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Nicotine metabolite ratio as an index of cytochrome P450 2A6 metabolic activity

Background: Nicotine and a variety of other drugs and toxins are metabolized by cytochrome P450 (CYP) 2A6. Our objective was to evaluate the use of oral nicotine with measurement of the *trans*-3'-hydroxycotinine (3HC)/cotinine (COT) metabolite ratio as a noninvasive probe of CYP2A6 activity.

Methods: Sixty-two healthy volunteers received an oral solution of deuterium-labeled nicotine (2 mg) and its metabolite cotinine (10 mg). Plasma nicotine and plasma and saliva cotinine and 3HC concentrations were measured over time.

Results: The 3HC/COT ratio derived from deuterium-labeled cotinine, measured in either plasma (2-8 hours after administration) or saliva (at 6 hours), was strongly correlated with the oral clearance of nicotine (r = 0.76-0.83, depending on the time of measurement). The 6-hour 3HC/COT ratio from nicotine derived from tobacco in 14 smokers was highly correlated with the ratio derived from deuterium-labeled nicotine (r = 0.88) and was also highly correlated with the oral clearance of nicotine (r = 0.90). Two subjects homozygous for inactive CYP2A6 alleles produced no 3HC, confirming the specificity of the metabolite ratio. The 3HC/COT ratio was also highly correlated with the clearance and half-life of cotinine, consistent with the fact that cotinine is also primarily metabolized by CYP2A6.

Conclusions: The 3HC/COT ratio derived from nicotine either administered as a probe drug or from tobacco use, measured in either plasma or saliva, is highly correlated with the oral clearance of nicotine. The ratio appears to be a useful noninvasive marker of the rate of nicotine metabolism (which is important in studying nicotine addiction and smoking behavior), as well as a general marker of CYP2A6 activity (which is important in studying drug and toxin metabolism). (Clin Pharmacol Ther 2004;76:64-72.)

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The liver enzyme cytochrome P450 (CYP) 2A6 is the major enzyme responsible for the metabolism of nicotine.[1,2] Individual differences in CYP2A6 activity may explain, at least in part, interindividual variability in nicotine intake and smoking-associated disease risk.[3,4] CYP2A6 also contributes to the metabolism of other drugs such as halothane, disulfiram, methoxyflurane, and valproic acid and participates in the activation of carcinogens such as aflatoxin B_1 and the nicotine-derived nitrosamines NNK (4-[methylnitrosoamino]-1-[3-pyridyl]-1-butanone), NNAL (4-[methylnitrosoamino]-1-[3-pyridyl]-1-butanol), and NNN (*N*'-nitrosonornicotine).[5,6]

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CYP2A6 levels and activity in the liver, as well as CYP2A6 activity as estimated by the metabolic clearance of nicotine, vary widely among individuals.[1,7,8] A number of *CYP2A6* alleles have been identified, many of which are associated with reduced activity and a gene duplication associated with increased activity.[3,9,10] The known genetic polymorphisms do not account for the wide population variability in CYP2A6 activity. Novel *CYP2A6* variant alleles are being rapidly discovered,[11] but genotype analysis remains incomplete. Thus a phenotypic marker of CYP2A6 would be useful for studying the biologic importance of individual differences in CYP2A6 activity. Ideally, this phenotypic probe could be used in smokers and nonsmokers.

In the past we have used the metabolic clearance of nicotine and, in particular, the clearance of nicotine via the cotinine pathway as a phenotypic marker of CYP2A6 activity.[12] Although we believe this is an excellent measure, this method requires intravenous administration of labeled nicotine and cotinine. This involves admission of patients to a research ward and cardiovascular monitoring during the infusion and thus is not practical for larger-scale studies of smoking behavior or smoking-related disease risk.

