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Short Communication

IN VITRO–IN VIVO CORRELATIONS OF HUMAN (*S*)-NICOTINE METABOLISM

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Abstract—The profile of (*S*)-nicotine metabolism in human liver microsomes was examined at concentrations approaching *in vivo* conditions (10 μM). At such concentrations, no (*S*)-nicotine *N*-1′-oxygenation was seen, and thus *C*-oxidation to the (*S*)-nicotine $\Delta^{1',5'}$-iminium ion was the sole product observed in the metabolic profile in the presence of the human liver microsomes. For simplicity of analysis, the (*S*)-nicotine $\Delta^{1',5'}$-iminium ion formed was converted to (*S*)-cotinine in the presence of exogenously added aldehyde oxidase. To explain the lack of (*S*)-nicotine *N*-1′-oxygenation at low (*S*)-nicotine concentrations, inhibition of flavin-containing monooxygenase (FMO) activity by (*S*)-cotinine was examined. Although (*S*)-cotinine was observed to inhibit pig FMO1 (K_i = 675 μM), partially purified cDNA-expressed adult human liver FMO3 was not inhibited by (*S*)-cotinine. We therefore concluded that the kinetic properties of the nicotine *N*′- and *C*-oxidases were responsible for the metabolic product profile observed. Kinetic constants were determined for individual human liver microsomal preparations from low (10 μM) and high (500 μM) (*S*)-nicotine concentrations by monitoring (*S*)-cotinine formation with HPLC. The mean $K_{m_{app}}$ and V_{max} for formation of (*S*)-cotinine by the microsomes examined were 39.6 μM and 444.3 pmol·min^{-1}·(mg protein)$^{-1}$, respectively. The formation of (*S*)-cotinine was strongly dependent on the previous drug administration history of each subject, and among the highest rates for (*S*)-cotinine formation were those of the barbiturate-pretreated subjects. The rate of (*S*)-cotinine formation at low (10 μM) concentration correlated well with immunoreactivity for cytochrome P450 2A6 (r = 0.89). *In vitro–in vivo* correlation of the results suggests that the low amount of (*S*)-nicotine *N*-1′-oxygenation and the large amount of (*S*)-cotinine formed in human smokers (i.e. 4 and 30% of a typical dose, respectively) are determined primarily by the kinetic properties of the human monooxygenase enzyme systems. However, additional non-hepatic monooxygenase(s) contributes to (*S*)-nicotine metabolism.

Key words: human (*S*)-nicotine metabolism; human liver flavin-containing monooxygenase; CYP 2A6; (*S*)-cotinine; (*S*)-nicotine *N*-1′-oxide

In the United States, approximately 75 million people smoke tobacco and, on the average, consume about 24 mg of (*S*)-nicotine per person each day. Because almost 65% of (*S*)-nicotine metabolism proceeds through the (*S*)-cotinine pathway, this suggests that the formation of (*S*)-nicotine $\Delta^{1',5'}$-iminium ion is one of the most prominent metabolic transformations in adult Americans [1–10]. Little is known about the pharmacological properties of (*S*)-nicotine $\Delta^{1',5'}$-iminium ion, but it is clear that a large percentage of humans worldwide are exposed to nicotine, and the determination of the metabolic pathways and enzymes responsible for iminium ion formation is important.

Considerable interindividual variability in both the amount of (*S*)-nicotine present and the type of metabolite formed [i.e. (*S*)-cotinine or (*S*)-nicotine *N*-1′-oxide] was observed in humans following intravenous, dermal, or free smoking routes of administration [8, 11]. The interindividual variability in the formation of human urinary (*S*)-nicotine metabolites may be a consequence of a number of factors including: (a) the fundamental kinetic properties of the metabolic enzymes involved, (b) the wide individual variability in subsequent transformation of (*S*)-nicotine metabolites [i.e., glucuronidation of (*S*)-cotinine], and (c) more complicated mechanisms involving (*S*)-nicotine metabolite inhibition of other biotransformation pathways. Currently, it is accepted that at least a portion of the human interindividual variability in glucuronidation of (*S*)-cotinine and (*S*)-nicotine and conversion to *trans*-3-hydroxy-(*S*)-cotinine is due to interindividual differences in UDP glucuronyltransferases and cytochrome P-450s, respectively [5, 11].

