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Research Article

Reproducibility of the Nicotine Metabolite Ratio in Cigarette Smokers

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Abstract

Background: The nicotine metabolite ratio (NMR or 3-hydroxycotinine/cotinine) has been used to phenotype *CYP2A6*-mediated nicotine metabolism. Our objectives were to analyze (i) the stability of NMR in plasma, saliva, and blood in various storage conditions, (ii) the relationship between NMRs derived from blood, plasma, saliva, and urine, and (iii) the reproducibility of plasma NMR in *ad libitum* cigarette smokers.

Methods: We analyzed data from four clinical studies. In studies 1 and 2, we assessed NMR stability in saliva and plasma samples at room temperature (~22°C) over 14 days and in blood at 4°C for up to 72 hours. In studies 2 and 3, we used Bland–Altman analysis to assess agreement between blood, plasma, saliva, and urine NMRs. In study 4, plasma NMR was measured on six occasions over 44 weeks in 43 *ad libitum* smokers.

Results: Reliability coefficients for stability tests of NMR in plasma and saliva at room temperature were 0.97 and 0.98, respectively, and 0.92 for blood at 4°C. Blood NMR agreed consistently with saliva and plasma NMRs but showed more variability in relation to urine NMR. The reliability coefficient for repeated plasma NMR measurements in smokers was 0.85.

Conclusion: The NMR is stable in blood, plasma, and saliva at the conditions tested. Blood, plasma, and saliva NMRs are similar whereas urine NMR is a good proxy for these NMR measures. Plasma NMR was reproducible over time in smokers.

Impact: One measurement may reliably estimate a smoker's NMR for use as an estimate of the rate of nicotine metabolism. *Cancer Epidemiol Biomarkers Prev; 21(7); 1105–14. ©2012 AACR.*

Introduction

The rate of nicotine metabolism, which influences nicotine dependence and smoking behavior (1, 2), is highly variable between individuals (3). Characterization of the rate of nicotine metabolism in smokers has important epidemiologic and pharmacologic applications. Because nicotine is metabolized primarily by hepatic cytochrome P450 (CYP) 2A6, one approach to measuring the rate of nicotine metabolism has been to genotype *CYP2A6* (4). However, genotyping *CYP2A6* alone does not account for environmental and regulatory influences on enzymatic activity. Cotinine (COT), the major proximate metabolite of nicotine, is further metabolized, essentially exclusively by *CYP2A6*, to trans-3′-hydroxycotinine (3-HC; refs. 5, 6). The ratio of the product 3-HC to the parent COT (3-HC/COT, also referred to as the nicotine metabolite ratio or NMR) is an indicator of *CYP2A6* enzymatic activity. Because cotinine has a relatively long half-life (averaging 16 hours) and 3-HC intrinsically has a shorter half-life (5 hours; refs. 7, 8), the elimination rate of the latter is formation-limited, meaning that the NMR is expected to be stable over time among regular smokers. The NMR can be measured in the saliva, urine, or plasma of smokers and has been validated as a biomarker of nicotine clearance (5, 9).

While the NMR is being used in a growing body of research to explore the influence of nicotine metabolism on smoking behaviors, dependence, and as a predictor of response to smoking cessation treatment (10–13), data on its reproducibility in *ad libitum* cigarette smokers are limited (14, 15). The long-term reproducibility of NMR measured in saliva or plasma of smokers has not been reported. In addition, there are limited data on the stability of the NMR in biologic samples at various storage temperatures. Lea and colleagues showed that saliva NMR was unaffected by storage at room temperature or at −20°C for up to 7 days (14).

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We investigated the stability of NMR in blood, plasma, and saliva stored at various temperatures to better understand how storage conditions after sample collection may affect the NMR. We analyzed the relationships between NMRs derived from whole blood, plasma, saliva, and urine. Finally, we assessed the reproducibility of plasma NMR over 44 weeks as well as the precision of plasma NMR with multiple replicate measurements in a well-defined population of *ad libitum* smokers.

Materials and Methods

Studies, subjects, and experimental protocols

Study 1. Details of this study are presented elsewhere (16). To investigate the stability of the NMR in plasma and saliva at room temperature over 14 days, we obtained previously frozen (−30°C) saliva and plasma samples collected during the same visit from 24 healthy smokers recruited by newspaper advertisement. Saliva and plasma samples were thawed and stored at room temperature (∼22°C) and aliquots of each were analyzed for 3-HC and cotinine on days 1, 4, 7, and 14.

Study 2. This study was a clinical trial of alternative therapies for smoking cessation treatment in smokers (results have not been published). Subjects were studied at the Centre for Addiction and Mental Health in Toronto, ON, Canada. After completing an initial telephone interview that determined preliminary eligibility, the subjects attended an intake session during which whole blood, saliva, and urine samples were collected. Plasma was extracted from one of the collected tubes of whole blood by centrifugation. The samples were frozen and stored at −30°C until analysis within 14 days from the collection date. An additional tube of whole blood was collected from 9 randomly selected subjects and one more subject whose urine and saliva were not available for NMR analysis. These 10 samples were stored at 4°C, aliquots were taken at 2, 18, 24, 48 and 72 hours, transferred to −30°C until all aliquots were collected and then analyzed in duplicate for the concentrations of 3-HC and cotinine.