A common approach to assessing a drug metabolism enzyme phenotype is to measure the ratio of the concentration of a metabolite produced by the pathway of interest to the concentration of the parent drug. Thus one might consider measuring the ratio of the metabolite cotinine (COT) to the parent nicotine. However, the half-life of nicotine is relatively short (2 hours), whereas the half-life of cotinine is long (16 hours).[12] As a consequence of the short half-life of nicotine, the ratio of cotinine to nicotine is highly dependent on the time of the last nicotine exposure. Nakajima et al[13] have described a CYP2A6 phenotyping test that uses the ratio of the concentrations of cotinine to nicotine in plasma after nicotine gum is chewed. To allow time for elimination of cotinine from the plasma, smokers had to stop smoking for 2 weeks before testing. The cotinine-to-nicotine ratio was shown to differ among individuals with different CYP2A6 genotypes,[14] but the ratio was not validated against a continuous, direct measure of CYP2A6 phenotype (such as nicotine clearance).

Cotinine is itself metabolized primarily by CYP2A6 to *trans*-3′-hydroxycotinine (3HC).[15] The half-life of 3HC administered alone is 5 to 6 hours.[16] However, when 3HC is generated from cotinine, its elimination half-life becomes generation-limited and is similar to that of cotinine. Therefore the ratio of 3HC to COT should be fairly constant over time.

We have evaluated the use of an oral nicotine probe for noninvasive assessment of CYP2A6 activity. Small doses of deuterium-labeled nicotine and cotinine were administered, with subsequent measurement of blood and saliva levels of nicotine and metabolites. We have examined the relationship between the nicotine metabolite ratio 3HC/COT measured in blood or saliva with the oral clearance of nicotine, in an attempt to identify a noninvasive marker of CYP2A6 activity.

METHODS

Subjects

The subjects comprised 62 healthy volunteers recruited from newspaper advertisements. These subjects are part of an ongoing 300-person study of racial-ethnic differences in nicotine metabolism. The subjects' mean age was 32 years (range, 19-52 years), and 32 (52%) were men. The racial-ethnic distribution was 40% white, 34% Asian, 13% Hispanic, and 13% black. Assessment of smoking status was based on saliva and plasma concentrations of cotinine at a screening visit; 16 of subjects (26%) were classified as cigarette smokers. One subject reported that he was a nonsmoker but had cotinine levels indicative of smoking. Two subjects by history were light smokers but were classified as nonsmokers on the basis of cotinine levels.

Written informed consent was obtained for each subject. The study was approved by the Committee on Human Research at the University of California, San Francisco.

Procedures

Subjects were asked to come to the General Clinical Research Center at San Francisco General Hospital in the morning, with instructions not to eat or use tobacco starting at 10 PM on the previous night. They were asked to refrain from grapefruit or grapefruit juice for 48 hours before and for the duration of the study. At approximately 8 AM, the subjects were given, in solution, 2 mg of deuterium-labeled nicotine (nicotine-3′-3′-d_2) and 10 mg of deuterium-labeled cotinine (cotinine-2,4,5,6-d_4). These labeled compounds were synthesized in our laboratory as described previously.[12,17] No impurities were detected on analysis of the compounds by gas chromatography–mass spectrometry (GC-MS) and by thin-layer chromatography. The 2-mg dose of nicotine was selected as a dose that is well tolerated by nonsmokers but results in plasma nicotine concentrations that are easily measurable. The 10-mg dose of cotinine was selected as a dose that would result in adequate saliva concentrations of cotinine over a 60-hour period, to allow us to determine the terminal

elimination half-life of cotinine. Two hours after dosing, breakfast was provided. Subjects were administered 1 g of ammonium chloride 1 hour before the oral dosing and again 4 hours later to acidify the urine and reduce the variability in the renal clearance of nicotine.

Blood samples were collected at 0, 0.5, 1, 1.5, 2, 3, 4, 6, and 8 hours after dosing for measurement of nicotine and metabolite concentrations. Blood was also collected for extraction of deoxyribonucleic acid for genotyping of the nicotine-metabolizing *CYP2A6* gene. Saliva (3 mL) was collected before and at 6, 12, 24, 36, 48, and 60 hours after nicotine and cotinine dosing. All saliva samples beyond 6 hours were collected at home and returned to the research center on a subsequent day. Smokers were permitted to smoke after lunch during the day while in the Clinical Research Center, but only 5 of the smokers did so. They were free to smoke as desired for the period of time when saliva samples were being collected at home.

