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DRUG METABOLISM & DISPOSITION

MICROSOMAL *N*-GLUCURONIDATION OF NICOTINE AND COTININE: HUMAN HEPATIC INTERINDIVIDUAL, HUMAN INTERTISSUE, AND INTERSPECIES HEPATIC VARIATION

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ABSTRACT:

Two of the abundant conjugates of human nicotine metabolism result from the *N*-glucuronidation of *S*-(−)-nicotine and *S*-(−)-cotinine, transformations we recently demonstrated in liver microsomes. We further studied these microsomal *N*-glucuronidation reactions with respect to human hepatic interindividual, human intertissue, and interspecies hepatic variation. The reactivities of microsomes from human liver (n = 12), various human tissues, and liver from eight species toward the *N*-glucuronidation of *S*-(−)-nicotine and *S*-(−)-cotinine, and also *R*-(+)-nicotine in human liver were examined. Assays with ^{14}C-labeled substrates involved radiometric high-performance liquid chromatography. For the human liver samples examined there were 13- to 17-fold variations in the catalytic activities observed toward *S*-(−)-nicotine, *R*-(+)-nicotine, and *S*-(−)-cotinine. Gender and smoking effects were studied, and after exclusion of an outlier a decrease in catalytic activity in females was observed. Significant correlations were observed between all three analytes, indicating that the same UDP-glucuronosyltransferase(s) enzyme is likely to be involved in these transformations. Catalytic activities were not observed for human gastrointestinal tract (colon, duodenum, ileum, jejunum, and stomach), kidney, or lung microsomes. For the seven animal species examined, activity was measurable only for monkey, guinea pig, and minipig, and only for *S*-(−)-nicotine *N*-glucuronidation and at rates 10- to 40-fold lower than humans. Activity was not measurable in the case of dog, mouse, rabbit, or rat, for the latter under five different treatment conditions for one of the strains. In conclusion, there are large hepatic interindividual variations in *N*-glucuronidation of *S*-(−)-nicotine and *S*-(−)-cotinine, in human extrahepatic metabolism seems limited, and none of the animal strains examined resembled human.

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Glucuronidation is an important route of nicotine metabolism in human. Three glucuronide metabolites have been identified that account for 25 to 30% of the urinary metabolites of nicotine after either inhalation or transdermal administration (Byrd et al., 1992; Caldwell et al., 1992; Benowitz et al., 1994). In summary, glucuronidation of natural *S*-(−)-nicotine, and its two major oxidative metabolites *S*-(−)-cotinine and *trans*-3′-hydroxycotinine, respectively, result in the quaternary ammonium-linked glucuronides of *S*-(−)-nicotine and *S*-(−)-cotinine (Fig. 1), and the *O*-glucuronide of *trans*-3′-hydroxycotinine. Most studies regarding these metabolic routes pertain to their in vivo formation in human. Recently, we investigated the two known human *N*-glucuronidation routes of metabolism of nicotine in vitro, including determination of the kinetics of formation in human liver microsomes and investigation of the UDP-glucuronosyltransferases (UGTs[1]) involved in catalysis. For the pooled human liver microsomes examined (n = 6) the apparent intrinsic clearance was 9-fold greater for *S*-(−)-nicotine *N*1-glucuronide than for *S*-(−)-cotinine *N*1-glucuronide, and

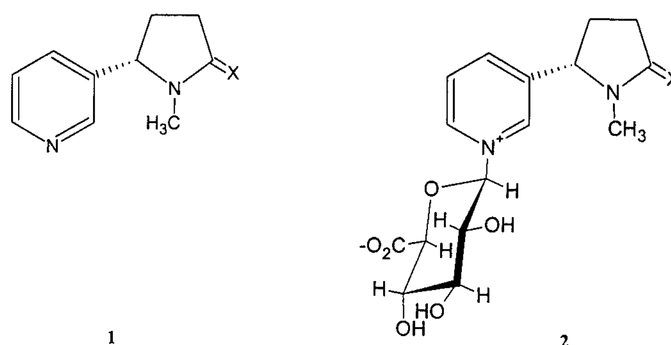

FIG. 1. *Chemical structures of S-(−)-nicotine (**1**, X = H_2), S-(−)-cotinine (**1**, X = O), S-(−)-nicotine N1-glucuronide (**2**, X = H_2), and S-(−)-cotinine N1-glucuronide (**2**, X = O).*

although none of the 10 expressed UGTs examined catalyzed the formation of these two *N*-glucuronide metabolites there was indirect indication that the same enzyme(s) was involved for both reactions (Ghosheh et al., 2001; Ghosheh and Hawes, 2002). In this article, we report on human hepatic interindividual variation, human intertissue variation, and interspecies hepatic variation of the microsomal *N*-glucuronidation of *S*-(−)-nicotine and *S*-(−)-cotinine.

