Research Article

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Nicotine Metabolite Ratio (3-Hydroxycotinine/Cotinine) in Plasma and Urine by Different Analytical Methods and Laboratories: Implications for Clinical Implementation

Julie-Anne Tanner[1,2], Maria Novalen[1,2], Peter Jatlow[3], Marilyn A. Huestis[4], Sharon E. Murphy[5], Jaakko Kaprio[6,7,8], Aino Kankaanpää[7], Laurence Galanti[9], Cristiana Stefan[10], Tony P. George[1,11,12], Neal L. Benowitz[13], Caryn Lerman[14], and Rachel F. Tyndale[1,2,12]

Abstract

Background: The highly genetically variable enzyme CYP2A6 metabolizes nicotine to cotinine (COT) and COT to *trans*-3′-hydroxycotinine (3HC). The nicotine metabolite ratio (NMR, 3HC/COT) is commonly used as a biomarker of CYP2A6 enzymatic activity, rate of nicotine metabolism, and total nicotine clearance; NMR is associated with numerous smoking phenotypes, including smoking cessation. Our objective was to investigate the impact of different measurement methods, at different sites, on plasma and urinary NMR measures from *ad libitum* smokers.

Methods: Plasma ($n = 35$) and urine ($n = 35$) samples were sent to eight different laboratories, which used similar and different methods of COT and 3HC measurements to derive the NMR. We used Bland–Altman analysis to assess agreement, and Pearson correlations to evaluate associations, between NMR measured by different methods.

Results: Measures of plasma NMR were in strong agreement between methods according to Bland–Altman analysis (ratios, 0.82–1.16) and were highly correlated (all Pearson $r > 0.96$, $P < 0.0001$). Measures of urinary NMR were in relatively weaker agreement (ratios 0.62–1.71) and less strongly correlated (Pearson r values of 0.66–0.98, $P < 0.0001$) between different methods. Plasma and urinary COT and 3HC concentrations, while weaker than NMR, also showed good agreement in plasma, which was better than that in urine, as was observed for NMR.

Conclusions: Plasma is a very reliable biologic source for the determination of NMR, robust to differences in these analytical protocols or assessment site.

Impact: Together this indicates a reduced need for differential interpretation of plasma NMR results based on the approach used, allowing for direct comparison of different studies. *Cancer Epidemiol Biomarkers Prev; 24(8); 1239–46. ©2015 AACR.*

Introduction

Interindividual differences in rates of nicotine metabolism and clearance, mainly mediated by hepatic enzyme cytochrome P450 2A6 (CYP2A6), alter numerous smoking behaviors, including cessation. CYP2A6, responsible for approximately 90% of nicotine's metabolism to cotinine (COT) and 100% of COT metabolism to *trans*-3′-hydroxycotinine (3HC; refs. 1, 2), exhibits highly variable interindividual metabolic activity largely resulting from genetic variation in *CYP2A6* (3), the gene encoding the enzyme. The ratio of these two nicotine metabolite concentrations (3HC/COT), known as the nicotine metabolite ratio (NMR), captures both genetic (4) and environmental (5) variation and can be used as a phenotypic biomarker of CYP2A6 enzymatic activity, the rate of nicotine metabolism, and total nicotine clearance (6).

[1]Campbell Family Mental Health Research Institute, Centre for Addiction and Mental Health (CAMH), Toronto, Ontario, Canada. [2]Department of Pharmacology and Toxicology, University of Toronto, Toronto, Ontario, Canada. [3]Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut. [4]Chemistry and Drug Metabolism, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, Maryland. [5]Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, Minnesota. [6]Department of Public Health, Hjelt Institute, University of Helsinki, Helsinki, Finland. [7]National Institute for Health and Welfare (THL), Helsinki, Finland. [8]Institute for Molecular Medicine Finland FIMM, University of Helsinki, Helsinki, Finland. [9]Department of Clinical Biology, Mont-Godinne University Hospital, Yvoir, Belgium. [10]Clinical Laboratory and Diagnostic Services, Centre for Addiction and Mental Health, Toronto, Canada. [11]Division of Brain and Therapeutics, Department of Psychiatry, University of Toronto, Toronto, Ontario, Canada. [12]Schizophrenia Division, Centre for Addiction and Mental Health (CAMH), Toronto, Ontario, Canada. [13]Division of Clinical Pharmacology and Experimental Therapeutics, Departments of Medicine and Bioengineering & Therapeutic Sciences, University of California, San Francisco, California. [14]Center for Interdisciplinary Research on Nicotine Addiction, Department of Psychiatry, University of Pennsylvania, Philadelphia, Pennsylvania.