Study 3. Study design has been described in a previous report (5). This was an outpatient study at the General Clinical Research Center at San Francisco General Hospital, San Francisco, CA. In the current article, we assessed the relationship between saliva and plasma NMR in a sample of smokers ($n = 67$) and nonsmokers ($n = 11$) exposed to tobacco smoke. Subjects were healthy volunteers recruited from newspaper advertisements. Afternoon plasma and saliva samples were collected and analyzed for cotinine and 3-HC.

Study 4. This was a clinical trial of reduced nicotine content cigarettes in which smokers were randomly assigned to a control or research arm after a 2-week baseline period in which they smoked their usual brand of cigarettes. The control group continued to smoke their usual brand of cigarettes for the duration of the study. Subjects were studied in a community-based clinic. Study design and subject inclusion/exclusion criteria have been described elsewhere (17). In the present analysis, we focused on the first 44 weeks of the control arm of this study to investigate the long-term reproducibility of plasma NMR in smokers who smoked *ad libitum*. Subjects were 43 healthy smokers of 10 or more cigarettes per day (CPD) recruited from newspaper advertisements. Plasma samples collected at weeks 1, 8, 16, 24, 32, and 44 and urine samples collected at baseline (week 1) were assayed for concentrations of cotinine and 3-HC. To compare plasma NMR measures between 2 testing laboratories, the NMR was analyzed in 15 split plasma samples at the University of California, San Francisco, California (UCSF) and the University of Toronto (UT), Toronto, ON, Canada.

Written informed consent was obtained from each participant. Studies 1, 3, and 4 were approved by the Institutional Review Board at the University of California, San Francisco (UCSF), and study 2 was approved by the IRB at the University of Pennsylvania (Philadelphia, PA), the Center for Addiction and Mental Health, and the University of Toronto.

Analytical chemistry

Analyses of plasma and saliva cotinine and 3-HC concentrations as well as urine cotinine, 3-HC, and their respective glucuronide metabolites for studies 1, 3, and 4 were carried out at UCSF by liquid chromatography/tandem mass spectrometry (LC/MS-MS) as described previously (18). In quality control (QC) tests of 176 QC plasma samples over a time span of approximately 6 years, the coefficients of variation (CV) for assays of plasma cotinine, 3-HC, and NMR were 8.7%, 10.7%, and 8.2%, respectively. Additional QC and precision data for the assays used are presented elsewhere (18). A variation of the method described earlier was used at University of Toronto to measure cotinine and 3-HC in 15 plasma samples from study 4 and whole blood, plasma, saliva, and urine samples from study 2. Specifically, 100 μL of biosamples was used and the volume was brought up to 1 mL with water and urine was diluted 1:20 with water prior to analysis. Assays at University of Toronto were run in duplicate. For analysis of total concentration of urinary metabolites the urine sample was deconjugated using incubation with β-glucuronidase (type H-1 from *Helix pomatia*, 20,000 units/mL in 0.5 mol/L ammonium acetate buffer pH 5.1) for 21 hours at 37°C.

Statistical analysis

Biomarker data for all studies were approximately log-normally distributed and therefore log-transformed. Also, log-transformed NMR has been shown to be more related to nicotine clearance than raw-scale NMR (19).

Study 1. To analyze the chemical/physical stability of biomarkers obtained from plasma and saliva samples stored up to 14 days at room temperature (∼22°C), we computed reliability coefficients, the ratio of between-sample variance to the sum of between- and within-sample variances, for plasma and saliva log-transformed cotinine, 3-HC, and NMRs from mixed-model analyses.

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We also conducted a repeated-measures mixed-effects analysis to assess changes of log-transformed plasma and saliva cotinine, 3-HC, and NMR over the 14-day period. We included a fixed effect for time in the models.

Study 2. The reproducibility coefficients and repeated measures analysis to assess the stability of log-transformed cotinine, 3-HC, and NMR in refrigerated whole blood were done as described earlier. We used a Bland–Altman analysis to assess the level of agreement between whole blood, plasma, saliva, and urine log-transformed NMR, cotinine, and 3-HC values, respectively. We defined a range of agreement as mean bias ±2 SD. The Bland–Altman analysis is based on examination of a scatter plot of variable means plotted on the horizontal axis and differences between variables plotted on the vertical axis (20). We used linear regression to examine the strength of the relationships between biomarkers in different biologic fluids.

Study 3. We conducted a Bland–Altman analysis to assess the agreement between saliva and plasma log-transformed cotinine, 3-HC, and NMRs obtained from smokers and nonsmokers as described earlier. We also fit a linear regression for saliva on plasma log-transformed NMR.

