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Journal

Nicotine & Tobacco Research, 22(2)

ISSN

1462-2203

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Publication Date

2020-02-06

DOI

10.1093/ntr/ntz034

Peer reviewed

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University of California

Nicotine & Tobacco Research, 2020, 288–292
doi:10.1093/ntr/ntz034
Original investigation
Received: October 8, 2018; Editorial Decision: February 26, 2019; Accepted March 6, 2019

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Original investigation

Urine Metabolites for Estimating Daily Intake of Nicotine From Cigarette Smoking

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Abstract

Introduction: Accurate measurement of nicotine exposure from cigarette smoke is important in studying disease risk and level of dependence. Urine total nicotine equivalents, the molar sum of nicotine and six metabolites (NE7), accounts for more than 90% of a nicotine dose and is independent of individual metabolic differences. However, measuring NE7 is technically difficult and costly. We compared NE7, the gold standard of nicotine intake, with different combinations of fewer urinary nicotine metabolites. We also examined the impact of individual differences in nicotine metabolic rate, sex, and race on strength of association with NE7.

Methods: Urine samples from 796 daily smokers, who participated across five clinical studies, were assayed for nicotine and/or metabolites. Associations with NE7 were assessed by regression and Bland–Altman analyses.

Results: Overall, the molar sum of urine [cotinine + 3′-hydroxycotinine (3HC)] (NE2) and [nicotine + cotinine + 3HC] (NE3) were strongly correlated with NE7 (r = .97 and .99, respectively). However, in slow metabolizers NE2 was less predictive of NE7, whereas NE3 was equally robust. Urine total cotinine was also strongly correlated with NE7 (r = .87).

Conclusions: Urine NE3 is a robust biomarker of daily nicotine intake, independently of individual metabolic differences, whereas NE2 is less accurate in slow metabolizers. Our findings inform the selection of more rigorous and cost-effective measures to assess nicotine exposure in tobacco research studies.

Implications: The molar sum of urine total nicotine, cotinine and 3HC (NE3) is a robust biomarker of daily nicotine intake, independently of individual metabolic differences, and performs as well as measuring seven nicotine metabolites (NE7). The sum of cotinine and 3HC (NE2) is less accurate in slow metabolizers. Our findings inform the selection of more rigorous and cost-effective measures to assess nicotine exposure in tobacco research studies.

Introduction

The daily systemic dose (intake) of nicotine reflects exposure to various tobacco-derived toxicants and is associated with the level of tobacco dependence.[1,2] Nicotine is extensively metabolized and has a short half-life (~2 hours), so nicotine metabolites have been used as biomarkers of nicotine intake. The concentration of cotinine, the major proximate metabolite of nicotine, measured in plasma, saliva, or urine is the most widely used biomarker of nicotine intake, but its

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accuracy as a predictor is limited by individual differences in nicotine metabolic pathways.[3,4] The best biomarker of daily nicotine intake is thought to be the total of nicotine and its metabolites in urine (urine total nicotine equivalents, TNE) measured during steady state dosing. Urine total nicotine equivalents (NE7), taken as the sum of nicotine and six metabolites (cotinine, 3′-hydroxycotinine (3HC), nicotine *N*-oxide, cotinine *N*-oxide, nornicotine, and norcotinine, including their glucuronide conjugates), accounts for 90% or more of the daily nicotine dose, and is not substantially influenced by individual metabolic differences.[5–7] Empirically, TNE has been demonstrated to be highly correlated with nicotine intake with known dosing.[8]

Analysis of the full panel of nicotine and six metabolites is technically difficult, time consuming and costly. The minor metabolites of nicotine are present in much lower concentrations than are nicotine, cotinine, and 3HC; and it takes more demanding analytical methods to measure low concentrations than higher concentrations. Significantly more time (labor cost) is required to include the four minor metabolites. This raises the question of whether measuring fewer metabolites will be adequate to estimate daily nicotine intake. An important question is whether measuring fewer metabolites will make NE7 less accurate owing to individual metabolic differences, such as race-related genetic differences or hormonally related sex differences in rates and pathways of nicotine metabolism.[9,10] The ratio of 3HC to cotinine (3HC/cotinine), known as the nicotine metabolite ratio (NMR) is a phenotypic marker of CYP2A6 metabolic activity and the rate of nicotine metabolism, and is a measure of individual metabolic differences that incorporates genetic, hormonal, and environmental influences.[11,12]

The major aim of our study was to compare the sum of nicotine and six metabolites (NE7) measured in urine as the gold standard for daily intake of nicotine with different combinations of fewer urine metabolites. We did this by both examining the strength of relationships (correlation analysis) and the limits of agreement between combinations of urine metabolites.[13,14] A second aim was to determine if individual differences in nicotine metabolic rates, either based on race (black vs. white), sex, or CYP2A6 activity, the latter assessed using the plasma ratio of 3HC/cotinine, influenced the strength of correlations with NE7.

