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Simultaneous and Sensitive Measurement of Anabasine, Nicotine, and Nicotine Metabolites in Human Urine by Liquid Chromatography–Tandem Mass Spectrometry

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Background: **Determination of nicotine metabolism/pharmacokinetics provides a useful tool for estimating uptake of nicotine and tobacco-related toxicants, for understanding the pharmacologic effects of nicotine and nicotine addiction, and for optimizing nicotine dependency treatment.**

Methods: **We developed a sensitive method for analysis of nicotine and five major nicotine metabolites, including cotinine, *trans*-3′-hydroxycotinine, nicotine-*N*′-oxide, cotinine-*N*-oxide, and nornicotine, in human urine by liquid chromatography coupled with a TSQ Quantum triple quadrupole tandem mass spectrometer (LC/MS/MS). Urine samples to which deuterium-labeled internal standards had been added were extracted with a simple solid-phase extraction procedure. Anabasine, a minor tobacco alkaloid, was also included.**

Results: **The quantification limits of the method were 0.1–0.2 μg/L, except for nicotine (1 μg/L). Cotinine-*N*-oxide, *trans*-3′-hydroxycotinine, nicotine, and anabasine in urine were almost completely recovered by the solid-phase extraction, whereas the mean extraction recoveries of nicotine-*N*′-oxide, cotinine, and nornicotine were 51.4%, 78.6%, and 78.8%, respectively. This procedure provided a linearity of three to four orders of magnitude for the target analytes: 0.2–400 μg/L for nicotine-*N*′-oxide, cotinine-*N*-oxide, and anabasine; 0.2–4000 μg/L for cotinine, nornicotine, and *trans*-3′-hydroxycotinine; and 1.0–4000 μg/L for nicotine. The overall interday method imprecision and recovery were 2.5–18% and 92–109%, respectively.**

Conclusions: **This sensitive LC/MS/MS procedure can be used to determine nicotine metabolism profiles of smokers, people during nicotine replacement therapy, and passively exposed nonsmokers. This method avoids the need for a time-consuming and labor-intensive sample enrichment step and thus allows for high-throughput sample preparation and automation.**

Nicotine is the primary alkaloid in tobacco products, is a major tobacco-specific component in both mainstream tobacco smoke and environmental tobacco smoke (ETS),[4] and is an active ingredient of most nicotine replacement therapeutic drugs. Determination of nicotine metabolism/pharmacokinetics provides a useful tool for estimating uptake of nicotine and tobacco-related toxicants, for understanding the pharmacologic effects of nicotine and nicotine addiction, and for optimizing nicotine dependency treatment. Metabolites of nicotine, such as cotinine, have been used as biomarkers of nicotine and tobacco smoke exposure *(1–4)*. Serum nicotine and urinary/saliva cotinine have been used to guide the dose of nicotine replacement therapy *(5, 6)*. Simultaneous measurement of urinary nicotine, cotinine, *trans*-3′-hydroxycotinine, and their conjugates can account for >80% of the total nicotine dose *(7)*, thereby providing a better estimate of nicotine

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[4] Nonstandard abbreviations: ETS, environmental tobacco smoke; GC, gas chromatography; LC/MS/MS, liquid chromatography–tandem mass spectrometry; LOQ, limit(s) of quantification; LOD, limit(s) of detection; QC, quality control; SPE, solid-phase extraction; SRM, selected-reaction monitoring; QC, quality control; and CI, confidence interval.

exposure and the extent of nicotine metabolism than the measurement of a single nicotine metabolite *(8)*.

Several methods, including RIAs, enzyme immunoassays, ELISA, gas chromatography (GC), and HPLC, have been developed for analysis of nicotine and its metabolites [summarized in Davis and Curvall *(9)*]. Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS), a major advance in analytical chemistry developed during the last decade, can provide a rapid, sensitive, and selective means for comprehensive measurement of nicotine and its metabolites. McManus et al. *(10)* described a thermospray LC/MS method for nicotine and 17 nicotine metabolites with detection limits ranging from 200 to 900 pg on column. Byrd et al. *(11)* modified the method of McManus et al. *(10)* and demonstrated the presence of glucuronidated conjugates of nicotine, cotinine, and *trans*-3′-hydroxycotinine in urine. More recently, LC/MS/MS methods have been reported to simultaneously measure nicotine and several nicotine metabolites in urine for clinical studies *(12–14)*. The limits of quantification (LOQ) for nicotine and its metabolites in these methods ranged from low μg/L to 10–20 μg/L, which are sufficient for determination of nicotine metabolism/pharmacokinetics in clinical studies. Tuomi et al. *(4)* developed a LC/MS/MS-based method for analysis of nicotine, cotinine, and *trans*-3′-hydroxycotinine in urine samples from ETS-exposed restaurant workers in which the limits of detection (LOD) were 10, 2, and 1 μg/L, respectively. More sensitive methods have been available when only one or two nicotine metabolites are the target analytes. A highly sensitive LC/MS/MS method has been developed for serum cotinine with a LOD of 0.05 μg/L *(2)*. Bentley et al. *(3)* described a method for measuring salivary cotinine and *trans*-3′-hydroxycotinine in nonsmokers, with LOQ of 0.05 μg/L for cotinine and 0.10 μg/L for *trans*-3′-hydroxycotinine.

