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Disposition Kinetics and Metabolism of Nicotine and Cotinine in African American smokers: Impact of *CYP2A6* Genetic Variation and Enzymatic Activity

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Abstract

Objective—The rate of nicotine metabolism, determined primarily by *CYP2A6* activity, influences tobacco dependence and smoking-induced disease risk. The prevalence of *CYP2A6* gene variants differs by race, with greater numbers in African American (AA) compared to Caucasians. We studied nicotine disposition kinetics and metabolism by *CYP2A6* genotype and enzymatic activity, as measured by nicotine metabolite ratio (NMR), in AA smokers.

Methods—Subjects were administered IV infusions of deuterium-labeled nicotine and cotinine. Plasma and urine concentrations of nicotine and metabolites were measured, and pharmacokinetic parameters estimated.

Results—Pharmacokinetic parameters and urine metabolite excretion data were analyzed by *CYP2A6* genotype and by NMR. A number of gene variants were associated with markedly reduced nicotine and cotinine clearances. NMR was strongly correlated with nicotine ($r=0.72$) and cotinine ($r=0.80$) clearances. Subjects with higher NMR excreted significantly greater nicotine C-oxidation and lower non C-oxidation products compared to lower NMR subjects.

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Conflict of Interest/Disclosure

NLB serves as a paid consultant to pharmaceutical companies that are developing or that market smoking cessation medications. He also has been a paid expert witness in litigation against tobacco companies, including on issues related to light cigarettes. RFT has served as paid consultant to pharmaceutical companies on unrelated topics. None of the other authors have any competing interests to declare.

Conclusions—*CYP2A6* genotype, NMR and nicotine pharmacokinetic data may inform studies of individual differences in smoking behavior and biomarkers of nicotine exposure.

Keywords

Nicotine; cotinine; smoking; genetics; *CYP2A6*; drug metabolism; pharmacokinetics

Introduction

African American smokers differ from non-Hispanic White smokers in several respects. African Americans on average smoke fewer cigarettes per day, take in more nicotine and tobacco smoke per cigarette, are more highly dependent and have a greater risk of lung cancer compared to White smokers [1–4]. They also differ in the average rate of nicotine metabolism [5]. The rate of nicotine metabolism is an important determinant of smoking behavior and its consequences, including the number of cigarettes smoked per day, the ease of quitting smoking and the risk of smoking-induced lung cancer [6, 7].

Nicotine is primarily metabolized by the liver enzyme *CYP2A6* to cotinine. Nicotine is also metabolized by glucuronidation (primarily by *UGT2B10*) and N-oxidation (primarily by *FMO3*), and there may be small contributions of other enzymes [8]. Cotinine is the major proximal metabolite of nicotine. Cotinine is metabolized exclusively by *CYP2A6* to trans-3' hydroxycotinine (3HC), and the ratio of 3HC/cotinine, known as the nicotine metabolite ratio (NMR), is a validated biomarker of *CYP2A6* enzymatic activity [9, 10]. Large racial/ethnic differences in the rate and pathways of nicotine and cotinine metabolism, as well as the frequency of *CYP2A6* gene variants, are known to exist [9, 11–13]. An understanding of the relationship between nicotine clearance and metabolism phenotypes and genotypes may be helpful in relating genotype and phenotype to smoking behavior and disease risk.

We previously published a detailed analysis of the relationship between the metabolism and disposition kinetics of nicotine and common variants in the *CYP2A6* gene, but that study was primarily among Caucasian smokers [14]. African Americans possess at reasonable frequencies some of the same alleles as Caucasians, such as *CYP2A6**9, but also a number of different *CYP2A6* gene variants, such as *CYP2A6**17, *20, *23–*27 and *35, which are generally not found in Caucasians. These various *CYP2A6* gene variants are associated with reduced NMR but have not been examined with respect to nicotine pharmacokinetics [15–17]. Given the underlying racial/ethnic differences in smoking patterns, lung cancer risk and *CYP2A6* gene variants, it is important to assess how these *CYP2A6* gene variants impact nicotine clearance. Thus, we performed the first investigation of *CYP2A6* genetic variation in association with nicotine pharmacokinetics and metabolism among AA smokers. The nicotine metabolite ratio has been assessed by quartiles in several clinical trials relating NMR to smoking cessation in response to pharmacotherapy [18–21]. Another aim of our study was to examine NMR quartiles in association with nicotine pharmacokinetics and metabolism in AA smokers.

Methods

Subjects

Seventy healthy African American smokers of 5 or more cigarettes per day were recruited and 60 completed the pharmacokinetic study. They were aged between 21 and 60 years (average 35) and smoked an average of 13.7 cigarettes per day (range 5 – 35). Participants were recruited via Craigslist and newspaper advertisements. They were screened for study participation by telephone. Exclusion criteria included pregnancy, use of known drug metabolism altering medications, uncontrolled hypertension or diabetes, heart, lung and cardiovascular disease, cancer, liver and kidney disease, and active substance abuse or dependence. The study was approved by the Institutional Review Boards at the University of California San Francisco and the University of Toronto.

Experimental Procedure

Subjects were asked to come to the Clinical Study Center at the San Francisco General Hospital for a four day pharmacokinetic study after 7 days at home during which they smoked their usual brand of cigarettes, which were supplied by the study. During these 7 days subjects smoked an average of 12.2 cigarettes per day (range 4–29). They came to the hospital the evening before and abstained from cigarettes starting at 10 PM on the night prior to infusion.