(*S*)-Nicotine undergoes extensive oxidative metabolism in humans, and 80–90% of a dose can be accounted for in terms of urinary metabolites [1–10]. The major excreted human urinary metabolites of (*S*)-nicotine are (*S*)-cotinine (10–15%) *trans*-(*S*)-nicotine *N*-1′-oxide (4%), *trans*-3-hydroxy-(*S*)-cotinine (39%) [11], and (*S*)-nicotine glucuronide and (*S*)-cotinine glucuronide [4, 5] (together approximately 30%) [11] (Fig. 1). In the presence of adult human liver microsomes, CYP§ 2A6 is the primary enzyme that transforms (*S*)-nicotine to (*S*)-nicotine $\Delta^{1',5'}$-iminium

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§ Abbreviations: CYP, cytochrome P450; FMO, flavin-containing monooxygenase; CL_{organ}, clearance from an organ; Q_{organ}, blood flow from an organ; and $CL_{intrinsic}$, intrinsic clearance.

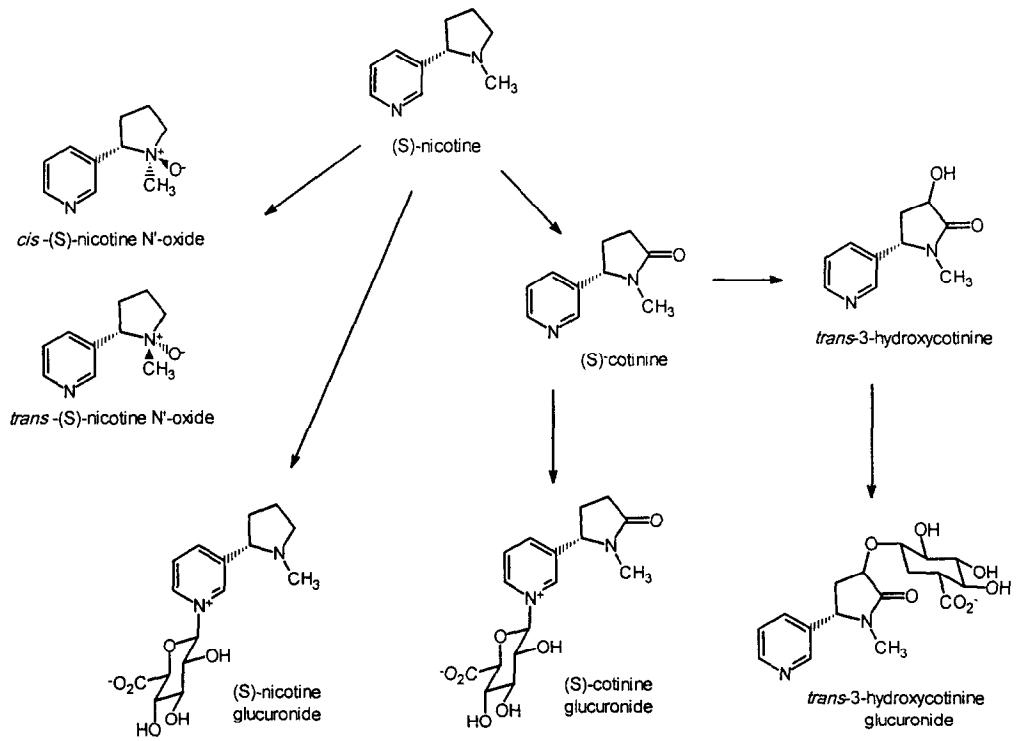

Fig. 1. Structures of the major metabolites of (*S*)-nicotine detected in human urine.