Although there have been no detailed reports about the interindividual variation of the *N*-glucuronidation of *S*-(−)-nicotine and *S*-(−)-cotinine in vitro there have been reports about in vivo variation after various modes of administration of nicotine to humans. Those reports compared urinary excretion of *N*-glucuronide metabolites ex-

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[1] Abbreviations used are: UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; HPLC, high-performance liquid chromatography.

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pressed as excretion per gram of creatinine, and molar fraction of either nicotine dose, recovered nicotine plus all metabolites, or parent drug plus the glucuronide (Benowitz et al., 1994, 1999). There have been no reports about the *N*-glucuronidation of *S*-(−)-nicotine or *S*-(−)-cotinine by extrahepatic tissues. With respect to interspecies differences in these *N*-glucuronidation reactions, there are only a few reports. Attempts to biosynthesize *S*-(−)-nicotine *N*1-glucuronide and *S*-(−)-cotinine *N*1-glucuronide from the respective precursor in monkey (marmoset) hepatic microsomes was successful only in the former case (Tsai and Gorrod, 1999). Also, the *N*-glucuronide of nicotine was found, at about 1% of the systemically administered dose, but the *N*-glucuronide of cotinine was not found as a biliary metabolite of racemic nicotine in rat (Seaton et al., 1993a,b). In the present work, the conversions of *S*-(−)-nicotine and *S*-(−)-cotinine [and *R*-(+)-nicotine in the former case] to their respective *N*-glucuronide metabolites were investigated in microsomes prepared from a range of human hepatic, human extrahepatic, and animal hepatic tissues to gain insight as to the extent of human interindividual, human intertissue, and interspecies variation of these metabolic routes.

Materials and Methods

Chemicals. *S*-(−)-Nicotine ditartrate, *S*-(−)-cotinine, UDP-glucuronic acid (UDPGA), 4-nitrophenol, 4-nitrophenol glucuronide, Tris base, magnesium chloride, and alamethicin were purchased from Sigma-Aldrich (St. Louis, MO). [*N*-Methyl-^{14}C]-*S*-(−)-cotinine (free base; specific activity, 52 mCi/mmmol), [*N*-Methyl-^{14}C]-*S*-(−)-nicotine (free base; specific activity, 55 mCi/mmmol), and [*N*-Methyl-^{14}C]-*R*-(+)-nicotine (free base; specific activity, 55 mCi/mmmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). *R*-(+)-Nicotine di-*p*-toluoyl tartrate and [glucuronyl-U-^{14}C]UDPGA (specific activity 380 mCi/mmmol) were purchased from ICN Pharmaceuticals (Costa Mesa, CA). Methanol and acetonitrile, both HPLC grade (EM Scientific, Gibbstown, NJ) and reagent-grade sodium phosphate (BDH Chemicals, Toronto, ON, Canada) were also used. Scintillation cocktail Ultima Flow-M was obtained from Packard BioScience (Meriden, CT). Double distilled water (18 ± 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 0.45-μm filters before use.

Preparation of Microsomes. For the study of interindividual variation, human livers (n = 12 white, equally distributed in terms of smoking habit and gender, as indicated in Fig. 2, a and b, respectively) were obtained from the International Institute for the Advancement of Medicine (Exton, PA). Microsomes were prepared from each individual liver by differential centrifugation as indicated previously (Vashishtha et al., 2002).

The human samples used in the comparison of intertissue catalytic activities were obtained from various sources: liver tissue (white; two females and four males), pooled ileum microsomes (two females and four males), and pooled kidney microsomes (two females and two males) were from Institute for the Advancement of Medicine; pooled (n = 3–5, mixed males and females) lung, stomach, duodenum, jejunum, ileum, and colon microsomes were from Human Biologics Institute (Scottsdale, AZ); and pooled lung microsomes (n = 4, one female and three males) were donated by Dr. T. Massey. In the case of liver, a pooled (equal weight taken from each liver) microsomal sample was prepared as indicated previously (Ghosheh et al., 2001). 4-Nitrophenol was used as a positive control for each human tissue, where the final incubation mixture (100 μl) comprised 1 mM 4-nitrophenol, 5 mM $MgCl_2$, 10 μg alamethicin/mg of protein, 50 mM Tris buffer, pH 7.4, 2 mM UDPGA (including 0.3 μCi of the labeled cofactor), and 0.5 mg of microsomal protein.