In a fasting state at about 9:00 AM, subjects received a simultaneous thirty minute infusion of deuterium-labeled nicotine-d2 (3',3'-dideuteronicotine) and cotinine-d4 (2, 4, 5, 6-tetradeterocotinine). The doses of nicotine and cotinine were 2.0 ug/kg/min. The synthesis of deuterium labeled compounds has been described previously [22]. Blood samples for measurement of plasma nicotine-d2 concentrations were collected at 0, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360 and 480 minutes, and then at 12, 16, 24, 48, 72 hours after infusion. We also measured cotinine and 3HC levels (unlabeled, d2 and d4) in the 360 minute plasma sample for determination of the plasma 3HC/cotinine ratio (NMR).

Genotyping of CYP variants was performed according to previously described protocols. Participants were genotyped for *CYP2A6* reduced/null activity alleles predominantly found in African populations, *CYP2A6**17, *20, *23-*28, *31, *35, and for those also found in other racial populations, *CYP2A6**2, *4, *9 and *12 [15, 23–27]. No participants with *12, *20, *23, or *28 alleles were identified.

Chemical analysis of nicotine and metabolites

Nicotine and cotinine concentrations in plasma were determined by gas chromatography-mass spectrometry [28]. Plasma concentration of 3HC and urine concentrations of nicotine, cotinine, 3HC and nicotine N-oxide were measured by liquid chromatography – tandem mass spectrometry [10]. Concentrations of nicotine, cotinine, and 3HC glucuronides in urine were measured as the difference in analyte concentrations after and before enzymatic hydrolysis, as described previously [29]. The limits of quantitation for nicotine, cotinine and 3HC in plasma were 0.1 ng/ml. For urine analytes, the limits of quantitation were 10 ng/ml

for nicotine, nicotine N-oxide, cotinine and 3HC and 1 ng/ml for minor species (nornicotine, cotinine N-oxide, norcotinine).

Pharmacokinetic analysis

Pharmacokinetic parameters were estimated from blood concentration and urinary nicotine and metabolite data by use of model independent methods, as described previously using Phoenix WinNonlin 6.3, Pharsight, Mountain View, CA [14].

Half-life was computed by least squares linear regression. We computed nicotine clearance as:

$$CL_{NIC} = [Dose_{NIC-d2}] / [AUC_{NIC-d2}],$$

where, Dose is the dose of nicotine-d2 infused and AUC is the area under the plasma nicotine-d2 concentration time curve extrapolated to infinity. Cotinine clearance was estimated the same way using cotinine-d4 dose and AUC. Fractional conversion of nicotine to cotinine (f) was estimated by use of blood levels of cotinine-d2 generated from infused nicotine-d2 and the clearance of cotinine, determined by infusion of cotinine-d4 as follows:

$$f = [AUC_{COT-d2} / Dose_{NIC-d2}] \times CL_{COT-d4}.$$

The metabolic clearance of nicotine by way of the cotinine pathway was computed as $[CL_{NIC-d2}] \times f$. Because metabolism of nicotine to cotinine is primarily mediated by CYP2A6, this measure is believed to reflect CYP2A6 enzymatic activity.

At steady state the ratio of CL_{COT}/f is the factor (K) that converts plasma cotinine concentration to daily intake of nicotine derived from tobacco use as follows: $D_{NIC} = [P_{COT}] \times [CL_{COT}/f] = P_{COT} \times K$, where D_{NIC} is the daily intake of nicotine from smoking (mg/24 hr) and P_{COT} is steady state or time-weighted average plasma cotinine (ng/ml)[22]. We computed this conversion factor and used the baseline plasma cotinine value, obtained during ad libitum smoking prior to study entry, to estimate daily intake of nicotine from each subject.

As mentioned previously, the plasma NMR was measured as a biomarker of CYP2A6 enzymatic activity. The NMR based on d2-labeled compounds measured at 360 min was used to group subjects into quartiles of enzymatic activity. We used the 360 min NMR to reduce the intersubject variability that may be seen with the NMR based on natural nicotine, which may be influenced by recency of smoking. We also did a similar analysis using quartiles based on unlabeled metabolites at 360 min to assess possible bias in using the labeled ratios. The results were nearly identical.

Urine metabolites were analyzed as a molar percentage of the sum of all labeled nicotine metabolites excreted within an eight hour collection, as well as ratios of sequential metabolites to the parent. In addition, we examined the molar sum of all metabolites formed by C-oxidation (cotinine + 3HC + their respective glucuronides, cotinine N-oxide,

nornicotine and norcotinine) and the sum of nicotine and metabolites generated via pathways other than by C-oxidation (nicotine + nicotine glucuronide and nicotine N-oxide) as a fraction of the total of all nicotine and metabolites excreted in urine.

Statistical analysis

Descriptive statistics were computed for pharmacokinetic parameters by genotype and by NMR quartile. Pharmacokinetic parameter means were compared using one-way ANOVA models, where group was the independent variable and each pharmacokinetic parameter was the dependent variable in separate models. Multiple comparisons were made between the four quartile groups, and p-values were adjusted by Tukey's method. The nonparametric Kruskal-Wallis analysis was also used to assess differences in pharmacokinetic parameters across quartiles (data not shown). The significance of the Kruskal-Wallis analysis concurred with the overall significance of the corresponding ANOVA. Pearson correlation coefficients were transformed using Fisher's z transformation to estimate population correlations between NMR from labeled cotinine vs natural cotinine, and NMR with various pharmacokinetic parameters. All analyses were carried out using SAS v. 9.3 (SAS Institute, Inc. Cary, NC, USA). Statistical tests were considered significant at $\alpha = 0.05$.

Results

Genotype frequency and phenotype classification

The frequency of *CYP2A6* genotypes and the phenotype-determined CYP2A6 enzymatic activity estimates (baseline NMR) are shown in table 1. The most prevalent was **1/*1* (62%) followed by **1/*17* (14%), **1/*35* (7%) and **1/*9* and **1/*4H* (3.7% each).