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J.-A. Tanner and M. Novalen contributed equally to this article.

Corresponding Author: Rachel F. Tyndale, 1 King's College Circle, University of Toronto, Medical Sciences Building, Room 4326, Toronto, Ontario, Canada, M5S 1A8. Phone: 416-978-6374; Fax: 416-978-6395; E-mail: r.tyndale@utoronto.ca

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NMR is often retrospectively used, and more recently prospectively used, in studies of treatment optimization for smoking cessation pharmacotherapies. Retrospective analyses have demonstrated that smokers with lower NMR (i.e., slow nicotine metabolism) have greater success in quitting smoking when treated with transdermal nicotine or placebo compared with those with higher NMR (i.e., faster nicotine metabolizers; refs. 7–9). In contrast, smokers treated with buproprion, not metabolized by CYP2A6, exhibited no differences in quit rates based on NMR (8). A recently completed prospective phase III clinical trial (NCT01314001) investigated the utility of NMR as a predictive biomarker of smoking cessation outcomes, specifically studying cessation success while on varenicline versus nicotine patch (10). Randomization to each treatment group was stratified prospectively based on the subject's NMR, and it was found that varenicline, compared with nicotine patch, was associated with greater quitting among normal nicotine metabolizers, whereas for slow metabolizers patch worked as well as varenicline, and had fewer side effects than varenicline for slow metabolizers (10). Results from this trial and others will aid in the translation of research to clinical practice, such that the most effective smoking cessation treatment strategy can be tailored using a smoker's NMR. In addition, NMR is used increasingly in case–control and –cohort studies of smokers, examining additional smoking phenotypes, including cigarettes smoked per day (11) and smoking topography (12).

In addition to the strong correlation of NMR with nicotine clearance and CYP2A6 activity, several other characteristics of NMR make this a useful biomarker. The relatively long half-life of COT (~16 hours) and formation dependence of 3HC promote stability over time of the relative COT and 3HC concentrations, and the resulting NMR, in daily smokers irrespective of heavy or light cigarette consumption, or sampling time of day (13–15). There is only minor variation in average daily NMR over a 7-day period for daily smokers (13), and NMR remains relatively stable over a 44-week range in regular daily smokers and in smokers who are reducing their smoking levels with the help of nicotine replacement therapy (NRT; refs. 14–16). This indicates that a reliable estimate of nicotine clearance rate can be obtained from a single sample, and that the rate of nicotine metabolism is not substantially altered over an extended period of time for regular smokers. In addition, beyond genetic variation in *CYP2A6*, several influences on NMR (gender, ethnicity, birth control use, hormone replacement therapy, body mass index, and cigarettes per day) together contribute only approximately 8% of the variation in NMR (17); thus these predictors are unlikely to substantially influence NMR nor alter its utility as a biomarker. NMR quantification requires relatively noninvasive procedures, such that COT and 3HC can be measured in saliva, plasma, blood, or urine samples, and strong correlations have been reported for NMR derived from blood, plasma, and saliva, whereas blood-derived compared with urine-derived NMR showed modestly lower correlations (16).

A potential source of variation in NMR that has yet to be examined is between different laboratory sites and analytical methods. Therefore, to further characterize utility of this nicotine clearance biomarker, we investigated the relationship between NMR measures performed in eight different laboratories utilizing similar (e.g., LCMS/MS) or different (e.g. LCMS/MS vs. HPLC-UV) analytical methods for plasma and urine samples from a population of treatment seeking *ad libitum* smokers who had been recruited for the Pharmacogenetics of Nicotine Addiction Treatment (PNAT2, NCT01314001) trial, described above (10).

Materials and Methods

Reagents and quality control samples

Cotinine and trans-3′-hydroxycotinine were purchased from Sigma-Aldrich Canada and Toronto Research Chemicals. Quality control (QC) samples were prepared in 0.01 mol/L hydrochloric acid by the addition of COT and 3HC to achieve known concentrations. Seven QC pools were created with a wide range of COT and 3HC concentrations of 1, 10, 100, 500, 1,000, 5,000, and 10,000 ng/mL. Before distributing, QC samples were analyzed by one of the participating laboratories (site using method 1A) to ensure preparation quality.