For the study of interspecies variation, various microsomal samples were either prepared in-house or purchased. Regarding the former, microsomes were prepared from livers of untreated, male dog (n = 1, beagle, 8.8 kg), guinea pig (n = 3, Dunkin-Hartley, 350–400 g), and rabbit (n = 1, New Zealand White, 2.9 kg) (in the case of guinea pig; pooled, equal amounts were taken from each liver) as reported previously (Vashishtha et al., 2002). The viability of all but the dog microsomes was demonstrated by the *N*-glucuronidation of 1-phenylimidazole (Vashishtha et al., 2002). Commercially available samples of the pooled microsomes, prepared from male animals were obtained from the companies indicated: mice (n = 30, CD1) and monkey (n = 2, cynomolgus) from Cedra Corporation (Austin, TX), and minipig (n = 2, Yucatan), monkey (n = 2, rhesus), and rats (n = 15, Wistar, Fischer 344, and Sprague-Dawley; the latter as five types, untreated or chronically treated with phenobarbital, 3-methylcholanthrene, dexamethasone, or β-naphthoflavone) were from In Vitro Technologies (Baltimore, MD). For human, the pooled sample from the intertissue study described above was used. All the above-mentioned tissues and microsomes were stored at −80°C until used.

Assays for the *N*-Glucuronidation of Nicotine and Cotinine. For each of the substrates, *S*-(−)-nicotine, *R*-(+)-nicotine, and *S*-(−)-cotinine, general assays for glucuronidation activities in various microsomal preparations were used. The incubation conditions were optimized for each substrate with the pooled sample of human liver microsomes (n = 6), as described previously (Ghosheh et al., 2001; Ghosheh and Hawes, 2002) 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 glucuronidation assay used for individual human liver microsomes was as follows. The final incubation mixture (100 μl) included 5 mM $MgCl_2$, 10 μg of alamethicin/mg of protein, 50 mM Tris buffer, pH 8.4, 2 mM UDPGA, 0.5 mg of microsomal protein, and substrate. The substrate concentrations used were 0.5 mM for *S*-(−)-nicotine and *S*-(−)-cotinine (including 0.1 μCi of the labeled compound), and 0.3 mM for *R*-(+)-nicotine (including 0.2 μCi of the labeled compound). The mixture was incubated for 45 min at 37°C. Protein was subsequently precipitated by adding 100 μl of methanol, and the mixture was then centrifuged at 9000*g* for 5 min. The supernatant (120 μl) was directly injected into the HPLC system for radiometric analysis.

For microsomes from different human tissues the general procedure for glucuronidation activity determinations was as follows. The final incubation mixture (100 μl) included 5 mM $MgCl_2$, 10 μg of alamethicin/mg of protein, 50 mM Tris buffer, pH 7.4 or 8.4, 2 mM UDPGA, 0.5 mg of microsomal protein, and 0.1 mM *S*-(−)-nicotine or *S*-(−)-cotinine (including 0.2 μCi of the labeled compound). The mixture was incubated for 60 min at 37°C. Subsequent sample treatment before analysis was as described above. In the case of ileum, kidney, and liver (all from Institute for the Advancement of Medicine source), a follow-up experiment was conducted with 0.02 and 0.5 mM substrate concentrations (including 0.2 μCi of the labeled compound) and buffer pH 7.4 or 8.4.

For liver microsomes from different species, the general procedure for glucuronidation activity determinations was as follows. The final incubation mixture (100 μl) included 5 mM $MgCl_2$, 10 μg of alamethicin/mg of protein, 50 mM Tris buffer, pH 7.4 or 8.4, 2 mM UDPGA, 0.5 mg of microsomal protein, and 0.1 mM *S*-(−)-nicotine, *R*-(+)-nicotine, or *S*-(−)-cotinine (including 0.2 μCi of the labeled compound). The mixture was incubated for 60 min at 37°C. Subsequent sample treatment before analysis was as described above.

The protein content of the microsomal samples prepared in our laboratory was determined by the method of Lowry et al. (1951) using bovine serum albumin as a reference standard. For microsomes obtained commercially or donated, the protein content was provided by the suppliers.