Development of highly sensitive and specific analytical methods for simultaneous measurement of urinary nicotine and multiple nicotine metabolites would greatly improve the estimation of nicotine exposure and dose for passively exposed nonsmokers *(9)*. The concentrations of nicotine and its metabolites in body fluids of active tobacco users or people during nicotine replacement therapy are two to three orders of magnitude higher than those in non-tobacco users *(13)*. Except for *trans*-3′-hydroxycotinine, the urinary concentrations of nicotine and other nicotine metabolites in nonsmokers usually range from <1 or 2 μg/L to 20 μg/L *(4, 13, 15)*. The concentration of *trans*-3′-hydroxycotinine in the urine of nonsmokers is usually <50 μg/L *(13)*. As indicated above, the LOQ of the previously reported LC/MS/MS methods were often insufficient to measure the concentrations found in nonsmokers if simultaneous analyses of nicotine and multiple metabolites were required. Some other limitations for the above-mentioned LC/MS/MS methods are (*a*) the inclusion of a time-consuming evaporation and solvent reconstitution step, and (*b*) dynamic linear calibration ranges that were usually suitable for detection of nicotine and nicotine metabolites in either smokers or nonsmokers but not both. We report here a new, highly sensitive LC/MS/MS method for simultaneous detection of nicotine and five major nicotine metabolites (cotinine, *trans*-3′-hydroxycotinine, nornicotine, nicotine-*N*′-oxide, and cotinine-*N*-oxide) across a wide range of concentrations in urine specimens after a simple solid-phase extraction (SPE). Anabasine, a minor tobacco alkaloid and a biomarker used to check compliance of nicotine replacement therapy, was also included in the assay.

Materials and Methods

STANDARDS AND REAGENTS

(−)-Nicotine (98%), (−)-cotinine (98%), nicotine-methyl-d_3 (99 atom % D), and cotinine-methyl-d_3 (99 atom % D) were obtained from Aldrich. ReagentPlus-grade ammonium acetate (99.99+%) and acetic acid (glacial, 99.99+%) were also obtained from Aldrich. *trans*-3′-Hydroxycotinine, *trans*-3′-hydroxycotinine-d_3, nicotine-*N*′-oxide, cotinine-*N*-oxide, and nornicotine were obtained from Toronto Research Chemicals. Methanol (B&J Brand, >99%) and water (High Purity, B&J Brand) were obtained from Honeywell Burdick & Jackson. Tripotassium phosphate (>98%) was obtained from Sigma.

CALIBRATORS AND QUALITY-CONTROL SAMPLES

Individual primary stock solutions (1 g/L) of nicotine, cotinine, *trans*-3′-hydroxycotinine, nicotine-*N*′-oxide, cotinine-*N*-oxide, nornicotine, and anabasine were prepared in methanol, from which three mixed working solutions (0.1, 10, and 100 mg/L) were prepared in methanol. Calibrators (0.2, 0.4, 1.0, 4.0, 10.0, 40.0, 100.0, 400.0, 1000.0, and 4000.0 μg/L) were freshly prepared by the addition of different aliquots of the working stock solution of the analytes to urine. Three urine voids with no detectable nicotine and nicotine metabolites were obtained and pooled from three individuals not exposed to tobacco or nicotine products. Three mixed quality-control (QC) working solutions (0.5, 5, and 150 mg/L) were prepared in methanol from separately prepared stock solutions (1 g/L). Purchased standards for nicotine and cotinine (1 g/L, in methanol; Sigma) were used to prepare the mixed QC working solutions. QC samples were prepared by adding the QC working solutions to the urine. Primary stock solutions of nicotine-methyl-d_3 (10 g/L), cotinine-methyl-d_3 (25 g/L), and *trans*-3′hydroxycotinine-d_3 (1 g/L) were prepared in methanol. The mixed working solution of the internal standards (5 mg/L) were prepared by diluting 10 μL of the nicotine-d_3 primary solution, 4 μL of the cotinine-d_3 primary solution, and 100 μL of the *trans*-3′-hydroxycotinine-d_3 primary solution in 20 mL of methanol. The primary and working solutions of the target analytes and the internal standards were stored at −10 °C in amber glass for up to 3 months.