Clinical study samples

The plasma and urine samples were collected as a part of the PNAT2 clinical trial for smoking cessation treatment (NCT01314001). The study was approved by the IRB at the University of Pennsylvania (Philadelphia, PA), the Center for Addiction and Mental Health (Toronto, Canada), MD Anderson Cancer Centre (Houston, TX), University at Buffalo (Buffalo, NY; the 4 recruitment sites), and at the University of Toronto (Toronto, Canada; the analytical site). Written, informed consent was obtained from each subject. Study details were described elsewhere (10, 18). Plasma ($n = 35$) and urine ($n = 35$) samples were frozen at −30°C until initial COT and 3HC analysis.

Plasma and urine samples, representing typical COT and 3HC concentrations found in smokers, were aliquoted in volumes of 0.125 to 1.5 mL. Depending on the laboratory's limit of quantification and the sample matrix, the specific assay was optimized for the laboratories receiving either plasma or urine, or both, as well as QC samples. The urine samples were not pretreated with glucuronidase, and only concentrations of free, nonconjugated metabolites were reported. The focus of this work was on the assessment of the contribution of different analytical methods. Deconjugation of urine samples was not expected to impact the agreement of metabolite measurements between different analytical methods, as this was consistent across all laboratories (i.e., all laboratories were sent the same nonconjugated samples). Similarly, urine concentrations were not corrected using creatinine. We did not expect correction of urine concentrations to affect the agreement and association of urine metabolite measures by different methods, as all laboratories were sent aliquots of the same urine sample. Laboratories were blinded to the analyte concentrations.

Analytical procedures

A summary of the analytical methods for each laboratory is in Table 1, with a more detailed method description in Supplementary Table S1. All methods used chromatography coupled to either mass spectrometer detector or UV; no immunoassay methods were used, as we are unaware of any which are specific for COT and separately for 3HC. Of the six LC/MS-MS methods, three utilized atmospheric-pressure chemical ionization (APCI; methods 1A–C) and three electrospray ionization (ESI; methods 2A–C). One gas chromatography (GC) MS (method 3) was utilized, and the final two methods used high-performance liquid chromatography (HPLC) with UV detection (methods 4A–B). All protocols used various extraction procedures and included an

Table 1. Summary of participating laboratories' analytical methods for analysis of biologic and quality control (QC) samples

Method ID	Analytical method	Internal standard(s)	Limit of quantification (ng/mL)	Type(s) of biologic samples analyzed
1A	APCI LC-MS/MS	Cotinine-D_3	1 ng/mL	Plasma, urine
		3′-Hydroxycotinine-D_3	1 ng/mL	
1B	APCI LC-MS/MS	Cotinine-D_9	1 ng/mL, 10 ng/mL	Plasma, urine
		3′-Hydroxycotinine-D_9	1 ng/mL, 10 ng/mL	
1C	APCI LC-MS/MS	Cotinine-D_3	1 ng/mL	Plasma
		3′-Hydroxycotinine-D_3	1 ng/mL	
2A	ESI LC-MS/MS	Cotinine-D_3	1 ng/mL	Plasma
		3′-Hydroxycotinine-D_3	1 ng/mL	
2B	ESI LC-MS/MS	Cotinine-D_3	1 ng/mL	Plasma
		3′-Hydroxycotinine-D_3	1 ng/mL	
2C	ESI LC-MS/MS	Cotinine-D_3	0.08 ng/mL	Plasma
		3′-Hydroxycotinine-D_3	0.2 ng/mL	
3	EI GC-MS	Cotinine-D_3	2.5 ng/mL	Plasma
		3′-Hydroxycotinine-D_3	2.5 ng/mL	
4A	HPLC-UV	2-phenylimidazole	10 ng/mL	Urine
4B	HPLC-UV	5-methylcotinine	12.5 ng/mL for COT 10 ng/mL for 3HC	Urine

NOTE: Method 1A, University of Toronto; method 1B, University of California, San Francisco; method 1C, Centre for Addiction and Mental Health; method 2A, Yale University; method 2B, National Institute on Drug Abuse; method 2C, University of Minnesota; method 3, National Institute for Health and Welfare (THL)/University of Helsinki; method 4A, Cliniques Universitaires UCL Mont-Godinne; method 4B, University of Toronto.

internal standard. All MS methods used deuterated COT and 3HC as internal standards, whereas the HPLC-UV methods used 2-phenylimidazole or 5-methylcotinine. Limits of quantification ranged from less than 1 to 12.5 ng/mL. Samples were sent to participating laboratories on dry ice in October or November 2012, and all results were returned by February 2013. Analyte concentrations were compared from the eight different laboratories that used seven methods for measurement from plasma and four methods for measurement from urine. Each site will hereafter be referred to by their method (1A–C, 2A–C, 3, 4A–B; Table 1). NMR was determined by each method using the ratio of 3HC/COT.

