A Rapid LC–MS–MS Method for the Determination of Nicotine and Cotinine in Serum and Saliva Samples from Smokers: Validation and Comparison with a Radioimmunoassay Method

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Abstract

The development and validation of a rapid liquid chromatography (LC)–tandem mass spectrometry (MS–MS) method for determination of nicotine and cotinine in smokers' serum is described. The method is based on solid-phase extraction in a 96-well plate format and requires only 100 µL of serum. Using normal-phase chromatography, both analytes elute in less than 1 min, which permits high sample throughput applications. The calibrated range is 2–100 ng/mL nicotine and 20–1,000 ng/mL cotinine. For known samples, recovery is 95–116% for nicotine and 93–94% for cotinine. The method is extended to rat serum and human saliva (cotinine only) using partial validation techniques. When compared with an existing radioimmunoassay method in our laboratory, the LC–MS–MS method gives improved accuracy, precision, and sample throughput.

Introduction

Nicotine absorbed during smoking appears rapidly in the bloodstream with serum concentration in smokers ranging from less than 1 ng/mL to more than 50 ng/mL during the day. Determination of nicotine concentrations in blood following smoking is an important tool in understanding smoking behavior (1,2). For example, in addition to the absolute rise in nicotine following smoking, the total amount of nicotine absorbed during cigarette smoking can be estimated from the area under the curve of a plot of serum nicotine concentration over time (3). Studies of smoking behavior often require blood samples at many time points to adequately describe this curve, resulting in large numbers of samples; for studies involving many subjects, thousands of samples can be generated. The primary nicotine metabolite, cotinine, also occurs in serum at approximately 10 times the concentration of unmetabolized nicotine and shows less variability because of its longer half-life in the body. Consequently, cotinine is useful in some contexts as a biomarker of exposure to tobacco smoke (4). Cotinine also appears in saliva at concentrations slightly higher than those in blood (5). Thus, to support studies of numerous subjects that generate both serum and saliva samples, high-throughput analytical techniques are required for efficient processing of samples for nicotine and cotinine.

A radioimmunoassay (RIA) method has been used in our laboratory as a high-throughput method to determine nicotine and cotinine in serum and other matrices since the early 1990s (6–8). RIA was initially chosen as a replacement for a gas chromatography–nitrogen phosphorous detection method (9) because it gave reasonable accuracy and precision in a high-throughput mode. Little sample preparation was required, the technology was simple and rugged, and no time-consuming extraction or chromatography steps were necessary. Consequently, RIA accommodated large numbers of samples. The disadvantages of RIA included occasional cross reactivity with matrix constituents, the inability to determine more than one analyte per assay, and the requirement for the use and disposal of radioactivity.

Liquid chromatography (LC)–tandem mass spectrometry (MS–MS) has moved into the high-throughput realm in recent years. The high specificity and sensitivity of MS–MS to isolate analyte signals in complex mixtures often precludes the need to chromatographically isolate the analyte from background components. This permits shorter chromatographic separations resulting in faster analysis times.

A high-throughput LC–MS–MS method developed for serum cotinine by Bernert et al. (10), and later extended to saliva (11), used multiple extraction and cleanup steps prior to LC–MS–MS analysis. With detection limits for cotinine at 0.05 ng/mL, this LC–MS–MS method was suitable for nonsmokers potentially exposed to environmental tobacco smoke and could analyze 100 serum samples per day for cotinine. Xu et al. (12) reported a rapid LC–MS–MS method for both nicotine and cotinine using liquid–liquid extraction of 1 mL of plasma and reversed-phase (C18) analytical chromatography. Moyer et al.

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(13) reported another LC–MS–MS method based on solid-phase extraction (SPE) of 1 mL of serum for nicotine, cotinine, and three additional analytes related to smoking with a total elution time of 7.5 min.

A method is described for nicotine and cotinine in serum that could accommodate large numbers of samples with minimal labor and be applicable for volume-limited samples such as those from rodent studies. This report describes the validation of a simple, rapid LC–MS–MS method for determination of both nicotine and cotinine in large numbers of serum samples in addition to cotinine in saliva samples. The method required only 100 µL of sample and was based on a single SPE in a 96-well plate format followed by rapid analysis using LC–MS–MS; both analytes eluted in less than 1 min. Normal-phase chromatography was used for separation. Quantitation was by the method of internal standards with trideuterated analogs (d_3-nicotine and d_3-cotinine) as the internal standards. The method validation procedures used in this study were based on FDA recommendations (14) and others (15,16). The method was extended to the same matrix in a different species (rat serum) and a different biological matrix in the same species (human saliva) using partial validation procedures. The results from the new LC–MS–MS method were compared with those from the RIA method using shared samples.

