Published in final edited form as:

Bioanalysis. 2011 April ; 3(7): 745–761. doi:10.4155/bio.11.42.

Nicotine exposure and metabolizer phenotypes from analysis of urinary nicotine and its 15 metabolites by LC–MS

Kannan Rangiah^{1,2}, Wei-Ting Hwang³, Clementina Mesaros^{1,2}, Anil Vachani^{2,4}, and Ian A Blair^{1,2,†}

¹ Centers for Cancer Pharmacology, Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6160, USA

² Excellence in Environmental Toxicology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6160, USA

³ Department of Biostatistics & Epidemiology, University of Pennsylvania School of Medicine, PA, USA

⁴ Division of Pulmonary Medicine, University of Pennsylvania School of Medicine, PA, USA

Abstract

Smokers who inhale less deeply are exposed to lower amounts of the toxic substances present in tobacco smoke. In order to more rigorously assess tobacco smoke exposure, it is necessary to have an accurate method for quantifying nicotine and all of its known metabolites.

Methods—A stable-isotope dilution LC–MRM/MS assay has been developed for quantification of urinary nicotine and the 15 possible metabolites that could arise from known metabolic pathways. Nicotine, cotinine, *trans*-3'-hydroxy-cotinine, nicotine-*N*-oxide, cotinine-*N*-oxide, nor nicotine, norcotinine and 4-hydroxy-4-(3-pyridyl)butanoic acid were quantified by direct analysis. The corresponding glucuronide metabolites were quantified after urine hydrolysis with β -glucuronidase.

Results—Nicotine and all 15 nicotine metabolites were quantified by LC MRM/MS in most urine samples from 61 tobacco smokers. Urinary nicotine and metabolite concentrations ranged from 7.9 to 337.8 μ M (mean $75.5 \pm 67.8 \mu$ M). Three nicotine metabolizer phenotypes were established as reduced metabolizers (ratio < 8), normal metabolizers (ratio 8–30), and extensive metabolizers (ratio > 30). 4-hydroxy-4-(3-pyridyl)butanoic acid, which has not been quantified previously, was an abundant metabolite in all three phenotypes.

© Ian A Blair

[†]Author for correspondence: Tel.: +1 215 573 9885, Fax: +1 215 573 9889, ian@mail.med.upenn.edu.

Supplementary data

To view the Supplementary Table that accompanies this paper please visit the journal website at: WWW.FUTURE-SCIENCE.COM/DOI/SUPPL/10.4155/BIO.11.42/SUPPL_FILE/SUPPL_TABLE_1.XLS.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

We acknowledge the support of NIH grants U01ES016004, P30ES013508, and Pennsylvania Department of Health Grant PA4100038714. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

Conclusion—Using this assay it will now be possible to determine whether there are relationships between nicotine exposure and/or metabolizer phenotype with exposure to toxic substances that are present in tobacco smoke and/or to biological response biomarkers to tobacco smoking. This will help in identifying individuals at high risk for developing smoking-related diseases as well as those amenable to smoking cessation programs.

Exposure to tobacco smoke is a leading cause of preventable death in the USA [1]. Tobacco smoke is composed of an extremely complex mixture including over 60 known carcinogens [2] such as nicotine-derived nitrosamines [3] and polycyclic aromatic hydrocarbons [4]. Smoking behavior impacts on exposure to tobacco smoke and the toxic chemicals that are present [5]. Therefore, a method for assessing an individual's exposure would facilitate the elucidation of inter-individual differences in risk for tobacco-related diseases [6–9]. This could help identify individuals for enrollment in smoking cessation programs [10] and/or initiation of preventative treatment [11]. Nicotine is the major addictive component of tobacco smoke, thus its rate of metabolism can affect smoking topography [5]. An important route of nicotine metabolism involves cytochrome P-450 (CYP) 2A6/aldehyde oxidase (AO)-mediated conversion to cotinine (Figure 1) [12,13]. This is thought to be followed by further CYP2A6-mediated oxidation of cotinine to *trans*-3'-hydroxy-cotinine [14–16]. Nicotine and cotinine are metabolized to *N*-glucuronides by uridine 5'-diphosphoglucuronosyltransferase (UGT) 2B10 and UGT1A4 [17–19]. In contrast, *trans*-3'-hydroxy-cotinine is converted to the *O*-glucuronide primarily by UGT2B7 [13]. It has been observed that there are significant inter-individual differences in the glucuronidation of nicotine and its metabolites [17–21]. CYP2A6 is thought to be the initial enzyme involved in the conversion of nicotine to 4-hydroxy-4-(3-pyridyl)butanoic acid [22]. Flavin monooxygenase-3 (FMO-3) converts nicotine to its corresponding *N*-oxide [23] and is probably involved in the formation of cotinine-*N*-oxide [13]. The two other minor metabolites, nor nicotine and norcotinine, are derived from CYP2A6-mediated *N*-demethylation of nicotine and cotinine, respectively [16,24] (Figure 1). CYP2A6 is a liver-specific [25], whereas CYP2A13 is found primarily in the nasal mucosa, trachea and lung [26,27]. CYP2A6-derived urinary metabolites are predicted to predominate over CYP2A13-derived metabolites because the liver has a larger metabolic capacity [28]. In keeping with this concept, approximately 60% of the variability in nicotine metabolism is heritable [29] and reduced activity and inactive variants of the *CYP2A6* gene are associated with slower nicotine clearance [10].

Urinary analyses afford the opportunity to establish nicotine metabolizer phenotypes by comparison of individual metabolite and nicotine concentrations [30–32]. **Stable-isotope dilution LC–MRM/MS** methodology provides the most sensitive and specific method available for the analysis of drugs and their metabolites [32]. Therefore, numerous LC–MRM/MS methods have been developed for the analysis of nicotine and its metabolites in urine [33–39]. However, none of them have included the 4-hydroxy-4-(3-pyridyl)butanoic acid metabolite [13,40]. As CYP2A6 is involved in the conversion of nicotine to 4-hydroxy-4-(3-pyridyl)butanoic acid [22], we reasoned that it could potentially be an abundant urinary nicotine metabolite. We report the development of a validated assay for urinary nicotine and its major known metabolites (including 4-hydroxy-4-[3-pyridyl]butanoic acid) and application of the assay to analysis of 61 urine samples from healthy cigarette smokers ($n = 61$).

Materials & methods

Chemicals

($\pm$)-nicotine-methyl- d_3 , (–)-(*S*)-cotinine, d_3 -($\pm$)-cotinine, *trans*-3'-hydroxy-cotinine, d_3 -*trans*-3'-hydroxy-cotinine, (*R*, *S*)-norcotinine, (*R*, *S*)-norcotinine-pyridyl- d_4 , ($\pm$)-*trans*-

nicotine-*N*-oxide, ($\pm$)-*trans*-nicotine-*N*-oxide-methyl- d_3 , (*S*)-cotinine-*N*-oxide, (*R*, *S*)-cotinine-*N*-oxide-methyl- d_3 , (*R*, *S*)-nornicotine, d_4 -(*R*, *S*)-nornicotine, ($\pm$)-4-hydroxy-4-(3-pyridyl)butanoic acid dicyclohexylamine salt, nicotine-*N*- β -glucuronide hydrate, nicotine-*N*- β -glucuronide-methyl- d_3 , cotinine-*N*- β -D-glucuronide, d_3 -cotinine-*N*- β -D-glucuronide, *trans*-3'-hydroxy-cotinine-*O*- β -D-glucuronide, ($\pm$)- d_3 -*trans*-3'-hydroxy-cotinine-*O*- β -D-glucuronide were obtained from Toronto Research Chemicals, Ontario, Canada. The purity of all analytes and deuterated internal standards was $\geq 98\%$. (–)-nicotine (99.8%), Type IX-A β -glucuronidase (1660,000 units/G solid), 50% formic acid (99.8%), sodium phosphate monobasic (99%), sodium phosphate dibasic (99.8%), sodium acetate (99.8%), sodium formate (99.9%) and HPLC-grade solvents (99.9%). They were purchased from Sigma Aldrich (Milwaukee, WI, USA). Oasis MCX (30 mg, 1 ml) cartridge was obtained from Waters Corp. (Milford, MA, USA).

