

Within-Subject Variation of the Salivary 3HC/COT Ratio in Regular Daily Smokers: Prospects for Estimating CYP2A6 Enzyme Activity in Large-Scale Surveys of Nicotine Metabolic Rate

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Abstract

Nicotine is the major addictive compound in tobacco and is responsible for tobacco dependence. It is primarily metabolized to cotinine (COT) and *trans*-3'-hydroxycotinine (3HC) by the liver enzyme cytochrome P-450 2A6 (CYP2A6). The 3HC/COT ratio measured in the saliva of smokers is highly correlated with the intrinsic hepatic clearance of nicotine and, therefore, may be a useful non-invasive marker of CYP2A6 activity and metabolic rate of nicotine. This study assessed within-subject variation in salivary 3HC/COT ratios in six regular daily smokers. Our data provide evidence that 1. variation in the 3HC/COT ratio is not dependent on the time of sampling during the day (i.e., morning vs. night) ($P > 0.1$) and 2. the average within-subject biological variation in the 3HC/COT ratio is approximately 26%. These findings should be useful for designing large-scale population surveys to assess the variation in the metabolic rate of nicotine (via CYP2A6) in smokers.

Introduction

Nicotine is primarily responsible for initiation and maintenance of cigarette smoking behavior. Smokers tend to smoke a fairly consistent number of cigarettes from day to day, presumably to maintain the desired pharmacological effects of nicotine. The number of cigarettes smoked per day and, thus, the level of systemic intake of nicotine is partly determined by the rate at which nicotine is metabolized (1,2). After nicotine is absorbed through the lungs it is primarily (~ 80%) metabolized to cotinine (COT) and then *trans*-3'-hydroxycotinine (3HC) by the liver enzyme cytochrome P-450 2A6 (CYP2A6) (2). Nicotine clearance via CYP2A6 is known to vary significantly among subjects and different racial/ethnic groups (3,4). Variation in

CYP2A6 activity may be an important factor influencing smoking initiation, degree of dependence, ability to quit, and, thus, tobacco-induced disease risk (3).

Variation in CYP2A6 enzyme activity is strongly influenced by genetic factors with a heritability of ~ 60% in Caucasians (5). Several DNA sequence polymorphisms in the CYP2A6 gene are known to affect the rate of metabolism of nicotine (6). However, the utility of the CYP2A6 genotype as a biomarker of tobacco dependence and susceptibility to smoking-related disease is currently limited. This is because the variants identified to date account for relatively little of the variation in the metabolic rate of nicotine (5). This prompted Dempsey and colleagues (7) to investigate a non-invasive approach to phenotyping CYP2A6 activity. They showed that the ratio of the 3HC and COT concentration in saliva is highly correlated with the oral clearance of nicotine in smokers ($r = 0.9$), which in turn reflects intrinsic metabolic clearance of nicotine by the liver via the CYP2A6 enzyme (7). These researchers proposed that the 3HC/COT ratio derived from a saliva sample is a reliable estimate of CYP2A6 activity and, hence, an indicator of the rate of hepatic metabolism of nicotine.

The present study examines the within-subjects variation in the salivary 3HC/COT ratio in a group of regular smokers to assess the utility of this non-invasive measure for use in large-scale population surveys of the metabolic rate of nicotine.

Methods

Participants and sampling

For this study, six regular daily smokers (five female and one male) were recruited from Wellington, New Zealand. The self-reported number of cigarettes smoked on an average day for this

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group ranged from 5 to 20 cigarettes. Each participant was asked to provide approximately 1–2 mL of saliva in a sterile, airtight plastic collection tube twice a day for seven days. One saliva sample for each day was obtained first thing in the morning, prior to eating, drinking, or tooth brushing. The second sample was obtained in the evening just prior to going to sleep. Therefore, each participant provided a total of 14 saliva samples for the 7-day period, allowing assessment of within- and between-day variation in the 3HC/COT ratio. All saliva samples were self-obtained and were stored in a freezer in the participant's home until collection for analytical chemistry testing. Written informed consent was obtained for each subject. The study was approved by the Central Region Ethics Committee of New Zealand.