Study 4. The reproducibility of plasma log-transformed cotinine, 3-HC, and NMR in smokers measured during 6 visits over 44 weeks was estimated as described earlier. To evaluate the sensitivity of reliability coefficient estimates to outlying observations, we excluded data from one subject who had the highest body mass index (BMI) in the sample (47.5) and whose plasma NMR and 3-HC concentrations were an order of magnitude lower than the mean of these variables for the sample.

We characterized changes in the precision of plasma NMR with multiple replicates of NMR measurements (i.e., sampling at different times). The variance of the mean of n replicated measurements taken on a single subject at different times is:

$$\text{Variance}_{\text{mean of } n \text{ replicates on subject}} = \text{variance}_{\text{between-subjects}} + \text{variance}_{\text{within-subject}}/n \quad \text{(A)}$$

We computed the reliability of the average of n measurements and the variance inflation factor of x replicate measurements relative to y replicate measurements, where x and y can be any finite number. The reliability of the average of n measurements is the ratio of the between-subjects variance to the total variance of the mean for that subject (Equation A). The variance inflation factor is the variance of x divided by the variance of y, and indicates how much larger a sample using x replicate measurements has to be to achieve the same precision in NMR as one using y replicate measurements.

As a further investigation of changes in plasma log-transformed cotinine, 3-HC, and NMR, respectively, over time, we used mixed effects repeated measures analysis with subjects as a random effect and included covariates age, gender, BMI, and CPD. Covariate data were all obtained at baseline. Age was treated as a continuous variable and CPD was as a categorical variable (1–10, 11–20, and >20 CPD). In addition, we computed Spearman correlation coefficients between baseline plasma cotinine, 3-HC, and NMR and age, BMI, CPD, time to first cigarette (TFC), Fagerström test for nicotine dependence (FTND), and nicotine dependence syndrome scale (NDSS) overall score and individual NDSS factor scores, all obtained at baseline. These analyses were done with and without data from the subject with outlying observations.

The agreement between urinary NMR derived from total 3-HC [$NMR_{total(3\text{-}HC)}$] and NMR derived from free 3-HC [$NMR_{free(3\text{-}HC)}$] were assessed by Bland–Altman analysis. The rationale for this analysis is that the NMR is intended to represent the product/parent drug ratio for the *CYP2A6* pathway. The parent is cotinine and 3-HC is the product. 3-HC is further metabolized to 3-HC-glucuronide, so accounting for all of 3-HC generated by cotinine metabolism via *CYP2A6* should include both free and conjugated species. All cotinine is not converted to 3-HC, so total cotinine is not appropriate as the denominator in the NMR. Thus, we used the free form of urine cotinine, as the substrate for *CYP2A6*, in NMR computations.

We did a Bland–Altman analysis to assess the level of agreement between the 2 testing sites' measurements of cotinine, 3-HC, and NMR in split plasma samples. All analyses were carried out with SAS v. 9.3 (SAS Institute, Inc.), and statistical tests were considered significant at $\alpha = 0.05$.

Results

NMR chemical/physical stability (studies 1 and 2)

The reliability coefficients for stability tests of log-transformed biomarkers in whole blood samples stored at 4°C were as follows: cotinine, 0.99; 3-HC, 0.96; and NMR, 0.92. Changes in whole blood log-transformed cotinine ($P = 0.687$), 3-HC ($P = 0.090$), and NMR ($P = 0.181$) over time were nonsignificant. The reliability coefficients for stability tests of log-transformed biomarkers in saliva at room temperature were as follows: cotinine, 0.99; 3-HC, 0.99; and NMR, 0.98, which were similar to the reliability coefficients for stability tests of biomarkers in plasma at room temperature: cotinine, 0.99; 3-HC, 0.99; and NMR, 0.97. Changes in both plasma ($P = 0.032$) and saliva ($P < 0.001$) NMR over time were significant. Changes in cotinine and 3-HC concentrations in these biologic fluids over time were relatively small but significant (all $P < 0.001$).

Comparison of plasma NMR between testing sites (study 4)

The 2 testing laboratories consistently provided similar measures of plasma NMR (Table 1, comparison between testing sites). Although plasma 3-HC measurements between the laboratories were on average closer than cotinine measurements (ratio of 0.95 vs. 0.89), we observed some discrepancies between the testing sites in plasma 3-HC measurements whereas there was consistent agreement in plasma cotinine measurements.