Materials and Methods

Study Procedures

Urine samples were collected from daily smokers who participated across five different research studies conducted at the University of California, San Francisco (UCSF) and University of Kansas. Details on these studies, including citations that describe study design for each study, are provided in Table 1. In brief summary, studies 1, 3, 4, and 5 were conducted in San Francisco. Study 1 (unpublished) was a study of the mechanisms of influence of nicotine metabolic rate on nicotine dependence. Study 2, conducted in Kansas City, was a smoking cessation in black light smokers (≤10 cigarettes per day). Study 3 (unpublished) was a pharmacokinetic study in black and white smokers. Study 4 was a clinical trial of reduced nicotine content cigarettes. Study 5 was a study comparing various biomarkers of tobacco smoke exposure in black and white smokers. Each participant provided a random spot urine sample at baseline before participating in the study. Blood samples were also obtained, although not always on the same day as the urine sample, for measurement of NMR. Urine and plasma samples were stored frozen after collection until the time of analysis.

Analytical Chemistry

Urine samples were assayed in the same laboratory for total nicotine, cotinine, 3HC, nornicotine, norcotinine, nicotine *N*-oxide, and cotinine *N*-oxide by liquid chromatography tandem mass spectrometry (LC-MS/MS).[18] The method, including sample preparation, is essentially the same as the published method[18] for cotinine and 3HC, but the liquid chromatography and mass spectrometric parameters have been modified to measure nicotine and additional metabolites.[7] All urine analyses were performed after treatment with a glucuronidase enzyme, so the metabolite concentrations represent the sum of free and conjugated compounds (ie, total nicotine) Plasma analyses were of free (unconjugated) cotinine and 3HC using the same LC-MS/MS method.[18] Urine concentrations were normalized by creatinine. Molar sums were computed for the following nicotine equivalent combinations: NE1 = cotinine; NE2 = cotinine + 3HC; NE3 = nicotine + cotinine + 3HC; NE7 = nicotine + cotinine + 3HC + nornicotine + norcotinine + nicotine *N*-oxide + cotinine *N*-oxide. NE7 is taken as the gold standard for daily nicotine exposure. Plasma NMR was determined as the ratio of 3HC/cotinine.

Data Analysis

The strength of the relationships for various biomarkers within participants was examined using Pearson linear regression. We also examined the Pearson correlations within race (black vs. white), sex, and quartile of NMR. The agreement between various biomarkers was assessed using Bland–Altman analysis.[13,14] Nicotine equivalents measures were log-transformed for the Bland–Altman analysis. The range of agreement was defined as mean difference between measures ± 2 standard deviations. Results obtained on the log-scale were back-transformed and presented as ratios between different forms of nicotine equivalents. Differences in characteristics between study populations whose data were used in our analyses were assessed using Kruskal–Wallis or Wilcoxon tests.

Results

Demographic data are shown in Table 1. Overall, participants averaged 42 (SD 12) years of age, 67% participants were black and 55% were female. Biomarker levels in participants across various studies are shown in Table 2. Participants smoked an average of 12.3 cigarettes per day. The median TNE was 50.2 nmol/mg creatinine and was similar for all study groups except for study 3, which was higher ($p < .001$). The median NMR was 0.35 and was similar across research study groups.

As shown in Table 3, for all participants, compared to NE7 as the gold standard, NE2 and NE3 were very highly correlated ($r = .97$ and 0.99, respectively), whereas NE1 was significantly but less strongly correlated with NE7 ($r = .89$).

Analyses performed within race (black or white) and comparing women and men showed similar strength of correlations (Table 3).