SAMPLE EXTRACTION PROCEDURE

We transferred 1 mL of the urine specimen into a 15-mL glass tube. After the addition of 5 μL of isotopically labeled internal standards, the sample was buffered with 1 mL of tripotassium phosphate solution (100 g/L, pH ~13). The mixture was then vortex-mixed briefly and centrifuged for 5 min at 2000*g* to pellet the salts. The sample was then loaded on an Oasis HLB SPE cartridge (3 mL/60 mg; Waters), which was preconditioned with 1 mL of methanol followed by 1 mL of water. If necessary, vacuum was applied to aspirate the sample through the SPE tubes in a dropwise flow. After the sample was loaded, the SPE column was washed with 1 mL of water (High Purity, B&J Brand) twice. The SPE column was air-dried application of vacuum (~15 psi) to the column for 5 min. The analytes were eluted with 1 mL of methanol–10 mmol/L ammonium acetate solution (90:10 by volume) in a dropwise flow rate. A 10-μL portion of each extract was injected and analyzed by HPLC/MS/MS.

HPLC CONDITIONS

Potential interference may occur when isobaric compounds are present in a sample matrix, although the selected-reaction monitoring (SRM) mode of a triple-quadrupole mass spectrometry provides great selectivity. For example, nicotine, anabasine, and norcotinine (a nicotine metabolite) all have a nominal molecular weight of 162, whereas *trans*-3′-hydroxycotinine, 5′-hydroxycotinine, and cotinine-*N*-oxide have a nominal molecular weight of 192. These compounds are structurally similar and may produce common product ions. Nicotine and anabasine have a common ion transition, *m/z* 163 → *m/z* 130, whereas *trans*-3′-hydroxycotinine and cotinine-*N*-oxide have a common ion transition, *m/z* 193 → *m/z* 80. These ion transitions were used to monitor nicotine and *trans*-3′-hydroxycotinine in this experiment (Fig. 1). Therefore, adequate chromatographic separation is important for simultaneous measurement of these compounds.

Column separation of anabasine, nicotine, and its metabolites was adapted from the method developed by Jacob et al. *(14)*. A Surveyor LC system (ThermoFinnigan) was used. The compounds were separated on a Supelco Discovery HS F5 column [15 cm × 4 mm (i.d.); particle size, 5 μm] by a 15-min HPLC gradient from 85% aqueous solution (10 mmol/L ammonia acetate, pH ~5) to 100% methanol at a flow rate of 0.6 mL/min (Fig. 1). The column was then equilibrated with 15% methanol–85% acetate for 5 min after the gradient. The Discovery HS F5 column exhibits reversed-phase behavior under low organic mobile phase conditions but normal-phase behavior under high organic mobile phase conditions. The retention times of cotinine-*N*-oxide, *trans*-3′-hydroxycotinine, nicotine-*N*′-oxide, cotinine, nornicotine, nicotine, and anabasine were 4.81, 6.51, 7.48, 8.31, 9.73, 10.87, and 12.54 min, respectively.

MS

The MS analysis was performed with a TSQ Quantum triple-quadrupole mass spectrometer (ThermoFinnigan) equipped with an electrospray ionization interface. The electrospray ionization interface was operated in positive-ion mode with a spray voltage of 4 kV. Capillary temperature was set at 320 °C. Nitrogen (99.99%; Airgas Inc.) was used as sheath gas at a pressure of 49 arbitrary units and auxiliary gas at a pressure of 5 arbitrary units. The analytes and the isotopic internal standards were measured by SRM. The most abundant ion transition for each analyte, except for anabasine, was selected for identification and quantification of the analyte (Fig. 1). Although *m/z* 163 → 130 was the most abundant transition for anabasine, the second abundant transition, *m/z* 163 → 118, provided a higher signal-to-noise ratio. Argon was used as the collision gas and maintained at a constant pressure of 1.0 mTorr. The collision energies for the analytes and internal standards are also listed in Fig. 1. Both Q1 and Q3 mass analyzers were operated under unit resolution (0.7 Da full width at half maximum). The described conditions were optimized to achieve the best sensitivity for each analyte. Xcalibur (Ver. 1.3; ThermoFinnigan) was used to control the HPLC/TSQ Quantum system and to acquire and process data.