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Table 1. Bland–Altman analysis of agreement between measures

Study	Comparison	Ratio (95% CI)	Range of agreement (ratio ± 2 SD)
A. Comparison between testing sites			
Study 4	UCSF vs. UT plasma 3-HC	0.95 (0.89–1.02)	0.74–1.22
	UCSF vs. UT plasma COT	0.89 (0.84–0.94)	0.72–1.10[b]
	UCSF vs. UT plasma NMR	1.06 (0.99–1.13)	0.84–1.34[b]
B. Comparison between NMR derived from total vs. free 3-HC			
Study 4	Urine total vs. free NMR	1.21 (1.18–1.25)	1.02–1.45
C. Comparison between biomarkers in various body fluids of smokers			
Study 2	Blood vs. plasma 3-HC	0.97 (0.90–1.04)	0.64–1.48[b]
	Blood vs. plasma COT	0.96 (0.91–1.01)	0.69–1.32
	Blood vs. plasma NMR	1.01 (0.95–1.06)	0.72–1.40[b]
	Blood vs. saliva 3-HC	0.96 (0.84–1.11)	0.44–2.13
	Blood vs. saliva COT	0.91 (0.83–1.00)	0.53–1.56
	Blood vs. saliva NMR	1.04 (0.95–1.15)	0.61–1.78[b]
	Urine vs. blood 3-HC[a]	40.7 (30.7–54.1)	7.6–217.8
	Urine vs. blood COT	4.8 (4.1–5.6)	1.94–11.9
	Urine vs. blood NMR[a]	8.5 (7.1–10.2)	2.89–24.8
	Saliva vs. plasma 3-HC	1.49 (0.87–1.15)	0.45–2.23
	Saliva vs. plasma COT	1.04 (0.95–1.14)	0.63–1.73
	Saliva vs. plasma NMR	0.96 (0.86–1.07)	0.52–1.77
	Urine vs. plasma 3-HC[a]	38.2 (28.9–50.5)	7.5–194.0
	Urine vs. plasma COT	4.6 (4.0–5.3)	2.05–10.3
	Urine vs. plasma NMR[a]	8.5 (7.1–10.1)	2.96–24.1
	Urine vs. saliva 3-HC[a]	36.4 (27.1–46.6)	6.9–190.8
	Urine vs. saliva COT	4.3 (3.7–5.0)	1.89–9.8
	Urine vs. saliva NMR[a]	8.6 (6.9–10.6)	2.60–28.2
D. Comparison between saliva and plasma in smokers and nonsmokers			
Study 3	Saliva vs. plasma 3-HC[a]	0.87 (0.81–0.95)	0.44–1.75
	Saliva vs. plasma COT	1.01 (0.94–1.08)	0.55–1.85
	Saliva vs. plasma NMR[a]	0.88 (0.83–0.94)	0.53–1.47

NOTE: Ratio, back-transformation of mean difference between measures on log-scale; 95% CI of ratio, back-transformation of 95% CI of the mean difference between measures on log-scale; range of agreement, back-transformation of the mean difference between measures on a log-scale ± 2 SDs from the mean difference.

[a]Urine total 3-HC was used in analysis.

[b]Measures are consistently similar, that is, measures were not observed outside the limit of agreement of mean bias ± 2 SDs.

Urine NMR derived from total 3-HC and free 3-HC (study 4)

The correlation between urine NMRs derived from total and free 3-HC from study 4 was strong ($r = 0.99$). Urine $NMR_{total(3\text{-}HC)}$ was on average 1.21 [95% confidence interval (CI), 1.18–1.25] times larger than urine $NMR_{free(3\text{-}HC)}$ and we show that the 2 methods of measuring NMR in urine did not consistently agree (Table 1, comparison between NMR derived from total vs. free 3-HC). The correlations between plasma NMR and urine $NMR_{total(3\text{-}HC)}$ ($r = 0.82$) and $NMR_{free(3\text{-}HC)}$ ($r = 0.84$) in that sample were similar.

Relationship between whole blood, plasma, saliva, and urine NMRs (studies 2 and 3)

On the basis of Bland–Altman analysis, blood-plasma and blood-saliva comparisons consistently provided similar measures of NMR (Table 1, study 2, comparison between NMR derived from total vs. free 3-HC). We observed discrepancies in saliva-plasma, urine-blood, urine-plasma, and urine-saliva NMR comparisons [results are for urine $NMR_{total(3\text{-}HC)}$ but similar observations were made for urine $NMR_{free(3\text{-}HC)}$].

Figure 1A–C summarizes the linear regression of plasma, saliva, and urine NMRs, respectively, on whole blood NMR measured in smokers enrolled in study 2. In addition, correlations between log-transformed NMRs in biologic fluids include: urine $NMR_{free(3\text{-}HC)}$ versus whole blood NMR, $r = 0.69$; urine $NMR_{total(3\text{-}HC)}$ versus saliva NMR, $r = 0.66$; urine $NMR_{free(3\text{-}HC)}$ versus saliva NMR, $r = 0.46$; urine $NMR_{total(3\text{-}HC)}$ versus plasma NMR, $r = 0.64$; urine $NMR_{free(3\text{-}HC)}$ versus plasma NMR, $r = 0.56$; and urine $NMR_{free(3\text{-}HC)}$ versus urine $NMR_{total(3\text{-}HC)}$, $r = 0.91$ (all $P < 0.01$).