HPLC Analysis. HPLC analysis was carried out on a chromatographic system consisting of a Waters 600 multisolvent delivery system (Waters, 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, Koyoto, Japan). Data acquisition and analysis were performed using Waters Millenium 32 (version 3.05.01) where data were collected from both ultraviolet and radiometric detectors. The separation and quantification of both nicotine isomers and their glucuronide metabolites were achieved by gradient reversed phase chromatography as described previously (Ghosheh et al., 2001). *S*-(−)-Cotinine and its glucuronide metabolite were separated and quantified by an isocratic reversed phase chromatographic method as described previously (Ghosheh and Hawes, 2002).

For the analysis of 4-nitrophenol *O*-glucuronide, gradient reversed phase chromatography was performed with a C_{18} Luna analytical column (ODS 4.6 × 250 mm; 5-μm-diameter particle) (Phenomenex, Torrance, CA). The analytical column was protected using Security Guard C_{18} cartridges (4 × 3 mm) (Phenomenex). The gradient system involved two solvents, A (5 mM sodium phosphate buffer, pH 4.5) and B (acetonitrile). The gradient elution

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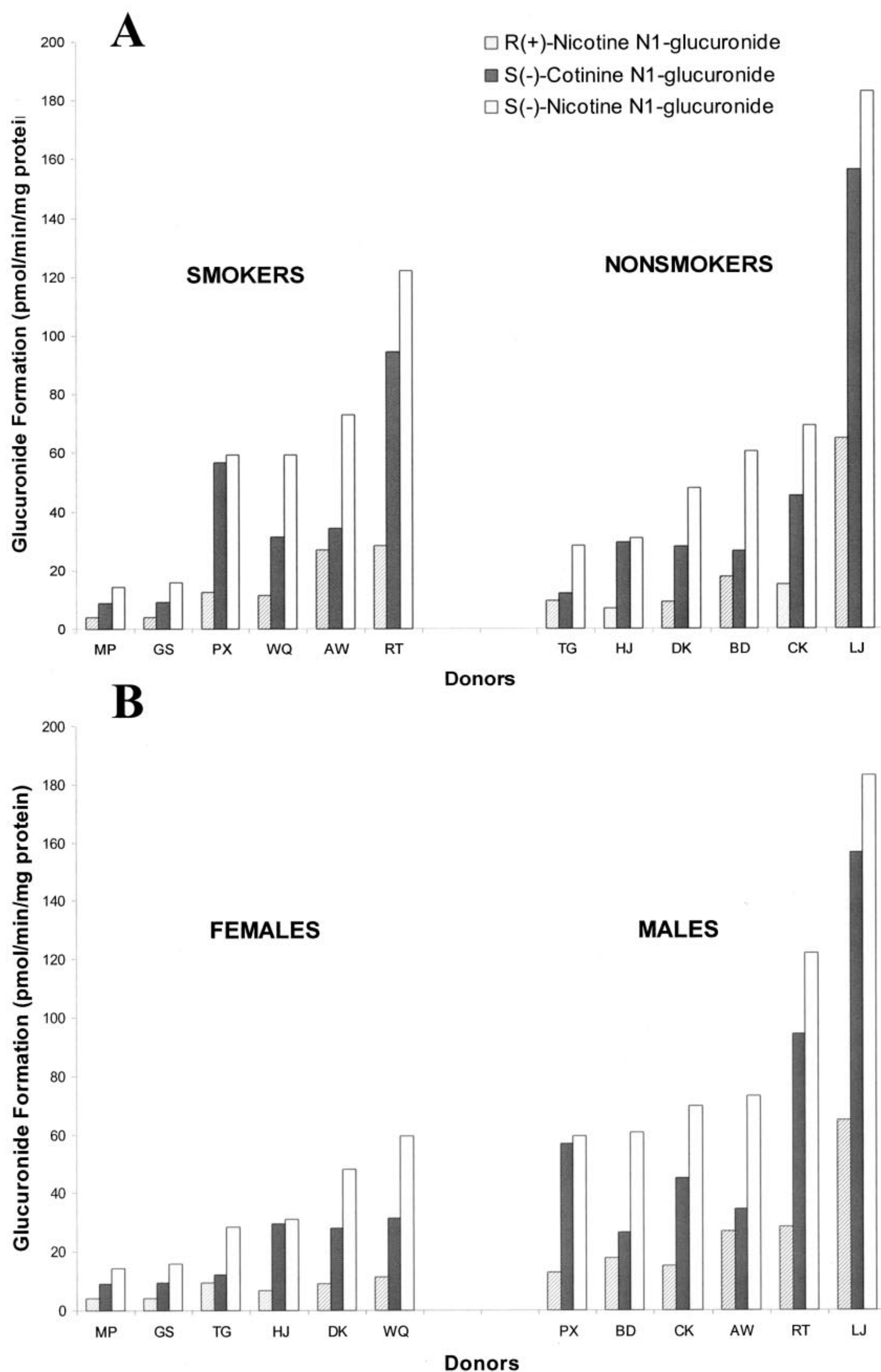