Analytical chemistry

A non-smoker technician, living in a non-smoking environment, carried out all extractions. All glassware used in the analysis was prerinsed with methanol to ensure that no COT was present. Saliva (0.5 mL) was pipetted into a prerinsed 7-mL silanized culture tube. Each sample was spiked with 50 μ L of internal standards solution for COT and 3HC and covered immediately using caps with clean Teflon liners. The samples and standards were then vortex mixed briefly, and the internal standards were allowed to equilibrate for 5 min. They were alkalized with 0.5 mL 2.0M potassium carbonate, and then 3 mL ethyl acetate was added. The tubes were recapped and mixed for 15 min on the vortex mixer. The tubes were centrifuged, and the ethyl acetate was transferred to clean culture tubes, which had been prerinsed with hexane. Glacial acetic acid (30 μ L) was added to each tube, and then the ethyl acetate was evaporated just to dryness in a Savant evaporator. The dry residue was reconstituted in 100 μ L 10:90 acetonitrile/deionized water. The samples and standards were briefly vortex mixed and then sonicated for 10 min.

Analyses were conducted using a Shimadzu 10AVP high-performance liquid chromatography (HPLC) system attached to an Applied Biosystems API 300 Triple Quadrupole mass spectrometer equipped with a TurboIonSpray ion^R source. The HPLC column used was a Phenomenex SynergiTM 4- μ Polar (75 \times 2.0-mm i.d.). The mobile phase was a gradient of acetonitrile and 5mM ammonium acetate. The transitions monitored were 177/80 (180/80) for cotinine and 193/80 (196/80) for 3HC; the numbers in parentheses are the corresponding transitions for the respective internal standards.

Quantitation

The analyte concentrations in the samples were determined using seven-point calibration lines with COT and 3HC concentrations ranging from 0 to 400 ng/mL. Linearity of the calibration lines was excellent, with typical r^2 values of 0.999. The interday coefficients of variation (CVs) for COT and 3HC at 150 ng/mL were 1.8% and 3.3%, respectively, based on six replicates on three different days. The corresponding accuracies were 102.4% and 100.8%, respectively.

Technical variation analysis

The variation in salivary 3HC/COT ratios attributable to tech-

nical sources (i.e., variation because of instrument repeat determinations and sample storage temperature and duration) was assessed by spiking 0.5 mL of Barnstead H₂O with set concentrations of COT (52.5 ng/mL) and 3HC (10 ng/mL). Values were chosen to reflect physiological concentrations of the 3HC/COT ratios, and based on these values, the expected 3HC/COT ratio for a test sample was 0.19. For this experiment, four tests were conducted with a separate sample prepared for each test (4 \times COT and 3HC suspended in H₂O). Two of the test samples were stored at room temperature (RT) and two samples were stored at -20°C . For each of the two storage temperatures, one sample was stored for one day and the other sample was stored for seven days. Duplicate HPLC measurements were subsequently taken for each of the four test samples.

Statistical analysis

CVs, the standard deviation/mean \times 100, were calculated to express the relative percent variability of data about the mean. Independent *t*-tests were performed to test for differences in repeat measures of the metabolic ratio or nicotine and the expected value in the technical variation experiment. A two-level repeated measures analysis of variance (ANOVA) was performed to assess within- and between-day variation in the measure. The Spearman's rho correlation coefficient and associated one-tailed *P*-value was calculated to test for positive linear association between the number of cigarettes smoked per day and the average metabolic ratio of nicotine among smokers. A *P*-value of 0.05 was considered statistically significant. All statistical analyses were performed using SPSS V12 and graphs were generated using GraphPad Prism V4.

Results

Technical variation of the 3HC/COT ratio

Analysis of technical variation of the salivary 3HC/COT ratio comparing samples stored for 1 and 7 days at RT and -20°C was performed by calculating the mean of the duplicate 3HC/COT ratios obtained for each of the four tests in the experiment. The results indicate that there were minor differences between the observed means and the expected value (0.19) for both the storage temperature and duration tests. Comparing the duplicate data of the four test groups to 0.19 did not provide evidence that any of the test means were significantly different from the expected value ($P > 0.1$). The CVs derived for the four tests were 1.99%, 7.78%, 7.32%, and 3.54% for day 1 (RT), day 1 (-20°C), day 7 (RT), and day 7 (-20°C), respectively. The CV for all tests combined was 4.16%. This value is an estimate of the sum total of all technical variation (error) in the 3HC/COT ratio measurement.