Nicotine and cotinine disposition kinetics

Total clearances and half-lives of nicotine and cotinine, clearance of nicotine via the cotinine pathway and the cotinine-d4 derived 3HC/cotinine ratios are shown by genotype in table 1 and figure 1. As expected there was considerable overlap between genotype groups, with the lowest clearance and NMR values in the *CYP2A6* variant/variant subjects^[15]. The lowest CYP2A6 activity as determined by the clearance of nicotine via the cotinine pathway and the NMR were seen in subjects with **9/*26* and **4/*17* genotypes.

Nicotine and Cotinine Disposition Kinetics by NMR Quartiles

The disposition kinetic profiles for nicotine and cotinine according to NMR quartiles are shown in table 2. In general nicotine and cotinine clearances increased and half-lives decreased with increasing NMR quartile. Statistical differences were consistently found comparing the first and fourth quartiles, with some but fewer statistical differences between second or third vs other quartiles. Pharmacokinetic parameters by NMR quartile are shown in Figures 2 and 3.

Relationship between Plasma Nicotine Metabolite Ratio and Pharmacokinetic Parameters

The NMR at 6 hr based on d4- and d2-labeled compounds were highly correlated ($r=0.91$) with nearly identical values (ratio 0.98, 95% C.I. 0.91 – 1.06). The d2-NMR was highly

correlated with ($r=0.88$) but significantly lower than the NMR based on metabolites generated from tobacco use (natural or d0-nicotine; ratio 0.60, 95% CI 0.48-0.72). Pearson correlation with Fisher's z transformation between labeled and natural NMR values, both unlogged and logged, and various pharmacokinetic parameters were examined (Table 3). The log of NMR is theoretically more closely related to clearances than non-logged values [30]. The log NMR (d2) was strongly correlated with total and non-renal clearance of nicotine ($r = 0.62$), clearance of nicotine to cotinine ($r=0.66$), total and non-renal clearance of cotinine ($r = 0.79$ and 0.82 , respectively) and inversely correlated with nicotine and cotinine half-lives ($r = -0.71$ and -0.59 , respectively). The degree of correlations of NMR and the PK variables was similar for unlogged vs logged NMR, and stronger for the d2 or d4-labeled vs unlabeled NMRs.

Impact of CYP2A6 activity on Cotinine as a Biomarker of Daily Nicotine Dose

As described in the methods and in our previous publication, one can use our pharmacokinetic data to compute a conversion factor to estimate daily intake of nicotine from tobacco (and by inference level of tobacco smoke exposure) from the steady state plasma cotinine concentration[22]. Based on our previous research the conversion factor, CL_{COT}/f , generally averages about 0.08 (derived primarily from studies of Caucasian smokers). Thus, a typical person with a cotinine concentration of 200 ng/ml would be taking in 200×0.08 or 16 mg nicotine per day. We computed the conversion factor in our subjects, as well as estimated daily nicotine intake derived from the baseline cotinine level and the conversion factor, as shown in table 1. While the average conversion factor ranged from 0.074 to 0.092, we saw some extreme values, ranging from 0.049 to 0.210. The two slowest metabolizers ($*4/*17$ and $*9/*26$ genotypes) had the highest conversion factors, meaning that their cotinine levels are much lower than expected for their daily nicotine intake – the result of not converting much nicotine to cotinine. On the other hand, most of the other subjects with reduced function *CYP2A6* variants had lower than normal conversion factors, which would be associated with higher than expected cotinine values, as previously observed among slow metabolizers as a result of reduced cotinine metabolism[31].

Urine metabolites

Eight hour urine metabolite excretion patterns are shown by genotype in table 4. In general subjects with faster metabolism excreted significantly more as total C-oxidation pathway products and less as nicotine plus non-C-oxidation pathway products compared to slower metabolizers. In the two slowest metabolizers ($*4/*17$ and $*9/*26$ genotypes) only 11–13% of total metabolites were generated via C-oxidation pathways and 87–89% excreted as nicotine or non-C-oxidation metabolites. In one subject nicotine and nicotine glucuronide accounted for 64% and in the other nicotine and nicotine N-oxide accounted for 58% of total metabolites. Table 5 shows urine metabolite data by NMR quartile. With increasing NMR quartile there were significant increases in 3HC and decreases in nicotine-N-oxide excretion, as well as increases in C-oxidation and decreases in non C-oxidation product excretion. Table 6 shows correlations between various NMR measures and urine metabolite excretion and urine metabolite ratios. Of note, there were no significant correlations between NMR and Nic-gluc/Nic, Cot-gluc/Cot or 3HC-gluc/3HC, suggesting no interaction between NMR and glucuronidation activity.

Discussion

We present novel data on the disposition kinetics and metabolism of nicotine and cotinine in relation to *CYP2A6* genotype and NMR-determined *CYP2A6* activity in African American smokers. We have published previously on similar associations in a predominantly Caucasian population [14]. African Americans have a higher prevalence of low frequency reduced and loss of function *CYP2A6* alleles, and slower nicotine and cotinine metabolism on average compared to Caucasians [5, 13]. The present study allows us to examine the functional significance of having a number of *CYP2A6* genetic variants found primarily in African Americans, as well as NMR, on nicotine and cotinine kinetics and metabolism.