Analytic chemistry

Measurement of nicotine levels in plasma and cotinine levels in saliva was performed by GC-MS, by use of methods previously reported by our laboratory.[18,19] We used GC-MS to measure low levels of nicotine because there is less interference from environmental (unlabeled) nicotine. During the course of a liquid chromatography–mass spectrometry (LC-MS) run (as described later), there is a significant amount of nicotine in the mobile phase. In contrast, with GC-MS, there is a negligible nicotine level in the helium carrier gas. 3'-Hydroxycotinine is much easier to analyze by LC-MS because it is not necessary to prepare a derivative.

Determination of cotinine, trans-3'-hydroxycotinine, and deuterium-labeled isotopomers. Concentrations of cotinine, 3HC, and their deuterium-labeled isotopomers (d_2 and d_4) in plasma and saliva (6-hour sample) were determined by liquid chromatography–tandem mass spectrometry. The method is similar to a published procedure for determining cotinine concentrations in serum of nonsmokers[20] but has been expanded to include the determination of 3HC and the deuterium-labeled analogs. Deuterium-labeled cotinine (cotinine-d_0) and deuterium-labeled 3HC (*trans*-3'-hydroxycotinine-d_0) were used as internal standards. The mass spectrometer was operated in the positive ion mode by use of atmospheric pressure chemical ionization. Quantitation was achieved via selected reaction monitoring of the transitions mass-to-charge ratio (*m/z*) 177 to *m/z* 80 for cotinine, *m/z* 179 to *m/z* 80 for cotinine-d_2, *m/z* 181 to *m/z* 84 for cotinine-d_4, *m/z* 193 to *m/z* 80 for 3HC, *m/z* 195 to *m/z* 80 for 3HC-d_2, and *m/z* 197 to *m/z* 84 for 3HC-d_4, as well as the transitions *m/z* 186 to *m/z* 84 and *m/z* 202 to *m/z* 84 for the respective internal standards. Calibration curves were constructed from peak area ratios of the analyte to its internal standard by linear regression. Standard curves were linear over the concentration range studied (0 to 500 ng/mL). Precision (between-run coefficient of variation, n = 6) ranged from 1.9% to 4.2% for cotinine and 1.3% to 5.5% for 3HC; accuracy (between-run mean percent of expected values, n = 6) ranged from 98% to 101% for cotinine and 98% to 103% for 3HC, for concentrations ranging from 5 to 100 ng/mL. The limit of quantitation was 0.2 ng/mL for both cotinine and 3HC.

Genotyping was performed by use of established assays for *CYP2A6 *2, *4, *7, *8,* and **10* alleles that have been previously characterized.[9,21] The novel assays that were used for **5, *6, *9, *11,* and **12* alleles were based on the same 2-step polymerase chain reaction approach (Schoedel K, unpublished observations, 2003). Results of the genotype-phenotype associations will be presented when the full 300-person study is reported. The relevance of genotyping in this study was to examine genotypes of individuals who did not generate 3HC to help us determine the specificity of 3HC generation as a function of CYP2A6 activity.

Data analysis

The main measure of nicotine metabolism was oral plasma clearance of nicotine-d_2, determined as Dose/Area under the plasma nicotine concentration–time curve extrapolated to infinity. The oral saliva clearance of cotinine was computed in a similar manner by use of the area under the saliva cotinine concentration–time curve. In a previous study, we have shown that the plasma and saliva clearances of cotinine are similar.[22] Half-lives of nicotine-d_2 and cotinine-d_4 were determined by nonlinear least squares fitting of the log concentration versus time by use of WinNonlin.[23] The 3HC/COT ratio was determined at multiple times in plasma and at 6 hours in saliva.

The relationship between various independent variables and the oral clearance of nicotine was determined by the Pearson correlation coefficient. Comparisons between smokers and nonsmokers were performed with unpaired *t* tests.