ion, which is converted to (*S*)-cotinine by the action of exogenously added aldehyde oxidase [12]. Others have reported that human CYP 2B6 cDNA expressed in mammalian cells is primarily responsible for (*S*)-nicotine $\Delta^{1',5'}$-iminium ion formation, but this study utilized a large, non-physiological millimolar substrate concentration [13]. The FMO from adult human liver (FMO3) is the principal enzyme that is responsible for exclusive formation of *trans*-(*S*)-nicotine *N*-1′-oxide [12]. In contrast, pig FMO1 catalyzes (*S*)-nicotine-*N*-1′-oxide formation with considerably less stereoselectivity [i.e. 49:51% *trans*:*cis* (*S*)-nicotine *N*-1′-oxide] than human FMO3 [12, 14]. For the cytochrome P450 enzymes examined, formation of (*S*)-nicotine *N*-1′-oxide occurred with a mean *trans*:*cis* ratio of 82:18 [8]. It is likely that the human liver cytochromes P450 do not possess the absolute stereoselectivity for the formation of (*S*)-nicotine *N*-1′-oxide observed *in vivo* and *in vitro* for human FMO3. Thus, formation of (*S*)-cotinine and (*S*)-nicotine *N*-1′-oxide in humans provides convenient markers of (*S*)-nicotine metabolism because both metabolites are chemically stable and because the metabolites are formed by different enzyme systems (i.e. CYP 2A6 and FMO3, respectively). As reported previously, *trans*-(*S*)-nicotine *N*-1′-oxide administered by intravenous infusion is not reduced appreciably *in vitro* or *in vivo* and does not represent a reservoir of (*S*)-nicotine that could reinforce the psychoactive properties of (*S*)-nicotine [8].

The objective of the present study was to examine whether (a) the kinetic properties of the principal human monooxygenases responsible for initial (*S*)-nicotine metabolism (i.e. CYP 2A6 and the FMO3 from adult human liver) contribute to the interindividual variability in (*S*)-nicotine metabolism, or (b) inhibition of one (*S*)-nicotine metabolic pathway by the product of another is responsible for the interindividual variability in human (*S*)-nicotine metabolism. The conclusion from this study is that the kinetic properties of the monooxygenases responsible for the initial carbon atom versus tertiary amine nitrogen atom oxidation and the kinetic properties of subsequent enzymes involved in metabolite formation are responsible for the profile of (*S*)-nicotine metabolites observed in humans.

Materials and Methods

Chemicals. (*S*)-Nicotine and (*S*)-cotinine were obtained from the Aldrich Chemical Co. (Milwaukee, WI). (*S*)- and (*R*)-Nornicotine was a gift of Peyton Jacobs, III (University of California, San Francisco). The *cis*- and *trans*-(*S*)-nicotine *N*-1′-oxides were prepared and purified as described previously [12]. All compounds of the NADPH-generating system were obtained from the Sigma Chemical Co. (St. Louis, MO). Chemicals, buffers, and other reagents were obtained from Fisher Scientific (Richmond, CA). All other chemicals were of the highest purity and were purchased from commercial sources.

Liver preparations. Adult human liver specimens were obtained [12] and liver microsomes were isolated according to the methods described before [15]; the specific content of microsomal cytochrome P450 protein concentration and the cytochrome P450 content were determined as described previously [16]. Each microsomal sample has been characterized thoroughly for all the major human liver cytochrome P450s including: 1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5 [17, 18]. Each microsomal sample has also been characterized thoroughly for human FMO1 and human FMO3 activity and protein content [12, 17] (see below). Aldehyde oxidase was obtained from the

supernatant of rat liver microsomes from untreated Sprague–Dawley rats.