The most prevalent minor allele in our subjects was the *17 variant which represents a nonsynonymous SNP in the coding region of the *CYP2A6* gene [32, 33]. Based on in vitro studies the *17 variant has substantially reduced enzymatic activity towards nicotine (40–47% activity remaining) consistent with in vivo activity where the *1/*17 and *17/*17 genotypes have 46–61% and 13–16% activity of those with *1/*1 normal metabolism [15, 17, 33]. In the current study, the *17 heterozygotes had only modestly and not significantly reduced nicotine and cotinine clearances compared to wild type. It may be that the wide variation within a genotype group, and the relatively small numbers of subjects with the *17 variant (n=8) genotype in the current pharmacokinetic study relative to the previous studies (N=13–54) contributed to this difference. The *17 variant in combination with other reduced functional alleles (i.e. compound heterozygotes with the *4, *31, *35 alleles) were observed to result in markedly reduced *CYP2A6* activity.

The next most prevalent minor alleles in our subjects were *9, *4 and *35. The *9 allele, which is quite common in both African Americans and Caucasians, is characterized by a SNP in the TATA box of the promoter region which decreases transcription; it is associated in vivo with moderately reduced *CYP2A6* activity (*1/*9 have 69–74% activity remaining). The *4 variant represents a gene deletion and is associated with null activity [15, 17]. The *35 variant contains a nonsynonymous SNP in the coding region resulting in decreased nicotine C-oxidation and thermal stability in vitro, and is associated with reduced activity in vivo [34]. Based on the NMR, subjects with the *35 variant were part of group 3 while the *1/*4 heterozygotes were part of group 2. The subject with the *4/*17 variant showed virtually no *CYP2A6* activity consistent with *4 being a null allele. The other variant present in two subjects was *26. This variant represents a nonsynonymous SNP in the coding region and is associated with markedly reduced activity in vitro and in vivo (i.e. *1/*26 had 54–55% activity remaining) [15, 17], consistent with the level of activity found in the single *1/*26 heterozygote and the subject with the *9/*26 variant who had virtually no *CYP2A6* activity, based on the NMR.

The NMR reflects the metabolism of cotinine to 3HC, a pathway thought to be mediated exclusively or nearly exclusively by *CYP2A6* [9]. The nicotine metabolite ratio can be conveniently measured in plasma, saliva or urine of regular tobacco users. The NMR has been recently proposed as a non-invasive biomarker to personalized pharmacotherapy for smoking cessation [20]. Most studies using NMR has grouped NMR by quartiles [18–20]. Few studies have specifically examined nicotine and cotinine pharmacokinetics and

metabolism by NMR. In the present study we found as expected that nicotine and cotinine clearances increased and half-lives decreased with increasing NMR quartiles. We present quantitative estimates of PK parameters by NMR quartiles that can be used to help explain the effects of NMR on smoking behavior and smoking cessation. The substantial magnitude of difference in nicotine half-life by genotype or NMR that we observed could affect smoking behavior and the time course of withdrawal symptoms; and differences in cotinine half-life could have implications for interpreting cotinine levels at various time intervals after smoking cessation.

As observed by us and other researchers, reduced CYP2A6 activity results in an alteration of urine nicotine metabolite excretion patterns [14, 35, 36]. Reduced CYP2A6 activity resulted in reduced excretion of nicotine C-oxidation products (the sum of cotinine, cotinine glucuronide, 3HC, 3HC glucuronide, cotinine N-oxide, norcotinine and nornicotine) and increased excretion of products generated by other pathways (the sum of nicotine, nicotine glucuronide and nicotine N-oxide). In the two subjects with the lowest level of CYP2A6 activity, 90% of nicotine was recovered as non-C-oxidation metabolites, primarily as nicotine and nicotine N-oxide.

In the present study we assessed the NMR based on deuterium-labeled cotinine measured six hours after infusion, as well as NMR based on non-labelled compounds derived from tobacco use. We confirmed the findings of our previous study that there was a strong correlation between NMR and nicotine clearance, and we also found strong correlations between NMR and cotinine clearance and the half-lives of nicotine and cotinine. Not surprisingly, the NMR derived from labeled nicotine or cotinine exhibited stronger correlations with nicotine and cotinine clearances and half-lives (also derived from labeled nicotine) compared to the NMR derived from nicotine from tobacco use. This is likely due to the concordant timing of the kinetics and NMR (i.e. both would be affected by any transitory impacts) and also because of the standardized time interval between dosing of labeled compounds and measurement of NMR. In the natural situation the time from last dose of tobacco (i.e. nicotine) can be quite variable, especially among lighter smokers. The NMR from labeled compounds is lower than the NMR from tobacco use, because 6 hours is an inadequate period of time to achieve full equilibrium between rates of 3HC formation and elimination.

Zhu et al reported that cotinine levels were disproportionately high for the same nicotine intake in people with reduced CYP2A6 enzymatic activity, as cotinine clearance was reduced to a greater extent than cotinine formation from nicotine^[31]; this was also observed among those with slower activity in the current study. In addition, we find that those smokers who are fully null (i.e. have no activity) have markedly reduced cotinine levels as they simply do not make much cotinine. Thus, we demonstrate that the relative changes in cotinine formation versus clearance, and therefore the conversion factor, vary substantially according to the particular genetic variant and possibly due to variability in activity of competing metabolic pathways (e.g. glucuronidation).

In conclusion, we present data on the wide variation in nicotine and cotinine pharmacokinetics and metabolism in African American smokers which is influenced by

genetic variation in *CYP2A6*. We also present data relating NMR quartile to pharmacokinetic data. Smokers in the lowest vs highest NMR quartiles demonstrate substantial differences in nicotine and cotinine pharmacokinetics, which are likely to influence smoking behavior and are important to consider in the interpretation of cotinine as a biomarker of tobacco smoke exposure.

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FIGURE 1.

CYP2A6 genotype-phenotype associations for baseline plasma trans-3'-hydroxycotinine/cotinine ratio (3HC/COT) (A), plasma nicotine clearance (B), and plasma cotinine clearance (C).

FIGURE 2.