FIG. 2. *Rates of* N-*glucuronidation of S-(−)-nicotine, S-(−)-cotinine, and R-(+)-nicotine for a panel of human liver microsomal samples (white, n = 12) categorized in terms of smoking habit (A) and gender (B).*

Assays were conducted using 0.5 mg of human liver microsomes, 0.5 mM substrate (including 0.1 μCi of labeled substrate), 50 mM Tris buffer, pH 8.4, 5 mM $MgCl_2$, 2 mM UDPGA, and 10 μg of alamethicin/mg of protein in a final volume of 100 μl that was incubated at 37°C for 45 min, except in the case of *R*-(+)-nicotine where instead 0.3 mM substrate (including 0.2 μCi of labeled substrate) was used.

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TABLE 1

Glucuronidation activity towards S-(−)-nicotine, S-(−)-cotinine, and 4-nitrophenol in microsomes prepared from human lung, liver, gastrointestinal tract (stomach, duodenum, jejunum, ileum, and colon), and kidney

Tissue	*S*-(−)-Nicotine *N*1-glucuronide	*S*-(−)-Cotinine *N*1-glucuronide	4-Nitrophenol-*O*-glucuronide
		V nmol/min/mg protein	
Lung	N.D.	N.D.	0.065–0.062[a]
Liver	0.094 ± 0.006	0.020 ± 0.001	3.80 ± 0.36
Gastrointestinal tract	N.D.	N.D.	0.10–1.49[b]
Kidney	N.D.	N.D.	3.08 ± 0.61

N.D., below the limit of detection (0.0015 nmol/min/mg protein for both *N*-glucuronides); V, UGT activity.
[a] Lung pools obtained from Dr. Massey (0.065 ± 0.023) and from Human Biologics Institute (0.062 ± 0.029 nmol/min/mg protein).
[b] Gastrointestinal tract pools of stomach (1.27 ± 0.29), duodenum (1.24 ± 0.05), jejunum (0.87 ± 0.21), ileum (Institute for the Advancement of Medicine, 0.70 ± 0.14; Human Biologics Institute, 1.49 ± 0.13), and colon (0.10 ± 0.04 nmol/min/mg protein).

programmed run was as follows: A (98 to 85%)/B (2 to 15%) changed over 0 to 9 min, changed to A (85 to 98%)/B (15 to 2%) over 9 to 15 min and maintained as such to the end of the 19-min run. The flow rate was maintained at 1.5 ml/min at all times. The retention time of the *O*-glucuronide metabolite was 4.6 min.

Calculations. Statistical correlations were calculated using JMP version 4.02 (SAS Institute Inc., Cary, NC). Data were obtained at least in triplicate and are given as mean ± S.E.M.

Results

Radiometric HPLC assays were used to determine the rate of formation of *S*-(−)-nicotine *N*1-glucuronide and *S*(−)-cotinine *N*1-glucuronide in microsomal preparations from various tissues and species, and of *R*-(+)-nicotine *N*1-glucuronide in the case of human liver preparations. The incubation conditions used were adopted or modified from those determined previously for human liver microsomes (Ghosheh et al., 2001; Ghosheh and Hawes, 2002). It is noteworthy that although an incubation pH of 8.4 was determined to be optimal in the previous work, the cautious step was taken of also performing incubations at pH 7.4 in studies of interspecies variation, and human tissue variation. However, in all cases any catalytic activity observed at pH 7.4 was more than 5-fold less that at pH 8.4. Thus, all the reported data for nicotine metabolism are for microsomal incubations at pH 8.4.