The NMR quartile cut points were 0.23, 0.35, and 0.51 with quartile median values of 0.17, 0.29, 0.42, and 0.68. As shown in Table 3, the notable effect of NMR was that in the lowest NMR quartile, NE1 and NE2 had weaker correlations with NE7 compared to correlations in higher NMR quartiles. NE3 was an equally robust predictor of NE7 in all quartiles. Further examination of the correlation between NE2 and NE7 at lower percentiles of plasma NMR demonstrated correlation coefficients as follows: 20th percentile, $r = .90$; 15th percentile, $r = .88$; 10th percentile, $r = .88$; 5th

Table 1. Study Population

Study	N	Age	Sex				Race									
		Mean (SD)	Male		Female		White		Black		AI/AN		Asian		Mixed	
			n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
1	134	36 (12)	83	62	51	38	98	73	36	27	0	0	0	0	0	0
2[15]	414	46 (12)	134	32	280	68	0	0	412	100	2	0	0	0	0	0
3	35	37 (10)	22	63	13	37	21	60	14	40	0	0	0	0	0	0
4[17]	87	37 (11)	50	57	37	43	62	71	8	9	2	2	5	6	10	11
5[1]	126	38 (11)	73	58	53	42	66	52	60	48	0	0	0	0	0	0
Full sample	**796**	**42 (12)**	**362**	45	**434**	55	**247**	31	**530**	67	**4**	1	**5**	1	**10**	1

AI/AN = American Indian or Alaska Native. Bold numbers indicate values for full sample.

Table 2. Biomarker Distribution

Study	CPD		pNMR		pCOT (ng/mL)		NE1 (nmol/mg cr)		NE2 (nmol/mg cr)		NE3 (nmol/mg cr)		NE7 (nmol/mg cr)	
	Mean	SD	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
1	13.7	6.3	0.39	0.25–0.68	176.2	113.4–263.2	11.8	6.2–21.6	33.9	18.6–55.4	43.9	23.5–67.0	53.1	27.7–79.4
2	8.1	2.2	0.34	0.21–0.51	225.8	147.0–309.2	10.6	6.3–16.5	32.5	19.7–50.3	41.6	26.7–61.2	48.8	32.0–71.2
3	16.4	5.6	0.30	0.25–0.43	285.6	201.5–345.0	22.6	14.5–36.2	59.0	45.3–93.6	67.3	51.1–99.7	77.3	56.8–115.2
4	20.0	7.2	0.40	0.28–0.53	231.3	163.8–330.1	6.0	3.8–8.7	27.7	19.1–39.3	33.3	25.1–48.6	39.5	30.1–56.2
5	18.0	9.3	0.34	0.23–0.44	206.1	124.5–270.6	14.8	9.3–25.1	42.7	25.0–65.1	49.4	32.3–78.1	55.1	35.3–88.2
Full sample	**12.3**	7.2	**0.35**	0.23–0.51	**218.7**	139.8–301.6	**11.0**	6.1–18.6	**33.8**	20.5–53.5	**42.2**	27.1–66.0	**50.2**	32.3–76.2

NE1: COT; NE2: COT + 3HC; NE3: COT + 3HC + NIC; NE7: COT + 3HC + NIC + norNIC +norCOT + NNO + CNO. COT = cotinine, CNO = cotinine *N*-oxide, 3HC = 3′-hydroxycotinine, IQR = interquartile range, CPD = cigarettes per day, NNO = nicotine *N*-oxide, pCOT = plasma cotinine, pNMR = plasma nicotine metabolite ratio. Bold numbers indicate values for full sample.

percentile, $r = .79$. Plasma NMR was not significantly correlated with NE7 in any of the group analyses.

Correlation analyses were also performed within study groups and showed similar strengths of correlations between NE subsets and NE7 (Supplementary Table 1).

Results of the Bland–Altman analysis are presented in Table 4. As expected, NE7 is higher than others, with Wratios of 1.18, 1.50, and 4.65 compared to NE3, NE2, and NE1, respectively. The confidence intervals of ratios were quite narrow for NE7/NE3, somewhat wider for NE7/NE2, and considerably wider for NE7/NE1.