Results and Discussion

RECOVERY OF SPE EXTRACTION

We evaluated the recovery for the SPE method by adding each analyte to human urine at three different concentrations. For nicotine, cotinine, nornicotine, and *trans*-3′-hydroxycotinine, the selected concentrations were 1, 50, and 1500 μg/L, whereas 1, 50, and 200 μg/L were chosen for cotinine-*N*-oxide, nicotine-*N*′-oxide, and anabasine. These concentrations were selected to represent low, medium, and high concentrations across the calibration range for each compound. The samples with added analyte were extracted in the same manner as the actual samples described above. Triplicate recovery reference solutions for each concentration were prepared by adding the calibrators and internal standards to 1 mL of mobile-phase solution. The reference solutions were injected directly and analyzed by the LC/MS/MS system without extraction.

The recoveries of the analytes from the pretreated urine and water samples are listed in Table 1. Relatively consistent recoveries were obtained for each analyte in urine across the selected three concentrations. The mean recoveries of cotinine-*N*-oxide, *trans*-3′-hydroxycotinine, nicotine, and anabasine were ~100% from both urine and water. Nornicotine showed incomplete recoveries from both urine (78%) and water (79%) solutions to which analyte had been added. The recoveries for cotinine (both native and d_3-form) and nicotine-*N*′-oxide from water were ~100% and ~78%, respectively, whereas from urine the recoveries were ~78% and ~51%, respectively. Two possible explanations for the lower responses for the two