Experimental

Materials and equipment

Nicotine was obtained from Eastman Kodak Company (Rochester, NY), *N*-methyl-d_3-nicotine (d_3-nicotine) from Toronto Research Chemicals (Toronto, Canada), cotinine from Sigma Chemical Company (St. Louis, MO), and *N*-methyl-d_3-cotinine (d_3-cotinine) from CDN Isotopes (Quebec, Canada). Water for solutions was from a Milli-Q A10 synthesis purification system (Millipore, Bedford, MA). Acetonitrile and methanol were from Burdick & Jackson (Muskegon, MI). Ammonium formate was from Acros Organics (Fairlawn, NJ) and Lyphochek drug-free serum (used for blank serum) was obtained from Bio-Rad Laboratories (Irvine, CA). SPEs were performed using 96-well Oasis HLB 10-mg plates (Waters, Milford, MA) on a 96-well plate manifold (Phenomenex, Torrance, CA) modified with a custom insert to adapt shallow 0.5-mL well plates (Agilent Technologies, Palo Alto, CA) for collection. Preslit silicon well caps (Agilent Technologies) were used to seal the plate after collection. A 12-channel pipettor (Costar, Cambridge, MA) was used for liquid handling with the plates. For RIA, nicotine and cotinine RIA kits were purchased from Brandeis University (Waltham, MA) and contained primary antinicotine and anticotinine antibodies, tritiated nicotine and cotinine, normal rabbit serum at two dilutions, and secondary goat antirabbit antibodies in concentrated forms. Tris-HCl, sodium chloride, sodium hydroxide, and gelatin were obtained from Sigma Chemical Company. Hydrochloric acid was purchased from Fisher Scientific (Fairlawn, NJ). A DPC-Mark 5 robotic dilutor/dispenser (Diagnostic Products Corporation, Los Angeles, CA) was used for sample preparation. Scintillation counting was performed with a Beckman Instrument model 6500IC liquid scintillation counter (Palo Alto, CA). Raw count-per-minute data from RIA calibration standards in each assay were converted by Immunofit EIA-RIA software (Beckman Instrument, Inc.) into a calibration curve using a logistic model. Sample counts-per-minute data were converted by interpolation from the calibration curve.

LC–MS–MS

Chromatography was performed on an Agilent 1100 high-performance liquid chromatographic (HPLC) binary pump system (Agilent Technologies) with a well-plate autosampler. The analytical column was a 2.1- × 50-mm Betasil Diol-100, 5-µm column (ThermoHypersil-Keystone, Bellefonte, PA) with a matching guard column and a 0.5-µm × 3-mm inline filter (Phenomenex). The system was operated isocratically with a flow of 1 mL/min of (A) 35% 2mM ammonium formate in methanol and (B) 65% acetonitrile. The flow was split 10:1 prior to the electrospray ionization (ESI) source. The MS used for detection was a Micromass Quattro Ultima triple stage MS (Manchester, U.K.); both HPLC and MS systems were controlled using Micromass MassLynx software, version 3.5. The MS was operated in the positive ion mode and used multiple reaction monitoring (MRM) for analyte and internal standard detection. In this mode, the first mass analyzer selects a specific precursor ion for each analyte or internal standard and transmits it to the collision cell; only specific product ions are monitored by the second mass analyzer. For nicotine and d_3-nicotine, cone voltage was 42 V with a collision-induced dissociation (CID) energy of 20 eV and the transitions monitored were *m/z* 163 → 130 and *m/z* 166 → 130, respectively. For cotinine and d_3-cotinine, cone voltage was 50 V with a CID energy of 25 eV and the transitions monitored were *m/z* 177 → 80 and *m/z* 180 → 80, respectively. The collision gas was argon at a pressure of 2.25×10^{-3} millibar. atmospheric pressure ionization gas flow (nitrogen) was 550–600 L/min and desolvation temperature was 400°C. For optimization of cone voltages and collision energies during method development and establishing fragmentation pathways, a 75-pg/µL solution of each analyte was infused into the source at 5 µL/min using a syringe pump (Pump 11, Harvard Apparatus, Holliston, MA). Product ion (daughter) mass spectra were recorded at different collision energies using the continuum averaging mode of operation. Fragmentation pathways were established in part using reactant (parent) ion spectra.