Participants

Urine samples were obtained from cigarette smokers ($n = 61$) who had smoked for a minimum of 6 years and a maximum of 58 years (mean = 33.8 years). Samples, which were provided during a clinic visit, were not collected at pre-determined times after the last cigarette had been smoked. Subjects were healthy individuals participating in an ongoing study approved by the University of Pennsylvania Institutional Review Board (Protocol # 800924). Smoking status was assigned based on questionnaires, which requested information on smoking history, packs/day and use of other tobacco products. All of the smoking subjects were cigarettes smokers except for one individual who also smoked one cigar/day. Urine samples were collected in 20-ml polypropylene tubes fitted with a screw cap. The tubes were capped, labeled and urine samples were stored at -80°C until analysis. 1-ml aliquots were taken from each tube and centrifuged for 3 min ($10,000 \times g$) to remove any precipitates.

Sample preparation

Volumes were measured using calibrated pipettes except for the addition of authentic standards when calibrated syringes were used. Urine (500 μl) was mixed with 1 ml of phosphate buffer (100 mM, pH 6.8) followed by the addition of 20 μl of internal standard solution (20 μl containing 100 $\mu\text{g}/\text{ml}$ of each standard). It was then separated into two 760 μl aliquots; one aliquot was incubated with β -glucuronidase enzyme (3000 units/ml) for 12 h at 37°C for the total (free nicotine + free and conjugated metabolites) analytes, the other aliquot was used to determine the free nicotine and metabolite concentrations. The internal standard solution (10 μl) was added to standard curve and QC samples. SPE columns (Oasis MCX) were pre-conditioned with 1 ml each of methanol, water and ammonium formate buffer (20 mM, pH 2.5). The pH of the samples was reduced to approximately 3 by adding 10 μl of 50% formic acid before loading on the SPE columns. The SPE columns were then washed with 1 ml of ammonium formate buffer (20 mM, pH 2.5) and analytes were eluted with 500 μl methanol/ammonium hydroxide (95/5). After elution, the pH of the eluate was reduced to approximately 4 by adding 30 μl of 50% formic acid, vortex-mixed, and 10 μl of the resulting solution was analyzed by LC–MRM/MS.

Efficiency of β -glucuronidase hydrolysis

Nicotine-*N*- β -glucuronide, cotinine-*N*- β -D-glucuronide, and *trans*-3'-hydroxy-cotinine-*O*- β -D-glucuronide (2 μg of each) were added to non-smoker's urine (250 μl). Sodium phosphate buffer (500 μl ; 100 mM, pH 6.8) was added and samples were incubated at 37°C with β -glucuronidase enzyme (3000 units/ml) for different times (0, 0.5, 1, 2, 4, 8, 12 and 24 h). Hydrolyses were stopped by reducing the pH to four through the addition of 50% formic acid (10 μl) and d_3 -nicotine-*N*- β -glucuronide, cotinine- d_3 -*N*- β -glucuronide and d_3 -3'-

hydroxy-cotinine-*O*- β -D-glucuronide- d_3 (2 μ g of each) were added. The samples were then extracted through SPE columns and analyzed by LC–MS.

LC–MS

An LTQ linear ion-trap MS (Thermo Scientific, San Jose, CA, USA) equipped with an ESI ion source was used in this study. It was connected to an Alliance HPLC system obtained from Waters Corp. and equipped with an autosampler and thermocontroller (set at 4°C). The analytical HS F5 Discovery pentafluorophenylpropyl bonded silica column (150 $\times$ 2.1 mm internal diameter, 5 μ m, 90 Å, Sigma) with a matching guard column (20 $\times$ 4 mm, 5 μ m; Sigma) was used at ambient temperature. The mobile phase (A) water (10 mM ammonium acetate, 0.1% TFA) and (B) acetonitrile (0.01% TFA) was used at a flow rate of 200 μ l/min with a binary gradient (0–3 min, 2% B; 4–20 min, 2–100% B; 21–25 min, 100% B; 26–35 min, 2% B). The injection volume was routinely 10 μ l. ESI–MS/MS conditions were as follows: spray voltage: 3000 V; sheath gas: nitrogen, 40 arbitrary units (gas pressure); auxiliary gas: nitrogen, 15 arbitrary units (gas pressure); ion transfer capillary temperature: 250° C; collision gas: helium; capillary voltage: 20 V; scan time: 100 msec; and, ion polarity: positive. For the MS/MS scan (50–300 Da) the precursor ions were selected (unit mass resolution width) and the product ions were generated by using different collision energy, the most intense product ion was selected for MRM scan as shown in Table 1. All MRM transitions were monitored with unit mass resolution for both precursor and product ions. Peak area ratios of target analytes and their respective internal standards were calculated by Xcalibur Quan software (version 2.1). The data were fitted to a linear least squares regression curve with an equal weighting factor.

Recoveries of nicotine & metabolites

The recovery of each analytes was determined at low, medium and high concentrations ($n = 5$). One set of spiked urine samples was extracted as described above but the internal standards were added after the elution from the SPE column. Samples were subsequently analyzed in the same way as described above. Another set of samples was prepared with neat analyte and internal standard mixed with the same volume methanol/ammonium hydroxide and the pH was reduced and analyzed as described previously. Recovery (%) was calculated by comparing the peak ratios of analyte to internal standard for the extracted and unextracted samples. There was minimal suppression (<5%) of the ESI signal by the purified urine extracts.

Calibration curves

Individual primary stock solutions of nicotine and seven unconjugated metabolites (1 mg/ml) were prepared in methanol and stored at -80°C . Working solutions were prepared by mixing equal amounts of corresponding stock solutions and performing serial dilutions with methanol. All seven deuterated internal standards (100 μ g/ml) were also prepared in methanol and stored at -80°C . One large urine sample (550 ml) was obtained from a non-smoker and used for the preparation of quality control (QC) samples. Calibration curves were prepared by spiking nicotine and the seven unlabeled metabolites in 250 μ l of urine from a never-smoker who had not been exposed to second-hand smoke with 500- μ l sodium phosphate buffer (pH 6.8). There is no commercially available deuterated internal standard for 4-hydroxy-4-(3-pyridyl)butanoic acid. However, because the retention time of 4-hydroxy-4-(3-pyridyl) butanoic acid (11.8 min) was close to that of *trans*-3'-hydroxycotinine (11.0 min), quantitation was conducted by using *trans*-3'-hydroxycotinine- d_3 as its internal standard (Figure 2). Nicotine and its metabolites were analyzed in the range 0.156 to 10 μ g/ml, except for nornicotine (0.156 to 5 μ g/ml) with all seven internal standards employed at 1 μ g/ml. Daily seven point calibration samples (0.156, 0.312, 0.625, 1.25, 2.5, 5 and 10 μ g/ml) for nicotine and its seven unconjugated metabolites were prepared and analyzed together

with two each of low, medium and high QC samples (LQC 0.625, MQC 4 and HQC 8 µg/ml, respectively). Concentrations are expressed as means ± standard deviation.

Validation

Rigorous bioanalytical validation was conducted using criteria established by the US FDA [41,42]. Inter- and intra-day precision and accuracy data for nicotine and its seven metabolites were determined with the LQC, MQC and HQC samples. Intra-day data were assessed by comparing data from within one run ($n = 5$ for each QC). Inter-day validation data were obtained from analyses conducted on four different days ($n = 5$). Accuracy of 85–115% and precision of $\pm 15\%$ were considered acceptable for LQC, MQC and HQC samples. Accuracy of 80–120% and precision of $\pm 15\%$ were considered acceptable for the LLOQ QC (LOQ) as recommended. Carryover, stability in the autosampler vial and freeze–thaw stability were also checked. Selected urine samples were analyzed over a 12-month period. This means that 12 months of stability data at -80°C are available to support the accuracy of the method for quantifying urinary nicotine and its 15 metabolites.