Disposition kinetics of nicotine based on NMR (d2) quartile grouping (mean and SD). Square brackets indicate significant differences between groups ($p < 0.05$).

FIGURE 3.

Disposition kinetics of cotinine and 3'-hydroxycotinine/cotinine (3HC/COT-d₂) ratio based on 6 hour NMR (d₂) quartile grouping (mean and SD). Square brackets indicate significant differences between groups (p<0.05).

CYP2A6 genotype activity, plasma clearance of nicotine and cotinine, and 3HC/COT ratio

TABLE 1

<i>CYP2A6</i> Genotype activity	n	Plasma 3HC/COT baseline [†]	Plasma 3HCd2/COTd2	CLNIC- COT (mL· min ⁻¹ · kg ⁻¹)	CLNIC- COT (mL· min ⁻¹ · kg ⁻¹)	Total CLCOT (mL· min ⁻¹ · kg ⁻¹)	NIC t1/2 (min)	COT t1/2 (min)	Total CLCOT/f	Baseline COT (ng/mL)	Estimated NIC intake (mg/24h)
*1/*1	37	0.45 (0.27)	0.19 (0.12)	17.6 (4.4)	12.4 (4.2)	0.57 (0.23)	124 (31)	977 (222)	0.092 (0.033)	223.4 (130.6)	20.3 (13.3)
*1/*17	8	0.29 (0.10)	0.12 (0.05)	16.3 (3.6)	10.7 (2.8)	0.42 (0.17)	134 (33)	1107 (131)	0.074 (0.016)	205.7 (60.9)	15.1 (3.4)
*1/*9	2	0.35 (0.21)	0.16	21.6 (4.6)	14.8 (2.3)	0.52 (0.01)	97 (12)	868 (119)	0.079	153.2 (27.5)	11.9 (0.5)
*1/*24	1	0.54	0.15	16.3	13.9	0.39	147	960	0.065	258.7	16.8
*1/*4H	2	0.22 (0.02)	0.14 (0.03)	13.8 (0.7)	8.8 (2.6)	0.45 (0.02)	152 (7)	1197 (128)	0.085 (0.008)	227.7 (22.8)	19.2 (0.2)
*1/*27	1	0.31	0.13	19.5	15.2	0.39	108	1092	0.067	67.3	4.4
*1/*2	1	0.25	0.13	14.6	11.6	0.41	151	1125	0.062	320.3	19.9
*1/*35	3	0.24 (0.06)	0.10 (0.06)	11.6 (5.5)	7.8 (3.1)	0.32 (0.11)	179 (35)	1387 (355)	0.048 (0.011)	211.0 (118.6)	9.4 (4.2)
*17/*5	1	0.25	0.12	13.7	10.7	0.31	167	1164	0.049	196.9	9.7
*17/*3	1	0.17	0.07	13.6	7.9	0.42	158	928	0.065	340.4	22.1
*1/*26	1	0.13	0.07	11.0	6.8	0.34	152	1216	0.059	240.4	14.2
*9/*26	1	0.03	0.18	6.6	2.3	0.29	210	1612	0.122	380.4	46.5
*4/*17	1	0.02	n/a	12.9	3.8	0.54	249	1644	0.210	154.5	32.4
All subjects	60	0.38 (0.25)	0.17 (0.11)	16.6 (4.6)	11.4 (4.2)	0.51 (0.21)	134 (37)	1052 (249)	0.086 (0.035)	221 (114)	18.8 (11.9)

Notes: Values are presented as mean (SD);

[†] the plasma 3HC/COT ratio from baseline screening sample

TABLE 2

Disposition kinetics of nicotine by nicotine-d2 metabolite ratio (NMR-d2) quartile

Variable	All subjects	Nicotine-d ₂ metabolite ratio (NMR-d ₂) quartile				Overall P value
		1 st Quartile	2 nd Quartile	3 rd Quartile	4 th Quartile	
n	60	17	14	15	14	
Body weight (kg)	80.0 (15.2)	78.6 (14.9)	80.9 (14.3)	82.2 (14.3)	78.3 (18.6)	0.89
A. Nicotine kinetics						
Total CL _{NIC} (mL · min ⁻¹ · kg ⁻¹)	16.6 (4.6)	14.2 (3.6)	15.6 (2.2)	15.6 (2.2)	21.6 (3.2) ^{b,c,d}	<0.001
Renal CL _{NIC} (mL · min ⁻¹ · kg ⁻¹)	0.69 (0.59)	0.68 (0.45)	0.73 (0.63)	0.34 (0.63)	1.05 (0.75) ^d	0.01
Nonrenal CL _{NIC} (mL · min ⁻¹ · kg ⁻¹)	15.9 (4.5)	13.5 (3.7)	14.9 (2.1)	15.3 (2.1)	20.5 (3.6) ^{b,c,d}	<0.001
CL _{NIC} → cot (mL · min ⁻¹ · kg ⁻¹)	11.4 (4.2)	8.9 (2.9)	10.7 (2.3)	11.2 (2.3)	16.1 (4.1) ^{b,c,d}	<0.001
Fractional conversion, <i>f</i>	0.68 (0.11)	0.62 (0.10)	0.68 (0.10)	0.71 (0.10)	0.73 (0.13)	0.053
Nicotine t _{1/2} (min)	134 (37)	164 (36)	141 (22)	128 (22) ^a	98 (15) ^{b,c,d}	<0.001
Nicotine V _{ss} (L/kg)	2.6 (0.7)	3.0 (0.6)	2.6 (0.7)	2.4 (0.7)	2.4 (0.6)	0.04
B. Cotinine kinetics						
Total CL _{COT} (mL · min ⁻¹ · kg ⁻¹)	0.51 (0.21)	0.39 (0.11)	0.44 (0.08)	0.50 (0.08)	0.77 (0.27) ^{b,c,d}	<0.001
Renal CL _{COT} (mL · min ⁻¹ · kg ⁻¹)	0.10 (0.05)	0.10 (0.04)	0.09 (0.05)	0.09 (0.05)	0.13 (0.07)	0.14
Nonrenal CL _{COT} (mL · min ⁻¹ · kg ⁻¹)	0.41 (0.19)	0.29 (0.12)	0.35 (0.06)	0.41 (0.06)	0.64 (0.25) ^{b,c,d}	<0.001
Cotinine t _{1/2} (min)	1053 (249)	1219 (256)	1055 (202)	994 (202) ^a	884 (175) ^b	0.002
Cotinine V _{ss} (L/kg)	0.68 (0.16)	0.64 (0.18)	0.64 (0.13)	0.66 (0.13)	0.85 (0.13) ^{b,c,d}	<0.001
Plasma 3HC/COT [†]	0.17 (0.11)	0.08 (0.02)	0.12 (0.01)	0.16 (0.01) ^a	0.30 (0.14) ^{b,c,d}	<0.001