RESULTS

Nicotine and metabolite levels in blood and saliva

Mean plasma concentrations of nicotine and metabolites over an 8-hour period after oral dosing are shown

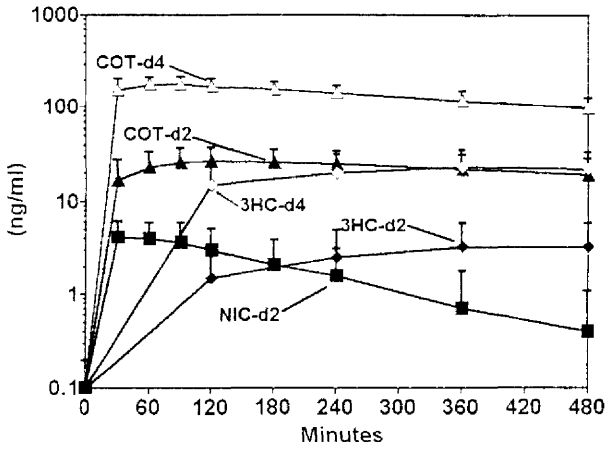

Fig 1. Mean deuterium-labeled nicotine and metabolite plasma concentrations over time (n = 62). *Solid squares*, Nicotine-d_2 (NIC-d2); *solid triangles*, cotinine (COT)–d_2; *solid diamonds*, *trans*-3'-hydroxycotinine (3HC)–d_2; *open triangles*, COT-d_4; *open diamonds*, 3HC-d_4. *Bars* indicate SDs.

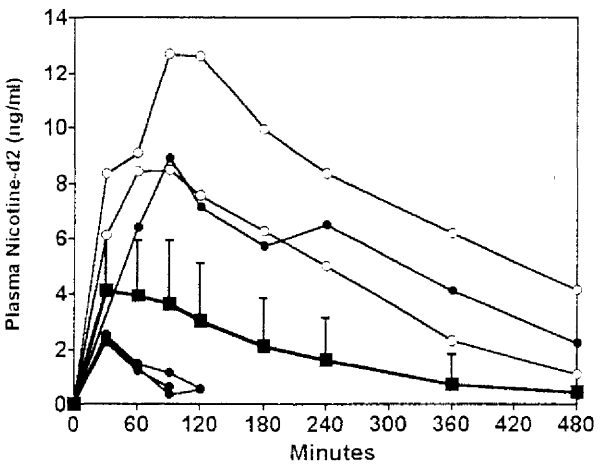

Fig 2. Plasma nicotine-d_2 concentrations after 2 mg oral nicotine-d_2. The *squares* represent the mean concentration for all subjects. The *circles* represent individual subjects. The *upper 3 curves* represent data from subjects with the slowest clearance of nicotine, and the *lower 3 curves* represent data from subjects with the fastest clearance. The *open circles* are subjects with the **4/*4* genotype. The other slow metabolizer had the **7/*7* genotype. Of the 3 fastest metabolizers, 2 had the **1/*1* genotype and deoxyribonucleic acid was not available in 1.

in Fig 1. The mean peak plasma nicotine-d_2 concentration was 4.7 ng/mL (95% confidence interval [CI], 4.1-5.3 ng/mL), occurring at a mean of 51 minutes (95% CI, 45-57 minutes) after dosing. To illustrate interindividual variability, Fig 2 shows plasma nicotine-d_2 concentration–time curves both as the mean for all subjects and for the 3 subjects with the slowest nicotine-d_2 clearance and the 3 subjects with the most rapid nicotine-d_2 clearance. The genotypes of these subjects are described in the legend to Fig 2. The mean peak plasma cotinine-d_4 concentration was 195 ng/mL (95% CI, 183-217 ng/mL). The mean cotinine-d_2 peak was 29 ng/mL (95% CI, 26-32 ng/mL). The peak plasma 3HC-d_4 concentration averaged 24.5 ng/mL (95% CI, 21-27 ng/mL). The mean saliva cotinine-d_2 and cotinine-d_4 concentrations over time are shown in Fig 3. The mean peak saliva cotinine-d_4 concentration averaged 120 ng/mL (95% CI, 110-131 ng/mL). Plasma and saliva cotinine levels were similar in smokers and nonsmokers.