Statistical analysis

COT and 3HC concentrations were nonnormally distributed and therefore were log-transformed for further analyses. In addition, logged, compared with the unlogged, NMR is a stronger surrogate of nicotine clearance rate (19). Bland–Altman analysis was used to test the level of agreement between methods used at each laboratory to measure log-transformed NMR, COT, and 3HC, respectively, in plasma and urine. The ratio computed by Bland–Altman analysis is a result of the back-transformation of the mean difference between measures on the log-scale. A ratio of 1 indicates the least difference between measures (complete agreement). The range of agreement is defined as the mean bias ± 2 SD. All methods were compared with one another for Bland–Altman analysis. Linear regression was used to determine the strength of associations between analyte measurements by each method compared with the reference method. Pearson correlation coefficients were computed between analyte measurements by each method compared with the reference method. Analyses were carried out with GraphPad Prism (version 6.0), and statistical tests were considered significant when $P < 0.05$.

Results

Comparison of plasma NMR measured by different methods

Plasma NMR, COT, and 3HC measurements ranged from 0.11 to 1.23, 56.0 to 538.1, and 15.0 to 236.0, respectively. Seven mass spectrometry approaches (1A–C, 2A–C, 3) for quantifying nicotine's plasma metabolites' concentrations consistently provided similar measures of log-transformed NMR according to Bland–Altman analyses of agreement between repeated measures (Table 2A). Bland–Altman ratios were between 0.82 and 1.16 when comparing plasma NMR between all methods, which is indicative of relatively strong agreement between NMR measurements across methods.

Measures of plasma NMR were highly correlated between methods, with all Pearson correlations (r) greater than 0.96 (all $P < 0.0001$; Table 2A, illustrated in Fig. 1A). Similar correlations were observed with non–log-transformed NMR (see legend to Fig. 1).

As a secondary analysis, we examined COT and 3HC individually. For plasma COT and 3HC concentrations measured by different methods (1A–C, 2A–C, 3), all Bland–Altman ratios were between 0.70 and 1.34 (Table 2B and C). Likewise, Pearson correlations (r) between log-transformed plasma COT and 3HC concentrations by different methods were high (all Pearson $r > 0.93$, all $P < 0.0001$; Table 2B and C; Fig. 1B and C). Similar correlations were observed with non-logged COT and 3HC concentrations from each method (see legend to Fig. 1).

Comparison of urine NMR measured by different methods

Urinary NMR, COT, and 3HC measurements ranged from 0.34 to 12.40, 100.3 to 3220.0, and 249.8 to 19409.8, respectively. Measures of urinary log-transformed NMR by four different methods (1A, 1B, 4A, 4B) were less consistent than that observed in plasma, according to Bland–Altman analyses of agreement between repeated measures (Table 2A). Bland–Altman ratios were between 0.62 and 1.71 when comparing urine NMR between all methods. Methods 1A and 1B are essentially the same, and both methods were used for the measurement of the NMR in urine and plasma; however, although agreement was best between methods 1A and 1B, the agreement was still poorer for urine than was observed by these methods in plasma (ratio and range of agreement of 1.05, 0.68–1.62 vs. 0.96, 0.83–1.10 for urine and plasma, respectively).

Table 2. Bland-Altman ratios (range of agreement) and Pearson correlations of (A) logNMR, (B) logCOT, and (C) log3HC measured in plasma and urine according to each method relative to all other methods

A.