Statistical analysis

Statistical significance (p -value < 0.05) using one-way analysis of variance (ANOVA) and standard deviations were determined using GraphPad Prism (v 5.01, GraphPad Software Inc., La Jolla, CA, USA). Metabolizer phenotypes were determined using a K-median cluster analysis (rather than a K-means analysis) because the metabolite ratios were not normally distributed [43,44].

Results

LC–MS analyses

Precursor protonated molecules and the most intense product ions were selected for MRM quantitation (Table 1). All three glucuronides had major product ions that arose through a neutral loss of m/z 176 to form a product ion that was identical with the protonated molecules of the corresponding free metabolites. *Trans*-3'-hydroxycotinine-*O*- β -D-glucuronide also revealed a signal corresponding to sodium adducts in both precursor and product ions (Table 1). Nicotine and all metabolites together with their respective internal standards were adequately separated on the LC column within 20 min. Two glucuronide conjugates (and internal standards), which eluted within the first 4 min from the column were a mixture of two diastereomers (Figure 3a). Therefore, they were quantified after urine hydrolysis with β -glucuronidase (Figure 3b). Both nicotine and cotinine glucuronide standards contained trace amount of free metabolites (Figure 3a). Each analytical run included an analysis to confirm that the hydrolysis was quantitative.

Validation

Nicotine and its metabolites were below the limit of detection when urine samples from nonsmokers who had not been exposed to second-hand smoke were analyzed [Data not shown]. A typical chromatogram of the non-smoker's urine sample used for the validation is shown in Figure 2. When standards and deuterated internal standards were added to the nonsmoker's urine sample, nicotine and its metabolites showed clear well-resolved peaks without any interference even at the LOQ (Figure 4). Intensities of all analyte peaks in the LOQ were > 20 -times higher than the background signals observed when analyzing the nonsmoker's urine sample. Recoveries for nicotine and its metabolites were $> 85\%$, except for nornicotine, which had a 75% recovery for LQC and MQC samples [Data not shown]. Calibration curves were generated from peak area ratio (standard/internal standard) versus the concentration of the standard. Simple linear regression lines were fitted to the data

because of the narrow concentration ranges. Calibration curves were linear for each analyte over the selected concentration range, with R^2 value >0.98 (Figure 5). Precision and accuracy ranged from 1.8 to 15.0% and from 87.0 to 112.0% [Data not shown]. Inter-day precision and accuracy, which were calculated from 20 samples (one standard curve and four sets of QC samples five each) over four separate analytical batches (four standard curves and 80 QC samples), ranged from 2.6 to 15.0% and from 87.0 to 109.0% (Table 2). Nicotine and its metabolites were analyzed in five selected urine study samples over a 12-month period. Precision and accuracy for these samples were within the acceptable range [Data not shown], confirming that there was no degradation of urine samples stored at -80°C . No carryover was observed, metabolites were quite stable in the autosampler vials during the analysis and all metabolites were stable for at least three freeze–thaw cycles [Data not shown].

Efficiency of β -glucuronidase

The hydrolysis of three glucuronides was greater than 90% complete within 6 h (Figure 6a). Similarly, the formation of all three free metabolites occurred primarily in the initial 6 h (Figure 6b). Hydrolysis of conjugated metabolites in study samples was conducted using an overnight incubation (12 h) at 37°C with β -glucuronidase. Complete hydrolysis under these conditions was confirmed by analyzing triplicate control urine samples to which the three deuterated internal standards had been added and correcting for the contribution of minor amounts of the non-conjugated glucuronides that were present in the authentic standards.

Urine analysis

In a typical smoker's urine sample, nicotine and all of its metabolites were observed before (Figure 7) and after hydrolysis with β -glucuronidase (Figure 8). There was an increase in the intensity of peaks derived from nicotine, cotinine and *trans*-3'-hydroxy-cotinine after treatment with the enzyme. Their presence was confirmed by LC–MS/MS analysis [Data not shown]. Concentrations of total nicotine and metabolites (after hydrolysis; Figure 9a), free nicotine and metabolites (before hydrolysis; Figure 9b), and conjugated nicotine and metabolites (the difference between the two; Figure 9c) from the 61 smoker's urine varied widely. Significant additional amounts of cotinine-*N*-oxide, 4-hydroxy-4-(3-pyridyl)butanoic acid, nicotine-*N*-oxide, norcotinine and nornicotine were detected in some of the smoker's urine samples after treatment with β -glucuronidase, indicating that they were also excreted as a glucuronide conjugates (Figure 9c). This was confirmed by LC–MS/MS analysis, which revealed the corresponding glucuronide parent ions at m/z 369 (retention time 3.26 min), m/z 358 (retention time 3.25 min), m/z 355 (retention time 3.24 min), m/z 339 (retention time 3.44 min) and m/z 325 (retention time 3.35 min), respectively. Each of these metabolites underwent the expected loss of a glucuronide moiety (176 Da) upon collision-induced dissociation and MS/MS analysis to reveal product ions at m/z 193, m/z 182, m/z 179, m/z 163 and m/z 149, respectively, together with other structurally significant product ions.

The concentrations of total urinary nicotine (free nicotine and its metabolites) ranged from 7.9 to 337.8 μM with a mean of $75.5 \pm 67.8 \mu\text{M}$. Metabolizer phenotypes for the 61 smoking subjects were assigned based on the total metabolite (free and conjugated)/free nicotine ratios as RMs (ratio <8) NMs (ratio 8–30) and EMs (ratio >30) (Figure 10a). This resulted in characterization of 18 RM, 33 NM and 10 EM phenotypes. Mean total urinary nicotine concentrations were not significantly different for each group $75.9 \pm 70.1 \mu\text{M}$ for RMs, $79.9 \pm 75.0 \mu\text{M}$ for NMs and $59.1 \pm 30.1 \mu\text{M}$ for EMs (Figure 10b).

Discussion

Nicotine is a weak base with a pKa of 8.0 [13] and so its rate of **renal clearance** is pH-dependent, varying substantially between conditions of urine alkalinity and acidity after a single dose of nicotine [45–47]. In acidic urine, reabsorption is minimal due to its ionization, which prevents its reabsorption and results in rapid secretion into the urine [48]. In contrast, a substantial amount of nicotine is reabsorbed as the urinary pH increases and the amount of ionized nicotine decreases. Genetic and environmental factors that influence the reabsorption of nicotine also play a significant role in its renal clearance after a single intravenous dose [48]. These factors also affect the renal clearance of cotinine [48]. However, in our study, the renal clearance rate was not considered to be an important determinant of concentrations of nicotine and its metabolites in the urine, because subjects were chronic smokers that were at steady state. Consistent with this concept, it has been estimated that daily urinary nicotine excretion reflects >90% of the nicotine dose that is retained by tobacco smokers [49,50]. In addition, consistently low urinary cotinine-to-nicotine ratios were found in smokers with inactive variants of the *CYP2A6* gene, providing further evidence that metabolism rather than urinary clearance is the most important determinant of urinary nicotine excretion [51,52]. Furthermore, urinary nicotine and metabolite concentrations have been widely used to determine nicotine equivalents as an estimate of the nicotine dose that is retained from smoking [53–58]. Thus, analysis of total urinary nicotine appears has proved to be a useful biomarker of tobacco smoke exposure.

