

## N-GLUCURONIDATION OF NICOTINE AND COTININE IN HUMAN: FORMATION OF COTININE GLUCURONIDE IN LIVER MICROSOMES AND LACK OF CATALYSIS BY 10 EXAMINED UDP-GLUCURONOSYLTRANSFERASES

OMAR GHOSHEH AND EDWARD M. HAWES

*Drug Metabolism and Drug Disposition Group, University of Saskatchewan, Saskatoon, Saskatchewan, Canada*

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### ABSTRACT:

Two predominant human glucuronide metabolites of nicotine result from pyridine nitrogen atom conjugation. The present objectives included determination of the kinetics of formation of *S*(-)-cotinine *N*1-glucuronide in pooled human liver microsomes and investigation of the UDP-glucuronosyltransferases (UGTs) involved in *N*-glucuronidation of nicotine isomers and *S*(-)-cotinine by use of recombinant enzymes (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B7, and UGT2B15). Quantification was by radiochemical high-performance liquid chromatography with use of radiolabeled substrates. *S*(-)-Cotinine *N*1-glucuronide formation in human liver microsomes was proven by comparing the chromatographic behaviors and electrospray ionization-mass spectral characteristics of the metabolite with a synthetic reference standard. This glucuronide was formed by one-enzyme kinetics with  $K_m$  and  $V_{max}$  values of 5.4 mM and 696

pmol/min/mg, respectively, and the apparent intrinsic clearance value ( $V_{max}/K_m$ ) was 9-fold less than that previously determined for *S*(-)-nicotine *N*1-glucuronide (0.13 versus 1.2  $\mu$ l/min/mg) using the same pooled microsomes. This comparison of values is consistent with the observation that on smoking cigarettes, although the average *S*(-)-cotinine plasma levels usually far exceed *S*(-)-nicotine levels, the urinary recovery of *S*(-)-cotinine *N*1-glucuronide only averages 3-fold greater than for *S*(-)-nicotine *N*1-glucuronide. None of the UGTs examined catalyzed the *N*-glucuronidation of *S*(-)-nicotine, *R*(+)-nicotine, and *S*(-)-cotinine, including UGT1A3 and UGT1A4, the only isoforms known to catalyze many substrates at a tertiary amine. Also, neither *S*(-)-nicotine or *S*(-)-cotinine affected enzyme inhibition of trifluoperazine, a UGT1A4 substrate. It would appear that the same, as yet unexamined, UGT catalyzes the *N*-glucuronidation of both cotinine and nicotine.

The major routes of metabolism of nicotine in human involve oxidation and glucuronidation. The former metabolic routes have been thoroughly investigated, including establishing that the predominant proportion of nicotine metabolism is accounted for by routes of metabolism involving the lactam oxidation product cotinine as an intermediate and that CYP2A6 dominates as the main cytochrome P450 enzyme involved in catalysis (Nakajima et al., 1996a, 1996b; Messina et al., 1997; Murphy et al., 1999). In contrast, most studies of the glucuronidation routes of nicotine metabolism involve investigation of *in vivo* formation in human. It is established that three glucuronide metabolites account for 25 to 30% of the urinary metabolites after either inhalation or transdermal administration of nicotine. These three metabolites are the quaternary ammonium-linked glucuronides of *S*(-)-nicotine and *S*(-)-cotinine (Fig. 1), and the *O*-glucuronide of *trans*-3'-hydroxycotinine (Byrd et al., 1992; Caldwell et al., 1992; Benowitz et al., 1994). Further knowledge has been lacking regarding these three glucuronide metabolites, including the

apparent formation kinetics and the UDP-glucuronosyltransferases (UGTs<sup>1</sup>) involved in catalysis. In the latter case, there have been no previous reports, although it is noteworthy that for *N*-glucuronidation of substrates at a tertiary amine an apparent specificity in catalysis by UGT1A3 and UGT1A4 has been indicated (Green et al., 1995, 1998; Green and Tephly, 1996; Tukey and Strassburg, 2000). With respect to the former, we recently reported the identification and the apparent kinetics of formation of nicotine *N*1-glucuronide in pooled human liver microsomes (Ghosheh et al., 2001; *n* = 6). There was marked stereoselectivity in the kinetics in that the apparent intrinsic clearance value ( $V_{max}/K_m$ ) for natural *S*(-)-nicotine was 4 times greater than for the *R*(+)-enantiomer reputedly formed during cigarette smoking (Klus and Kuhn, 1977; Crooks et al., 1992). In the present study, we report further *in vitro* investigations of the *N*-glucuronides involved in nicotine metabolism. The formation of the *N*-1 glucuronide of *S*(-)-cotinine in the same pooled human liver microsomes was demonstrated and the kinetics of formation determined. Also by use of 10 commercially available recombinant enzymes, the UGT(s) responsible for the formation of the *N*-1 glucuronides of nicotine isomers and *S*(-)-cotinine were investigated.

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**Address correspondence to:** Dr. Edward M. Hawes, Drug Metabolism and Drug Disposition Group, College of Pharmacy and Nutrition, 110 Science Place, University of Saskatchewan, Saskatoon, SK, S7N 5C9, Canada. E-mail: [hawes@duke.usask.ca](mailto:hawes@duke.usask.ca)

<sup>1</sup> Abbreviations used are: UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; HPLC, high-performance liquid chromatography; ESI, electrospray ionization; HLM, human liver microsomes.