Serum and saliva samples

Blank serum for the standards and quality control (QC) samples in this study was from a commercial source of drug-free lyophilized serum from nonsmokers. Serum and saliva samples from smokers were obtained from the Smoking Behavior and Physiology Research Laboratory at R.J. Reynolds Tobacco Company (Winston-Salem, NC) in addition to six samples of serum and saliva from nonsmokers used to assess matrix effects. The study protocols were approved by an internal human research resources review committee and all subjects gave informed consent after learning the details of the study. For rat serum, samples from exposed and unexposed

rats were supplied by the Inhalation Toxicology Laboratory at R.J. Reynolds Tobacco Company using standard operating procedures. All samples were stored at –80°C and thawed just prior to analysis.

Preparation of primary standards and working solutions

Primary standards for nicotine (~ 10 ng/µL), cotinine (~ 100 ng/µL), d_3-nicotine (~ 10 ng/µL), and d_3-cotinine (~ 100 ng/µL) were prepared in water. Two working standards were prepared by diluting with water the nicotine and cotinine primary standards to give a solution with 0.005 ng/µL nicotine and 0.050 ng/µL cotinine and another solution at 10 times these concentrations. An internal standard solution was prepared from the deuterated primary standards at 0.020 ng/µL d_3-nicotine and 0.200 ng/µL d_3-cotinine.

Preparation of calibration standards, QC samples, and analytical samples

All calibration standards, QC samples, blanks, and analytical samples, were prepared in 96-well plates with 0.5-mL wells. Six nonzero standards were prepared by spiking 100 µL of blank serum at 2, 5, 10, 20, 50, and 100 ng/mL nicotine and 10 times these concentrations for cotinine. In addition, a zero standard was prepared in blank serum with no added nicotine or cotinine but with internal standards; a double zero standard was prepared with neither analytes nor internal standards added. QC samples were prepared at the limit of quantitation (LOQ), 2.5 × LOQ, and at 50% and 95% of the calibrated range by spiking the blank serum with working solutions independently prepared from those used to make the calibration standards. Analytical samples were prepared by adding 100 µL of serum to a well, followed by 200 µL of water. Internal standard solution (100 µL) was added to each well to give d_3-nicotine at 20 ng/mL and d_3-cotinine at 200 ng/mL. The same procedure was used for saliva samples with the exception that blank saliva (from a nonsmoker not exposed to tobacco smoke or nicotine) was used to prepare the calibration standards and QC samples.

Extraction

An extraction plate was prepared by positioning it on the vacuum manifold and adding 400 µL methanol to wet each well (opening to vacuum briefly to pull the methanol through), followed by 400 µL of water to condition each well. All wells in the sample preparation plate were mixed by repeatedly (five times) aspirating and dispensing 200 µL of the contents of each well using the 12-channel pipettor. After mixing, the contents of each row were transferred to the extraction plate and pulled through under vacuum. Each well was then washed with 200 µL of 10% methanol in water, and air was pulled through for approximately 1 min to dry each well. A collection plate was positioned in the manifold chamber, and each well was eluted under vacuum with 200 µL of a solvent mixture of 65% acetonitrile–35% methanol. The collection plate was capped with a silicon sealing mat and taken directly to the well plate autosampler on the LC–MS–MS system.

Results and Discussion

MS of nicotine and cotinine

Nicotine and cotinine were characterized under positive ion ESI conditions by optimizing the cone voltage for formation of $(M+H)^+$ and taking product ion spectra at different collision energies. Figures 1 and 2 are the product ion profiles for $(M+H)^+$ of nicotine and cotinine, respectively. Proposed fragmentation schemes for $(M+H)^+$ of each analyte are shown in Figures 3 and 4, respectively. For nicotine, abundance of the $(M+H)^+$ ion at *m/z* 163 maximized at a cone voltage of 42 V. The product ion spectrum of *m/z* 163 gave a fragment at *m/z* 132 at low collision energy, which corresponds to loss of CH_3NH_2. This fragment abundance decreased at higher energies and *m/z* 130 increased, suggesting a further loss of H_2. A minor competitive process was loss of 15 Da (CH_3·) from *m/z* 132 to yield *m/z* 117. Two other minor fragmentation pathways shown in Figure 3 occurred through the loss of C_3H_7· (*m/z* 120)