Numerous LC–MRM/MS methods have been developed for the analysis of urinary nicotine and its metabolites [34–39,51,59]. This has made it possible to study the effects of smoking behavior [15], ethnicity [60], genetic and environmental factors [29], and metabolizer phenotype [61] on the urinary excretion of nicotine and its metabolites. However, none of the previous studies have included all of the known metabolites. The LC–MRM/MS method that we have developed involves a single extraction step using an Oasis MCX cartridge, which contains both reversed-phase and strong cation exchange properties and is used to isolate molecules from urine. Importantly, all three glucuronide metabolite standards and 4-hydroxy-4-(3-pyridyl)butanoic acid bound to the MCX stationary phase, in contrast to many other solid-phase extraction cartridges. This made it possible to routinely monitor the efficiency of glucuronidase hydrolysis and to identify minor glucuronide metabolites. A particular advantage of the MCX cartridge is that the final eluate can be injected directly on the LC–MS system after reducing the pH approximately 4, which simplifies sample handling. In order to minimize potential ion suppression effects from analytes with similar precursor and product ions, gradient elution of the mobile phase was conducted over 20 min. As such, no interfering peaks were observed from the matrix (Figure 4). All analytes were well-resolved in this 20 min gradient (Figure 2), which permitted rigorous validation to be conducted (Table 2).

The smoking subjects were in steady state after many years of smoking (mean of 33.8 years) and so the concentrations of nicotine and nicotine metabolites in urine can serve as a measure of their chronic nicotine exposure. This takes into account inter-individual differences in smoking behavior as well as differences in absorption and metabolism. Absolute concentrations of nicotine and its metabolites in plasma, serum and saliva are more dependent upon the acute dose of cigarette smoke [62–64]. There was a tremendous range of the total nicotine concentrations ($75.5 \pm 67.8 \mu\text{M}$) in the urine samples (Figure 10b). Furthermore, there were significant differences in the amount of nicotine that was metabolized. In our cohort of urine from 61 tobacco smokers there were 18 RMs, 33 NMs and 10 EMs (Figure 10a). In the EM phenotype, free nicotine was the ninth most abundant of the nicotine-related compounds in the urine corresponding to a mean of $2.2 \pm 0.6\%$ of the 16 analytes that were quantified (Figure 1, Supplementary Table). There was significantly

more free nicotine in the NM phenotype so that it was the sixth most abundant ($7.7 \pm 2.2\%$). In contrast, free nicotine was the most abundant ($22.8 \pm 10.3\%$) of the nicotine-related compounds in the urine of RM phenotypes.

The most abundant metabolite in all subjects was *trans*-3'-hydroxy-cotinine with its glucuronide derivative increasing from the seventh most abundant metabolite in RMs to the second most abundant in EMs (Figure 1, Supplementary Table). The ratio of urinary *trans*-3'-hydroxy-cotinine/cotinine was lowest in the RMs (2.5), higher in NMs (4.4) and highest in EMs (5.3) (Supplementary Table). Surprisingly, 4-hydroxy-4-(3-pyridyl)butanoic acid was the third most abundant metabolite in the RM ($10.2 \pm 4.2\%$) and NM ($9.7 \pm 5.4\%$) phenotypes (Figure 1, Supplementary Table). This provided compelling evidence for the need to analyze the butanoic acid metabolite in order to fully assess nicotine exposure. Schwartz and McKennis suggested that it could arise through intermediate formation of cotinine, which involves initial oxidation at CYP2A6-mediated oxidation at C-5' [65]. Subsequent studies by the Hecht group showed that the butanoic acid metabolite was actually formed through initial CYP2A6-mediated hydroxylation at C-2' rather than through hydroxylation at C-5' (Figure 1) [66]. Previous bioanalytical studies have employed α -methyl-benzyl isocyanate derivatization together with relatively large urine volumes (4.8 ml) [22]. The use of Oasis MCX cartridges coupled with LC-MRM/MS methodology employing a pen-tafluorophenylpropyl-bonded LC column has made the analysis of this challenging but important analyte relatively routine. Unfortunately, no heavy isotope analog was available for use as an internal standard for 4-hydroxy-4-(3-pyridyl) butanoic acid. Therefore, d_3 -*trans*-3'-hydroxycotinine, which elutes with a similar retention time, was employed as the internal standard. Unexpectedly, 4-hydroxy-4-(3-pyridyl)butanoic acid was also present as a glucuronide conjugate in many of the subjects (Figure 9C). Nicotine-*N*-oxide, which has been often been regarded as relatively unimportant, was the third most abundant metabolite in RMs ($10.2 \pm 5.9\%$) and the second most abundant metabolite in NMs ($15.4 \pm 13.7\%$; Figure 1, Supplementary Table), whereas (as expected) free cotinine-*N*-oxide was present in urine at significantly lower concentrations in all metabolizer phenotypes. Free nornicotine, free norcotinine, and nicotine glucuronide were also observed at lower concentrations together with glucuronides of nicotine, nornicotine, nicotine-*N*-oxide, norcotinine and cotinine-*N*-oxide (Figure 1, Supplementary Table).

Previous studies have shown that the *trans*-3'-hydroxy-cotinine/cotinine ratio is highly correlated with the oral clearance of nicotine and so this ratio has been employed as a surrogate to establish nicotine metabolizer phenotypes [67]. The *trans*-3'-hydroxy-cotinine/cotinine ratio is stable [61] and relatively easy to use for assessing metabolizer phenotypes in plasma [62], serum [63], saliva [64] and urine [51]. The resulting metabolizer phenotypes been particularly useful for determining CYP2A6 genotypes [5,68], which in turn have been successfully applied to selecting subjects for individualized smoking cessation studies [10]. However, metabolizer phenotypes determined by the ratio of *trans*-3'-hydroxy-cotinine to cotinine only account for the conversion of nicotine to cotinine and its subsequent conversion to *trans*-3'-hydroxy-cotinine (Figure 1). Cotinine and *trans*-3'-hydroxy-cotinine represented a mean of only 31.2% of the nicotine-derived compounds in the urine from RMs, a mean of 35.4% for the EMs, and a mean of 48.0% for the EMs (Table 3).

Conclusion

Metabolism rather than renal clearance is the most important determinant of the urinary excretion of nicotine and its metabolites by chronic smokers [49–52]. Therefore, total urinary nicotine equivalents have been widely used as an index of nicotine exposure [53–58]. Thus, analysis of total urinary nicotine appears has proved to be a useful biomarker of tobacco smoke exposure. However, no previous studies have reported the analysis of urinary

metabolites from all of the known pathways of nicotine metabolism (Figure 1). The ability to conduct such analyses, including the important 4-hydroxy-4-(3-pyridyl)butanoic acid metabolite, which was not quantified previously, will now make it possible to determine the nicotine equivalents from cigarette smoke exposure for individual smokers with greater rigor than has been possible previously. The stable-isotope dilution LC–MRM/ MS assay that we have developed has made it possible to identify three nicotine metabolizer phenotypes: RMs (ratio < 8), NMs (ratio 8–30) and EMs (ratio > 30). It is noteworthy that a recent study by Church *et al.* showed that there was a significant variability of urinary nicotine concentrations in individuals over time [69]. However, this study did not determine whether the variability in nicotine excretion affected the nicotine metabolizer phenotype or the nicotine equivalents that were excreted in the urine. Therefore, it will require additional longitudinal studies to establish the relationship between these parameters and urinary nicotine concentrations. The method that we have developed will permit such studies to be rigorously conducted.

Future perspective

The LC–MS assay that we have developed for total urinary nicotine will make it possible in to more rigorously establish exposure to nicotine from tobacco smoke. Consequently, a biomarker of smoking behavior will be available both for future studies and for samples that have already been collected in epidemiology studies. This will provide a more rigorous quantitative assessment of inter-individual differences in tobacco smoke exposure than has been possible previously. Concomitant quantification of various urinary biological response biomarkers of smoking including, nicotine-derived nitrosamines, oxidatively damaged DNA bases and oxidized lipids can also be conducted [31,32]. This could ultimately provide the means to identify individuals at increased risk for smoking-related diseases, making it possible to implement life-style changes such as quitting smoking [70], increasing exercise [71], modifying diet [72,73], and/or the implementation of pharmacological interventions [74,75]. The more comprehensive metabolizer phenotype that we have determined could also have potential utility in identifying individuals that would benefit from personalized smoking cessation programs in addition to those identified from their CYP2A6 genotypes [10].