Bland-Altman ratios

Correlations		Plasma							Urine			
		1A	1B	1C	2A	2B	2C	3	1A	1B	4A	4B
Plasma	1A		0.96 (0.83–1.10)	1.04 (0.89–1.22)	0.97 (0.86–1.11)	0.94 (0.81–1.09)	0.89 (0.76–1.04)	1.09 (0.86–1.37)				
	1B	r = 0.99 P < 0.0001		0.92 (0.80–1.06)	1.02 (0.92–1.13)	0.98 (0.84–1.15)	1.07 (0.95–1.21)	0.88 (0.72–1.08)				
	1C	r = 0.98 P < 0.0001	r = 0.99 P < 0.0001		0.94 (0.86–1.03)	0.91 (0.78–1.05)	1.16 (1.06–1.27)	0.96 (0.77–1.19)				
	2A	r = 0.99 P < 0.0001	r = 0.99 P < 0.0001	r = 1.00 P < 0.0001		1.04 (0.94–1.15	1.09 (1.02–1.17)	0.90 (0.73–1.10)				
	2B	r = 0.99 P < 0.0001	r = 0.99 P < 0.0001	r = 0.99 P < 0.0001	r = 0.99 P < 0.0001		1.05 (0.93–1.19)	0.87 (0.70–1.08)				
	2C	r = 0.98 P < 0.0001	r = 0.99 P < 0.0001	r = 0.99 P < 0.0001	r = 1.00 P < 0.0001	r = 0.99 P < 0.0001		0.82 (0.68–1.00)				
	3	r = 0.97 P < 0.0001	r = 0.97 P < 0.0001	r = 0.97 P < 0.0001	r = 0.97 P < 0.0001	r = 0.97 P < 0.0001	r = 0.98 P < 0.0001					
Urine	1A									1.05 (0.68–1.62)	0.62 (0.22–1.75)	0.99 (0.75–1.30)
	1B								r = 0.94 P < 0.0001		1.71 (0.70–4.20)	1.07 (0.72–1.59)
	4A								r = 0.66 P < 0.0001	r = 0.74 P < 0.0001		0.64 (0.24–1.72)
	4B								r = 0.98 P < 0.0001	r = 0.94 P < 0.0001	r = 0.67 P < 0.0001	

B.

Bland-Altman ratios

Correlations		Plasma							Urine			
		1A	1B	1C	2A	2B	2C	3	1A	1B	4A	4B
Plasma	1A		0.98 (0.83–1.16)	0.98 (0.86–1.13)	1.02 (0.87–1.20)	1.06 (0.92–1.22)	0.85 (0.72–1.01)	1.01 (0.75–1.36)				
	1B	r = 0.99 P < 0.0001		1.00 (0.89–1.12)	1.05 (0.86–1.27)	1.08 (0.95–1.23)	1.15 (0.99–1.33)	0.97 (0.69–1.37)				
	1C	r = 0.99 P < 0.0001	r = 0.99 P < 0.0001		1.04 (0.90–1.21)	1.07 (0.97–1.18)	1.15 (1.04–1.28)	0.98 (0.71–1.35)				
	2A	r = 0.99 P < 0.0001	r = 0.98 P < 0.0001	r = 0.99 P < 0.0001		0.97 (0.85–1.11)	1.20 (1.05–1.37)	1.02 (0.76–1.36)				
	2B	r = 0.99 P < 0.0001	r = 0.99 P < 0.0001	r = 1.00 P < 0.0001	r = 0.99 P < 0.0001		1.24 (1.10–1.39)	1.05 (0.78–1.42)				
	2C	r = 0.99 P < 0.0001	r = 0.99 P < 0.0001	r = 0.99 P < 0.0001	r = 0.99 P < 0.0001	r = 1.00 P < 0.0001		0.85 (0.63–1.15)				
	3	r = 0.96 P < 0.0001	r = 0.94 P < 0.0001	r = 0.95 P < 0.0001	r = 0.96 P < 0.0001	r = 0.95 P < 0.0001	r = 0.96 P < 0.0001					
Urine	1A									1.07 (0.52–2.20)	1.40 (0.43–4.56)	0.59 (0.23–1.49)
	1B								r = 0.82 P < 0.0001		0.76 (0.22–2.64)	1.81 (0.60–5.48)
	4A								r = 0.56 P = 0.0006	r = 0.53 P = 0.0012		2.31 (0.52–10.30)
	4B								r = 0.70 P < 0.0001	r = 0.59 P = 0.0002	r = 0.35 P = 0.044	

(Continued on the following page)

Table 2. Bland-Altman ratios (range of agreement) and Pearson correlations of (A) logNMR, (B) logCOT, and (C) log3HC measured in plasma and urine according to each method relative to all other methods (Cont'd)

C.