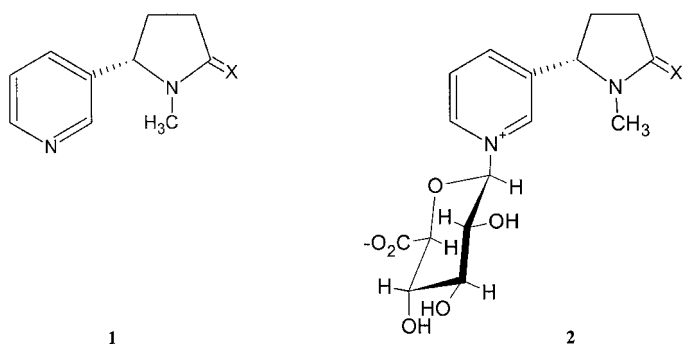


FIG. 1. Chemical structures of *S*(-)-nicotine (1,  $X = H_2$ ), *S*(-)-cotinine (1,  $X = O$ ), *S*(-)-nicotine *N*1-glucuronide (2,  $X = H_2$ ), and *S*(-)-cotinine *N*1-glucuronide (2,  $X = O$ ).

### Materials and Methods

**Chemicals.** *S*(-)-Nicotine ditartrate, *S*(-)-cotinine, trifluoperazine dihydrochloride, 4-nitrophenol, 7-hydroxy-4-trifluoromethylcoumarin, UDP-glucuronic acid (UDPGA), Tris base, magnesium chloride,  $\beta$ -glucuronidase (type 1X-A; 1,560,000 units/g, pH 6.8, from *Escherichia coli*), alamethicin, and D-saccharic acid 1,4-lactone were purchased from Sigma-Aldrich (St. Louis, MO). [*N*-Methyl- $^{14}C$ ]-*S*(-)-cotinine (free base; specific activity, 52 mCi/mmol), [*N*-Methyl- $^{14}C$ ]-*S*(-)-nicotine (free base; specific activity, 55 mCi/mmol), and [*N*-methyl- $^{14}C$ ]-*R*(+)-nicotine (free base; specific activity, 55 mCi/mmol) were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). *S*(-)-Nicotine *N*1-glucuronide (Vashishtha et al., 2000) and trifluoperazine *N*4'-glucuronide, (Luo et al., 1992) were synthesized by modification of previously reported procedures, and *S*(-)-cotinine *N*1-glucuronide was purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). *R*(+)-Nicotine di-*p*-toluoyl tartrate and [glucuronyl- $U$ - $^{14}C$ ] UDPGA (specific activity, 252 and 380 mCi/mmol) were purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Methanol and acetonitrile, both HPLC grade (EM Science, Gibbstown, NJ) and reagent-grade sodium phosphate (BDH Chemicals, Toronto, ON, Canada) were also used. Scintillation cocktail Ultima Flow-M was obtained from PerkinElmer Life Sciences (Boston, MA). Double-distilled water ( $18 \pm 0.05$  ohm cm), deionized, and purified by Milli-QTM Water System (Millipore Corporation, Bedford, MA) was used. HPLC mobile phase solvents were filtered through Millipore 0.45- $\mu$ m filters prior to use.

**Preparation of Liver Microsomes.** Human livers (white; 2 female and 4 males) were obtained from the International Institute for the Advancement of Medicine (Exton, PA). Microsomes were prepared from both individual and pooled livers (equal weight taken from each liver) by differential centrifugation as previously indicated (Ghosheh et al., 2001). The microsomes were stored at  $-80^\circ C$  until used. The protein content of the microsomal suspension was determined by the method of Lowry et al. (1951) using bovine serum albumin as a reference standard.

**Biosynthesis of *S*(-)-Cotinine *N*1-Glucuronide in Human Liver Microsomes.** The reaction mixture (500  $\mu$ l) that consisted of  $MgCl_2$  (10 mM), alamethicin (25  $\mu$ g), UDPGA (3 mM), human liver microsomes (1 mg), Tris buffer (50 mM, pH 7.4), and *S*(-)-cotinine (1.25 mM) was incubated for 120 min at  $37^\circ C$ . The reaction was stopped by cooling on ice and adding acetonitrile (1.5 ml). The resultant mixture was centrifuged at 9,000g for 15 min. The supernatant was evaporated under nitrogen. The residue was dissolved in 50% aqueous methanol and analyzed by electrospray ionization (ESI)-mass spectrometry.

ESI-mass spectra of the biosynthesized sample and synthesized reference standard (dissolved in 50% aqueous methanol) were acquired on a Thermoquest Finnigan TSQ7000 mass spectrometer (Thermo Finnigan MAT, San Jose, CA), operated in the positive ion mode. Desolvation of solvent droplets was aided by a heated capillary temperature of  $250^\circ C$ , and sheath and auxiliary gas pressures were set at 80 and 40 psi, respectively. Data acquisition and reduction was carried out using Xcalibur (version 1.2; Thermo Electron Corporation, Waltham, MA) loaded onto an NT workstation. The data were acquired over the mass range of  $m/z$  150 to 400, at a scan time of 1 s. Samples dissolved in 50% aqueous methanol were infused into the mass spectrometer at a rate of 10  $\mu$ l/min.

**Assay for *N*-Glucuronidation of Cotinine.** The incubation conditions of pooled microsomes for *S*(-)-cotinine initially were optimized with respect to pH, latency disrupting agent concentration, and time of incubation and protein concentration required to give a linear rate of formation of the glucuronide. The effect of pH on the rate of glucuronidation was studied in the range of 5.5 to 9.5 (5.5, 6.5, 7, 7.6, 8, 8.4, 9, and 9.5). Alamethicin was used as the latency disrupting agent, and its concentration was varied over the range 0 to 50  $\mu$ g/mg of protein (0, 2.5, 5, 10, 15, 20, 25, and 50  $\mu$ g/mg of protein). The time of incubation and the protein concentration were varied from 15 to 120 min (15, 30, 45, 60, 90, and 120 min) and 125 to 625  $\mu$ g/ml (125, 250, 375, 500, and 625  $\mu$ g/ml), respectively.