Key Terms

Aldehyde oxidase	An enzyme that generates carboxylic acids from aldehydes and 2-pyrrolidinones (such as cotinine) from 2-pyrrolidinols or the corresponding iminium ion intermediate
CYP2A6	The CYP2A6 gene is part of a large cluster of CYP genes from the CYP2A, CYP2B and CYP2F subfamilies on chromosome 19q
UGT1A4	UDP-glucuronosyltransferase 1A4 (UGT1A4) transforms small lipophilic molecules, such as steroids, hormones and drugs, into water-soluble, excretable metabolites
UGT2B7	Enzyme that is primarily responsible for the formation of <i>trans</i> -3'-hydroxy-cotinine-glucuronide
FMO-3	Human FMO-3 is the most prominent flavin monooxygenase in adult human liver
CYP2A13	The CYP2A13 gene is expressed primarily in the nasal mucosa, trachea and lung. CYP2A13 and CYP2A6 are 93.5% identical and can both metabolize nicotine and cotinine

Stable-isotope dilution	The addition of a stable isotope analog to the biological fluid of interest. Therefore, the endogenous unlabeled analyte is 'diluted' by its identical stable-isotope analog
β-glucuronidase	Enzyme that hydrolyses β -d-glucuronide conjugates to their corresponding nonconjugated metabolites
Renal clearance	Measurement of the renal excretion rate for a particular substance

Bibliography

1. Mokdad AH, Marks JS, Stroup DF, Gerberding JL. Actual causes of death in the United States, 2000. *JAMA*. 2004; 291(10):1238–1245. [PubMed: 15010446]
2. Hoffmann D, Hoffmann I, El-Bayoumy K. The less harmful cigarette: a controversial issue. A tribute to Ernst L Wynder. *Chem Res Toxicol*. 2001; 14(7):767–790. [PubMed: 11453723]
3. Hecht SS. Progress and challenges in selected areas of tobacco carcinogenesis. *Chem Res Toxicol*. 2008; 21(1):160–171. [PubMed: 18052103]
4. Wogan GN, Hecht SS, Felton JS, Conney AH, Loeb LA. Environmental and chemical carcinogenesis. *Semin Cancer Biol*. 2004; 14(6):473–486. [PubMed: 15489140]
5. Strasser AA, Malaiyandi V, Hoffmann E, Tyndale RF, Lerman C. An association of CYP2A6 genotype and smoking topography. *Nicotine Tob Res*. 2007; 9(4):511–518. [PubMed: 17454707]
6. Kandel DB, Hu MC, Schaffran C, Udry JR, Benowitz NL. Urine nicotine metabolites and smoking behavior in a multiracial/multiethnic national sample of young adults. *Am J Epidemiol*. 2007; 165(8):901–910. [PubMed: 17322544]
7. Shopland DR. Tobacco use and its contribution to early cancer mortality with a special emphasis on cigarette smoking. *Environ Health Perspect*. 1995; 103 (Suppl 8):131–142. [PubMed: 8741773]
8. Bach PB, Kattan MW, Thornquist MD, et al. Variations in lung cancer risk among smokers. *J Natl Cancer Inst*. 2003; 95(6):470–478. [PubMed: 12644540]
9. van Zandwijk N. Chemoprevention of lung cancer. *Lung Cancer*. 2001; 34 (Suppl 3):S91–S94. [PubMed: 11741000]
10. Lerman C, Jepson C, Wileyto EP, et al. Genetic variation in nicotine metabolism predicts the efficacy of extended-duration transdermal nicotine therapy. *Clin Pharmacol Ther*. 2010; 87(5): 553–557. [PubMed: 20336063]
11. Pastorino U. Lung cancer screening. *Br J Cancer*. 2010; 102(12):1681–1686. [PubMed: 20424610]
12. Gorrod JW, Hibberd AR. The metabolism of nicotine-delta 1' (5')-iminium ion, *in vivo* and *in vitro*. *Eur J Drug Metab Pharmacokinet*. 1982; 7(4):293–298. [PubMed: 7166181]
13. Hukkanen J, Jacob P 3rd, Benowitz NL. Metabolism and disposition kinetics of nicotine. *Pharmacol Rev*. 2005; 57(1):79–115. [PubMed: 15734728]
14. Nakajima M, Yamamoto T, Nunoya K, et al. Characterization of CYP2A6 involved in 3'-hydroxylation of cotinine in human liver microsomes. *J Pharmacol Exp Ther*. 1996; 277(2):1010–1015. [PubMed: 8627511]
15. Johnstone E, Benowitz N, Cargill A, et al. Determinants of the rate of nicotine metabolism and effects on smoking behavior. *Clin Pharmacol Ther*. 2006; 80(4):319–330. [PubMed: 17015050]
16. Brown KM, von Weyarn LB, Murphy SE. Identification of *N*-(hydroxymethyl) norcotinine as a major product of cytochrome P450 2A6, but not cytochrome P450 2A13-catalyzed cotinine metabolism. *Chem Res Toxicol*. 2005; 18(12):1792–1798. [PubMed: 16359169]
17. Berg JZ, von Weyarn LB, Thompson EA, et al. UGT2B10 genotype influences nicotine glucuronidation, oxidation, and consumption. *Cancer Epidemiol Biomarkers Prev*. 2010; 19(6): 1423–1431. [PubMed: 20501767]
18. Berg JZ, Mason J, Boettcher AJ, Hatsukami DK, Murphy SE. Nicotine metabolism in African-Americans and European-Americans: variation in glucuronidation by ethnicity and UGT2B10 haplotype. *J Pharmacol Exp Ther*. 2010; 332(1):202–209. [PubMed: 19786624]