Bland-Altman ratios

Correlations		Plasma							Urine			
		1A	1B	1C	2A	2B	2C	3	1A	1B	4A	4B
Plasma	1A		0.94 (0.81–1.09)	1.02 (0.88–1.18)	1.00 (0.85–1.17)	0.99 (0.85–1.17)	0.76 (0.65–0.89)	1.09 (0.81–1.47)				
	1B	*r* = 0.99 *P* < 0.0001		0.92 (0.82–1.03)	1.07 (0.90–1.26)	1.06 (0.93–1.22)	1.23 (1.11–1.37)	0.86 (0.64–1.14)				
	1C	*r* = 0.99 *P* < 0.0001	*r* = 1.00 *P* < 0.0001		0.98 (0.86–1.11)	0.97 (0.86–1.10)	1.34 (1.21–1.47)	0.94 (0.70–1.25)				
	2A	*r* = 0.99 *P* < 0.0001	*r* = 0.99 *P* < 0.0001	*r* = 0.99 *P* < 0.0001		1.00 (0.86–1.17)	1.31 (1.14–1.50)	0.91 (0.69–1.22)				
	2B	*r* = 0.99 *P* < 0.0001	*r* = 0.99 *P* < 0.0001	*r* = 1.00 *P* < 0.0001	*r* = 0.99 *P* < 0.0001		1.30 (1.16–1.47)	0.91 (0.67–1.23)				
	2C	*r* = 0.99 *P* < 0.0001	*r* = 1.00 *P* < 0.0001	*r* = 1.00 *P* < 0.0001	*r* = 0.99 *P* < 0.0001	*r* = 1.00 *P* < 0.0001		0.70 (0.53–0.97)				
	3	*r* = 0.98 *P* < 0.0001	*r* = 0.97 *P* < 0.0001	*r* = 0.97 *P* < 0.0001	*r* = 0.97 *P* < 0.0001	*r* = 0.97 *P* < 0.0001	*r* = 0.97 *P* < 0.0001					
Urine	1A									1.12 (0.46–2.78)	0.84 (0.24–2.94)	0.58 (0.22–1.53)
	1B								*r* = 0.88 *P* < 0.0001		1.35 (0.40–4.57)	1.93 (0.63–5.94)
	4A								*r* = 0.74 *P* < 0.0001	*r* = 0.74 *P* < 0.0001		1.40 (0.35–5.66)
	4B								*r* = 0.86 *P* < 0.0001	*r* = 0.79 *P* < 0.0001	*r* = 0.64 *P* = 0.0002	

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.
All methods (1A-C, 2A-C, 3) use mass spectrometry approaches except for methods 4A and 4B, which use HPLC-UV approaches.