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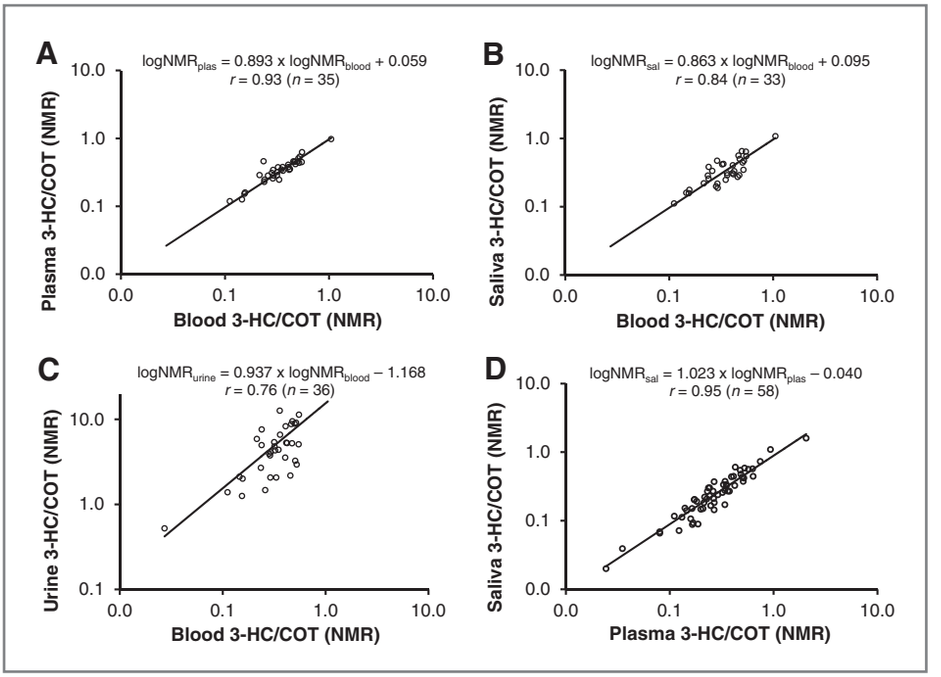

Figure 1. Regression of plasma 3-hydroxycotinine to cotinine (3-HC/COT) ratio or NMR in plasma versus whole blood (plot A); saliva versus whole blood (plot B); urine versus whole blood (using urine total 3-HC/free COT; plot C) in a sample of smokers; and saliva versus plasma in a sample of smokers and nonsmokers combined (plot D).

Saliva and plasma log-transformed NMR from a sample of smokers and nonsmokers in study 3 were highly correlated ($r = 0.95$) and is shown in Fig. 1D. However, Bland–Altman analysis indicated that plasma and saliva log-transformed NMR from this sample were not consistently similar (Table 1, comparison between NMR derived from total vs. free 3-HC). Similarly, correlations between plasma and saliva log-transformed 3-HC ($r = 0.95$) and plasma and saliva log-cotinine ($r = 0.97$) were high but we also observed disagreement between these measures in saliva and plasma in Bland–Altman analyses (Table 1, comparison between NMR derived from total vs. free 3-HC).

Plasma NMR reproducibility with multiple measurements over time (study 4)

Demographic data, cigarette consumption, and various measures of nicotine dependence for participants in study 4 are presented in Table 2. Controlling for age, gender, and BMI, we observed small changes in CPD over time ($P = 0.044$).

The geometric means and 95% CIs of plasma NMR at each measurement occasion for the full sample and plasma NMRs for individual subjects are presented in Fig. 2A. Geometric means and 95% CI for plasma cotinine and 3-HC are presented in Fig. 2B. All participants had biomarker data for 5 of the 6 visits whereas 38 participants had data for all 6 visits over the 44-week study period. The reliability coefficients for repeated measurements of these variables were as follows: cotinine, 0.68; 3-HC, 0.79; and NMR, 0.85. When outliers were omitted, the reliability coefficients were reduced primarily because the between-subjects variance decreased whereas within-subjects variance was unchanged: NMR, 0.70; 3-HC, 0.63; and cotinine, 0.67. To investigate whether the variability in NMR was higher at larger NMRs, we determined the trend in the mean of NMRs over 6 visits for each individual versus the SD of the NMR for that person. The positive linear trend was significant ($P = 2.7 \times 10^{-7}$).