The general procedure for kinetic determinations in pooled human liver microsomes under optimized conditions is now given. The final incubation mixture (100  $\mu$ l) included  $MgCl_2$  (5 mM), alamethicin (10  $\mu$ g/mg of protein), Tris buffer (50 mM, pH 8.4), UDPGA (2 mM), and pooled human liver microsomes (500  $\mu$ g). Variable concentrations of the substrate were added (16–0.04 mM, including 0.2  $\mu$ Ci of labeled cotinine). The individual human liver microsomes, with respect to the determination of glucuronidation activities, were treated similarly except that only a 0.5 mM *S*(-)-cotinine concentration was used. The mixture was incubated at  $37^\circ C$  for 45 min, and protein was then precipitated by adding 100  $\mu$ l of methanol followed by centrifugation at 9,000g for 5 min. The supernatant (120  $\mu$ l) was directly injected into the HPLC for radiochemical analysis. In all experiments, incubations were carried out in triplicate, and in the case of the determination of kinetic constants, the experiment was repeated 4 to 6 times at each substrate concentration.

**$\beta$ -Glucuronidase Hydrolysis.**  $\beta$ -Glucuronidase treatment of samples obtained under kinetic determination conditions was studied. Incubated mixtures, as described above (i.e., 100  $\mu$ l; optimized conditions, 0.04 mM *S*(-)-cotinine and 45 min of incubation), were centrifuged (9000g for 10 min) and then further incubated at  $37^\circ C$  for 24 h after the addition of an *E. coli* preparation (1500 U) as an enzyme source and adjustment to pH 7.4. The incubated mixtures were then treated by the usual work-up of addition of methanol and centrifugation prior to HPLC analysis. The control samples were treated in the same way, except that no  $\beta$ -glucuronidase was added.

**Identification of Human UGT Enzyme(s) Catalyzing the *N*-Glucuronidation of Cotinine and Nicotine.** Commercially available human UGT enzymes produced in baculovirus insect cell expression systems were employed: UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, and UGT2B15 from BD Gentest (Woburn, MA), and UGT1A7, UGT1A10, and UGT2B7 from PanVera Corp. (Madison, WI). Protein content data were given and appropriate control preparations were obtained from these companies. The expression systems were validated in-house for their glucuronidation activity using 7-hydroxy-4-trifluoromethylcoumarin and 4-nitrophenol at 0.1 and 1 mM substrate concentration, respectively, and buffer pH 7.4. Also the catalysis of trifluoperazine by UGT1A4 was examined at 1 mM substrate concentration and buffer pH 8.4. The control products were used as negative controls for the respective UGT procedures. For general expressed UGT screening, the final incubation mixture (100  $\mu$ l) included the substrate (0.04, 0.5, and 5 mM, including 0.2  $\mu$ Ci of labeled substrate),  $MgCl_2$  (5 mM), alamethicin (10  $\mu$ g/mg of protein), Tris buffer (50 mM at both pH 8.4 and 7.4), UDPGA (2 mM), and cellular protein (100  $\mu$ g). Mixtures (with and without added D-saccharic acid 1,4-lactone) were incubated for 45 min at  $37^\circ C$ . After terminating the reaction by keeping the tube at  $4^\circ C$  and adding 100  $\mu$ l of methanol, the cellular mixture was centrifuged at 9000g for 5 min. The supernatant (120  $\mu$ l) was then analyzed using HPLC, as described below.

**Inhibition of Trifluoperazine *N*-Glucuronidation.** A typical incubation mixture (100  $\mu$ l) included the substrate trifluoperazine (1 mM),  $MgCl_2$  (5 mM), alamethicin (10  $\mu$ g/mg of protein), Tris buffer (50 mM, pH 8.4), UDPGA (2 mM, including 0.3  $\mu$ Ci of the labeled cofactor), and cellular protein (expressed UGT1A4, 100  $\mu$ g or pooled human liver microsomes, 0.5 mg). The effect of *S*(-)-nicotine, *S*(-)-cotinine, 1-phenylimidazole, and imipramine on trifluoperazine *N*-glucuronidation was investigated by adding them separately at 0.5 mM concentration to the incubations of both expressed UGT1A4 and pooled human liver microsomes. Mixtures were preincubated 1 min with the purported inhibitors before addition of the trifluoperazine. The final mixtures were incubated for 60 min at  $37^\circ C$ . Termination of the reaction and injection into the HPLC was as described earlier. A further study was conducted in a similar manner to examine the effects of 0.5 and 2.5 mM

*S*(-)-nicotine and 5 and 25 mM *S*(-)-cotinine concentrations. In this case, duplicate samples were examined both with and without preincubation of the nicotine alkaloid.

**HPLC Analysis.** HPLC analysis was carried out on a chromatographic system consisting of a Waters 600 multisolvent delivery system (Waters Corp., Milford, MA) connected to a variable wavelength absorbance detector adjusted at 254 nm (Waters model 486) and a Packard 150TR flow scintillation analyzer. Samples were injected via an autosampler SCL-10A (Shimadzu Corp., Koyoto, Japan). Data acquisition and analysis were performed using Waters Millennium 32 (version 3.05.01), where data for quantification was collected from the radiochemical detector. The ultraviolet detector was used especially in assay development and in qualitative analysis. The separation and quantification of each nicotine isomer and its glucuronide metabolite were achieved by gradient reversed phase chromatography as described previously (Ghosheh et al., 2001). In the case of the quantification of cotinine and its glucuronide metabolite by isocratic reversed phase chromatography, a Supelco (Bellefonte, PA) Supelcosil LC-SCX analytical column (4.6 × 250 mm; 5- $\mu$ m diameter particle) was used. The analytical column was protected using Phenomenex (Torrance, CA) Security Guard C<sub>18</sub> cartridges (4 × 3 mm). The mobile phase consisted of 95% 5 mM sodium phosphate buffer (pH 4.5) and 5% acetonitrile in which the run period and the flow rate were 19 min and 1.5 ml/min, respectively. The retention times of cotinine and the *N*-glucuronide metabolite were 11.3 and 3.2 min, respectively.