19. Chen G, Giambone NE, Dluzen DF, et al. Glucuronidation Genotypes and Nicotine metabolic phenotypes: importance of functional UGT2B10 and UGT2B17 polymorphisms. *Cancer Res.* 2010; 70(19):7543–7552. [PubMed: 20876810]
20. Nakajima M, Yokoi T. Interindividual variability in nicotine metabolism: C-oxidation and glucuronidation. *Drug Metab Pharmacokinet.* 2005; 20(4):227–235. [PubMed: 16141602]
21. Lessov-Schlaggar CN, Benowitz NL, Jacob P, Swan GE. Genetic influences on individual differences in nicotine glucuronidation. *Twin Res Hum Genet.* 2009; 12(5):507–513. [PubMed: 19803778]
22. Hecht SS, Hatsukami DK, Bonilla LE, Hochalter JB. Quantitation of 4-oxo-4-(3-pyridyl)butanoic acid and enantiomers of 4-hydroxy-4-(3-pyridyl)butanoic acid in human urine: a substantial pathway of nicotine metabolism. *Chem Res Toxicol.* 1999; 12(2):172–179. [PubMed: 10027795]
23. Park SB, Jacob P 3rd, Benowitz NL, Cashman JR. Stereoselective metabolism of (S)-(-)-nicotine in humans: formation of trans-(S)-(-)-nicotine N-1'-oxide. *Chem Res Toxicol.* 1993; 6(6):880–888. [PubMed: 8117928]
24. Murphy SE, Johnson LM, Pullo DA. Characterization of multiple products of cytochrome P450 2A6-catalyzed cotinine metabolism. *Chem Res Toxicol.* 1999; 12(7):639–645. [PubMed: 10409404]
25. Di YM, Chow VD, Yang LP, Zhou SF. Structure, function, regulation and polymorphism of human cytochrome P450 2A6. *Curr Drug Metab.* 2009; 10(7):754–780. [PubMed: 19702528]
26. Ding X, Kaminsky LS. Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annu Rev Pharmacol Toxicol.* 2003; 43:149–173. [PubMed: 12171978]
27. Smith BD, Sanders JL, Porubsky PR, Lushington GH, Stout CD, Scott EE. Structure of the human lung cytochrome P450 2A13. *J Biol Chem.* 2007; 282(23):17306–17313. [PubMed: 17428784]
28. Bao Z, He XY, Ding X, Prabhu S, Hong JY. Metabolism of nicotine and cotinine by human cytochrome P450 2A13. *Drug Metab Dispos.* 2005; 33(2):258–261. [PubMed: 15528319]
29. Swan GE, Lessov-Schlaggar CN, Bergen AW, He Y, Tyndale RF, Benowitz NL. Genetic and environmental influences on the ratio of 3' hydroxycotinine to cotinine in plasma and urine. *Pharmacogenet Genomics.* 2009; 19(5):388–398. [PubMed: 19300303]
30. Meyer UA, Zanger UM. Molecular mechanisms of genetic polymorphisms of drug metabolism. *Annu Rev Pharmacol Toxicol.* 1997; 37:269–296. [PubMed: 9131254]
31. Hecht SS. Human urinary carcinogen metabolites: biomarkers for investigating tobacco and cancer. *Carcinogenesis.* 2002; 23(6):907–922. [PubMed: 12082012]
32. Ciccimaro E, Blair IA. Stable-isotope dilution LC–MS for quantitative biomarker analysis. *Bioanalysis.* 2010; 2(2):311–341. [PubMed: 20352077]
33. Tuomi T, Johnsson T, Reijula K. Analysis of nicotine, 3-hydroxycotinine, cotinine and caffeine in urine of passive smokers by HPLC–tandem mass spectrometry. *Clin Chem.* 1999; 45(12):2164–2172. [PubMed: 10585349]
34. Moyer TP, Charlson JR, Enger RJ, et al. Simultaneous analysis of nicotine, nicotine metabolites and tobacco alkaloids in serum or urine by tandem mass spectrometry, with clinically relevant metabolic profiles. *Clin Chem.* 2002; 48(9):1460–1471. [PubMed: 12194923]
35. Xu X, Iba MM, Weisel CP. Simultaneous and sensitive measurement of anabasine, nicotine and nicotine metabolites in human urine by liquid chromatography–tandem mass spectrometry. *Clin Chem.* 2004; 50(12):2323–2330. [PubMed: 15472033]
36. Heavner DL, Richardson JD, Morgan WT, Ogden MW. Validation and application of a method for the determination of nicotine and five major metabolites in smokers' urine by solid-phase extraction and liquid chromatography–tandem mass spectrometry. *Biomed Chromatogr.* 2005; 19(4):312–328. [PubMed: 15651085]
37. Hoofnagle AN, Laha TJ, Rainey PM, Sadrzadeh SM. Specific detection of anabasine, nicotine, and nicotine metabolites in urine by liquid chromatography–tandem mass spectrometry. *Am J Clin Pathol.* 2006; 126(6):880–887. [PubMed: 17074683]
38. Kataoka H, Inoue R, Yagi K, Saito K. Determination of nicotine, cotinine and related alkaloids in human urine and saliva by automated in-tube solid-phase microextraction coupled with liquid

- chromatography-mass spectrometry. *J Pharm Biomed Anal.* 2009; 49(1):108–114. [PubMed: 19004590]
39. Miller EI, Norris HR, Rollins DE, Tiffany ST, Wilkins DG. A novel validated procedure for the determination of nicotine, eight nicotine metabolites and two minor tobacco alkaloids in human plasma or urine by solid-phase extraction coupled with liquid chromatography–electrospray ionization–tandem mass spectrometry. *J Chromatogr B.* 2010; 878(9–10):725–737.
 40. Upadhyaya P, Kenney PM, Hochalter JB, Wang M, Hecht SS. Tumorigenicity and metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol enantiomers and metabolites in the A/J mouse. *Carcinogenesis.* 1999; 20(8):1577–1582. [PubMed: 10426810]
 41. Bansal S, DeStefano A. Key elements of bioanalytical method validation for small molecules. *AAPS J.* 2007; 9(1):E109–E114. [PubMed: 17408234]
 42. Viswanathan CT, Bansal S, Booth B, et al. Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays. *Pharm Res.* 2007; 24(10):1962–1973. [PubMed: 17458684]
 43. Forgy EW. cluster analysis of multivariate data – efficiency vs interpretability of classifications. *Biometrics.* 1965; 21(3):68.
 44. Everitt, BS.; Landau, S.; Leese, M.; Stahl, D. *Cluster Analysis* (Wiley Series in Probability and Statistics). 5. Vol. Chapter 5. John Wiley and Sons; Chichester, UK: 2011.
 45. Feyerabend C, Russell MAH. Effect of urinary pH and nicotine excretion rate on plasma nicotine during cigarette-smoking and chewing nicotine gum. *Br J Clin Pharmacol.* 1978; 5(4):293–297.
 46. Matsukura S, Sakamoto N, Takahashi K, Matsuyama H, Muranaka H. Effect of pH and urine flow on urinary nicotine excretion after smoking cigarettes. *Clin Pharmacol Ther.* 1979; 25(5):549–554. [PubMed: 35297]
 47. Benowitz NL, Jacob P. Nicotine renal excretion rate influences nicotine intake during cigarette-smoking. *J Pharmacol Exp Ther.* 1985; 234(1):153–155. [PubMed: 4009497]
 48. Benowitz NL, Lessov-Schlaggar CN, Swan GE. Genetic influences in the variation in renal clearance of nicotine and cotinine. *Clin Pharmacol Ther.* 2008; 84(2):243–247. [PubMed: 18388871]
 49. Benowitz NL, Jacob P, Fong I, Gupta S. Nicotine metabolic profile in man – comparison of cigarette-smoking and transdermal nicotine. *J Pharmacol Exp Ther.* 1994; 268(1):296–303. [PubMed: 8301571]
 50. Feng S, Kapur S, Sarkar M, Muhammad R, Mendes P, Roethig HJ. The relationship between nicotine retention and urinary excretion of nicotine and its five major metabolites in adult male smokers. *Clin Pharmacol Ther.* 2007; 8:S42–S43.
 51. Yamanaka H, Nakajima M, Nishimura K, et al. Metabolic profile of nicotine in subjects whose CYP2A6 gene is deleted. *Eur J Pharm Sci.* 2004; 22(5):419–425. [PubMed: 15265511]
 52. Nakajima M, Fukami T, Yamanaka H, et al. Comprehensive evaluation of variability in nicotine metabolism and CYP2A6 polymorphic alleles in four ethnic populations. *Clin Pharmacol Ther.* 2006; 80(3):282–297. [PubMed: 16952495]
 53. Liang Q, Roethig HJ, Lipowicz PJ, Jin Y, Mendes PE. The effect of cigarette burn time on exposure to nicotine and carbon monoxide in adult smokers. *Regul Toxicol Pharmacol.* 2008; 50(1):66–74. [PubMed: 17931761]
 54. Mendes P, Liang QW, Frost-Pineda K, Munjal S, Walk RA, Roethig HJ. The relationship between smoking machine derived tar yields and biomarkers of exposure in adult cigarette smokers in the US. *Regul Toxicol Pharmacol.* 2009; 55(1):17–27. [PubMed: 19481578]
 55. Roethig HJ, Munjal S, Feng SX, et al. Population estimates for biomarkers of exposure to cigarette smoke in adult US cigarette smokers. *Nicotine Tob Res.* 2009; 11(10):1216–1225. [PubMed: 19700523]
 56. Derby KS, Cuthrell K, Caberto C, et al. Exposure to the carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in smokers from 3 populations with different risks of lung cancer. *Int J Cancer.* 2009; 125(10):2418–2424. [PubMed: 19598261]
 57. Berg JZ, von Weymarn LB, Thompson EA, et al. UGT2B10 genotype influences nicotine glucuronidation, oxidation, and consumption. *Cancer Epidemiol Biomarkers Prevent.* 2010; 19(6):1423–1431.