Using repeated measures analysis and controlling for age, gender, BMI, and CPD, we observed some variation in plasma NMR over the 44-week period ($P = 0.006$). BMI ($P < 0.001$) had a significant effect on plasma NMR but the effects of age ($P = 0.545$), gender ($P = 0.640$), and CPD ($P = 0.165$) were not significant (covariates were collected at baseline). Plasma 3-HC also had small but significant changes over time ($P = 0.013$; Fig. 2B). The effects of BMI ($P < 0.001$), age ($P = 0.049$), and CPD ($P = 0.001$) on plasma 3-HC were significant whereas the gender effect was nonsignificant ($P = 0.556$). Plasma cotinine did not change significantly over time ($P = 0.266$). Age ($P = 0.038$) and CPD ($P < 0.001$) had significant effects on plasma cotinine whereas the effects of BMI ($P = 0.089$) and gender ($P = 0.140$) were nonsignificant. The results of these analyses were consistent when outliers were omitted but the effect of BMI on plasma NMR and 3-HC, respectively, did not achieve significance. Plasma NMR was not significantly associated to demographic, CPD, and nicotine dependence scales (Table 3).

Precision of plasma NMR with replication (study 4)

The variance in plasma NMR measurements decreased with increasing replicates, which translates to increased precision of plasma NMR (Table 4). The variance of plasma NMR measurements was lower (i.e., precision higher) when outliers are excluded. Table 4 further

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Table 2. Demographic, cigarette consumption, and nicotine dependence of participants enrolled in study 4, which investigated the variation of plasma NMR in *ad libitum* smokers over 44 weeks

Variable	Descriptive statistics
N	43
Sex	
Female, *n* (%)	14 (32.6)
Male, *n* (%)	29 (67.4)
Race	
Asian, *n* (%)	4 (9.3)
Black, *n* (%)	4 (9.3)
Mixed, *n* (%)	5 (11.6)
White, *n* (%)	30 (69.8)
Age	
Mean (SD)	37.6 (12.1)
Range	20–61
BMI, kg/m^2	
Mean (SD)	25.6 (5.7)
Range	16.5–47.3
CPD	
Mean (SD)	19.2 (7.7)
Range	10–40
≤10, *n* (%)	3 (7.0)
11–20, *n* (%)	28 (65.1)
21–30, *n* (%)	9 (20.9)
>31, *n* (%)	3 (7.0)
TFC	
Mean (SD)	17.0 (16.9)
Range	1–60
FTND	
Mean (SD)	5.6 (2.0)
Range	1–10
NDSS overall score	
Mean (SD)	−0.08 (0.97)
Range	−1.93–1.89

illustrates a comparison of precision obtained when using x replicate measurements of plasma NMR relative to 2 or 3 replicates. For example, if 1 plasma NMR measurement is used rather than 2, the variance in NMR estimate is increased by 8%. Removal of outliers increased the variance inflation of x replicates relative to y replicates. Thus, with outliers removed the variance of plasma NMR when 1 measurement is used instead of 2 increases from 8% to 18%.

Discussion

Main observations

We make several observations in this study that may help guide the application and use of the NMR in epidemiologic studies and in treatment of nicotine addiction. First, we show that the NMR is stable in whole blood at 4°C over a 72-hour period and in plasma and saliva at room temperature over a 14-day period. Second, we show that whole blood NMR consistently provided similar measures as plasma or saliva NMRs and that urine NMR can serve as a reasonably good proxy for blood, plasma, and saliva NMR. Finally, in a 44-week study of *ad libitum* smokers, the longest such study to date, our results indicate that a single measurement of plasma NMR is relatively reliable (reliability coefficient was 0.85 in the full sample).

NMR chemical/physical stability

NMR, cotinine, and 3-HC had relatively high stability in biologic fluids at the conditions tested. The small within-sample variability in plasma and saliva cotinine and 3-HC concentrations observed may be attributable to small degradations over time at room temperature. Within-sample variability accounted for a higher fraction of the total variability in whole blood NMR than that of plasma and saliva (8% vs. 3% and 2%, respectively). This may be due to the smaller number of samples used for whole blood stability tests (10 whole blood samples vs. 24 pairs of saliva and plasma samples). To minimize variability in NMR estimates, we recommend that blood, plasma, and saliva samples be refrigerated after sample collection as soon as is logistically feasible in field studies and all samples should be kept frozen during storage and/or transportation to analytical laboratories. Limitations of our findings include assessing the effects of only one storage condition per biologic fluid and not assessing the stability of biomarkers in urine. Also, we did not assess the impact of freeze-thaw cycles on the stability of biomarkers at the conditions tested.

Urine NMR derived using free and total 3-HC and plasma NMR

We showed that although urine $NMR_{total(3\text{-}HC)}$ and urine $NMR_{free(3\text{-}HC)}$ are highly correlated, the 2 methods did not provide similar measures of NMR. Urine $NMR_{total(3\text{-}HC)}$ is more biologically reasonable as it incorporates all 3-HC generated from cotinine, however urine $NMR_{free(3\text{-}HC)}$ explains reasonably well the variation in nicotine metabolism and is technically less time-consuming to measure. Both forms of urine NMR were similarly well correlated to plasma NMR, suggesting that both forms would serve as good proxies for plasma NMR.