In the case of the quantification of trifluoperazine *N*4'-glucuronide by radiochemical analysis, the same analytical column and security guard C<sub>18</sub> cartridges were used with a gradient system of two solvents A (10 mM sodium phosphate buffer, pH 6.5) and B (acetonitrile). The gradient elution programmed run was as follows: A (70%)/B (30%) from 0 to 5 min, changed to A (35%)/B (65%) over 5 to 9 min, changed to A (70%)/B (30%) over 19 to 21 min, and maintained as such to the end of the 25 min run. The flow rate was maintained at 1.5 ml/min at all times. The retention times of trifluoperazine and the *N*-glucuronide metabolite were 22.0 and 9.5 min, respectively.

**Calculations.**  $V_{\max}$  and  $K_m$  values were calculated according to Michaelis-Menten equations for one- and two-enzyme kinetics by nonlinear least squares regression analysis (Graph Pad Prism; Graph Pad Software, San Diego, CA). Student's *t* tests and statistical correlations were calculated using Excel 97 (Microsoft Corp., Redmond, WA). The  $V_{\max}/K_m$  ratios were determined as a rough calculation of intrinsic clearance. Except where indicated, data were obtained at least in triplicate and are given as mean  $\pm$  S.E.M.

## Results

A polar metabolite that was isolated from the incubation of *S*(-)-cotinine with activated human liver microsomes was identified as *S*(-)-cotinine *N*1-glucuronide. This conclusion was reached as the isolated metabolite and a synthetic standard were comparable in both HPLC-ultraviolet retention times under various chromatographic conditions and positive ion ESI-mass spectrometry [ $M^+$ , 353; ( $M + Na$ )<sup>+</sup>, 375]. Furthermore, the daughter ion mass spectrum of the latter molecular ion peak gave a peak at 177 mass units, indicative of the characteristic cleavage of the glycosidic bond ( $M-176$ )<sup>+</sup> with transfer of a proton from the glucuronic acid moiety to the aglycone. Also further evidence that the peak subsequently measured in HPLC radiochemical assays was due to *S*(-)-cotinine *N*1-glucuronide was the similarity in retention time irrespective of whether microsomal incubations were carried out with the <sup>14</sup>C label on *S*(-)-cotinine or UDPGA. Finally, after further incubation of incubated mixtures for assay with  $\beta$ -glucuronidase, the HPLC peak for the *N*1-glucuronide could not be detected. The radiochemical chromatographic method for the kinetic and other studies that used [<sup>14</sup>C]*S*(-)-cotinine showed a complete resolution of the peaks of concern and was reproducible and sensitive for the range of substrate concentrations required.

The incubation conditions for the formation of *S*(-)-cotinine *N*1-glucuronide in the pooled human liver microsomes (*n* = 6) were optimized with respect to pH, latency disrupting agent, protein concentration, and incubation time. *N*-Glucuronidation of *S*(-)-cotinine

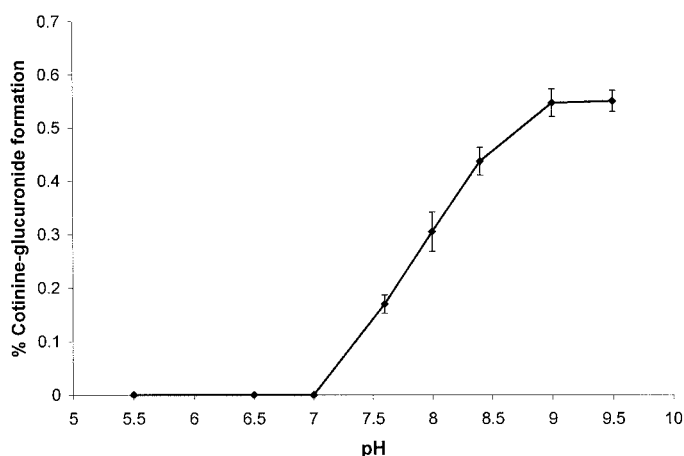


Fig. 2. pH profile for the formation of *S*(-)-cotinine *N*1-glucuronide.

Assays were conducted using 0.5 mg pooled human liver microsomes, 0.25 mM cotinine (including 0.1  $\mu$ Ci of labeled substrate), 5 mM MgCl<sub>2</sub>, 2 mM UDPGA, and 10  $\mu$ g alamethicin/mg of protein in a final volume of 100  $\mu$ l that was incubated at 37°C for 1 h.

was not measurable at or below pH 7.0 but showed 3-fold increase in catalysis over the pH 7.6 to 9.0 range with no change between pH 9.0 and 9.5 (Fig. 2). A pH value of 8.4 was used in all subsequent studies as only 1.3-fold increase in activity over the pH 8.4 to 9.0 range was observed, and this pH value has been used in previous studies of *N*-glucuronidation at a tertiary amine, including nicotine (Green et al., 1995, 1998; Ghosheh et al., 2001). Also since alamethicin has been used successfully with respect to the activation of *N*-glucuronidation at an aromatic tertiary amine of other substrates, including nicotine (Ghosheh et al., 2001; Vashishtha et al., 2001), this pore-forming peptide was investigated as a latency disrupting agent. In comparison to control values, there was a 2.2- to 2.8-fold increase in the glucuronidation rate of *S*(-)-cotinine at alamethicin concentrations of 2.5 to 50  $\mu$ g/mg of protein. An alamethicin concentration of 10  $\mu$ g/mg of protein was used in subsequent experiments; the optimum observed concentration and the concentration employed in the previous nicotine study (Ghosheh et al., 2001). The protein concentration and incubation time were linear up to 0.5 mg of protein/incubation and 45 min, respectively.