Pharmacokinetic analyses

Table I provides the pharmacokinetic parameters for nicotine-d_2, derived from plasma concentration data, and for cotinine-d_4, derived from saliva concentration data. There was no difference in the peak nicotine-d_2 concentration between smokers and nonsmokers, but the mean time to peak nicotine-d_2 concentration tended to be longer in smokers compared with nonsmokers (60

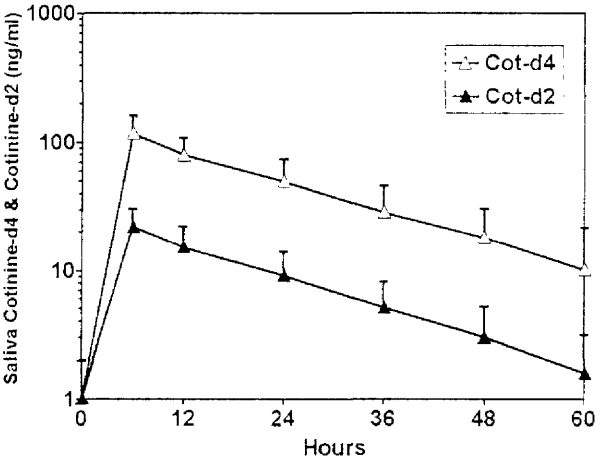

Fig 3. Mean saliva concentrations of COT-d_4 (*open triangles*) and COT-d_2 (*closed triangles*) after oral administration of 10 mg COT-d_4 and 2 mg nicotine-d_2. *Bars* indicate SDs.

versus 48 minutes, $P = .09$). When men and women were compared, there was a significant difference in time to peak nicotine-d_2 plasma concentration (45 versus 57 minutes, $P = < .05$). The half-life of cotinine-d_4 was significantly longer in men than in women (1076

Table I. Pharmacokinetics of oral nicotine-d_2 and cotinine-d_4

	All subjects (n = 62) (mean and 95% CI)	*Nonsmokers (n = 46) (mean and 95% CI)*	*Smokers (n = 16) (mean and 95% CI)*
Nicotine-d_2 (plasma data)			
C_{max} (ng/mL)	4.7 (4.1-5.3)	4.7 (3.94-5.41)	4.9 (3.88-5.94)
t_{max} (min)	51 (45-57)	48 (41-54)	60* (47-73)
Cl/F (mL/min)	3564 (2802-4327)	3821 (2852-4790)	2824 (1769-3880)
$t_{1/2}$ (min)	116 (104-128)	112 (98-126)	127 (98-156)
Cotinine-d_4 (saliva data)			
C_{max} (ng/mL)	120 (110-131)	118 (106-130)	127 (102-152)
Cl/F (mL/min)	68 (58-77)	67 (58-76)	69 (38-100)
$t_{1/2}$ (min)	950 (841-1058)	957 (820-1095)	927 (760-1095)

CI, Confidence interval; C_{max}, mean peak plasma concentration; t_{max}, time to mean peak plasma concentration; Cl/F, systemic clearance/bioavailability; $t_{1/2}$, elimination half-life.
*$P = .087$, compared with nonsmoker.

Table II. 3HC/COT ratios in plasma and saliva and their correlation with nicotine and cotinine clearance and cotinine half-life

	Saliva	*Plasma*			
	6 h	*2 h*	*4 h*	*6 h*	*8 h*
3HC-d_4/COT-d_4 ratio	0.19	0.09	0.15	0.21	0.24
95% CI	0.16-0.23	0.08-0.10	0.13-0.17	0.18-0.24	0.20-0.28
3HC-d_2/COT-d_2 ratio	0.22	0.10	0.17	0.21	0.25
95% CI	0.19-0.26	0.08-0.11	0.14-0.19	0.17-0.24	0.21-0.29
3HC-d_0/COT-d_0† ratio	0.38	0.46	0.46	0.45	0.46
95% CI	0.18-0.59	0.20-0.73	0.18-0.73	0.19-0.71	0.17-0.75
*Correlation (r) between kinetic parameter and 3HC-d_4/COT-d_4 ratios**					
Nicotine-d_2 Cl/F	0.78	0.82	0.83	0.79	0.76
Cotinine-d_4 Cl/F	0.71	0.62	0.62	0.65	0.74
Cotinine-d_4 $t_{1/2}$	–0.50	–0.52	–0.53	–0.53	–0.52
*Correlation (r) between nicotine clearance and 3HC-d_2/COT-d_2 ratios**					
Nicotine-d_2 Cl/F	0.68	0.74	0.78	0.77	0.77
Correlation (r) between nicotine clearance and 3HC-d_0/COT-d_0 ratios†*					
Nicotine-d_2 Cl/F	0.95	0.70	0.74	0.90	0.90
*Correlation (r) between d_4 and d_2 3HC/COT ratios**					
3HC/COT ratio	0.91	0.83	0.93	0.91	0.96
Correlation (r) between d_4 and d_0 3HC/COT ratios†*					
3HC/COT ratio	0.92	0.94	0.87	0.88	0.87
Correlation (r) between d_2 and d_0 3HC/COT ratios†*					
3HC/COT ratio	0.90	0.83	0.96	0.86	0.86