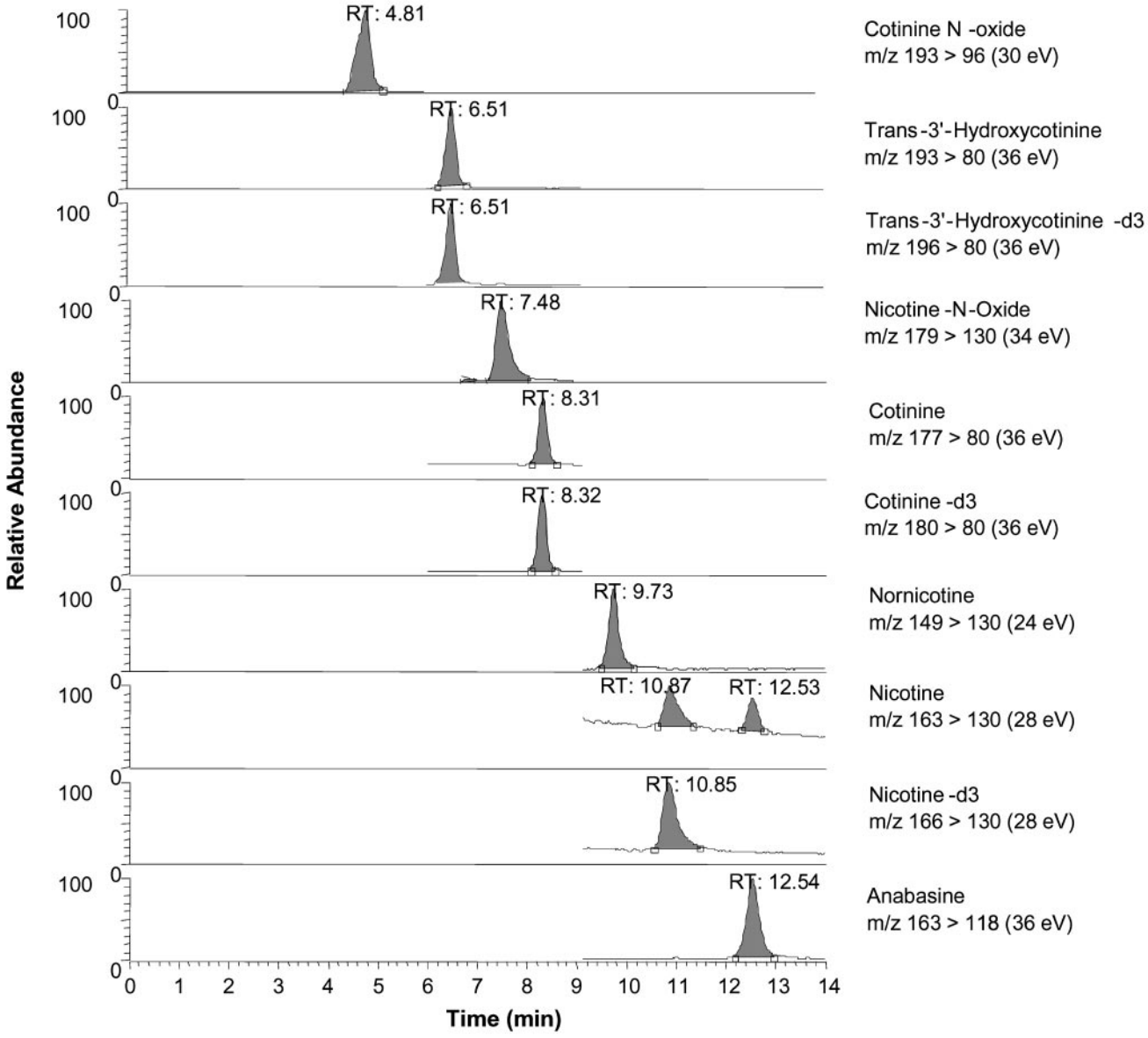

Fig. 1. SRM chromatograms for anabasine, nicotine, and nicotine metabolites.
RT, retention time.

compounds in urine are lower extraction efficiency for a urine matrix and ion suppression of analyte signals. To evaluate the latter possibility, we processed urine not containing any of the target compounds through the SPE column and added known amounts of all the analytes to 1 mL of the final eluant immediately before analysis. Any reduction in MS/MS responses relative to reference solutions prepared by adding the same amounts of the analytes to 1 mL of mobile phase solution would be attributable to ionization suppression. No apparent ion suppression was observed for cotinine (4–7%), whereas ion suppression was 12% for nicotine-*N*′-oxide (Table 1). Thus, the lower recovery of cotinine from urine was mainly attributable to lower SPE efficiency for urinary cotinine, whereas both lower SPE efficiency and ionization suppression caused the reduction in response of nicotine-*N*′-oxide from urine relative to from water. No differences in recoveries were observed between the unlabeled nicotine, cotinine, and *trans*-3′-hydroxycotinine and their corresponding deuterium-labeled compounds (Table 1). As indicated previously, calibration curves were prepared by adding the target compounds to urine and processing it through the SPE column rather than mathematically adjusting for recovery differences.

Our preliminary experiments suggested that use of the Oasis HLB SPE column provided higher and more stable recoveries than traditional silica-based reversed-phase columns because the Oasis column is usable at pH 13, and

Table 1. Recoveries for the SPE step and ion suppression.

	Recovery,[a] %					
	Urine					
Compound	Level 1	Level 2	Level 3	Mean (SD)	Water (50 μg/L), mean (SD)	Ion suppression,[b] %
CNO[c]	94.9	92.5	106.2	97.9 (7.3)	105.5 (4.2)	9.7
t3HC	114.0	88.4	97.6	100.0 (13.0)	104.9 (6.1)	6.3
t3HC-d_3	103.6	90.9	92.5	95.6 (6.9)	101.7 (6.3)	9.0
NNO	47.2	60.6	46.4	51.4 (8.0)	78.2 (5.2)	12.0
COT	78.5	72.0	85.2	78.6 (6.6)	103.3 (3.1)	4.1
COT-d_3	80.7	73.4	80.4	78.1 (4.1)	105.6 (2.7)	6.7
NNIC	74.5	76.8	85.2	78.8 (5.6)	79.3 (1.8)	1.1
NIC	104.4	96.6	109.6	103.5 (6.6)	100.5 (2.7)	0.0
NIC-d_3	104.6	102.6	103.7	103.6 (1.0)	105.6 (3.1)	0.0
ANAB	107.2	93.4	104.7	101.8 (7.4)	98.7 (4.3)	0.0

[a] The recoveries of the compounds in urine were evaluated at three concentrations: level 1 (1 μg/L); level 2 (50 μg/L); and level 3 (200 or 1500 μg/L). The recoveries of the compounds in water were evaluated at a concentration of 50 μg/L.

[b] Ion suppression of the analytes was evaluated by adding known amounts of the analytes into the final extract (1 mL) of blank urine at a concentration of 50 μg/L immediately before analysis. Three replicates were prepared for each concentration.

[c] CNO, cotinine-*N*-oxide; t3HC, *trans*-3′-hydroxycotinine; NNO, nicotine-*N*′-oxide; COT, cotinine; NNIC, nornicotine; NIC, nicotine; ANAB, anabasine.

the packing remains solvated for several minutes even after the column runs dry, providing consistent recovery. In addition, this simple SPE extraction method can be easily automated and allows for high sample throughput because the method does not include a time-consuming solvent evaporation and reconstitution procedure, which is often required by other reported SPE *(3, 4, 13)* and liquid-liquid extraction methods *(2, 14)*.

LOQ

For all target compounds, the LOQ (Table 2), defined as a signal-to-noise ratio of 10:1 *(16)*, was generally <0.2 μg/L, an improvement of one to two orders of magnitude over previous methods that analyzed multiple nicotine metabolites *(12–14)*. The excellent sensitivity of the proposed method makes it applicable for the determination of metabolic profiles of nicotine in clinical studies and studies on exposure to ETS.