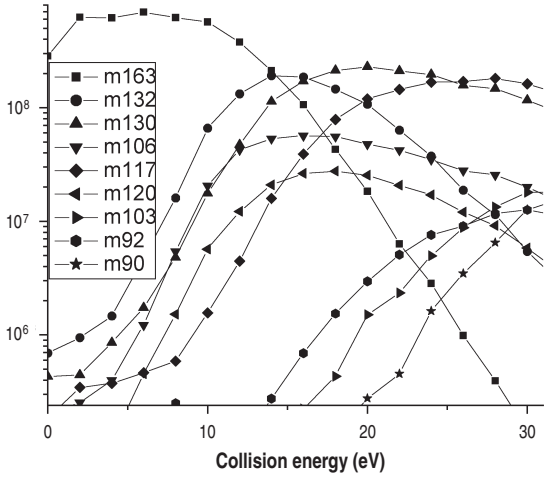

Figure 1. Plot of major fragment abundances from the protonated molecular ion $(M+H)^+$ of nicotine versus collision energy.

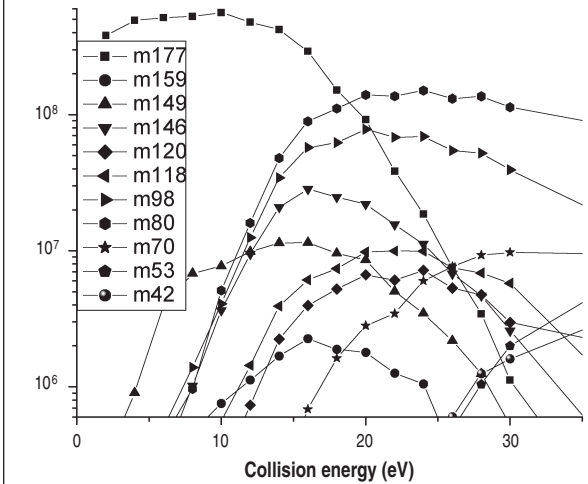

Figure 2. Plot of major fragment abundances from the protonated molecular ion $(M+H)^+$ of cotinine versus collision energy.

and loss of C_3H_7N (*m/z* 106). The most abundant transition, *m/z* 163 → 130, was selected for quantitation of nicotine with the analogous transition of *m/z* 166 → 130 selected for d_3-nicotine. For cotinine, the $(M+H)^+$ ion abundance remained relatively constant over a broad range of cone voltages (1–100 V); a cone setting of 50 V was selected. The product ion profile (Figure 2) showed two abundant small mass fragments. The transition that produced *m/z* 80 corresponded to loss of the methylpyrrolidinone ring, leaving the charge with protonated pyridine. Conversely, loss of pyridine to produce protonated methylpyrrolidinone at *m/z* 98 was a competing pathway. A minor fragmentation pathway shown in Figure 4 was loss of CH_3NH_2 to produce *m/z* 146. The most abundant transition, *m/z* 177 → 80, was selected for quantitation with the analogous transition *m/z* 180 → 80 selected for d_3-cotinine.

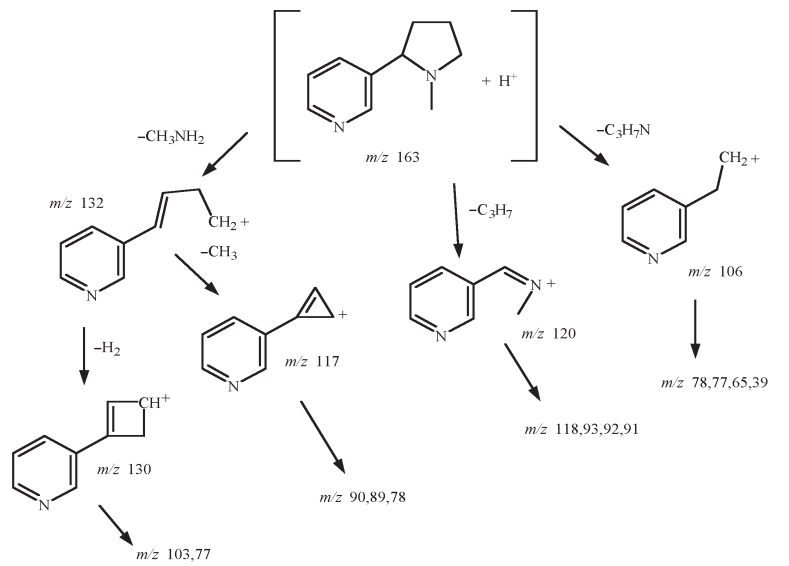

Figure 3. Proposed fragmentation scheme for $(M+H)^+$ of nicotine.