58. Wang JZ, Roethig HJ, Appleton S, Werley M, Muhammad-Kah R, Mendes P. The effect of menthol containing cigarettes on adult smokers' exposure to nicotine and carbon monoxide. *Regul Toxicol Pharmacol*. 2010; 57(1):24–30. [PubMed: 20025920]
59. Fan Z, Xie FW, Xia QL, Wang S, Ding L, Liu HM. Simultaneous determination of nicotine and its nine metabolites in human urine by LC–MS–MS. *Chromatographia*. 2008; 68(7–8):623–627.
60. Derby KS, Cuthrell K, Caberto C, et al. Nicotine metabolism in three ethnic/racial groups with different risks of lung cancer. *Cancer Epidemiol Biomarkers Prev*. 2008; 17(12):3526–3535. [PubMed: 19029401]
61. Mooney ME, Li ZZ, Murphy SE, Pentel PR, Le C, Hatsukami DK. Stability of the nicotine metabolite ratio in ad libitum and reducing smokers. *Cancer Epidemiol Biomarkers Prev*. 2008; 17(6):1396–1400. [PubMed: 18559554]
62. Ho MK, Mwenifumbo JC, Al KN, et al. Association of nicotine metabolite ratio and CYP2A6 genotype with smoking cessation treatment in African-American light smokers. *Clin Pharmacol Ther*. 2009; 85(6):635–643. [PubMed: 19279561]
63. Rubinstein ML, Benowitz NL, Auerback GM, Moscicki AB. Rate of nicotine metabolism and withdrawal symptoms in adolescent light smokers. *Pediatrics*. 2008; 122(3):e643–e647. [PubMed: 18762498]
64. Levi M, Dempsey DA, Benowitz NL, Sheiner LB. Prediction methods for nicotine clearance using cotinine and 3-hydroxy-cotinine spot saliva samples II. Model application *J Pharmacokinet Pharmacodyn*. 2007; 34(1):23–34.
65. Schwartz SL, McKennis H Jr. Studies on the degradation of the pyrrolidine ring of (–)-nicotine *in vivo*. Formation of γ -(3-pyridyl)- γ -oxobutyric acid. *J Biol Chem*. 1963; 238:1807–1812. [PubMed: 13987394]
66. Hecht SS, Hochalter JB, Villalta PW, Murphy SE. 2'-Hydroxylation of nicotine by cytochrome P450 2A6 and human liver microsomes: formation of a lung carcinogen precursor. *Proc Natl Acad Sci USA*. 2000; 97(23):12493–12497. [PubMed: 11050152]
67. Schnoll RA, Patterson F, Wileyto EP, Tyndale RF, Benowitz N, Lerman C. Nicotine metabolic rate predicts successful smoking cessation with transdermal nicotine: a validation study. *Pharmacol Biochem Behav*. 2009; 92(1):6–11. [PubMed: 19000709]
68. Dempsey D, Tutka P, Jacob P 3rd, et al. Nicotine metabolite ratio as an index of cytochrome P450 2A6 metabolic activity. *Clin Pharmacol Ther*. 2004; 76(1):64–72. [PubMed: 15229465]
69. Church TR, Anderson KE, Le C, et al. Temporal stability of urinary and plasma biomarkers of tobacco smoke exposure among cigarette smokers. *Biomarkers*. 2010; 15(4):345–352. [PubMed: 20374068]
70. Penning TM, Lerman C. Genomics of smoking exposure and cessation: lessons for cancer prevention and treatment. *Cancer Prev Res (Phila Pa)*. 2008; 1(2):80–83.
71. Alfano CM, Klesges RC, Murray DM, et al. Physical activity in relation to all-site and lung cancer incidence and mortality in current and former smokers. *Cancer Epidemiol Biomarkers Prev*. 2004; 13(12):2233–2241. [PubMed: 15598785]
72. Kubik A, Zatloukal P, Tomasek L, Pauk N, Petruzelka L, Plesko I. Lung cancer risk among nonsmoking women in relation to diet and physical activity. *Neoplasma*. 2004; 51(2):136–143. [PubMed: 15190423]
73. Buchner FL, Bueno-de-Mesquita HB, Ros MM, et al. Variety in fruit and vegetable consumption and the risk of lung cancer in the European prospective investigation into cancer and nutrition. *Cancer Epidemiol Biomarkers Prev*. 2010; 19(9):2278–2286. [PubMed: 20807832]
74. Fant RV, Buchhalter AR, Buchman AC, Henningfield JE. Pharmacotherapy for tobacco dependence. *Handb Exp Pharmacol*. 2009; (192):487–510. [PubMed: 19184660]
75. Marquez-Garban DC, Chen HW, Goodglick L, Fishbein MC, Pietras RJ. Targeting aromatase and estrogen signaling in human non-small cell lung cancer. *Ann N Y Acad Sci*. 2009; (1155):194–205. [PubMed: 19250205]

Executive summary

- Metabolism rather than renal clearance is the most important determinant of the urinary excretion of nicotine and its metabolites by chronic smokers
- A stable-isotope dilution LC–MRM/MS assay has been developed for quantification of urinary nicotine and the 15 possible metabolites that could arise from known metabolic pathways.
- Nicotine, cotinine, *trans*-3'-hydroxy-cotinine, nicotine-*N*-oxide, cotinine-*N*-oxide, norcotinine, norcotinine and 4-hydroxy-4-(3-pyridyl) butanoic acid were quantified by direct analysis.
- Glucuronide metabolites were quantified after urine hydrolysis with β -glucuronidase.
- Total urinary nicotine concentrations ranged from 7.9 to 337.8 μ M (mean $75.5 \pm 67.8 \mu$ M) in the 61 subjects.
- Three nicotine metabolizer phenotypes have been identified using the new LC–MRM/MS assays: reduced metabolizers (RMs; ratio < 8), normal metabolizers (NMs; ratio 8–30) and extensive metabolizers (EMs; ratio > 30).
- 4-hydroxy-4-(3-pyridyl)butanoic acid, which has not been quantified previously, was an abundant metabolite in all three phenotypes.
- Metabolizer phenotypes determined by the ratio of *trans*-3'-hydroxy-cotinine to cotinine only account for the conversion of nicotine to cotinine and its subsequent conversion to *trans*-3'-hydroxy-cotinine.
- Cotinine and *trans*-3'-hydroxy-cotinine represented a mean of only 31.2% of the nicotine-derived compounds in the urine from RMs, a mean of 35.4% for the EMs, and a mean of 48.0% for the EMs.

Figure 1. Conversion of nicotine to its major urinary metabolites

Mean relative amounts (%) of nicotine and its metabolites (order reduced, normal and extensive metabolizers) found in the urine of 61 smokers are shown in parentheses.

Figure 2. LC-MRM/MS chromatogram of nicotine, its metabolites, and internal standards extracted from a non-smoker's urine sample to which analytes had been added at LOQ
 NL: Normalized level.

Figure 3. LC-MRM/MS analysis of d₃-nicotine-*N*-glucuronide, d₃-cotinine-*N*-glucuronide, and d₃-3'-hydroxy-cotinine-*O*-glucuronide

(A) Before hydrolysis with β -glucuronidase. Trace amounts of free metabolites [nicotine (0.6%), d₃-nicotine (1.4%), cotinine (0.3%) and d₃-cotinine (2.2%)] were present in the glucuronide conjugates. (B) After hydrolysis with β -glucuronidase.