The interindividual study was performed with liver microsomes from 12 individuals. The extent of interindividual variation in the catalytic activities for *S*-(−)-nicotine, *S*-(−)-cotinine, and *R*-(+)-nicotine were observed to be 13- (14.3–183 pmol/min/mg of protein), 17- (9.0–156 pmol/min/mg of protein), and 15-fold (4.2–64.9 pmol/min/mg of protein), respectively. For all individuals the rate of formation followed the order *S*-(−)-nicotine *N*1-glucuronide > *S*-(−)-cotinine *N*1-glucuronide. Comparison with *R*-(+)-nicotine *N*1-glucuronide is not viable because the assay conditions for this analyte differed from the other two analytes in that to enhance the sensitivity of the radiometric method a lower amount of substrate (0.3 versus 0.5 mM) with more label (0.2 versus 0.1 μCi) was used. Nevertheless, correlations were found between all analytes, namely, *S*-(−)-nicotine *N*1-glucuronide and *S*-(−)-cotinine *N*1-glucuronide ($r = 0.967$), *S*-(−)-nicotine *N*1-glucuronide and *R*-(+)-nicotine *N*1-glucuronide ($r = 0.956$), and *S*-(−)-cotinine *N*1-glucuronide and *R*-(+)-nicotine *N*1-glucuronide ($r = 0.924$). The data for the three analytes are presented as both Fig. 2, a and b, to facilitate comparison of the catalytic activities for the six smokers with the six nonsmokers, and the six females with the six males. The mean catalytic activities observed for *S*-(−)-nicotine, *S*-(−)-cotinine, and *R*-(+)-nicotine for smokers and nonsmokers were 57.3 ± 16.3 versus 70.0 ± 23.5 pmol/min/mg of protein, 39.3 ± 7.9 versus 49.8 ± 21.8 pmol/min/mg of protein, and 14.7 ± 4.4 versus 20.5 ± 9.0 pmol/min/mg of protein, respectively. For females and males the values were 32.9 ± 7.3 versus 94.7 ± 20.0 pmol/min/mg of protein, 20.0 ± 4.4 versus 69.1 ± 20.0 pmol/min/mg of protein, and 7.6 ± 1.2 versus 27.6 ± 7.9 pmol/min/mg of protein, respectively. Both the smoking ($p = 0.935$) and gender ($p = 0.0564$) effects were not statistically significant. However, reanalysis of the data without that of an outlier (LJ) indicated that, in fact, a gender effect was present ($p = 0.0135$).

The intertissue study involved comparison of the *N*-glucuronidation catalytic activity of pooled human liver microsomes with pooled microsomes obtained from human kidney, lung, and various parts of the gastrointestinal tract, namely, stomach, duodenum, jejunum, ileum, and colon. No detectable microsomal catalysis of the *N*-glucuronidation of either *S*-(−)-nicotine or *S*-(−)-cotinine was observed for any of the extrahepatic tissues examined (Table 1). In comparison, the microsomes of all these tissues catalyzed the *O*-glucuronidation of the nonspecific UGT substrate 4-nitrophenol (King et al., 2000).

None of the seven animal species had liver microsomal catalytic activities for the *N*-glucuronidation of *S*-(−)-nicotine and *S*-(−)-cotinine that resembled those of humans (Table 2). In fact, for all seven species for *S*-(−)-cotinine and for all but three species for *S*-(−)-nicotine, either no activity or detectable but not measurable activity was found. Values for *N*-glucuronidation of *S*-(−)-nicotine by liver microsomes of guinea pig (Dunkin-Hartley), minipig (Yucatan), and monkey (cynomolgus and rhesus) were approximately 10- to 40-fold less than for human (2.5–10.0 versus 94.6 pmol/min/mg of protein). No measurable catalytic activities were found for liver microsomes from dog (beagle), mouse (CD1), rabbit (New Zealand White), or rat. For rat variation was examined for three different strains (Wistar, Fischer 344, and Sprague-Dawley), and untreated and four different enzyme inducer-treated animals for the Sprague-Dawley strain.