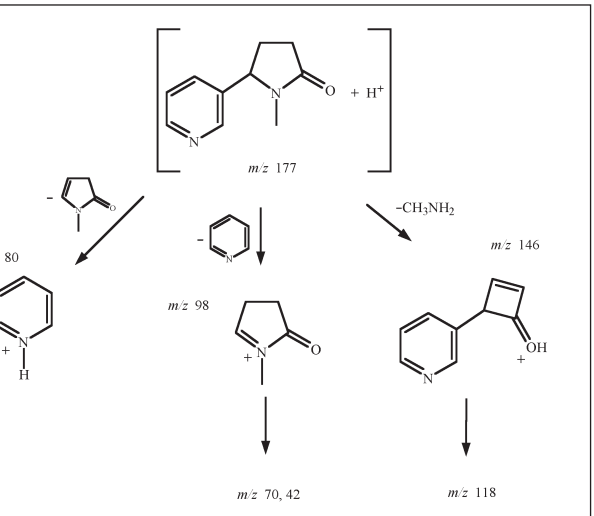

Figure 4. Proposed fragmentation scheme for $(M+H)^+$ of cotinine.

Chromatography of nicotine and cotinine

Because of the specificity offered by the MRM transitions, a relatively short isocratic normal-phase chromatography method was developed. A diol column was used for separation with an organic mobile phase that was similar in composition to the SPE eluent. Figure 5 contains MRM traces for the analytes and internal standards in an extract from a smoker's serum sample. Cotinine eluted at approximately 0.25 min and nicotine at 0.50 min.

Specificity and matrix effects

Specificity of the assay was evaluated on six serum and saliva samples from non-smokers not exposed to tobacco smoke or nicotine to check for interfering substances with the assay. The response for nicotine or cotinine did not exceed that of the LOQ response in any of the samples. Matrix effects in serum were evaluated using a secondary MRM transition for each analyte and comparing the ratio with the analytical transition in authentic serum samples from smokers, spiked blank serum, and simple solutions (unextracted samples prepared in water and eluent). With cone voltages and collision gas pressure the same as described in the Procedure section, the secondary transitions monitored were *m/z* 163 → 117 for nicotine (25 eV collision energy) and *m/z* 177 → 98 for cotinine (25 eV collision energy). The results are listed in Table I. For nicotine and cotinine, none of the response ratio means were significantly different from the other means based on an independent *t*-test. Thus, the original analytical transitions chosen were specific for these analytes in the human serum matrix.

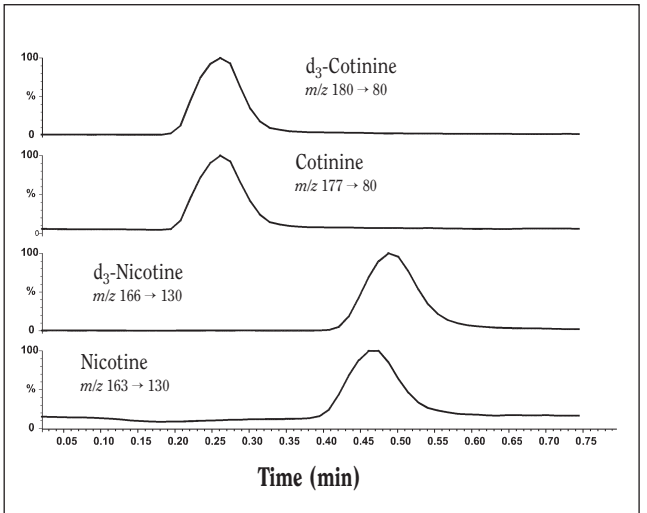

Figure 5. MRM chromatograms for d_3-cotinine, cotinine, d_3-nicotine, and nicotine in a serum extract from a smoker.