Under linear reaction conditions (0.5 mg of protein/incubation; 45 min incubation time) the glucuronidation of *S*(-)-cotinine in pooled liver microsomes conformed to single  $K_m$  Michaelis-Menten kinetics (Fig. 3). Nonlinear transformation of the data yielded apparent  $K_m$  and  $V_{\max}$  values of 5.4 mM and 696 pmol/min/mg of protein, respectively, and the apparent intrinsic clearance ( $V_{\max}/K_m$ ) was determined to be 0.13  $\mu$ l/min/mg of protein (Table 1). The catalytic activities of the individual liver microsomes (*n* = 6) determined at one concentration under the optimum conditions for the pooled sample were found to vary 5.6-fold (28.0 to 156 pmol/min/mg of protein).

None of the 10 expressed UGT enzymes examined were found to catalyze the *N*-glucuronidation of *S*(-)-nicotine, *R*(+)-nicotine, and *S*(-)-cotinine. Appropriate positive control data were obtained for each UGT enzyme, including in the cases of UGT1A3 and UGT1A4 with the substrates 4-nitrophenol and trifluoperazine, respectively, where the respective turnover values of 610  $\pm$  81.3 and 271.0  $\pm$  11.3 pmol/min/mg of protein were similar to previously reported values (Green et al., 1995, 1998; Green and Tephly, 1996). Further investigation of the UGT1A4 enzyme was conducted by use of the suggested probe substrate trifluoperazine (Dehal et al., 2001). Thus the effect was investigated of *S*(-)-nicotine and *S*(-)-cotinine, as well as the established UGT1A4 substrates imipramine (Green et al., 1995) and 1-phenylimidazole (Vash-

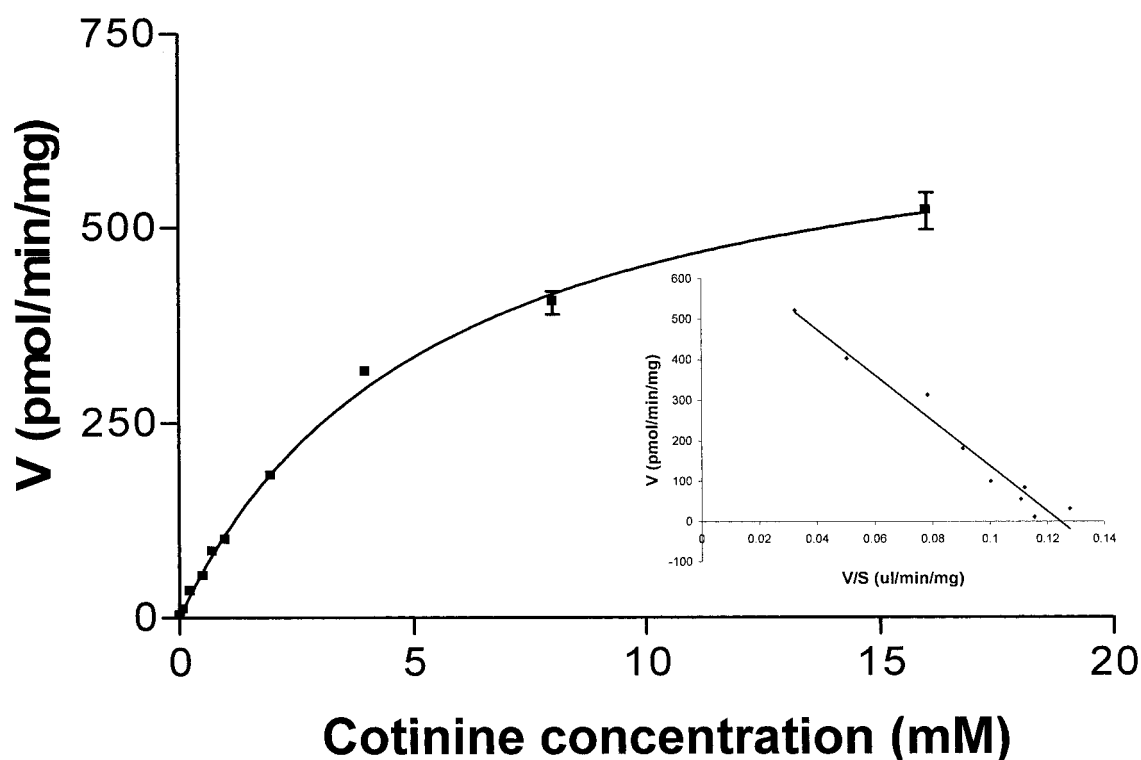


FIG. 3. Michaelis-Menten kinetics of glucuronidation of *S*(-)-cotinine by human liver microsomes.