Incubation systems and analytical methods. Incubations were done by the methods described previously [8, 12]. Careful time–course studies (i.e. 1.5, 3.0, 5.0, 7.5, 10.0, and 20 min) were done for each microsomal preparation to define the linear portion of the initial rate. FMO3 activity was prepared as described before [19, 20] and further purified by column chromatography by applying the protein solution to a DEAE-Sephacel column as described previously [21]. Active fractions were collected [i.e. those fractions exhibiting 10-(*N*,*N*-dimethylaminopentyl)-2-(trifluoromethyl)phenothiazine *N*-oxygenation activity and anti-guinea pig liver FMO3 immunoreactivity] and combined, dialyzed and concentrated by ultrafiltration. On the basis of SDS–PAGE, the recombinant enzyme was greater than 45% pure.

The profile of (*S*)-nicotine metabolites was determined by HPLC analysis of 2-propanol/dichloromethane extracts from incubations done in the presence of pig FMO1, recombinant human FMO3, and adult human liver microsomes. For (*S*)-cotinine determinations, the extracts were evaporated to a minimum volume, taken up in methanol, and separated on an IBM model 9533 HPLC system interfaced to an HP model 3396 Series II integrator with a UV detector set at 255 nm, fitted with a 5 μm, 25 cm × 4.5 mm AXXIOM silica gel analytical column (Richard Scientific, Novato, CA). The mobile phase consisted of CH_3CN/MeOH/$HClO_4$ (60%) (50:50:0.03, by vol.). (*S*)-Nicotine and (*S*)-cotinine were efficiently separated by this system with retention volumes of 16.4 and 7.3 mL, respectively. Quantification of substrate and metabolite was determined from peak areas of the chromatogram.

(*S*)-Nicotine and *trans*-(*S*)-nicotine *N*-1′-oxide were separated and quantified by a Rainin HPLC using a Knauer variable wavelength monitor set at 255 nm interfaced to an HP 3392A integrator and fitted with a 5 μm, 25 cm × 4.6 mm Microsorb C-18 reversed phase analytical column (Rainin Instrument Co., Inc., Emeryville, CA). The mobile phase consisted of 70% A and 30% B, where A was 10 mM potassium phosphate buffer (pH 7.0) with 0.1% (v/v) triethylamine, and B was CH_3CN/2-propanol/triethylamine (70:30:0.1, by vol.). (*S*)-Nicotine and *trans*-(*S*)-nicotine-*N*-1′-oxide were efficiently separated by this system with retention volumes of 8.3 and 5.6 mL, respectively. Quantification of substrate and metabolite was determined from peak areas of the chromatogram.

Results and Discussion

In vitro (S)-*Nicotine metabolism: Formation of* (S)-*cotinine in human liver microsomes.* The chemical synthesis, purification and analysis of prominent metabolites of (*S*)-nicotine from human liver preparations have been reported previously [12] and were used as standards for characterization of enzymatic and microsomal (*S*)-nicotine metabolism. The metabolism of a low concentration (i.e. 10 μM) of (*S*)-nicotine that more closely reflects the amount of (*S*)-nicotine expected to be present in the livers of smokers that previously studied was examined. In the previous studies, aerobic incubation of (*S*)-nicotine (500 μM) in the presence of adult human liver microsomes and NADPH resulted in the formation of (*S*)-cotinine and *trans*-(*S*)-nicotine *N*-1′-oxide [12]. For the incubations described here, (*S*)-nicotine $\Delta^{1',5'}$-iminium ion was directly converted into (*S*)-cotinine by the action of exogenously added rat liver cytosolic aldehyde oxidase to simplify the analysis.