Notes: NMR-d2: 1st quartile, 0.11; median, 0.14; 3rd quartile, 0.19^a p<0.05, 3rd quartile significantly different from 1st quartile^b p<0.05, 4th quartile significantly different from 1st quartile^c p<0.05, 4th quartile significantly different from 2nd quartile^d p<0.05, 4th quartile significantly different from 3rd quartile[†] Plasma 3HC/COT is based on d2 analytes.

TABLE 3

Correlation between NMR and pharmacokinetic parameters

Variable	Pearson Correlation with Fisher's z transformation					
	NMR(d ₀)	logNMR(d ₀)	NMR(d ₂)	logNMR(d ₂)	NMR(d ₄)	logNMR(d ₄)
Total CL _{NIC} (mL · min ⁻¹ · kg ⁻¹)	0.52 *	0.56 *	0.55 *	0.62 *	0.64 *	0.72 *
Nonrenal CL _{NIC} (mL · min ⁻¹ · kg ⁻¹)	0.51 *	0.55 *	0.55 *	0.62 *	0.65 *	0.72 *
Renal CL _{NIC} (mL · min ⁻¹ · kg ⁻¹)	0.14	0.12	0.03	0.04	0.03	0.06
CL _{NIC} → cor (mL · min ⁻¹ · kg ⁻¹)	0.59 *	0.66 *	0.59 *	0.66 *	0.65 *	0.73 *
Total CL _{cor} (mL · min ⁻¹ · kg ⁻¹)	0.75 *	0.59 *	0.82 *	0.79 *	0.84 *	0.80 *
Nonrenal CL _{cor} (mL · min ⁻¹ · kg ⁻¹)	0.79 *	0.62 *	0.84 *	0.82 *	0.85 *	0.82 *
Renal CL _{cor} (mL · min ⁻¹ · kg ⁻¹)	0.12	0.09	0.23	0.19	0.27	0.20
Nicotine T _{1/2} (min)	-0.65 *	-0.74 *	-0.62 *	-0.71 *	-0.70 *	-0.76 *
Cotinine T _{1/2} (min)	-0.57 *	-0.67 *	-0.50 *	-0.59 *	-0.64 *	-0.71 *

* P < 0.001

TABLE 4

Nicotine and cotinine urine metabolites and metabolite ratios by *CYP2A6* genotype activity

CYP2A6 Genotype activity	n	Nicotine (NIC)	NIC-gluc (COT)	Cotinine (COT)	COT- gluc	3HC	3HC- gluc	Nicotine N-oxide (NNO)	Cotinine N-oxide (CNO)	Normicotine (NNIC)	Norcotinine (NCOT)
*1/*1	37	26.0 (18.4)	4.6 (2.9)	20.0 (7.1)	3.8 (2.6)	15.2 (10.0)	2.7 (2.2)	24.8 (10.0)	1.6 (0.7)	0.7 (0.3)	0.7 (0.3)
*1/*17	8	17.2 (6.7)	4.1 (3.1)	24.2 (3.4)	2.6 (2.6)	13.4 (5.9)	4.5 (4.0)	30.4 (8.1)	1.8 (0.7)	0.8 (0.2)	1.0 (0.3)
*1/*9	2	10.4 (1.0)	5.9 (4.0)	23.0 (5.5)	4.8 (1.0)	16.3 (3.3)	3.3 (1.9)	32.8 (4.7)	1.8 (0.4)	0.9 (0.5)	0.8 (0.1)
*1/*24	1	11.6	1.9	20.5	0.8	25.0	6.2	28.7	3.0	1.0	1.4
*1/*4H	2	28.0 (21.8)	10.3 (2.5)	15.8 (3.3)	5.2 (1.8)	10.6 (7.8)	1.4 (1.5)	24.9 (7.7)	1.7 (0.8)	1.3 (0.8)	0.7 (0.6)
*1/*27	1	39.3	3.2	13.1	2.6	11.6	2.8	24.3	1.7	0.7	0.6
*1/*2	1	7.0	6.3	24.9	4.2	19.7	4.4	26.0	5.3	1.2	1.1
*1/*35	3	35.8 (4.8)	1.7 (0.2)	11.9 (3.6)	0.4 (0.6)	10.3 (6.7)	2.5 (2.6)	34.6 (10.2)	1.4 (0.4)	0.8 (0.2)	0.6 (0.3)
*17/*35	1	4.0	9.0	11.2	4.7	19.5	5.4	40.6	3.2	0.9	1.5
*17/*31	1	47.6	1.6	15.1	1.0	4.1	0.5	27.3	1.6	0.7	0.3
*1/*26	1	36.2	0.9	22.8	0.1	4.0	0.8	33.0	0.6	1.1	0.6
*9/*26	1	15.5	15.7	7.8	1.6	0.8	0.0	57.9	0.1	0.5	0.1
*4/*17	1	45.0	19.1	6.8	2.5	0.0	0.0	23.0	0.0	3.0	0.4
All subjects	60	24.9 (16.8)	5.0 (3.9)	19.5 (6.9)	3.3 (2.5)	14.0 (9.1)	2.9 (2.5)	27.4 (10.1)	1.6 (0.9)	0.8 (0.4)	0.7 (0.3)