TABLE 1

Apparent kinetic parameters for the *N*-glucuronides of *S*(-)-cotinine and *S*(-)-nicotine in pooled human liver microsomes

Substrate	$K_m$	$V_{max}$	$V_{max}/K_m$
	mM	pmol/min/mg	$\mu\text{L}/\text{min}/\text{mg}$
<i>S</i> (-)-Cotinine	$5.43 \pm 0.32$	$695.6 \pm 18.9$	$0.13 \pm 0.06$
<i>S</i> (-)-Nicotine <sup>a</sup>	$0.11 \pm 0.017$	$132 \pm 4.5$	$1.2 \pm 0.27$

<sup>a</sup> Data previously reported for the same pooled human liver microsomes (Ghosheh et al., 2001).

ishtha et al., 2000), on the *N*-glucuronidation of trifluoperazine in expressed UGT1A4 and pooled human liver microsomes. The trifluoperazine *N*4'-glucuronide formed was quantified by a radiochemical HPLC method that was verified by demonstrating that the retention time was identical to that of a synthetic reference standard and that the peak was absent without added trifluoperazine and when control baculovirus insect cells expressing no UGTs were tested. At 1 mM trifluoperazine concentration, *N*-glucuronidation by either expressed-UGT1A4 or pooled human liver microsomes was significantly inhibited ( $p < 0.05$ ) by imipramine and 1-phenylimidazole (expressed UGT1A4,  $5.33 \pm 0.41$  and  $8.20 \pm 2.50$ ; human liver microsomes (HLM),  $70.4 \pm 3.0$  and  $56.2 \pm 6.1\%$  of control values, respectively) but not by *S*(-)-nicotine and *S*(-)-cotinine (expressed UGT1A4,  $96.3 \pm 5.8$  and  $102.5 \pm 3.6$ ; HLM,  $106.6 \pm 2.8$  and  $103.3 \pm 7.6\%$  of control values, respectively) at the 0.5 mM concentration examined (Fig. 4). In a similarly conducted study, but with equal and greater concentrations of *S*(-)-nicotine (0.5 and 2.5 mM) and *S*(-)-cotinine (5 and 25 mM), the mean trifluoperazine *N*4'-glucuronide concentrations were found to be 94 to 115% of the control values.

### Discussion

The *N*1-glucuronide was identified as a metabolite of *S*(-)-cotinine in human liver microsomes by comparing the HPLC and ESI-mass spectrometry characteristics with an authentic synthetic reference

standard. Originally, this metabolite was unequivocally identified as a major metabolite of nicotine in smoker's urine (Caldwell et al., 1992). The aromatic pyridine nitrogen atom is the site of glucuronidation for both cotinine and nicotine. In previous work, we demonstrated the formation of *S*(-)- and *R*(+)-nicotine *N*1-glucuronides in human liver microsomes (Ghosheh et al., 2001). Various similarities were found between *S*(-)-cotinine and *S*(-)-nicotine when optimizing the incubation conditions for glucuronidation. The pH profiles were similar with little or no detectable activity at or below pH 7 and with marked increase in activity thereafter to pH 9.0. Also on investigating an appropriate concentration of alamethicin to remove the latency for glucuronidation, for both substrates a 2- to 3-fold increase in glucuronidation catalysis was present over the 2.5 to 50  $\mu\text{g}/\text{mg}$  of protein range as compared with control values.

The enzyme catalysis of the glucuronidation of *S*(-)-cotinine conformed to single apparent  $K_m$  Michaelis-Menten kinetics. The determination of the kinetics using the same pooled liver microsomes as used in the earlier study of nicotine *N*-glucuronidation enabled comparison of the two metabolic reactions (Table 1). The apparent  $K_m$  and  $V_{max}$  values for *S*(-)-cotinine as compared with those for *S*(-)-nicotine were approximately 50- and 5-fold greater, respectively; hence, the apparent intrinsic clearance was approximately 10-fold less for *S*(-)-cotinine than *S*(-)-nicotine. This latter comparison of the glucuronidation efficiencies of *S*(-)-cotinine and *S*(-)-nicotine is consistent with observations in vivo regarding the plasma levels and the urinary concentrations of metabolites after nicotine intake. That is, after smoking cigarettes and transdermal administration of nicotine, almost invariably the plasma levels of *S*(-)-cotinine are more than an order of magnitude greater than *S*(-)-nicotine levels yet the urinary excretions of *S*(-)-cotinine glucuronide and *S*(-)-nicotine glucuronide approximate 14 and 5%, respectively (Benowitz et al., 1994, 1997). However, comparison of the currently determined in vitro kinetics with in vivo kinetics awaits investigation of the latter with



