A4 cell lysate compared with that by UGT1A9 Supersomes. The activity with V79 UGT1A9 cell lysate was more than 2-fold higher, when expressed per milligram of total protein. This is in contrast to the 2-fold lower rate of propofol glucuronidation by V79-expressed UGT1A9 compared with UGT1A9 Supersomes. The amount of UGT1A protein in the UGT1A9 V79-cell lysate was 1/13 that present in UGT1A9 Supersomes, suggesting that there may be significant inactive protein present in Supersomes. If this is true, then the relative activity of the V79-expressed UGT1A9 cell lysate is 17 times that of UGT1A9 Supersomes (Table 3). This difference in activity between expression systems may be due to differences in membrane compo-

sition or possibly the proprietary addition of reagents to increase Supersome solubility. It has been previously reported that the phospholipid environment may influence the rate-limiting step of glucuronidation (Magdalou et al., 1982; Nakajima et al., 2002b).

Despite all the questions this study has raised, it is clear that UGT1A4 catalyzes the glucuronidation of both nicotine and cotinine. However, due to lipophilicity issues discussed above, it is difficult to determine whether nicotine is actually a better substrate than is cotinine, even though we report a higher rate of UGT1A4 catalysis of nicotine glucuronidation. Inhibition studies reported here and those by Nakajima et al. (2002c) with imipramine support a role for UGT1A4 in the glucuronidation of both substrates. A possible role for UGT1A9 catalysis is also suggested by our data and those of Nakajima et al. (2002c).

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