Relationship between whole blood, plasma, saliva, and urine NMR

Our results suggest that whole blood NMR can be substituted for plasma and saliva NMR as the measures were consistently similar. On the other hand, urine NMRs did not consistently provide similar NMRs as whole blood, plasma, and saliva but it can serve as a reasonably good proxy for these measures based on moderate to high correlations between these variables. We also show that saliva and plasma NMRs are not consistently similar.

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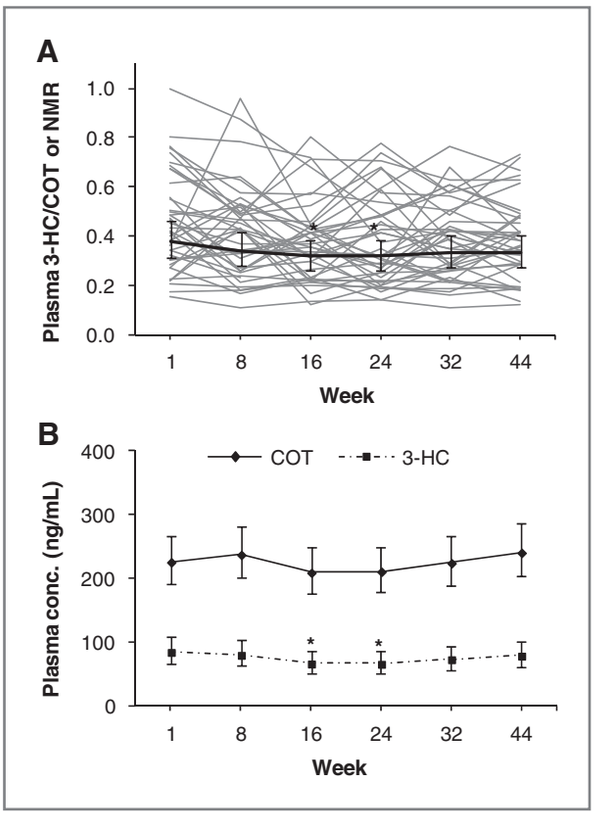

Figure 2. Plasma NMR or 3-HC/COT for individual subjects and geometric mean and 95% CI (plot A) and 3-hydroxycotinine (3-HC) and cotinine (COT) concentrations (geometric means and 95% CI; plot B) in *ad libitum* smokers ($n = 43$) over 44 weeks. *, significantly different from week 1 (baseline; $P < 0.05$, adjusted by the Tukey's method).

Nonetheless, plasma and saliva NMRs are strongly related, indicating that saliva NMR can be used to estimate plasma NMR with reasonably high accuracy.

Plasma NMR reproducibility with multiple measurements over time

The variability within subjects accounted for about 15% to 30% of the total variability observed in plasma NMR measurements (full sample and outliers excluded, respectively) and the variability in NMR increased with increasing NMR. Several sources of intraindividual variability in NMR have been proposed. First, because the stability of NMR depends on steady state level of cotinine and therefore smoking, abrupt changes in cigarette consumption and smoking pattern in the days before sample collection could have led to 3-HC not reaching steady state and incorrect estimation of the NMR (14). There was evidence of slight within-subjects changes in cigarette consumption (CPD) during the study ($P = 0.044$). But because within-subjects plasma cotinine ($P = 0.266$) remained stable whereas plasma 3-HC changed during the study ($P = 0.013$), changes in plasma NMR were probably not related to variation in CPD. Instead, this indicates that there were times when plasma 3-HC was not at steady state. Other sources of intraindividual variation in plasma NMR over time include the effects of food, drugs, or other environmental exposures on *CYP2A6* activity and other enzymes involved in the rates of cotinine clearance from other competing pathways such as glucuronidation or renal clearance (3). Variations in laboratory measurements of these biomarkers may also contribute to the overall variation in plasma NMR. We saw a linear trend of increasing within-subject SD

Table 3. Spearman rank correlation coefficients between baseline demographic and nicotine dependence and baseline plasma cotinine, 3-HC, and NMR (3-HC/COT)

Variable	COT	3-HC	3-HC/COT
Age	**0.30** (0.047)	**0.35** (0.023)	0.14 (0.359)
BMI	−0.11 (0.472)	−0.10 (0.526)	−0.11 (0.485)
FTND	**0.46** (0.002)	0.23 (0.141)	−0.21 (0.169)
CPD	**0.37** (0.013)	0.28 (0.064)	−0.04 (0.804)
TFC	−**0.46** (0.002)	−0.21 (0.168)	0.21 (0.183)
NDSS[a]			
Overall score	0.26 (0.094)	0.11 (0.491)	−0.18 (0.253)
Continuity	0.00 (0.976)	0.08 (0.595)	0.06 (0.721)
Drive	0.15 (0.330)	−0.01 (0.956)	−0.20 (0.191)
Priority	−0.07 (0.670)	0.09 (0.586)	0.18 (0.261)
Stereotypy	0.01 (0.949)	0.03 (0.855)	0.00 (0.999)
Tolerance	**0.32** (0.034)	**0.29** (0.055)	0.01 (0.966)

[a]NDSS factors: Continuity, which taps the regularity of smoking; drive, which captures craving and withdrawal in abstinence; priority, which reflects the behavioral preference of smoking over other reinforcers; stereotypy, which gets at the invariance or the "sameness" of smoking; and tolerance, which measures the reduced sensitivity to smoking's effects (Shiffman and colleagues; ref. 29). Significant correlations are in **bold** font.