Assay linearity, LOQ, and recovery

Linearity was assessed by preparing each of the six standard (non-zero) concentrations five times and analyzing them. As shown in Table II, all of the mean concentrations were well within the acceptable limits of ± 15% (± 20% at LOQ) (14) of the prepared concentration when back-calculated from the regression equation. The relative standard deviations (RSDs) at each level were all within the acceptable limits of less than 15% (< 20% at LOQ) (14). LOQ, defined as 10 standard deviations of a blank sample (16), was 1.8 ng/mL nicotine and 7.7 ng/mL cotinine. LOQs were conservatively set at 2 ng/mL nicotine and 20 ng/mL cotinine. Testing for accuracy and precision on a set of independently prepared samples at these LOQs showed that the recommended validation criteria (14) of back-calculated concentrations ± 20% of the prepared concentration with RSD < 20% were met. Recovery was assessed using six replicates of spiked serum samples at LOQ and 50% and 95% of the calibrated range and determining their concentrations using a calibration curve of *non-extracted* standards. Analyte recovery, as a percentage, was expressed as the measured amount divided by the amount added to the sample. Recoveries were 95–116% for nicotine and 93–94% for cotinine.

Accuracy and precision

Intrabatch accuracy and precision were determined on three separate batches of spiked blank serum samples prepared at LOQ and 50% and 95% of the calibrated range as shown in Table III. For the six replicates at each level, accuracy (%Delta) was within ± 15% for each analyte in each batch. Precision, as expressed by RSD, was less than 15% (< 20% at LOQ) for each analyte in each batch. Interbatch accuracy and precision were determined on the mean concentrations from all three batches as listed at the bottom of Table III. The interbatch assay accuracy was within ± 15% of the prepared concentration with RSDs less than 15% for both analytes at all three concentration levels.

Dilution effects

In order to determine any effects of dilution on samples whose concentrations fall above the calibrated range, a serum sample was prepared with nicotine and cotinine at approximately twice the calibrated range (215 ng/mL nicotine and 1,780 ng/mL cotinine). Six replicate 4-fold dilutions of this sample were prepared using water as the diluent. Table IV summarizes the results and shows that the determined concen-

Table I. Comparison of Ratios for Two Transitions of Nicotine and Cotinine in Three Different Types of Samples to Assess Specificity*

Samples	Nicotine response ratios†	Cotinine response ratios‡
Spiked serum	0.844 ± 0.025	0.523 ± 0.012
Authentic serum	0.836 ± 0.015	0.528 ± 0.01
Simple solutions	0.838 ± 0.018	0.528 ± 0.013

* Three independently spiked serum and simple solution samples at LOQ, 50% of the calibrated range, and 95% of the calibrated range were analyzed. Authentic serum samples were duplicates taken from five different smokers.
† (Response *m/z* 163 → 117)/(Response *m/z* 163 → 130).
‡ (Response *m/z* 177 → 98)/(Response *m/z* 177 → 80).

Table II. Linearity Data for Nicotine and Cotinine are Shown*

Nicotine				Cotinine			
Prepared (ng/mL)	Measured mean ± SD	RSD	Prepared %Delta	(ng/mL)	Measured mean ± SD	RSD	%Delta
2	2.0 ± 0.3	13.7%	1.0%	20	20.2 ± 1.1	5.4%	1.1%
5	5.2 ± 0.4	6.8%	3.1%	50	49.9 ± 1.7	3.5%	–0.2%
10	9.8 ± 0.2	2.3%	–1.8%	100	101 ± 3	3.1%	1.5%
20	19.4 ± 0.6	2.8%	–3.3%	200	195 ± 11	5.9%	–2.8%
50	50.4 ± 0.8	1.7%	0.8%	500	499 ± 12	2.5%	–0.2%
100	100.3 ± 1.7	1.7%	0.3%	1000	1005 ± 20	2.0%	0.5%

* Each standard was prepared 5 times. %Delta is the difference in the prepared and mean measured (back-calculated) concentration from the linear regression equation for each analyte.