3HC, *Trans*-3′-hydroxycotinine; COT, cotinine.
*All correlations, $P < .01$.
†Smokers only, n = 14. d_0 refers to unlabeled compounds.

versus 844 minutes, $P < .05$). Nicotine-d_2 clearance and cotinine-d_4 clearance were significantly correlated ($r = 0.59$, $P < .01$), as was cotinine-d_4 half-life with nicotine-d_2 clearance ($r = -0.59$, $P < .01$).

Metabolite ratios

Data for the ratio of 3HC/COT for cotinine-d_4 (3HC/COT-d_4) are shown in Table II. The ratios measured in plasma and saliva at 6 hours were highly correlated ($r = 0.88$, $P < .001$), as shown in Fig 4. The 3HC/COT-d_4 ratios were highly correlated with the ratios derived from nicotine-d_2, shown in Table II. The 3HC/COT-d_4 ratio was strongly correlated with the oral clearance of nicotine, with correlation coefficients ranging from 0.76 to 0.83, depending on the time of measurement (Table II). The 3HC/COT-d_4 ratio mea-

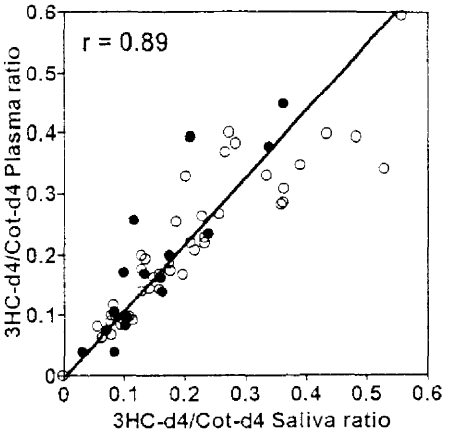

Fig 4. Correlation between 3HC-d4/COT-d4 ratio in saliva and in plasma, collected 6 hours after oral cotinine dosing. *Open circles* represent nonsmokers; *solid circles* represent smokers. The *solid line* is the regression curve.

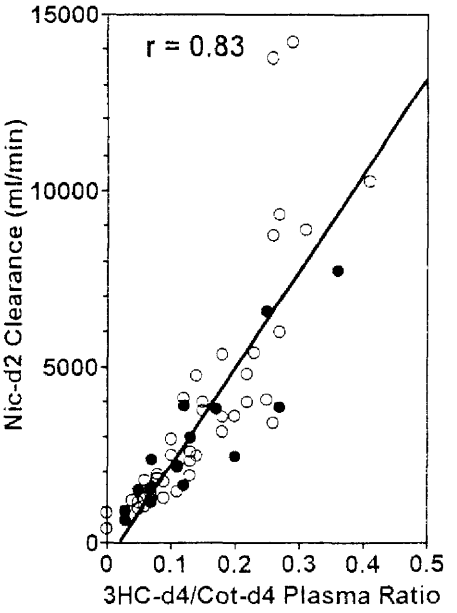

Fig 5. Correlation between nicotine-d_2 (Nic-d2) oral clearance and the 3HC-d4/COT-d4 ratio in plasma, collected 4 hours after oral cotinine dosing. *Open circles* represent nonsmokers; *solid circles* represent smokers. The *solid line* is the regression curve.

sured at various times was strongly correlated with cotinine-d_4 clearance and cotinine-d_4 half-life (Table II). Data showing the correlation between nicotine clearance and plasma 3HC/COT-d_4 ratio at 4 hours are shown in Fig 5. There was no difference in ratios when smokers and nonsmokers were compared.