Bernet et al. *(2)* and Bentley et al. *(3)* developed highly sensitive methods for cotinine (LOQ ~0.05 μg/L) in serum and *trans*-3′-hydroxycotinine (LOQ ~0.1 μg/L) in saliva that use a high-percentage organic mobile phase for LC. The lower sensitivity for the two compounds obtained for our current method, which was optimized for separation and detection of nicotine and five of its major metabolites, may be explained by the following two reasons. The ionization and detection in LC/MS/MS can be improved by use of a mobile phase with a high percentage of organic solvent. The analytes may elute at shorter retention times when a high-percentage organic mobile phase is used. Consequently, better peak shapes (or shaper peaks), usually associated with improved signal-to-noise ratios, can be obtained. However, the use of a high-percentage organic mobile phase for LC did not provide baseline separation of all target compounds in this study.

Other analytical methods for measurement of nicotine and nicotine metabolites have included a solvent evaporation step and a reconstitution step to reduce the final sample volume and to improve the method LOQ. Typically, the ratio of the volume of the processed biological sample (urine, blood, or saliva) to the final organic extract volume is 5–10 *(2–4, 13, 14, 17)*. To improve the throughput of sample pretreatment, we did not include the sample volume reduction and concentration step. If greater sensitivity is required and additional labor and time per analysis are acceptable, then a sample enrichment step could be incorporated into the current method.

Table 2. LOQ and linearity.

Compound	LOQ, μg/L	Calibration range, μg/L	Calibration equation	R^2
Cotinine-*N*-oxide	0.1	0.2–400	$y = 0.00348x - 0.000750$	0.9992
trans-3′-Hydroxycotinine	0.2	0.2–4000	$y = 0.0665x + 0.00766$	0.9962
Nicotine-*N*′-oxide	0.2	0.2–400	$y = 1.03x + 0.0263$	0.9918
Cotinine	0.1	0.2–4000	$y = 0.0389x + 0.00794$	0.9983
Nornicotine	0.1	0.2–4000	$y = 0.0438x - 0.00398$	0.9984
Nicotine	1.0	1.0–4000	$y = 0.0553x - 0.0118$	0.9983
Anabasine	0.2	0.2–400	$y = 0.0230x + 0.00618$	0.9999

CALIBRATION CURVE AND DYNAMIC LINEAR RANGE

We performed a linear regression of the peak-area ratios vs concentrations with a $1/x$ weighting. Because isotope-labeled nicotine-*N*′-oxide, cotinine-*N*-oxide, nornicotine, and anabasine are not commercially available, we used cotinine-d_3 as the internal standard for these compounds. Although nornicotine, anabasine, and nicotine are structurally similar, our results showed that use of cotinine-d_3 as the internal standard for nornicotine and anabasine provided higher regression coefficients and a wider linear range for the calibration curves of these two compounds than does use of nicotine-d_3 as the internal standard. The LC/MS/MS method provided excellent linearity over three to four orders of magnitude for the target analytes (Table 2). The smaller linear ranges for nicotine-*N*′-oxide, cotinine-*N*-oxide, and anabasine were probably attributable to the absence of coeluting isotopic internal standards for these compounds. The wide linear range and high sensitivity for this method allow its application to samples from smokers, people being treated with nicotine replacement therapy, and people exposed to environmental tobacco smoke.

IMPRECISION AND RECOVERY

Using QC samples at two or three concentrations, we evaluated the intra- and interday imprecision and recovery of this method, expressed as the relative standard deviation (CV) and the percentage of expected values, respectively (Table 3). We prepared six replicates at each concentration, and analyzed the samples over 3 consecutive days. The intraday imprecision was generally <5%, except at concentrations near the LOQ, which had CVs <10% or, in a few cases, <20%, whereas the intraday recoveries were 85–117%. The overall interday imprecision and recovery for the method ranged from 2.5% to 18% and from 92% to 109%, respectively.