Discussion

Most studies of the interindividual variation of the metabolism of nicotine have involved the oxidative routes of metabolism. Discovery that CYP2A6 is the major cytochrome P450 enzyme involved in the major routes of metabolism of nicotine has resulted in various recent studies to relate interindividual differences in metabolism to the genetic polymorphism of the *CYP2A6* gene (Inoue et al., 2000; Nakajima et al., 2001; Oscarson, 2001; Raunio et al., 2001; Tyndale and Sellers, 2001). In contrast, the few published studies of interindividual variation in the glucuronidation routes of metabolism of nicotine have been based on urinary excretion data (Benowitz et al., 1994, 1999). The present pilot study of the interindividual variation in the catalytic activities of the microsomal glucuronidation of *S*-(−)- and *R*-(+)-nicotine, and *S*-(−)-cotinine involved 12 in-house prepared microsomal samples of Caucasian liver with equal distribution in both gender and smoking habit. The extent of interindividual variation was found to vary 13- to 17-fold for the three analytes, variabilities that are

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TABLE 2

Glucuronidation activity towards S-(−)-nicotine and S-(−)-cotinine in liver microsomes prepared from eight different species

Species		S-(−)-Nicotine N1-glucuronide	S-(−)-Cotinine N1-glucuronide
		V pmol/min/mg protein	
Human		94.6 ± 6.4	19.9 ± 1.1
Monkey			
	Rhesus	9.97 ± 3.81	N.D.
	Cynomolgus	6.84 ± 2.04	tr
Minipig	Yucatan	2.50 ± 0.42	N.D.
Dog	Beagle	N.D.	N.D.
Rabbit	New Zealand White	tr	tr
Mouse	CD1	N.D.	N.D.
Guinea pig	Dunken-Hartley	3.91 ± 0.13	tr
Rat			
	Fischer 344	N.D.	N.D.
	Wistar	N.D.	N.D.
	Sprague-Dawley*	N.D.	N.D.

N.D., below the limit of detection (1.5 pmol/min/mg protein for each *N*-glucuronide); tr, below the limit of quantification (2.0 pmol/min/mg protein for each *N*-glucuronide); V, UGT activity.

* Untreated or chronically treated with phenobarbital, 3-methylcholanthrene, dexamethasone, or β-naphthoflavone.

similar to those reported for the glucuronidation of various other substrates by human liver microsomes (Furlan et al., 1999; Court et al., 2001). Statistical analysis of the present pilot study data indicated that smoking had no significant effect on the *N*-glucuronidation of *S*-(−)-nicotine and *S*-(−)-cotinine, which is in agreement with a recent in vivo study (Benowitz and Jacob, 2000). Although the analysis also indicated that there was not a gender effect, with deletion of an outlier there was significant decreased catalytic activity in women. The indication of a possible gender effect merits further investigation involving a greater number of liver microsomal samples. Gender differences in glucuronidation have been noted infrequently, although a decreased clearance in females has been noted for a few substrates (Liston et al., 2001). In the case of nicotine, there has been no report of gender differences in glucuronidation, including in a relatively large population study ($n = 108$) where the urinary excretion of the *N*-glucuronides of *S*-(−)-nicotine and *S*-(−)-cotinine was quantified (Benowitz et al., 1999).

N-Glucuronidation activity of cotinine or nicotine was not detected for any of the extrahepatic microsomal preparations examined. In contrast, in the cases of the kidney and the gastrointestinal tract, glucuronidation activity has been found for a wide array of compounds that in some cases was comparable with or greater than liver on a per milligram of microsomal protein basis (McGurk et al., 1998; Fisher et al., 2001; Shipkova et al., 2001; Soars et al., 2001b; Tukey and Strassburg, 2001). Also there are a few reports of glucuronidation at an aliphatic tertiary amine by microsomes of kidney (Soars et al., 2001b), colon (Strassburg et al., 1999), and duodenum, ileum, and jejunum (Strassburg et al., 2000). In those studies the reported catalytic activities by extrahepatic tissues for the *N*-glucuronidation of imipramine and amitriptyline were comparable with those for hepatic microsomes.

Because the *N*-glucuronides of *S*-(−)-nicotine and *S*-(−)-cotinine are substantive metabolites in human it is important to determine the specific UGT enzyme(s) involved in their formation. In the previous in vitro studies conducted in these laboratories, although this issue was not resolved, various observations indicated that the same enzyme was involved in both reactions (Ghosheh et al., 2001; Ghosheh and Hawes, 2002). That suggestion was especially based on the strong correlation observed in the catalytic activities between *S*-(−)-nicotine and *S*-(−)-cotinine within individuals. Furthermore, there is report of a correlation for the excretion of the two *N*-glucuronide metabolites in vivo ($n = 12$; Benowitz et al., 1994). The present interindividual and intertissue studies reinforce the suggestion that the same enzyme was involved in both reactions. This present support includes the strong correlation of the two catalytic activities in the interindividual study, and that neither metabolic reaction could be detected in any of the extrahepatic tissues examined. Another important conclusion of the latter study is that because study was made of microsomes of not only kidney and various regions of the gastrointestinal tract, but also lung, extrahepatic *N*-glucuronidation in the metabolism of nicotine is likely to be unimportant irrespective of the mode of administration. Consequently, there is clear indication that hepatic UGT(s) largely catalyze these metabolic reactions.