The 3HC/COT ratios derived from nicotine-d_2 (3HC/COT-d_2) were likewise highly correlated with the oral clearance of nicotine, although in 12 subjects the concentrations of 3HC-d_2 fell below the limit of quantitation by 8 hours.

Among 16 smokers, plasma and saliva samples were assayed for 3HC and cotinine derived from natural nicotine (from cigarettes), as well as from labeled cotinine. In 2 smokers the cotinine concentrations were extremely low (presumably reflecting not smoking for a few days before the study), so 3HC/COT ratios could not be determined. The ratios based on nicotine derived from tobacco (unlabeled nicotine) were higher than those from labeled nicotine (Table II). The ratios derived from unlabeled and labeled nicotine were highly correlated. The correlation coefficients between the 3HC/COT ratio from natural (unlabeled) nicotine (3HC/COT-d_0) and the 3HC/COT-d_4 ratio ranged from 0.87 to 0.94 (all $P < .01$), as presented in Table II. The correlation coefficients between 3HC/COT-d_0 and 3HC/COT-d_2 ratio ranged from 0.83 to 0.96 (all $P < .01$). The 3HC/COT-d_0 ratio in smokers was highly correlated with the oral clearance of nicotine, with correlation coefficients ranging from 0.70 to 0.95 (Table II).

Two subjects had no detectable 3HC. These subjects were determined to have null alleles for the *CYP2A6* gene (*CYP2A6*4/*4*). These 2 subjects had the slowest and third-slowest oral clearances of nicotine (382 and 829 mL/min, respectively) (Fig 2). These subjects did generate small amounts of cotinine from nicotine.

DISCUSSION

Our report describes a noninvasive method for assessing the clearance of nicotine, which reflects the metabolic activity of CYP2A6. We provide novel data showing that the ratio of 3HC/COT, measured in plasma or in saliva, is a good predictor of the oral clearance of nicotine and, therefore, of CYP2A6 activity.

Deuterium-labeled nicotine and cotinine were administered to our subjects so that we could study both smokers (who have unlabeled nicotine and metabolites in their body derived from cigarette smoking) and nonsmokers. The dose of nicotine, 2 mg, was small and was well tolerated by all subjects. The cotinine dose of 10 mg was higher than that of nicotine so that we could measure adequate concentrations of cotinine in the saliva for as long as 60 hours.

The main measure for CYP2A6 activity was taken as the oral clearance of nicotine. Oral clearance reflects systemic clearance/bioavailability. Thus it reflects both systemic clearance and first-pass metabolism. Our prior research found that the oral bioavailability of nicotine averages 30% to 40%, indicating substantial presystemic metabolism.[24] Nicotine is known to be a high-extraction drug, and most of its presystemic metabolism can be accounted for by the expected contribution of hepatic metabolism. CYP2A6 is present in small amounts in intestinal tissue, so intestinal first-pass metabolism could contribute to some extent.[25,26] However, intestinal first-pass metabolism is likely to be minor compared with hepatic first-pass metabolism. If we assume that absorption is complete and that elimination is primarily via liver metabolism (which is thought to be the case for nicotine), the oral clearance of a drug may be taken as a measure of intrinsic hepatic clearance.[27]

Nicotine is metabolized primarily by CYP2A6, although there is also metabolism by other pathways including *N*-oxidation via flavin monooxygenase and glucuronidation.[28] Because most of the metabolism is via CYP2A6, it is reasonable to use the oral clearance of nicotine as a marker of CYP2A6 activity.

In support of this proposition, the 2 subjects with inactive *CYP2A6* alleles (*CYP2A6*4/*4*) had higher peak nicotine levels and lower oral nicotine clearances than most other subjects. Similarly, higher plasma nicotine levels after oral nicotine were found by Xu et al[9] in subjects with *CYP2A6*4/*4* variants and by Sellers et al[29] after treating subjects with 8-methoxypsoralen, an inhibitor of CYP2A6. Cotinine is metabolized by CYP2A6 to 3HC.[15] Cotinine is also metabolized by *N*-oxidation and by glucuronidation.[28] Because cotinine is metabolized much more slowly than nicotine, there is little first-pass metabolism and the oral bioavailability of cotinine is approximately 100%.[30] In addition, plasma and saliva cotinine concentrations have been shown to be highly correlated.[22] Therefore the clearance of cotinine administered orally and measured in saliva should be highly correlated with the clearance of cotinine measured after intravenous dosing. As we reported previously, nicotine clearance and cotinine clearance were significantly correlated, reflecting the contributions of CYP2A6 to the metabolism of both.[12]