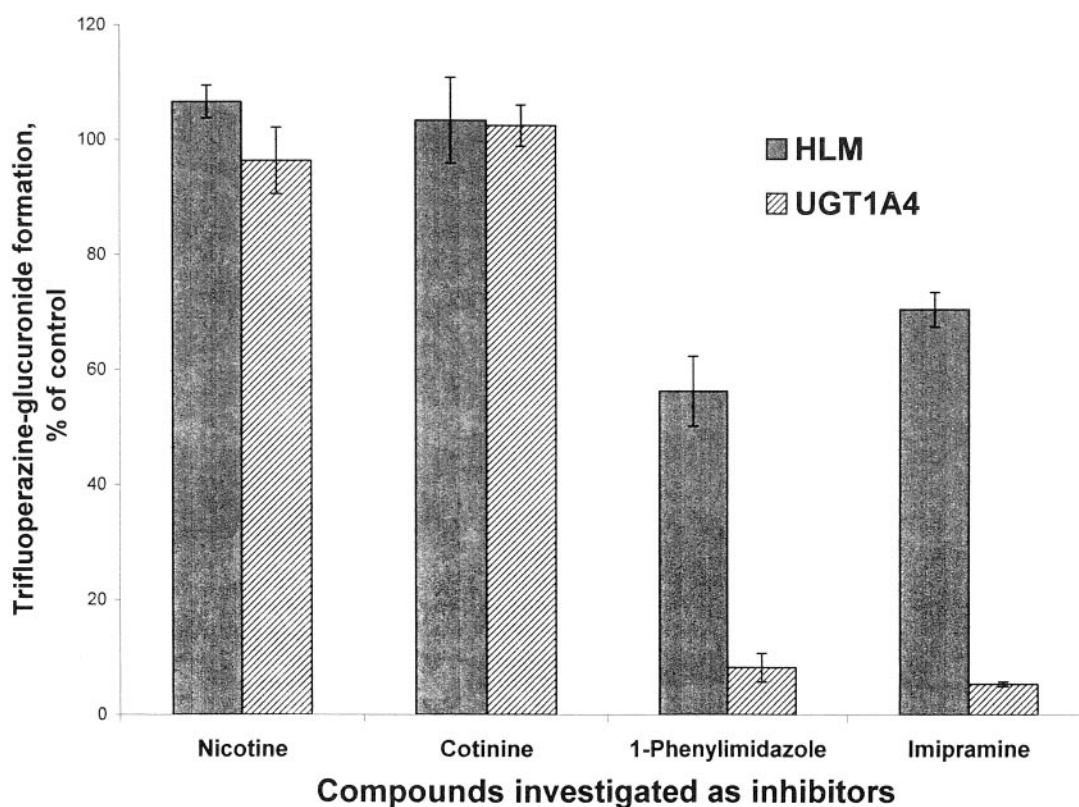


FIG. 4. Trifluoperazine *N*4'-glucuronide inhibition by various substrates in pooled HLM and expressed UGT1A4.

The inhibition is expressed as percent of formation of the glucuronide as compared with control samples where only trifluoperazine was incubated. Incubations included 1 mM trifluoperazine and 2 mM UDPGA (including 0.3  $\mu$ Ci of labeled cofactor), with and without 0.5 mM of investigated compound as described under *Materials and Methods*.

respect to the *N*-glucuronides of nicotine, including determination of metabolic hepatic clearance values.

The major objective of the present study was to determine the human UGT enzyme(s) that can catalyze the tertiary amine *N*-glucuronidation of nicotine and *S*(-)-cotinine. That none of the 10 recombinant UGTs examined affected detectable catalysis of *S*(-)-nicotine, *R*(+)-nicotine, and *S*(-)-cotinine was unexpected. To expedite detection of the metabolites, 14-C-labeled substrates were employed, which allows detection of very low levels of the glucuronide metabolites. In fact, the screen for all examined UGTs was run at not only typically employed substrate concentrations of 0.5 and 5 mM but also at a 0.04 mM concentration in which only manufacturer-supplied labeled substrate was employed so as to boost assay sensitivity. Also, all UGTs were examined in incubations at both pH 7.4 and 8.4 and at both of these pH values with and without added D-saccharic acid 1,4-lactone as a  $\beta$ -glucuronidase inhibitor. In particular, the lack of activity of UGT1A3 and UGT1A4 was the most surprising in view of the previously mentioned apparent isoform selectivity of these enzymes in the catalysis of the *N*-glucuronidation of tertiary amine substrates. Since UGT1A4 has been speculated to be the more important of the two enzymes regarding this general reaction type in vivo (Green and Tephly, 1998), a study was conducted with a different approach to determine the involvement of this enzyme in nicotine metabolism. The results of this enzyme inhibition study conducted with both expressed UGT1A4 and pooled human liver microsomes also indicated that the *N*-glucuronidation of neither *S*(-)-cotinine or *S*(-)-nicotine is catalyzed by UGT1A4. Neither of these compounds inhibited the *N*-glucuronidation of the UGT1A4 probe substrate, trifluoperazine (Dehal et al., 2001). In contrast, imipramine and 1-phe-

nylimidazole, established UGT1A4 substrates, where *N*-glucuronidation, respectively occurs at an aliphatic and aromatic tertiary amine, affected significant inhibition of the *N*-glucuronidation of trifluoperazine. Nonetheless, although these observations of a lack of catalysis of pyridine nitrogen atom glucuronidation of nicotine and *S*(-)-cotinine by UGT1A3 and UGT1A4 may appear surprising, examination of the substrates where catalysis by these UGT enzymes occurs at a tertiary amine (Green et al., 1995, 1998; Green and Tephly, 1996; Breyer-Pfaff et al., 2000; Vashishtha et al., 2001) reveals that in the vast majority of cases glucuronidation occurs at an aliphatic tertiary amine, and only in the cases of lamotrigine and various 1-substituted imidazoles have catalysis at an aromatic tertiary amine been reported (Green et al., 1995; Vashishtha et al., 2001).

For all three substrates, *S*(-)-cotinine, *S*(-)-nicotine, and *R*(+)-nicotine, the enzyme kinetics for the pooled microsomes examined supported the hypothesis that one enzyme was involved in *N*-glucuronidation. That the same UGT enzyme may be involved in these catalyzes was indicated by not only the same negative UGT screening result for the three substrates, but also the previously mentioned similarities observed when optimizing the incubation conditions for the pooled liver microsomes, and the fact that when the microsomes of the six individuals were examined with respect to the glucuronidation rates of *S*(-)-nicotine (Ghosheh et al., 2001) and *S*(-)-cotinine a significant correlation ( $r = 0.98$ ;  $p < 0.05$ ) was found. This latter observation needs verification using microsomes from more individuals. In conclusion, the *N*-glucuronidation of *S*(-)-cotinine was demonstrated in pooled human liver microsomes and was found to occur 10-fold less efficiently than for *S*(-)-nicotine. Although catalysis of nicotine and *S*(-)-cotinine by none of the 10 UGT enzymes examined

was demonstrated, there was indication that the same enzyme was involved in each case.