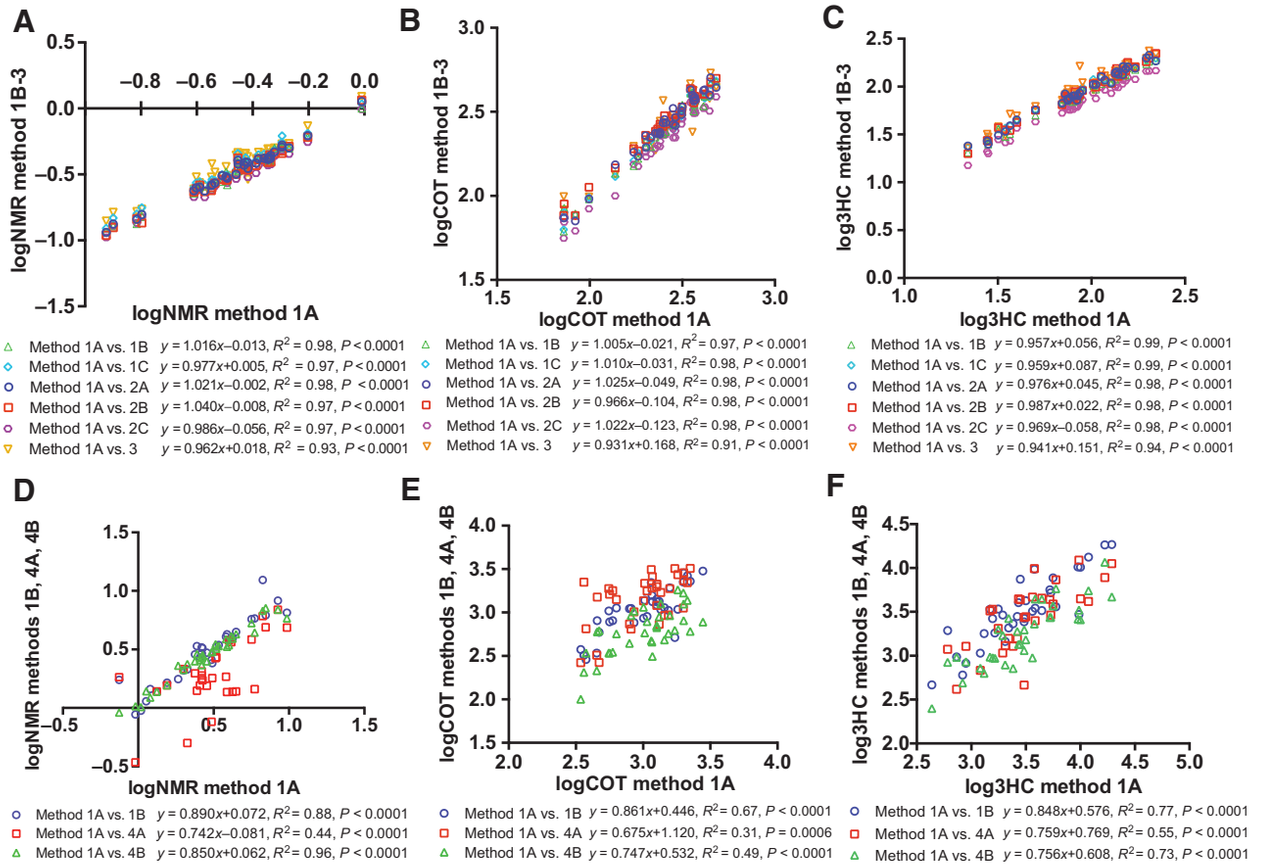

Figure 1.
Correlation and linear regression of measures of metabolites from plasma and urine ($n = 35$) from 8 methodologic approaches (methods 1A–C, 2A–C, 3, 4A–B). Each point on the plot represents an individual measurement of a sample by one test method compared with a reference method (method 1A). Plasma log-corrected (A) 3-hydroxycotinine over cotinine ratio (log3HC/COT, logNMR), (B) cotinine (logCOT) and (C) 3-hydroxycotinine (log3HC), measured by methods 1–3. Urine log-corrected (D) 3-hydroxycotinine over cotinine ratio (log3HC/COT, logNMR), (E) cotinine (logCOT) and (F) 3-hydroxycotinine (log3HC), measured by methods 1A, 1B, 4A, and 4B. Pearson correlations were similar among logged and non-logged NMR, COT, and 3HC (Pearson r values for plasma NMR, COT, and 3HC concentrations ranged from 0.92–0.99, all $P < 0.0001$; Pearson r values for urine NMR, COT, and 3HC measurements ranged from 0.55 to 0.95, all $P < 0.0009$).

Measures of urine NMR were also less strongly correlated between methods, with Pearson correlations (r) ranging from 0.66 to 0.98, although still statistically significant ($P <$ 0.0001, Table 2A, illustrated in Fig. 1D). NMR determined using the essentially identical methods 1A and 1B exhibited weaker correlation in urine compared with plasma (Pearson r values of 0.94 vs. 0.99 for urine and plasma, respectively). Similar correlations were observed with non–log–transformed NMR.

In relation to the findings for NMR, individual metabolite concentrations in urine had larger variation. Bland–Altman ratios were between 0.58 and 2.31 for urine COT and 3HC measures by methods 1A, 1B, 4A, and 4B. Pearson correlations (r) between log-transformed urine COT and 3HC measures by different methods ranged from 0.35 to 0.88 (all $P < 0.05$, Table 2B and C; Fig. 1E and F). Similar correlations were observed with non-logged COT and 3HC concentrations from each method.

Discussion

Using Bland–Altman analyses and Pearson correlations, we determined the extent of agreement and association between plasma and urinary NMR measurements by different analytical methods. Plasma NMR concentrations were consistent across all methods examined and appear robust to differences in analytical methods used for detection in different laboratories. Together this suggests that plasma NMR measured by any of these methods will provide comparable data.