Measuring metabolite ratios is an attractive way by which to noninvasively assess drug metabolism phenotype. Ideally, the metabolite should reflect metabolism by one enzyme only, and the ratio of the metabolite to parent drug should be stable over time. Measuring the cotinine/nicotine ratio is not optimal because cotinine can be produced from nicotine, at least in small amounts, even in the absence of CYP2A6,[31] and more importantly, the ratio of cotinine/nicotine is quite variable over time, depending on the time of last nicotine dosing.

The 3HC/COT ratio is a much better candidate to be a metabolic marker of CYP2A6 activity. We show in this report that 2 subjects homozygous for CYP2A6 null alleles, which results in a complete lack of CYP2A6 protein, generated no 3HC, supporting the specificity of 3HC as a marker of CYP2A6 activity. When 3HC is generated from cotinine, its elimination is generation-limited. Therefore, after 3HC levels peak, the curves of cotinine and 3HC decline in parallel over time. Our data showed that the ratio increases over an 8-hour period after dosing, but the rate of rise between 6 and 8 hours is slow. We also found that the 3HC/COT ratio was similar and highly correlated in plasma and saliva at 6 hours, indicating that saliva can be used as the biofluid for measurement of the ratio, making the test entirely noninvasive.

We found that the 3HC/COT ratios from unlabeled nicotine in smokers were higher than but highly correlated with the ratio from the labeled compounds. Correspondingly, the 3HC/COT ratio derived from natural nicotine was highly correlated with the oral clearance of nicotine. This means that in smokers the 3HC/COT ratio derived from natural nicotine can be used as a marker of CYP2A6 activity, without the administration of any test compound.

The most likely explanation for higher metabolite ratios derived from natural nicotine compared with those derived from labeled nicotine or cotinine is that the former ratios represent steady-state values whereas the latter ratios have not yet reached steady state. Although the generation rate of 3HC is expected to parallel the concentration of cotinine in the blood, the elimination half-life of 3HC itself averages about 6 hours.[16] Therefore it would be expected that 3HC levels would continue to increase over 3 to 4 half-lives (18-24 hours) before reaching steady state. With regular smoking, blood levels of 3HC (derived from natural cotinine) achieve steady state. Therefore the ratio of 3HC/COT derived from natural nicotine is expected to be higher than that observed 8 hours after administration of a labeled test dose of nicotine or cotinine, at which time labeled 3HC levels are not yet at steady state.

Although the 3HC/COT ratio is highly correlated with the oral clearance of nicotine, the ratio accounts for only 69% ($r = 0.83$, $r^2 = 0.69$) of the variation in oral nicotine clearance. The reason the metabolite ratio

does not account for a higher percentage of variance is most likely because the oral clearance of nicotine is contributed to by some renal excretion of nicotine, as well as the metabolism of nicotine via other minor pathways, as mentioned previously. Of note, however, is that the extent of correlation between the nicotine metabolite ratio and oral nicotine clearance is similar to that of other well-established metabolite ratios, such as the urine caffeine metabolite ratio and its correlation with caffeine clearance, which is widely used as a marker of CYP1A2 activity.[32]

In summary, we provide data indicating that the ratio of 3HC/COT measured in plasma or saliva is highly correlated with the oral clearance of nicotine. We propose that this ratio is useful as a noninvasive marker of the rate of nicotine metabolism (which can be of use in smoking and addiction studies) and as a general marker of CYP2A6 activity (which may be useful in studies of the metabolism of other drugs and carcinogens). In studying a general population that includes persons who do use tobacco and those who do not use tobacco, we recommend oral administration of labeled nicotine or cotinine with measurement of the 3HC/COT ratio in plasma at 2 to 8 hours after dosing or in saliva at 6 hours after dosing. Of course, the time of sampling should be standardized for all subjects within a particular study. If the study population consists of smokers exclusively, then the 3HC/COT ratio derived from natural nicotine in tobacco can be used.

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