In agreement with our previous study utilizing microsomes and under the experimental conditions employed herein, we did not observe formation of (*S*)-nornicotine [12]. In contrast to our previous results, experiments using a concentration of 10 μM (*S*)-nicotine failed to yield detectable levels of (*S*)-nicotine *N*-1′-oxide formation. This was not due to enzymatic or nonenzymatic reduction of the (*S*)-nicotine *N*-1′-oxide because the *N*-1′-oxide was found to be stable under these conditions. In the presence of adult human liver microsomes supplemented with aldehyde oxidase, the sole metabolite observed from (*S*)-nicotine was (*S*)-cotinine (Table 1). As shown in Table 1, production of (*S*)-cotinine was strongly dependent on the previous drug administration history of the subject from whom the hepatic microsomes were isolated. Among the highest rates of (*S*)-cotinine formation that occurred in the presence of human liver microsomes were those from barbiturate-pretreated subjects (i.e. subjects E and I, Table 1). As before, the rate of (*S*)-cotinine formation was found to correlate with the levels for CYP 2A6 as determined by immunoblot analysis ($r = 0.88$, patient N microsomal sample excluded) (Table 1). In agreement with our previous study, neither gender, age nor smoking history provided a direct relation between (*S*)-cotinine formation and CYP 2A6 levels. CYP 2A6-catalyzed formation of 7-hydroxycoumarin from coumarin correlated very well with the ability of adult human liver microsomes to produce (*S*)-nicotine $\Delta^{1',5'}$-iminium ion ($r = 0.75$, patient N microsomal sample excluded; $r = 0.58$, patient N microsomal sample included) at the low substrate concentration used. The differences in the correlation coefficients were probably due to the unusually large $V_{max}/K_{m_{app}}$ value for sample N.

From careful time–course studies that provided initial rate data for each microsomal preparation at low (i.e. 10 μM) and high (i.e. 500 μM) (*S*)-nicotine oxidation, kinetic constants for *C*-oxidation were calculated from the rate of (*S*)-cotinine formation by the HPLC procedures described in Materials and Methods. The $K_{m_{app}}$ and V_{max} values obtained from double-reciprocal plots of velocity versus substrate concentration are listed in Table 1. The mean $K_{m_{app}}$ and V_{max} values (N = 9) were calculated to be 39.6 μM and 444.3 pmol·min^{-1}·(mg protein)$^{-1}$, respectively. Most samples gave $K_{m_{app}}$ values between 20 and 75 μM, but samples A, B, L, and N yielded substantially different values and were not used in the correlation because the $K_{m_{app}}$ values observed were approximately 10-fold different than the mean value. Data obtained with sample C were unreliable. Possibly these samples had substantially different profiles of monooxygenases present.

The determination of adult human liver microsomal Michaelis–Menten kinetic constants can, in principle, allow the determination of *in vitro–in vivo* correlations useful in simulating the relationship among hepatic metabolism and plasma and liver clearance of (*S*)-nicotine [22]. For chemicals such as (*S*)-nicotine that are efficiently metabolized by the liver and that are not extensively protein bound, the clearance from the liver ($CL_{metabolic}$) may approximate the blood flow (Q_{liver}) after consideration of the intrinsic clearance ($CL_{intrinsic}$) (see equation 1) [23].

$$CL_{metabolic} = Q_{liver} \cdot f_{UB} \cdot CL_{intrinsic} / (Q_{liver} + f_{UB} \cdot CL_{intrinsic}) \quad (1)$$

Q_{liver} is 1450 mL·min^{-1}, f_{UB} is the unbound fraction of (*S*)-cotinine in blood (i.e. 95.1%), and $CL_{intrinsic}$ is calculated assuming the kinetic constants of Table 1 and that 2% of adult human liver (i.e. 1.8 kg) is microsomal protein (P).