Also, that UGT1A3 and UGT1A4, the only UGTs known to catalyze glucuronidation at a tertiary amine (Green et al., 1995, 1998; Green and Tephly, 1996; Tukey and Strassburg, 2000) are not the enzymes involved in these reactions was as indicated by the previously reported inactivity with expressed enzymes (Ghosheh and Hawes, 2002), and also indirectly by the present study. Thus, inactivity was observed for extrahepatic tissues where UGT1A3 and/or UGT1A4 are documented to be expressed, including stomach, duodenum, jejunum, ileum, and colon (Strassburg et al., 2000; Tukey and Strassburg, 2001). Furthermore, although knowledge of the UGT enzymes of human kidney is limited, it is noteworthy that the UGT1A3 and UGT1A4 substrate imipramine is *N*-glucuronidated by microsomal preparations (Green et al., 1998; Soars et al., 2001b).

Based on the results obtained for hepatic microsomes, none of seven animal species examined seems to be appropriate to model the *N*-glucuronidation metabolism of nicotine metabolism in humans. However, a limitation of the study is that, apart from one species, the rat, only one microsomal sample from one or two strains was examined. In the case of monkey, two strains were examined and although they gave the highest observed catalytic activity at approximately 7 to 10% of human regarding *S*-(−)-nicotine, like all animal species examined, no measurable activity was observed for *S*-(−)-cotinine. It is conceivable that other primates might be appropriate to model the *N*-glucuronidation of nicotine metabolism. The marmoset, for example, has received attention as a potential model species for drug glucuronidation and, as previously noted, has been used successfully in *S*-(−)-nicotine *N*1-glucuronide biosynthesis (Tsai and Gorrod, 1999; Soars et al., 2001a).

That the catalytic activities observed for the *N*-glucuronidation of nicotine metabolism by guinea pig and rabbit microsomes is low at best is not consistent with the accepted use of these species as models to study glucuronidation at a tertiary amine of substrates (Lehman et al., 1983; Remmel and Sinz, 1991; Chiu and Huskey, 1998). Hence it seems that differences between nicotine and various other substrates with respect to glucuronidation at a tertiary amine occur with other species, and not just humans. Nicotine is similar to other substrates regarding tertiary amine glucuronidation with respect to the observed null activity for dog, mouse, and rat, because almost invariably this route of metabolism has been observed to be a minor route or absent with these species (Hucker et al., 1978; Chiu and Huskey, 1998; Li et al., 2001; Soars et al., 2001b). Because the rat has been commonly used in nicotine experimentation and there is a report of the detection and quantification of racemic nicotine *N*1-glucuronide as a biliary metabolite in the Sprague-Dawley strain (Seaton et al., 1993a,b), various microsomal samples were examined for this species. The lack of formation of *N*-glucuronide irrespective of strain of rat or enzyme inducer treatment of the Sprague-Dawley strain is not inconsistent with the previous report of racemic nicotine *N1*-glucuronide as a biliary metabolite, as in that case the metabolite was found to be a trace metabolite under the artificial condition of continuous bile

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collection. Finally, evidence that the minipig metabolized *S*-(−)-nicotine, albeit at low catalytic activity is of interest, because *N*-glucuronidation has not been previously reported for this species.

In conclusion, the current studies with the microsomes of the liver of eight species and various tissues of human has given various insights into the *N*-glucuronidation of *S*-(−)-nicotine and *S*-(−)-cotinine. The indication of a gender effect in a pilot study of hepatic interindividual variation in human merits follow-up with a much larger group of individuals. The correlation found between the catalytic activities of the two routes of metabolism, as well as other observations made in the present and previous studies, indicates that for both metabolic routes the same UGT enzyme(s) is involved in catalysis. However, indirect evidence indicated that human UGT1A3 and UGT1A4 do not play a significant role in such catalysis, reinforcing previous observation in these regards. Clear indication was obtained that in human, extrahepatic *N*-glucuronidation in nicotine metabolism is limited. Also, due to limited enzymatic catalyzes at most, none of the strains of the seven animal species examined seem appropriate to model the *N*-glucuronidation routes of human nicotine metabolism.

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