ACCURACY

We evaluated the method accuracy for nicotine and cotinine by two different approaches. The method recovery of nicotine and cotinine was based on commercial standard solutions of nicotine and cotinine (Sigma) added to urine QC samples at different concentrations (0.5, 50, and 1500 μg/L) compared with calibration curves prepared from pure compounds (Aldrich). The method recovery (Table 3) is therefore similar to the method accuracy across a wide range of concentrations. We also evaluated the method accuracy for nicotine and cotinine by comparing the total nicotine and cotinine (free and conjugated forms) in 10 urine samples at high concentrations analyzed by the LC/MS/MS method with the values for these two compounds in the urine samples analyzed independently by a GC/MS method after liquid–liquid extraction with methylene chloride *(18)*. The urine specimens were collected from nine smokers who were undergoing nicotine replacement therapy. β-Glucuronidase was used to hydrolyze the urine samples based on a modified method of Curvall et al. *(19)*. The results obtained by the LC/MS/MS method appeared to agree very well with those obtained by the GC/MS method (Fig. 2). We evaluated the statistical equivalency between the urinary concentration data obtained with the two

Table 3. Intra- and interday recovery and reproducibility for the analytes at different concentrations.[a]

		Day 1 (n = 6)			Day 2 (n = 6)			Day 3 (n = 6)			Overall (n = 18)		
Compound	Actual concentration, μg/L	Mean, μg/L	CV, %	Recovery, %	Mean, μg/L	CV, %	Recovery, %	Mean, μg/L	CV, %	Recovery, %	Mean, μg/L	CV, %	Recovery, %
CNO[b]	0.5	0.51	5.1	101	0.43	9.9	86	0.45	9.9	90	0.46	10.2	92
	50.0	49.4	3.4	99	47.3	3.6	95	46.9	4.3	94	47.9	5.3	96
t3HC	0.5	0.50	6.5	99	0.54	21	107	0.47	4.7	94	0.50	18	100
	50.0	52.6	1.9	105	52.9	3.6	106	52.5	2.1	105	52.7	4.6	105
	1500	1602	2.5	107	1460	2.2	97	1594	4.7	106	1552	5.1	103
NNO	0.5	0.43	9.0	86	0.45	10	90	0.55	9.0	110	0.48	13	95
	50.0	52.5	4.2	105	52.0	7.5	104	47.3	5.5	95	50.6	7.9	101
COT	0.5	0.49	5.9	97	0.59	5.1	117	0.56	2.7	112	0.54	6.5	109
	50.0	50.9	1.2	102	52.5	1.8	105	50.6	2.7	101	51.3	3.4	103
	1500	1572	2.3	105	1508	2.9	101	1568	2.3	105	1549	2.7	103
NNIC	0.5	0.48	9.9	96	0.56	15	112	0.43	3.8	87	0.49	17	98
	50.0	54.0	4.7	108	49.1	11	98	44.6	10.9	89	49.2	13	98
	1500	1558	3.1	104	1505	3.1	100	1427	2.1	95	1497	3.4	100
NIC	1.5	1.71	3.4	114	1.33	5.3	89	1.62	11	108	1.56	12	104
	50.0	45.5	2.6	91	47.1	1.5	94	52.4	1.9	105	48.3	3.5	97
	1500	1465	3.2	98	1417	1.6	94	1453	1.6	97	1445	3.1	96
ANAB	0.5	0.43	16	85	0.52	16	105	0.44	2.0	87	0.46	17	92
	50.0	51.8	2.1	104	46.7	1.9	93	51.9	2.2	104	50.1	2.5	100

[a] Method imprecision and recovery are expressed as the relative standard deviation (CV, %) and percentage of expected values, respectively.
[b] CNO, cotinine-*N*-oxide; t3HC, *trans*-3′-hydroxycotinine; NNO, nicotine-*N*′-oxide; COT, cotinine; NNIC, nornicotine; NIC, nicotine; ANAB, anabasine.