CYP2A6 Genotype activity	n	NIC-gluc/total NIC	COT-gluc/total COT	3HC-gluc/total 3HC	NIC-gluc/NIC	COT-gluc/COT	3HC-gluc/3HC	3HC/COT	[3HC + 3HC-gluc]/COT	Non C-oxidation	C-oxidation
*1/*1	37	20.4 (15.5)	16.4 (12.0)	14.1 (8.5)	32.1 (36.0)	22.8 (23.8)	17.5 (11.8)	0.83 (0.69)	0.97 (0.77)	55.5 (16.3)	44.5 (16.3)
*1/*17	8	18.3 (13.5)	9.0 (8.5)	22.5 (7.6)	25.7 (22.7)	10.8 (10.7)	30.2 (13.7)	0.56 (0.24)	0.74 (0.36)	51.8 (11.1)	48.2 (11.1)
*1/*9	2	33.8 (14.2)	17.9 (6.6)	16.3 (5.3)	54.6 (33.1)	22.2 (9.8)	19.7 (7.6)	0.71 (0.03)	0.85 (0.02)	49.1 (9.7)	50.9 (9.7)
*1/*24	1	14.0	3.7	19.9	16.3	3.8	24.9	1.22	1.52	42.2	57.8
*1/*4H	2	31.1 (13.3)	24.5 (2.7)	10.4 (4.1)	48.0 (28.6)	32.5 (4.8)	11.8 (5.1)	0.63 (0.36)	0.72 (0.44)	63.2 (16.6)	36.8 (16.6)
*1/*27	1	7.5	16.4	19.2	8.1	19.6	23.7	0.88	1.09	66.9	33.1
*1/*2	1	47.3	14.4	18.3	89.9	16.8	22.4	0.79	0.97	39.2	60.8
*1/*35	3	4.7 (1.0)	2.7 (3.4)	17.2 (7.5)	4.9 (1.1)	2.9 (3.7)	21.4 (11.4)	0.79 (0.38)	0.98 (0.52)	72.1 (13.6)	27.9 (13.6)
*17/*35	1	69.4	29.7	21.8	226.6	42.3	27.9	1.74	2.23	53.5	46.5
*17/*31	1	3.3	6.2	11.0	3.5	6.7	12.3	0.27	0.31	76.6	23.4
*1/*26	1	2.3	0.5	16.0	2.4	0.5	19.1	0.17	0.21	70.0	30.0
*9/*26	1	50.4	17.1	0.0	101.8	20.6	0.0	0.10	0.10	89.1	10.9

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CYP2A6	Genotype	activity	n	NIC-gluc/total NIC	COT-gluc/total COT	3HC-gluc/total 3HC	NIC-gluc/NIC	COT-gluc/COT	3HC-gluc/3HC	3HC/COT	[3HC + 3HC-gluc]/COT	Non C-oxidation	C-oxidation
*4/*17			1	29.8	26.9	0.0	42.5	36.8		0.00	0.00	87.2	12.8
All subjects			60	21.1 (16.8)	14.8 (11.3)	15.5 (8.3)	35.0 (42.0)	19.9 (20.6)	19.5 (12.1)	0.76 (0.60)	0.90 (0.69)	57.2 (16.2)	42.8 (16.2)

Notes: Non C-oxidation is [NIC + NIC-gluc + NNO]/TNE × 100; C-oxidation is [COT + COT-gluc + 3HC + 3HC-gluc + CNO + NNIC + NCOT]/TNE × 100

TABLE 5

Nicotine and cotinine urine metabolites (A) and nicotine and cotinine urine metabolite ratios (B) by nicotine-d2 metabolite ratio (NMR-d2) quartile