From a knowledge of the K_m, V_{max}, and the fact that 2% of human liver becomes microsomes, a value for the intrinsic clearance that is related to the extraction ratio can be obtained by employing equation 2 [23].

$$CL_{intrinsic} = V_{max} \cdot P / K_{m_{app}} \quad (2)$$

$CL_{metabolic}$ was calculated to be 404 mL·min^{-1} or 4.33 mL·min^{-1}·kg^{-1} for a 70 kg human. $CL_{metabolic}$ represents only 23% of the total clearance of (*S*)-nicotine in humans [22], a value considerably lower than that expected based on the essentially complete metabolism of (*S*)-nicotine in humans [1–10]. The discrepancy may arise from alternative

Table 1. Kinetic values for oxidation of (*S*)-nicotine and coumarin by human liver microsomes*

Patient code	Gender	Age	Drug history†	CYP 2A6 immunoreactivity‡	Metabolite formed [pmol·min^{-1}(mg protein)$^{-1}$]		Cotinine formation		
					(*S*)-Cotinine	7-OH-Coumarin	$K_{m_{app}}$ (μM)	V_{max} (pmol·min^{-1}·mg^{-1})	$V_{max}/K_{m_{app}}$ (μL·min^{-1}·mg^{-1})
A	M	25	None	100	9.4	330	808.5	769	0.952
B	M	50	None	143	11.4	280	341.3	400	1.17
C	M	22	Ethanol	139	14.5	230			
D	M	31	None	389	100.6	740	52.4	629	12.0
E	M	14	Pentobarbital	355	90.8	600	34.2	402	11.7
F	F	50	Ethanol	395	69.8	520	45.1	385	8.53
G	F	48	Teldrin	264	53.6	410	51.5	330	6.40
H	F	28	None	192	89.4	330	23.2	298	12.8
I	M	43	Phenobarbital	718	258.0	780	20.2	781	38.5
J	F	55	None	139	77.9	360	75.6	435	5.74
K	M	23	Ethanol	569	116.5	940	29.2	457	15.6
L	F	58	Dopamine	31	10.8	110	297.0	331	1.12
M	M	18	Ethanol	224	70.5	400	24.8	246	9.89
N	M	21	Ethanol	132	246.0	430	2.7	313	115

* Incubations were performed as described previously [12]. Values are means of 5–6 determinations.
† For a summary of the smoking history of each subject, refer to Ref. 12.
‡ Relative percent immunoquantitation was determined with human cytochrome P450 form-selective antibodies [17].

or subsequent metabolic processes. For example, *trans*-3-hydroxy-(*S*)-cotinine must be catalyzed in a monooxygenase-dependent process distinct from that of (*S*)-cotinine formation and, likely, at another site distinct from the liver because we did not observe significant levels of *trans*-3-hydroxy-(*S*)-cotinine formation in human liver microsomes. Likewise, formation of glucuronides is likely non-monooxygenase dependent and possibly occurring in other organs and, thus, (*S*)-cotinine formation and clearance *in vivo* will be determined by a number of additional metabolic and enzymatic parameters.

To examine the molecular basis for the lack of *trans*-(*S*)-nicotine *N*-1′-oxide formation in the presence of adult human liver microsomes at the low (*S*)-nicotine substrate concentration used (i.e. 10 μM), we next investigated the influence of (*S*)-cotinine as an inhibitor of FMO-dependent *trans*-(*S*)-nicotine *N*-1′-oxide formation activity.

Effect of (S)-*cotinine on* (S)-*nicotine* N-1′-*oxide formation.* As described above, no detectable amount of (*S*)-nicotine *N*-1′-oxide was observed to be formed in the presence of 10 μM (*S*)-nicotine and adult human liver microsomes despite significant human FMO3-dependent conversion of 10-(*N*,*N*-dimethylaminopentyl)-3-(trifluoromethyl)phenothiazine to the corresponding tertiary amine *N*-oxide (data not shown). A possible explanation for the variable (*S*)-cotinine:*trans*-(*S*)-nicotine *N*-1′-oxide ratios is that (*S*)-cotinine inhibits human FMO3 responsible for *trans*-(*S*)-nicotine *N*-1′-oxide formation. This could explain the fact that, on the average, a typical human dose of (*S*)-nicotine results in excretion of only 4% as *trans*-(*S*)-nicotine *N*-1′-oxide.