Discussion

The gold standard for daily nicotine intake is the sum of nicotine and all of its metabolites excreted in urine during steady state intake conditions. We demonstrate that measurement of fewer metabolites, that is, total [nicotine + cotinine + 3HC] (NE3) is highly correlated with NE7 and can therefore be used as a valid biomarker of nicotine intake. This is not surprising because the minor metabolites account for only a small fraction of nicotine dose, and individual metabolic differences in these pathways would have only a small impact on the overall estimate of dose.[5,6]

In most cases NE2, the sum of total [cotinine + 3HC] is also highly correlated with NE7, but the range of agreement is wider (indicative of poorer agreement between NE7 and NE2). The strength of this correlation is weaker in people with reduced CYP2A6 metabolic activity, as determined by the bottom quartile of NMR, and even weaker for smokers in the bottom 5% of NMR. This is expected because with reduced CYP2A6 activity, a smaller proportion of nicotine is metabolized to cotinine, and more nicotine is excreted as unchanged nicotine and nicotine glucuronide; and NE2 does not measure nicotine or nicotine glucuronide.[16,19] NE3 measures total nicotine as well, so this metabolic difference is accounted for.

Blacks, on average, metabolize nicotine more slowly than whites, owing to a higher prevalence of reduced activity CYP2A6 and uridine 5'-diphospho-glucuronyltransferase (UGT) gene variants.[16,20,21] Women on average metabolize nicotine more rapidly than men because estrogen induces CYP2A6 to increase its metabolic activity.[9,22] However, in group comparisons neither race nor sex affected the strength of association between NE2 or NE3 with TNE7. The independence of the predictive values of various NE measures with NE7 supports the generalizability of our findings. Because of the small number, we could not analyze NE correlations in Asians, who have much higher prevalence of reduced metabolism CYP2A6 gene variants than do whites; and our studies should be repeated in that population.[10]

One limitation of our study is that we did not measure two minor metabolites of nicotine: 4-oxo-4-(3-pyridyl)-butanoic acid and 4-hydroxy-(3-pyridyl)-butanoic acid; however, these metabolites are thought to contribute less than 10% of total nicotine metabolism, so the impact on our conclusions is likely to be minimal.[6,23] Another limitation is that our study population was a convenience sample, with a higher proportion of black smokers than in the general population. In addition, we did not include Asian smokers in our analysis. Asians are genetically slow metabolizers of nicotine and cotinine, similar to blacks, so we would expect that our observations in blacks would hold for Asians.[10,20] Nonetheless, our findings should be confirmed in a nationally representative sample of smokers.

Urine nicotine equivalents can be used to estimate daily intake of nicotine from various tobacco products. If we assume that NE7 represents 90% of all nicotine metabolites, then:

$$D = \frac{NE7_{24}}{0.9} \quad (1)$$

where D (mg) is the daily dose of nicotine and $NE7_{24}$ (mg nicotine/day) is excretion of nicotine metabolites expressed as mg nicotine

Table 3. Biomarker Correlation with NE7

Correlation	Full sample *n* = 796	Blacks *n* = 530	Whites *n* = 247	Females *n* = 434	Males *n* = 362	NMR Q1 *n* = 200	NMR Q2 *n* = 200	NMR Q3 *n* = 198	NMR Q4 *n* = 198
NE1	0.89*	0.91*	0.87*	0.90*	0.85*	0.86*	0.95*	0.92*	0.83*
NE2	0.97*	0.97*	0.96*	0.97*	0.95*	0.89*	0.98*	0.97*	0.98*
NE3	0.99*	0.99*	0.99*	0.99*	0.99*	0.99*	0.99*	0.99*	0.99*
pNMR	0.06	0.05	0.03	0.06	0.05	0.02	0.03	0.07	0

NMR = nicotine metabolite ratio, pNMR = plasma NMR.
*$p < .001$.

Table 4. Bland–Altman Analysis of Agreement Between Various Forms of Nicotine Equivalents (NE)

Comparison	Ratio (95% CI)	Range of agreement (Ratio ± 2SD)
NE7 vs. NE3	1.18 (1.17 to 1.19)	1.00–1.39
NE7 vs. NE2	1.50 (1.47 to 1.53)	0.81–2.77
NE7 vs. NE1	4.65 (4.51 to 4.79)	1.95–11.07
NE3 vs. NE2	1.27 (1.25 to 1.30)	0.74–2.18
NE3 vs. NE1	3.95 (3.83 to 4.07)	1.65–9.42
NE2 vs. NE1	3.10 (3.00 to 3.20)	1.23–7.83