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### References

- Benowitz NL, Jacob P III, Fong I, and Gupta S (1994) Nicotine metabolic profile in man: comparison of cigarette smoking and transdermal nicotine. *J Pharmacol Exp Ther* **268**:296–303.
- Benowitz NL, Zevin S, and Jacob P III (1997) Sources of variability in nicotine and cotinine levels with use of nicotine nasal spray, transdermal nicotine and cigarette smoking. *Br J Clin Pharmacol* **43**:259–267.
- Breyer-Pfaff U, Mey U, Green MD, and Tephly TR (2000) Comparative *N*-glucuronidation kinetics of ketotifen and amitriptyline by expressed human UDP-glucuronosyltransferases and liver microsomes. *Drug Metab Dispos* **28**:869–872.
- Byrd GD, Chang K-M, Greene JM, and deBethizy JD (1992) Evidence for urinary excretion of glucuronide conjugates of nicotine, cotinine and trans-3'-hydroxycotinine in smokers. *Drug Metab Dispos* **20**:192–197.
- Caldwell WS, Greene JM, Byrd GD, Chang KM, Uhrig MS, deBethizy JD, Crooks PA, Bhatti BS, and Riggs RM (1992) Characterization of the glucuronide conjugate of cotinine: a previously unidentified major metabolite of nicotine in smokers' urine. *Chem Res Toxicol* **5**:280–285.
- Crooks PA, Godin CS, and Pool WF (1992) Enantiomeric purity of nicotine in tobacco smoke condensate. *Med Sci Res* **20**:879–880.
- Dehal SS, Gange PV, Crespi CL, and Patten CJ (2001) Characterization of a probe substrate and an inhibitor of UDP-glucuronosyltransferase 1A4 activity in human liver microsomes and cDNA-expressed UGT-enzymes (Abstract). *Drug Metab Rev* **33**:162.
- Ghosheh O, Vashishtha SC, and Hawes EM (2001) Formation of the quaternary ammonium-linked glucuronide of nicotine in human liver microsomes: identification and stereoselectivity in the kinetics. *Drug Metab Dispos* **29**:1525–1528.
- Green MD, Bishop WP, and Tephly TR (1995) Expressed human UGT1.4 protein catalyzes the formation of quaternary ammonium-linked glucuronides. *Drug Metab Dispos* **23**:299–302.
- Green MD, King CD, Mojarrabi B, Mackenzie PI, and Tephly TR (1998) Glucuronidation of amines and other xenobiotics catalyzed by expressed human UDP-glucuronosyltransferase 1A3. *Drug Metab Dispos* **26**:507–512.
- Green MD and Tephly TR (1996) Glucuronidation of amines and hydroxylated xenobiotics and endobiotics catalyzed by expressed human UGT1.4 protein. *Drug Metab Dispos* **24**:356–363.
- Green MD and Tephly TR (1998) *N*-Glucuronidation of xenobiotics symposium, glucuronidation of amine substrates by purified and expressed UDP-glucuronosyltransferase proteins [ASPET Symposium]. *Drug Metab Dispos* **26**:860–867.
- Klus H and Kuhn H (1977) A study of the optical activity of smoke nicotine. *Fachliche Mitt Oesterr Tabakregie* **17**:331–336 (through Chem Abs **87**:180692e).
- Lowry DH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265–275.
- Luo H, Hawes EM, McKay G, and Midha KK (1992) Synthesis and characterization of quaternary ammonium-linked glucuronide metabolites of drugs with aliphatic tertiary amine group. *J Pharm Sci* **81**:1079–1083.
- Messina ES, Tyndale RF, and Sellers EM (1997) A major role for CYP2A6 in nicotine C-oxidation by human liver microsomes. *J Pharmacol Exp Ther* **282**:1608–1614.
- Murphy SE, Johnson LM, and Pullo DA (1999) Characterization of multiple products of cytochrome P450 2A6-catalyzed cotinine metabolism. *Chem Res Toxicol* **12**:639–645.
- Nakajima M, Yamamoto T, Nunoya K-I, Yokoi T, Nagashima K, Inoue K, Funae Y, Shimada N, Kamataki T, and Kuroiwa Y (1996a) Role of human cytochrome P450 2A6 in C-oxidation of nicotine. *Drug Metab Dispos* **24**:1212–1217.
- Nakajima M, Yamamoto T, Nunoya K-I, Yokoi T, Nagashima K, Inoue K, Funae Y, Shimada N, Kamataki T, and Kuroiwa Y (1996b) Characterization of CYP 2A6 involved in 3'-hydroxylation of cotinine in human liver microsomes. *J Pharmacol Exp Ther* **277**:1010–1015.
- Tukey RH and Strassburg (2000) Human UDP-glucuronosyltransferases: metabolism, expression and disease. *Annu Rev Pharmacol Toxicol* **40**:581–616.
- Vashishtha SC, Hawes EM, McKay G, and McCann DJ (2000) Synthesis and identification of the quaternary ammonium-linked glucuronide of 1-phenylimidazole in human liver microsomes and investigation of the human UDP-glucuronosyltransferases involved. *Drug Metab Dispos* **28**:1009–1013.
- Vashishtha SC, Hawes EM, McKay G, and McCann DJ (2001) Quaternary ammonium-linked glucuronidation of 1-substituted imidazoles: studies of human UDP-glucuronosyltransferases involved and substrate specificities. *Drug Metab Dispos* **29**:1290–1295.