Several careful Lineweaver–Burk studies of the effect of (*S*)-cotinine on cDNA-expressed adult human liver FMO3-mediated (*S*)-nicotine *N*-1′-oxygenation failed to show any significant level of inhibition. Thus, reciprocal plots of velocity of (*S*)-nicotine *N*-1′-oxide formation versus substrate concentration in the presence of (*S*)-cotinine (500 μM) failed to change the observed $K_{m_{app}}$ and V_{max} values [i.e. 100 μM and 14.1 nmol·min^{-1}·(mg protein)$^{-1}$, respectively [for cDNA-expressed adult human liver FMO3. We concluded that human FMO3 is not inhibited by (*S*)-cotinine and would not be anticipated to alter *trans*-(*S*)-nicotine *N*-1′-oxide formation under physiological conditions *in vivo*. Thus, *trans*-(*S*)-nicotine *N*-1′-oxide formation is determined by the Michaelis–Menten kinetic parameters and not inhibition by (*S*)-cotinine [i.e. at a low (*S*)-nicotine concentration, a low amount of *trans*-(*S*)-nicotine *N*-1′-oxide is formed]. Interestingly, in the presence of pig FMO1, (*S*)-cotinine was a competitive inhibitor of (*S*)-nicotine *N*-1′-oxide formation (i.e. K_i value of 675 μM, $r = 0.98$). This result suggests that FMO1 and FMO3 are functionally distinct with regard to (*S*)-nicotine metabolism.

It is important to study chemical or drug metabolism at sub-$K_{m_{app}}$ concentrations to determine how a substrate is handled under *in vivo* conditions. With a set of physiologically relevant biochemical parameters, a pharmacokinetic model can be constructed that stimulates the concentration of chemical or metabolite in the blood and organs. In addition, a comparison of the intrinsic clearance and the clearance of a chemical from an organ can reveal in a predictive sense the requirement for new metabolic pathways or additional sites of metabolism. The calculated metabolic clearance of (*S*)-nicotine via the (*S*)-cotinine pathway was only about 23% of the total clearance. The difference in total versus liver metabolic clearance is probably due to extensive non-monooxygenase-dependent metabolism in the liver including glucuronidation of (*S*)-nicotine and (*S*)-cotinine [1–6, 11]. We made no attempt to reconstitute the glucuronidation pathway in the studies reported here. In addition, the major urinary metabolite of (*S*)-nicotine in smokers (i.e. *trans*-3-hydroxy-(*S*)-cotinine, at 39% of a dose, [11]) was formed in extremely low levels in the adult human liver preparations used in this study [i.e. 0–8 pmol·min^{-1}·(mg protein)$^{-1}$ in the presence of 500 μM (*S*)-nicotine] (data not shown). Thus, we predict that *trans*-3-hydroxy-(*S*)-cotinine is formed in other tissues, possibly in the lung or intestine [24, 25], because we could not observe any significant levels of (*S*)-nicotine or (*S*)-cotinine metabolism in the adult human liver or kidney microsomes we examined (unpublished results).

Our data indicate that there is a 26-fold variability in formation of (*S*)-cotinine, approaching physiologic concentrations of (*S*)-nicotine in the presence of a set of selected adult human liver microsomes. The sources of variability of human (*S*)-nicotine metabolism undoubtedly arise from variability in human UDP glucuronyltransferase activity and non-hepatic and non-renal formation of *trans*-3-hydroxycotinine. However, it is possible that variant forms of CYP 2A6 are also present that could contribute to the metabolic variability observed. For example, adult human liver microsomal samples A, B, and L (Table 1) had unusually high $K_{m_{app}}$ values for (*S*)-nicotine *C*-oxidation and may have unusual forms of CYP 2A6 present. It is possible that the difference between total metabolic clearance and hepatic clearance for (*S*)-nicotine biotransformation derives from a (yet to be discovered) metabolite or non-hepatic metabolic pathway of (*S*)-nicotine.

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