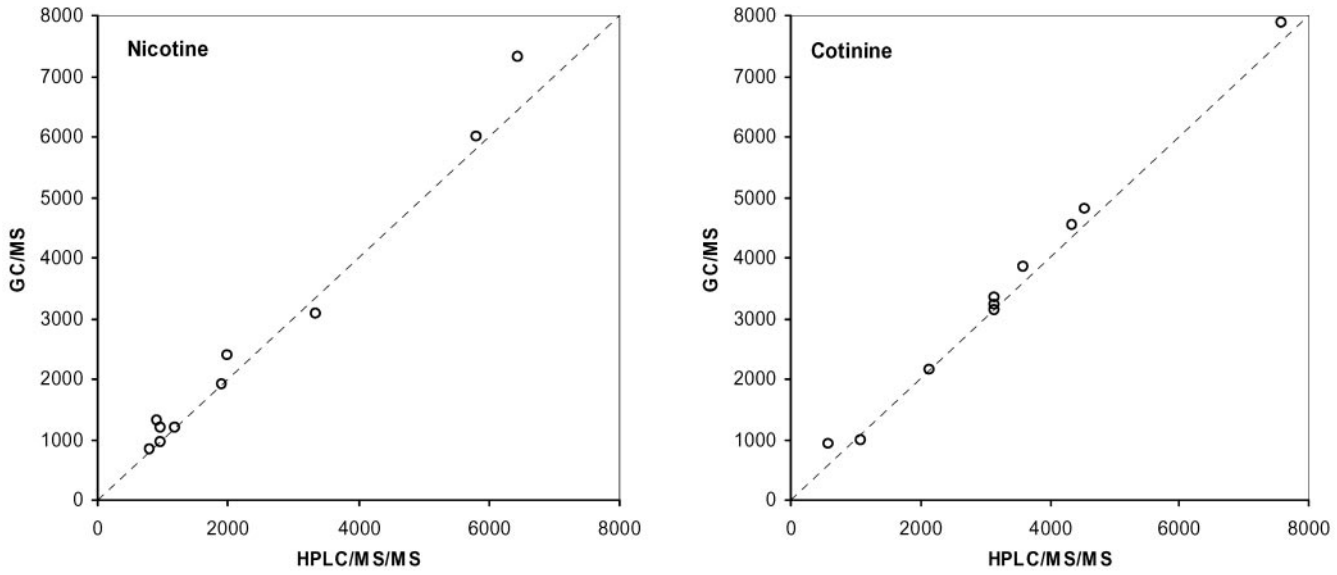

Fig. 2. Comparison of urinary nicotine (*left*) and cotinine (*right*) concentrations measured by LC/MS/MS and GC/MS methods in 10 urine samples collected from nine smokers undergoing nicotine replacement therapy.

The *dashed line* represents the 1:1 line.

methods by a regression procedure developed by Passing and Bablok *(20)* (available in Analyze-it, Analyze-It Software, Ltd., Leeds, UK). The 95% confidence intervals (CIs) of the slopes of the regression lines for nicotine and cotinine were 0.91–1.21 and 0.98–1.17, respectively, indicating no proportional systematic differences between the two methods as evidenced by the inclusion of unity in the 95% CIs of the slopes. The 95% CIs of the intercepts of the regression lines were −217 to 247 for nicotine and −466 to 279 for cotinine. Thus, the intercepts of the regression lines were not statistically different from zero ($\alpha = 0.05$), suggesting no constant systematic difference between the LC/MS/MS method and the GC/MS method. Paired *t*-tests also showed that there was no significant overall difference between these two methods for nicotine and cotinine ($\alpha = 0.05$).

Table 4. Concentrations of anabasine, nicotine, and nicotine metabolites in urine samples from nine smokers during nicotine replacement therapy.

	Concentration, μg/L						
Individual	**CNO[a]**	**t3HC**	**NNO**	**COT**	**NNIC**	**NIC**	**ANAB**
1	455	6820	432	1241	67.1	400	12.8
2	23.0	102	148	256	19.0	658	1.0
3	223	2970	1040	970	42.6	275	3.1
4	1290	22700	2050	2097	265	5180	27.9
5	468	4280	495	1267	91.0	1570	12.2
6	55.2	398	161	425	21.9	599	3.3
7	135	1330	225	959	51.6	1440	6.5
8	321	2420	715	1282	125	5470	20.3
9-1[b]	300	2910	867	740	29.9	637	NF
9-2	517	4980	957	1340	87.7	2580	12.8
Mean	378	4890	709	1060	80.1	1880	11.1
SD	362	6600	574	522	73.4	1940	8.8

[a] CNO, cotinine-*N*-oxide; t3HC, *trans*-3′-hydroxycotinine; NNO, nicotine-*N*′-oxide; COT, cotinine; NNIC, nornicotine; NIC, nicotine; ANAB, anabasine; NF, not found.

[b] Two urine samples were collected from individual 9 at different times.

APPLICATION TO URINE SAMPLES FROM SMOKERS

The measured concentrations of anabasine, nicotine, and its metabolites (free forms) in the 10 urine samples are listed in Table 4. The mean values of the determined nicotine/metabolite concentrations for the small group of individuals are consistent with reported concentrations in smokers' urine [reviewed in Davis and Curvall *(9)*]. Urinary anabasine can be used as a biomarker of tobacco use to monitor compliance to nicotine replacement therapy. Patients abstaining from tobacco use typically have a urinary anabasine concentration <2 μg/L *(13, 14)*. On the basis of this cutpoint, only individual 2 appeared to have stopped using tobacco products at the time when the urine sample was collected. The anabasine concentrations in individuals 3 and 7 were slightly higher than the cutpoint, indicating that these two people had minimally used tobacco products. Finally, we must note that further evaluation of this method with urine samples relevant to passive smoking will be helpful, although this method has been validated with QC samples with target analytes added at various concentrations.

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