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Table 4. Precision of plasma NMR with replication of NMR measurements

n/subject	Variance of the mean of *n* replicates	Reliability	Variance inflation relative to $n = 2$	Variance inflation relative to $n = 3$
A. All subjects				
1	0.080	0.85	1.08	1.11
2	0.074	0.92	1.00	1.03
3	0.072	0.94	0.97	1.00
4	0.071	0.96	0.96	0.99
10	0.069	0.98	0.94	0.96
∞	0.068	1.00	0.92	0.94
B. Outliers removed				
1	0.039	0.70	1.18	1.25
2	0.033	0.82	1.00	1.06
3	0.031	0.87	0.94	1.00
4	0.030	0.90	0.91	0.97
10	0.029	0.96	0.86	0.91
∞	0.027	1.00	0.82	0.87

NOTE: *n*, number of replicate measurements per subject; variance of the mean of *n* replicates, $\text{variance}_{\text{between-subjects}} + \text{variance}_{\text{within-subjects}}/n$; Reliability, $\text{variance}_{\text{between-subjects}}$/total variance; variance inflation of *x* relative to *y*, variance of *x* replicates/variance of *y* replicates.

of NMRs with increasing mean NMRs. However, it is common to see increasing SD with increasing mean values for analytical chemistry assays.

Precision of plasma NMR

We show that replication of NMR measurements (i.e., taking samples on separate occasions) is one method to increase the precision of NMR estimates. For example, the variance in plasma NMR is reduced from 0.080 to 0.072 (on log-scale) by increasing the number of replicate measurements from 1 to 3 (Table 4, all subjects). We illustrate the extent to which smaller sample sizes are needed to attain the same precision in plasma NMR measurements when the number of replicates is increased. The precision of NMR estimates is sensitive to the presence of outlying observations in a sample. We believe that as a rule one should not exclude observations simply because they are extreme or influential. The presence of outliers in a sample, however, may suggest within-study differences or errors in measurement or data handling that render some observations incommensurable with the others. If such problems can be identified, then it is appropriate to investigate all observations and exclude those that are not comparable on that basis, but never simply because they are outliers. Nevertheless there is value in conducting statistical analyses both including and excluding influential or extreme observations, as a form of sensitivity analysis.

Correlates of plasma NMR

Baseline plasma NMR was not significantly correlated with demographic data, CPD, and nicotine dependence scores (Table 3). Several studies have reported significant correlations between the NMR and cigarette consumption (9, 10, 15), whereas others have not found significant correlations (11, 21, 22). These conflicting results are not surprising because CPD is not always predictive of nicotine intake due to variations in puff frequency and depth of inhalation (23). On the other hand, the NMR has been shown to be correlated with total cigarette puff volume (24, 25). Plasma NMR and cotinine were not significantly correlated in this study. Furthermore, we did not find significant correlations between plasma NMR and commonly used scores of nicotine dependence, FTND, and NDSS. These results are similar to other published studies (11, 13, 22). In contrast, genetic variation in *CYP2A6* has been shown to be significantly associated to FTND scores (26). It should be noted that the currently available scales of nicotine dependence have their limitations and are not likely to be valid predictors of all aspects of smoking behavior (27). For example, these scores are less predictive of quit attempts than the NMR (11, 28).

Conclusion

Our data show relatively high stability of NMR derived from plasma and saliva samples stored at room temperature over a 14-day period and in refrigerated whole blood over a 72-hour period. Whole blood provides similar measures of NMR as plasma and saliva whereas urine NMR is a reasonably good proxy for plasma NMR measurement in whole blood, plasma, and saliva. Finally, a single measurement of plasma NMR may reliably

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estimate a smoker's true homeostatic rate of nicotine metabolism.

Disclosure of Potential Conflicts of Interest

T.P. George reports that in the past 2 years, he has received contract support from Pfizer, is a consultant to Novartis, Astra-Zeneca, Eli Lilly, Memory Pharmaceuticals, Evotec, Janssen, and Pfizer. R.F. Tyndale has consulted for Novartis and McNeil and holds shares in Nicogen Research Inc., a company that is focused on novel smoking cessation treatment approaches; no Nicogen funds were used in this work. N.L. Benowitz consults with Pfizer on smoking cessation medications and has provided paid expert testimony concerning nicotine addiction in litigation against tobacco companies. No potential conflicts of interest were disclosed by other authors.

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Collection of blood, urine, and saliva samples to be used for NMR analysis: A. Aziziyeh

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