NL: Normalized level.

Figure 4. LC-MRM/MS chromatogram of nicotine metabolites from the nonsmoker's urine samples used in the validation process

The arrow shows the retention time of normic-d4.

NL: Normalized level.

Figure 5. Standard curves and regression lines for analysis of nicotine and seven non-conjugated metabolites

Figure 6. Time course for β -glucuronidase-mediated hydrolysis of a glucuronide derivative of nicotine, cotinine and 3'-hydroxy-cotinine
(A) Loss of glucuronide conjugates. (B) Formation of free metabolites.

Figure 7. LC–MRM/MS analysis of free nicotine and free metabolites in a smoker's urine sample before hydrolysis with β -glucuronidase
NL: Normalized level.

Figure 8. LC-MRM/MS analysis of total (free + conjugated) nicotine and metabolites in a smoker's urine sample after hydrolysis with β -glucuronidase

NL: Normalized level.

Figure 9. Concentrations of nicotine and metabolites in smoker's urine (n = 61)
(A) Total nicotine (free and conjugated) and total (free and conjugated) metabolites. **(B)** Free nicotine and free metabolites. **(C)** Conjugated nicotine and conjugated nicotine metabolites. Horizontal lines show the mean values.

Figure 10. Nicotine metabolizer characteristics of 61 smokers

(A) Metabolizer phenotypes based on the ratio of total urinary nicotine metabolites (free and conjugated)/free nicotine. Horizontal dashed lines separate reduced metabolizers (RMs; ratio < 8), normal metabolizers (NMs; ratio 8–30) and extensive metabolizers (EMs; ratio > 30).

(B) Total urinary nicotine (free nicotine + free and conjugated metabolites) for RMs, NMs, and EMs. Horizontal lines show the mean values.

RM: Reduced metabolizer; NM: Normal metabolizer; EM: Extensive metabolizer.

Table 1

LC–MRM/MS parameters for analysis of nicotine and its metabolites.

	Metabolites	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Collision energy (V)
1	Nic	163.2	132.1	21
	Nic-d ₃	166.2	132.1	21
2	Cot	177.3	146	22
	Cot-d ₃	180.3	146	22
3	Hcot	193.3	134	23
	Hcot-d ₃	196.3	134	23
4	Nic-oxi	179.3	132.1	30
	Nic-oxi-d ₃	182.3	132.1	30
5	Cot-oxi	193.2	162	30
	Cot-oxi-d ₃	196.2	162	30
6	Norcot	163.2	146.1	22
	Norcot-d ₄	167.2	150.1	22
7	Nornic	149.2	132.1	25
	Nornic-d ₄	153.2	136.1	25
8	HyPyBut	182.2	164	25
9	Nic-Glu	339.2	163.1	12
	Nic-Glu-d ₃	342.2	166.1	12
10	Cot-Glu	353.1	177.1	12
	Cot-Glu-d ₃	356.1	180.1	12
11	Hcot-Glu	369.1	193	15
	Hcot-Glu-d ₃	372.1	196	15
12	Hcot-Glu(Na ⁺)	391.1	215	15
	Hcot-Glu-d ₃ (Na ⁺)	394.1	218	15

Table 2

Validation for LC–MRM/MS analysis of nicotine and major metabolites in urine on four separate days.

	LOQ	LQC	MQC	HQC
<i>Nicotine</i>				
µg/ml	0.312	0.625	4.000	8.000
Interday mean	0.321	0.643	3.761	8.043
% CV (n = 4)	7.3	7.0	7.9	9.9
Accuracy (%)	102.7	102.9	94.0	100.5
<i>Cotinine</i>				
µg/ml	0.312	0.625	4.000	8.000
Interday mean	0.307	0.604	3.786	7.760
% CV (n = 4)	8.8	7.5	6.9	7.3
Accuracy (%)	98.4	96.6	94.6	97.0
<i>Hydroxycotinine</i>				
µg/ml	0.312	0.625	4.000	8.000
Interday mean	0.325	0.618	3.978	8.272
% CV (n = 4)	4.7	4.2	2.7	2.4
Accuracy (%)	104.1	98.9	99.5	103.4
<i>Nicotine-N-oxide</i>				
µg/ml	0.625	1.000	4.000	8.000
Interday mean	0.683	0.882	3.616	8.241
% CV (n = 4)	2.6	5.6	4.6	5.3
Accuracy (%)	109.3	88.2	90.4	103.0
<i>Cotinine-N-oxide</i>				
µg/ml	0.312	0.625	4.000	8.000
Interday mean	0.321	0.615	3.980	7.987
% CV (n = 4)	5.3	4.1	2.6	2.2
Accuracy (%)	102.8	98.5	99.5	99.8
<i>Norcotinine</i>				
µg/ml	0.312	0.625	4.000	8.000
Interday mean	0.342	0.629	3.927	7.872
% CV (n = 4)	4.4	5.4	5.6	8.3
Accuracy (%)	109.6	100.7	98.2	98.4
<i>Nornicotine</i>				
µg/ml	0.312	0.625	2.000	4.000
Interday mean	0.309	0.564	1.741	4.108
% CV (n = 4)	11.5	9.5	15.0	12.5
Accuracy (%)	99.1	90.2	87.1	102.7
<i>Hydroxy-4-(3-pyridyl)butanoic acid</i>				
µg/ml	0.312	0.625	4.000	8.000

	LOQ	LQC	MQC	HQC
Interday mean	0.316	0.592	4.122	8.662
% CV (n = 4)	8.5	9.8	5.6	9.2
Accuracy (%)	101.4	94.8	103.1	108.3

HQC: High quality control; LQC: Low quality control; MQC: Medium quality control.

Table 3

Relative amounts of urinary nicotine and metabolites in reduced, normal and extensive metabolizers phenotypes.

	Nic	Hcot	Cot	HyPyBu	Nic-Oxi	Cot-Glu	Hcot-Glu	Nornic	Cot-oxi	Nic-Glu	Norcot	HyPyBu-Glu	Nic-Oxi-Glu	Cot-Oxi-Glu	Norcot-Glu	Nornic-Glu	Hcot + Cot	Hcot/Cot
Reduced metabolizers (mean MR = 4.1)																		
Mean (%)	22.8	20.8	10.4	10.2	10.2	8.0	6.9	3.6	2.8	1.8	1.3	0.7	0.3	0.1	0.1	0.1	31.2	2.5
SD (%)	10.3	9.7	5.5	4.2	5.9	5.9	5.5	2.6	1.4	2.4	1.0	1.4	0.4	0.3	0.1	0.1	11.9	1.7
Rank	1	2	3	4	4	6	7	8	9	10	11	12	13	14	14	14	NA	NA
Normal metabolizers (mean MR = 13.3)																		
Mean (%)	7.7	26.8	8.6	9.7	15.4	7.3	9.7	6.9	3.3	1.3	2.0	0.7	0.2	0.3	0.1	0.1	35.4	4.4
SD (%)	2.2	14.9	4.2	5.4	13.7	7.2	8.4	7.1	2.2	1.6	1.7	1.3	0.4	0.5	0.1	0.1	14.6	3.8
Rank	6	1	5	3	2	7	3	8	9	11	10	12	14	13	15	15	NA	NA
Extensive metabolizers (mean MR = 48.8)																		
Mean (%)	2.2	38.7	9.3	7.7	10.4	7.6	11.0	4.7	3.6	2.1	1.5	0.6	0.1	0.4	0.1	<0.1	48.0	5.3
SD (%)	0.6	14.1	4.4	3.8	11.0	7.4	5.6	5.5	1.5	3.3	1.2	0.8	0.2	0.7	0.1	<0.1	12.9	3.3
Rank	9	1	4	5	3	6	2	7	8	10	11	12	14	13	15	16	NA	NA

MR: Metabolite Ratio; SD: Standard deviation.