Ratio: Back-transformation of mean differences 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 standard deviations from the mean difference (narrower range of agreement indicates greater agreement between variables). NE7 = nicotine, cotinine, 3′-hydroxycotinine, nornicotine, norcotinine, nicotine *N*-oxide, and cotinine *N*-oxide; NE3 = nicotine, cotinine, and 3′-hydroxycotinine; NE2 = cotinine and 3′-hydroxycotinine; NE1 = cotinine. CI = confidence interval.

equivalents over 24 hours. NE7 in nmol/mg creatinine can be converted to dose of nicotine as follows:

$$NE7_{24} = NE7 \times 162.2 \times UCE \quad (2)$$

where NE7 is in nmol/mg creatinine, 162.2 g/mol (or ng/nmol) is the molar mass of nicotine, and UCE (mg creatinine/24 hr) is 24 hour urinary creatinine excretion. Urinary creatinine excretion varies from person to person in relation to lean muscle mass, race, sex, age, and dietary protein.[24] On average, however, UCE (mg creatinine per 24 hr) = body weight (kg) × 24 mg creatinine/24hr/kg in men, and body weight (kg) × 21 mg creatinine/24hr/kg in women.[25]

As an illustration of a computation of daily intake of nicotine, we assume that NE7 = 50 nmol/mg creatinine in a 70 kg man. Then, the product of NE7 × 162.2 from Equation 2 is 50 nmol/mg creatinine x 162.2 ng/nmol, which equals 8110 ng/mg or 8.1mg nicotine/mg creatinine. For a 70 kg man, UCE is estimated to be 70 kg × 24 mg creatinine/24 hr/kg, which equals 1680 mg, or 1.68 g creatinine per 24 hr. Completing Equation 2, $NE7_{24}$ is 8.1 mg nicotine/gm creatinine × 1.68 g creatinine, which equals a 24-hour nicotine equivalent excretion of 13.6 mg. Using Equation 1, the daily intake of nicotine is estimated to be 15.1 mg. The Bland–Altman analysis provides ratios of NE7 to other potential biomarkers. Thus, if NE3, NE2, or NE1 are used as biomarkers for nicotine intake, one needs to multiply by 1.18, 1.50, and 4.65, respectively, to estimate NE7, which can then be converted to daily intake of nicotine as described earlier. These computations will be useful for computing average nicotine exposure in groups of smokers, but would be less useful for individuals due to individual variations in UCE and in the ratios of various NE measures to NE7.

In conclusion, our data demonstrate that the use of the molar sum of total [nicotine + cotinine + 3HC] (NE3) is highly correlated with the sum of nicotine and all metabolites, and can be used a valid biomarker of daily nicotine intake in cigarette smokers. The analytical measurement of NE3 is technically easier and less costly than that of NE7. The sum of total [cotinine + 3HC] (NE2) also does well in the general population of smokers, but is less accurate for smokers who are slow metabolizers of nicotine via CYPA6. Although our study focused on cigarette smokers, the findings should be applicable to users of other forms of nicotine, so long as the intake of nicotine was consistent from day to day so that urine metabolite concentrations would approximate steady state. Our findings will be useful in designing more cost-effective studies of nicotine and tobacco exposure for clinical experimental and epidemiological studies. We also present an approach to estimate daily intake of nicotine using urine NE measurements.

Supplementary Material

Supplementary data are available at *Nicotine and Tobacco Research* online.

Funding

Research that contributed to this article was supported by the National Institute on Drug Abuse (NIDA) under award numbers P30 DA012393, R01 DA002277, R01 DA031193, U01 DA020830 (to NLB); the National Cancer Institute (NCI) under award numbers R01 CA 078603 (to NLB) and R01 CA091912 (to LC) and the National Center for Research Resources (NCRR) under award S10 RR026437 (to PJ). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Acknowledgments

The authors acknowledge Newton Addo for statistical and editorial contributions and the many chemists in the Clinical Pharmacology Laboratory at UCSF who performed the analytical chemistry.

Declaration of Interests

NLB is a consultant to Pfizer and Achieve Life Sciences, companies that market or are developing smoking cessation medications, and has been a paid expert witness in litigation against tobacco companies. The other authors have no conflicts to disclose.

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