Within-subject variation analysis

ANOVA was performed to assess whether variation in 3HC/COT ratios were dependent on daily time of sampling (i.e., a.m. vs p.m.). These data are presented in Table I. The analyses indicated that the ratios averaged over the seven-day

sampling period did not differ significantly between a.m. or p.m. for these subjects, when considered independently or as a group ($P > 0.01$).

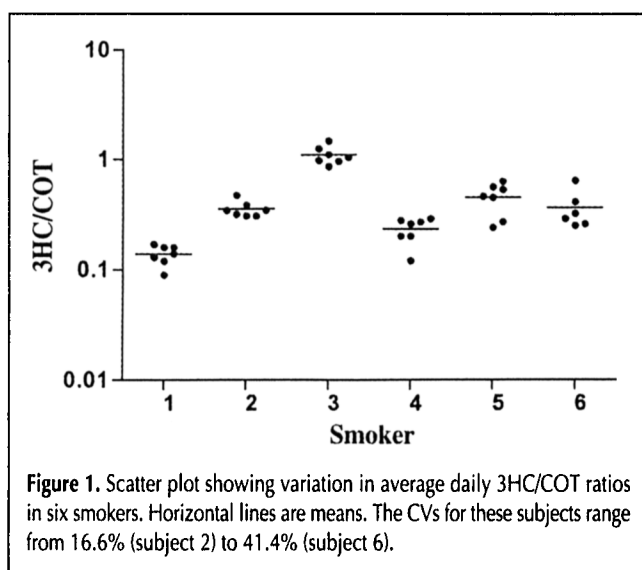
Because daily time of sampling did not seem to have a significant effect on 3HC/COT ratio in this group of smokers, we combined a.m. and p.m. values for each day to give average daily 3HC/COT ratios for each subject. These values were used to assess day-to-day variation for the six smokers as illustrated in Figure 1. The CVs for the average daily 3HC/COT ratio for subjects 1–6 were 19.4%, 16.6%, 18.8%, 27.3%, 32.3%, and 41.4%, respectively.

There was a moderately positive correlation between the number of cigarettes smoked per day and the average daily metabolic ratio of nicotine, but this was not statistically significant for this small sample ($r = 0.46$, $P = 0.18$).

Table I. Mean 3HC/COT Ratios for Each of the Six Smokers by Time of Sampling (i.e., a.m. or p.m.)

Subject	Time*	Mean ($n = 7$)	SD
1	a.m.	0.14	0.03
	p.m.	0.14	0.03
2	a.m.	0.41	0.13
	p.m.	0.31	0.08
3	a.m.	1.09	0.17
	p.m.	1.12	0.29
4	a.m.	0.24	0.09
	p.m.	0.23	0.07
5	a.m.	0.47	0.23
	p.m.	0.42	0.15
6	a.m.	0.44	0.16
	p.m.	0.29	0.16
Mean (Overall)		0.43	0.31

* For each subject mean ratios did not differ significantly between a.m. and p.m. ($P > 0.05$).



Discussion

The metabolism of COT to 3HC is mediated primarily by the liver enzyme CYP2A6. (2). The ratio of 3HC/COT measured from saliva has been proposed as a non-invasive marker of CYP2A6 activity and, correspondingly, as a marker of the rate of nicotine metabolism (7). The present study provides novel information on the storage procedure, circadian variability, and weekly variability of subjects in the 3HC/COT ratio measured in saliva. We provide evidence that 1. the 3HC/COT ratio is unaffected by storage at room temperature (as opposed to freezing) and unaffected by storage for up to seven days; 2. the 3HC/COT ratio is independent of the time of sampling during the day (i.e., morning vs. night); and 3. the 3HC/COT ratio is relatively stable for subjects when measured daily over a one-week period.