Urine metabolite or ratio	All subjects	Nicotine-d ₂ metabolite ratio (NMR-d ₂) quartile				Overall P value
		1 st Quartile	2 nd Quartile	3 rd Quartile	4 th Quartile	
n	60	17	14	15	14	
A. Percent (%) of nicotine and metabolites recovered						
Nicotine (NIC)	24.9 (16.8)	27.1 (15.0)	28.4 (19.5)	14.8 (8.1)	29.3 (19.8)	0.06
NIC glucuronide	5.0 (3.9)	5.7 (5.1)	5.4 (3.5)	4.6 (3.7)	4.1 (2.7)	0.67
Cotinine (COT)	19.5 (6.9)	18.0 (5.6)	18.6 (7.9)	22.2 (6.7)	19.1 (7.5)	0.35
COT glucuronide	3.3 (2.5)	2.3 (2.2)	3.6 (2.5)	3.1 (2.0)	4.5 (3.1)	0.11
3-Hydroxycotinine (3HC)	14.0 (9.1)	8.5 (5.2)	11.5 (6.7)	16.7 (6.6) ^a	20.1 (12.4) ^{b,c}	<0.001
3HC glucuronide	2.9 (2.5)	1.8 (1.7)	2.2 (1.7)	4.3 (3.3) ^a	3.4 (2.4)	0.02
Nicotine N-oxide (NNO)	27.4 (10.1)	33.5 (7.9)	27.1 (7.6)	30.6 (11.1)	16.8 (4.1) ^{b,c,d}	<0.001
Cotinine N-oxide (CNO)	1.6 (0.9)	1.5 (0.8)	1.7 (1.3)	1.9 (0.7)	1.4 (0.7)	0.45
Nornicotine (NNIC)	0.8 (0.4)	1.0 (0.6)	0.8 (0.3)	0.8 (0.4)	0.5 (0.2)	0.07
Norcotinine (NCOT)	0.7 (0.3)	0.7 (0.3)	0.7 (0.4)	0.9 (0.3)	0.7 (0.3)	0.13
B. Nicotine and cotinine urine metabolite ratios						
NIC-gluc/total NIC × 100 (%)	21.1 (16.8)	21.1 (19.1)	22.3 (18.9)	23.9 (13.3)	17.1 (15.9)	0.75
COT-gluc/total COT × 100 (%)	14.8 (11.3)	10.9 (9.6)	17.0 (13.7)	12.6 (7.6)	19.5 (12.7)	0.14
3HC-gluc/total 3HC × 100 (%)	15.5 (8.3)	15.8 (6.5)	15.0 (8.9)	17.6 (10.0)	13.3 (7.9)	0.58
NIC-gluc/NIC × 100 (%)	35.0 (42.0)	36.0 (42.6)	40.9 (58.4)	35.7 (26.8)	27.3 (38.9)	0.87
COT-gluc/COT × 100 (%)	19.9 (20.6)	13.6 (13.0)	24.4 (26.7)	15.3 (10.2)	28.1 (27.0)	0.15
3HC-gluc/3HC × 100 (%)	19.5 (12.1)	19.5 (9.7)	18.8 (12.5)	23.0 (15.2)	16.2 (10.5)	0.51
3HC/COT	0.76 (0.60)	0.46 (0.26)	0.74 (0.69)	0.75 (0.30)	1.14 (0.85) ^b	0.02
[3HC + 3HC-g-luc]/COT	0.90 (0.69)	0.55 (0.32)	0.87 (0.75)	0.94 (0.40)	1.33 (0.96) ^b	0.02
Percent non C-oxidation (%)	57.2 (16.2)	66.3 (11.8)	60.8 (14.1)	50.0 (14.4) ^a	50.2 (19.0) ^b	0.006
Percent C-oxidation (%)	42.8 (16.2)	33.7 (11.8)	39.2 (14.1)	50.0 (14.4) ^a	49.8 (19.0) ^b	0.006

Notes: NMR-d2: 1st quartile, 0.11; median, 0.14; 3rd quartile, 0.19^a p<0.05, 3rd quartile significantly different from 1st quartile

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c $p < 0.05$, 4th quartile significantly different from 1st quartile

c $p < 0.05$, 4th quartile significantly different from 2nd quartile

d $p < 0.05$, 4th quartile significantly different from 3rd quartile

Percent non C-oxidation is $[NIC + NIC-gluc + NNO]/TNE \times 100$

Percent C-oxidation is $[COT + COT-gluc + 3HC + 3HC-gluc + CNO + NNIC + NCOT]/TNE \times 100$

TABLE 6

Correlation between NMR and urine metabolites and metabolite ratios

Variable	Pearson Correlation with Fisher's z transformation					
	NMR(d ₀)	logNMR(d ₀)	NMR(d ₂)	logNMR(d ₂)	NMR(d ₄)	logNMR(d ₄)
Nicotine (NIC)	-0.01	-0.06	-0.12	-0.12	-0.14	-0.09
NIC glucuronide	-0.24	-0.43***	-0.04	-0.002	-0.23	-0.21
Cotinine (COT)	0.001	0.16	-0.01	0.05	0.14	0.20
COT glucuronide	0.24	0.28*	0.29*	0.37**	0.30*	0.36**
3-Hydroxycotinine (3HC)	0.60***	0.59***	0.67***	0.64***	0.71***	0.66***
3HC glucuronide	0.38**	0.40**	0.41**	0.41**	0.42***	0.40**
Nicotine N-oxide (NNO)	-0.60***	-0.57***	-0.55***	-0.60***	-0.59***	-0.70***
Cotinine N-oxide (CNO)	0.08	0.22	0.04	0.04	0.13	0.11
Nomicotinine (NNIC)	-0.33**	-0.46***	-0.32*	-0.28*	-0.36**	-0.23
Norcotinine (NCOT)	0.11	0.20	0.08	0.09	0.13	0.13
NIC-gluc/total NIC	-0.11	-0.14	0.01	0.03	-0.07	-0.10
COTgluc/total COT	0.21	0.17	0.27*	0.35**	0.19	0.26*
3HC-gluc/total 3HC	0.01	0.06	-0.01	-0.03	0.01	0.06
NIC-gluc/NIC	-0.11	-0.13	-0.02	-0.02	-0.09	-0.16
COT-gluc/COT	0.21	0.18	0.25	0.31*	0.19	0.24
3HC-gluc/3HC	0.01	0.06	-0.01	-0.02	-0.01	0.04
3HC/COT	0.56***	0.53***	0.58***	0.54***	0.57***	0.50***
[3HC + 3HC-gluc]/COT	0.57***	0.54***	0.60***	0.55***	0.59***	0.51***
Non C-oxidation	-0.44***	-0.51***	-0.48***	-0.51***	-0.57***	-0.58***
C-oxidation	0.43***	0.51***	0.48***	0.51***	0.57***	0.58***

Notes: Non C-oxidation is [NIC + NIC-gluc + NNO]/TNE; C-oxidation is [COT + COT-gluc + 3HC + 3HC-gluc + CNO + NNIC + NCOT]/TNE;

* p<0.05;

** p<0.01;

*** p<0.001