Although it was not anticipated that plasma and urine NMR measurements would be in complete agreement, as seen before (16), we anticipated that there would be minimal within-fluid variation between different methods. However, although measurements of NMR by essentially the same method (1A and 1B) at different locations were in high agreement for plasma, they were in weaker agreement in urine, suggesting that urine NMR may be a less precise biomarker of nicotine clearance, *CYP2A6* phenotype, and genotype compared with plasma. As only two participating laboratories used HPLC-UV approaches, it may be of value in the future to analyze samples using additional HPLC-UV approaches, to test the effect of UV detection on the measured concentrations of COT, 3HC, and NMR in plasma and urine. The data presented here suggest that more care may be needed in selecting the method of analysis when evaluating NMR in urine. Discrepancies in

agreement between urine NMR measures by different methods may also result from different approaches to sample preparation for plasma compared with urine, or through the use of different internal standards. Two of the four methods that measured analytes in urine used structural analogs as internal standards (HPLC-UV methods 4A and 4B), whereas, all of the methods for plasma (LC/MS-MS or GC-MS approaches) used the deuterated form of the analyte. Deuterated forms are preferred over structural analogs as internal standards as they have identical chemical properties to the analyte, thus meeting the goal of closely mimicking the extraction and chromatographic characteristics of the analyte (20). Together, our results suggest that plasma NMR can be measured with confidence across these different methods, due to strong correlations between plasma NMR measures, while urinary NMR was more variable. Consistent with this, NMR and *CYP2A6* genotype associations are more variable when metabolites were measured from urine as opposed to plasma (21).

Similar to the trends observed for NMR, there was less variation in COT and 3HC plasma than urine concentrations, with different methodologic approaches. There was modest agreement and moderate to strong correlations between plasma metabolite concentrations measured by different methods, whereas for urine analyte concentrations, agreement and correlations were weaker, indicating that plasma COT and 3HC measurements are reproducible irrespective of method employed.

One limitation to this study is the assessment of urine and plasma NMR, but not saliva NMR. Nicotine's metabolite concentrations in saliva and plasma provide consistent pharmacokinetic information (22, 23), and saliva sampling, compared with plasma and urine sampling, is the least invasive approach available to determine NMR. In addition, saliva, like plasma, is stable over a 14-day period at room temperature (16), making this a desirable matrix for metabolite measurement. Another study limitation was that NMR was only measured in urine by four methods (two of which were essentially the same), whereas, seven methods (again, two being essentially the same) were utilized for plasma measurements. Given that measurements of NMR from urine showed less consistency relative to measurements from plasma, it would be useful going forward to examine more methods to establish whether the methodologic approach or biologic fluid is the primary reason for the observed inconsistencies. In addition, the HPLC-UV methods that were used in the measurement of urine metabolites were not performed for plasma. This is a limitation, as it does not allow for the comparison of plasma-LCMS and plasma-HPLC-UV methods, and thus this is an area for future investigation.

We found that plasma NMR measurements are in good agreement and strongly correlated between methods and sites. Our results provide greater confidence that plasma NMR measures, regardless of method used here, are consistent. These findings reduce the need for differential interpretation of plasma NMR results based on approach used to conduct the analysis.

Disclosure of Potential Conflicts of Interest

J. Kaprio is a consultant/advisory board member for Pfizer. T.P. George reports receiving commercial research grant from Pfizer, Inc. and is a consultant/advisory board member for Novartis. N.L. Benowitz is a consultant/advisory board member for Pfizer and GlaxoSmithKline. C. Lerman reports receiving commercial research grant from Pfizer and is a consultant/advisory board member for Gilead. R.F. Tyndale received honoraria from speakers bureaus for seminars and Associate Editor CPT, and is a consultant/advisory board member for pharmaceutical companies. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: M. Novalen, C. Lerman, R.F. Tyndale
Development of methodology: A. Kankaanpää, L. Galanti, R.F. Tyndale
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Novalen, P. Jatlow, M.A. Huestis, S.E. Murphy, J. Kaprio, A. Kankaanpää, L. Galanti, C. Stefan, T.P. George, N.L. Benowitz, C. Lerman, R.F. Tyndale
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.-A. Tanner, M. Novalen, S.E. Murphy, J. Kaprio, C. Stefan, T.P. George, R.F. Tyndale
Writing, review, and/or revision of the manuscript: J.-A. Tanner, M. Novalen, P. Jatlow, S.E. Murphy, J. Kaprio, A. Kankaanpää, L. Galanti, C. Stefan, T.P. George, N.L. Benowitz, C. Lerman, R.F. Tyndale
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.-A. Tanner, P. Jatlow, N.L. Benowitz
Study supervision: R.F. Tyndale
Other (review and editing of the manuscript): M.A. Huestis
Other (quality control and quality assurance of data produced): C. Stefan

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