The utility of the 3HC/COT ratio as a marker of CYP2A6 activity is based on the idea that conversion of cotinine to 3HC is a function of CYP2A6 activity and that the elimination rate of 3HC is formation-limited. The latter factor is expected because the half-life of COT is approximately 16–18 h, and the half-life of 3HC is 5 h (8). Because 3HC is eliminated much more rapidly than is its parent metabolite, COT, the levels of 3HC in the blood (or urine or saliva) would be proportional to its formation rate and independent of its own elimination rate. Thus, 3HC and COT levels would decline in parallel over time, and the ratio would remain constant.

There are several potential sources of subject variability in the 3HC/COT ratio that should be considered. First is that CYP2A6 activity may change over time, particularly in relationship to the effects of food, drugs, or other environmental exposures (2). Second, because the conversion from COT to 3HC depends upon CYP2A6 activity in relation to other competing clearance pathways (e.g., oxidation, glucuronidation, or renal clearance), changes in the clearance of COT via these competing pathways could also affect the ratio (2). Third, the stability of the 3HC/COT ratio in subjects depends upon reaching a steady state. When a person is an occasional smoker or has changed their smoking rate markedly, the level of 3HC may not have reached its steady state, and the ratio may be underestimated. Other sources of variability not investigated include the effects of saliva composition or flow rate on COT or 3HC levels and the stability of actual saliva samples in storage.

The present study provides the first data assessing natural variation in the salivary 3HC/COT ratio in smokers. We have provided evidence that, for smokers with fairly constant smoking habits, 3HC/COT ratios do not vary significantly from morning to night and vary by about 26% from day-to-day. At this level of within-subject variation, a reasonably good estimate of the true homeostatic value can be obtained from a single saliva sample. Our data, considered in conjunction with the previous findings of Dempsey et al. (7), suggest that the salivary 3HC/COT ratio as a non-invasive index of CYP2A6 activity is useful for large-scale population surveys of the metabolic rate of nicotine in smokers. One caveat with respect to the present study is that the smokers we examined were generally moderate smokers, who smoked on average between 5 and 20 cigarettes per day. The ratio of 3HC/COT might be more variable in light

or occasional smokers. Thus, further investigation of the stability of the ratio in such smokers is needed.

References

1. N.L. Benowitz, O.F. Pomerleau, C.S. Pomerleau, and P. Jacob, III. Nicotine metabolite ratio as a predictor of cigarette consumption. *Nicotine Tob. Res.* **5**(5): 621–624 (2003).
2. J. Hukkanen, P. Jacob, III, and N.L. Benowitz. Metabolism and disposition kinetics of nicotine. *Pharmacol Rev.* **57**(1): 79–115 (2005).
3. K.A. Schoedel, E.B. Hoffmann, Y. Rao, E.M. Sellers, and R.F. Tyndale. Ethnic variation in CYP2A6 and association of genetically slow nicotine metabolism and smoking in adult Caucasians. *Pharmacogenetics* **14**: 615–626 (2004).
4. N.L. Benowitz, E.J. Perez-Stable, B. Herrera, and P. Jacob, III. Slower metabolism and reduced intake of nicotine from cigarette smoking in Chinese-Americans. *J. Natl. Cancer Inst.* **94**(2): 108–115 (2002).
5. G.E. Swan, N.L. Benowitz, C.N. Lessov, P. Jacob, III, R.F. Tyndale, K. Wilhelmsen. Nicotine metabolism: the impact of CYP2A6 on estimates of additive genetic influence. *Pharmacogenet. Genomics* **15**(2): 115–125 (2005).
6. V. Malaiyandi, E.M. Sellers, and R.F. Tyndale. Implications of CYP2A6 genetic variation for smoking behaviors and nicotine dependence. *Clin. Pharmacol Ther.* **77**(3): 145–158 (2005).
7. D.T.P. Dempsey, P. Tutka, P. Jacob, III, F. Allen, K. Schoedel, R.F. Tyndale, and N.L. Benowitz. Nicotine metabolite ratio as an index of CYP2A6 metabolic activity. *Clin. Pharmacol. Ther.* **76**(1): 64–72 (2004).
8. N.L. Benowitz and P. Jacob, III. trans-3'-Hydroxycotinine: disposition kinetics, effects and plasma levels during cigarette smoking. *Br. J. Clin. Pharmacol.* **51**(1): 53–59 (2001).

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