Table III. Accuracy and Precision Data for Three Batches with Samples Prepared at LOQ and 50% and 95% of the Calibrated Range*

	Nicotine				Cotinine			
	Prepared (ng/mL)	Measured mean ± SD	RSD	%Delta	Prepared (ng/mL)	Measured mean ± SD	RSD	%Delta
Batch 1	2	2.1 ± 0.2	9.2%	5.8%	20	17.6 ± 0.2	1.3%	–11.9%
(*N* = 6)	50	49.8 ± 0.9	1.7%	–0.5%	500	475 ± 11	2.3%	–5.0%
	95	96.5 ± 0.9	1.0%	1.6%	950	914 ± 22	2.4%	–3.8%
Batch 2	2	2.0 ± 0.1	6.0%	1.7%	20	20.8 ± 0.7	3.2%	3.9%
(*N* = 6)	50	50.4 ±1.7	3.3%	0.9%	500	501 ± 16	3.1%	0.2%
	95	98.2 ± 3.7	3.7%	3.4%	950	969 ± 26	2.7%	2.0%
Batch 3	2	1.9 ± 0.3	16.6%	–5.0%	20	20.3 ± 2.5	12.3%	1.3%
(*N* = 6)	50	47.9 ± 0.7	1.5%	–4.2%	500	504 ± 20	3.9%	0.8%
	95	90.3 ± 1.9	2.1%	–5.0%	950	964 ± 24	2.5%	1.4%
Interbatch	2	2.0 ± 0.1	4.9%	0.8%	20	19.1 ± 1.7	8.9%	–4.7%
(*N* = 3)	50	49.4 ± 0.5	1.0%	–1.3%	500	485 ± 16	3.3%	–3.1%
	95	95.0 ± 1.4	1.5%	0.0%	950	931 ± 30	3.3%	–2.0%

* %Delta is the relative difference in the measured and prepared concentrations.

tration multiplied by the dilution factor was within ± 15% of the prepared concentration with RSD less than 15%. Thus, the effects of dilution with water were not found to significantly alter the accuracy and precision of the method.

Validation for rat serum

Rat serum constitutes a different matrix than human serum and it was necessary to validate the method in rat serum in order to apply the method to rodent studies. A partial validation was performed that included a demonstration of specificity based on blank samples from six different unexposed rats and accuracy and precision data on spiked samples prepared in blank rat serum. The serum samples from six unexposed rats showed no responses above the LOQ for either nicotine or cotinine. The upper part of Table V shows the results for a set of blank rat serum samples spiked with nicotine and cotinine using a calibration curve prepared in blank (unexposed) rat serum. A set of blank human serum samples spiked with the same amounts of nicotine and cotinine were analyzed at the same time using the calibration curve prepared in rat serum. The results for human serum are listed in the lower portion of Table V. For the six replicates at each level in both sets of data, the accuracy (%Delta) was within ± 15% for each analyte in each batch. Precision (RSD) was less than 15% (< 20% at LOQ) for each analyte in each batch. Thus, accuracy and precision of the method in rat serum satisfied the validation criteria (14). In addition, no biases were observed between rat and human serum samples.

Validation for human saliva (cotinine only)

Although human saliva from smokers contains both nicotine and cotinine, the nicotine derives principally from direct absorption of nicotine from smoke in the mouth and does not reflect concentrations in the blood as does saliva cotinine. Thus, only cotinine is of interest in the saliva of smokers. To extend the method to this matrix, a partial validation was performed that included a demonstration of specificity by analyzing blank samples from six nonsmokers and accuracy and precision data in spiked blank saliva. The saliva samples from six nonsmokers showed no responses above the LOQ for cotinine. Table VI shows the results for a set of blank human saliva samples spiked with cotinine using a calibration curve prepared in blank human saliva. For the six replicates at each level in saliva, the accuracy (%Delta) was within ± 15% for the analyte in each batch. Precision (RSD) was less than 15% (< 20% at LOQ) for the analyte. Thus, accuracy and precision of the method in saliva satisfied the validation criteria (14).

Comparison with RIA

Several samples analyzed by RIA within the past year were rethawed and analyzed using the LC–MS–MS method in order to compare the two methods. Selection of samples was based on availability at the time; no RIA serum cotinine data were available for comparison. The sample sets included nicotine only in 322 human serum samples representing 13 different

Table IV. Data for Determinations of Six Replicate Diluted (4-Fold) Samples*

Sample	Nicotine (ng/mL)	Cotinine (ng/mL)
1	234	1840
2	232	1879
3	228	1873
4	242	1880
5	218	1786
6	224	1804
Avg.	230	1844
STD	8	41
RSD	3.7%	2.2%
%Delta	7.0%	3.6%

* Each sample was prepared at 215 ng/mL nicotine and 1,780 ng/mL cotinine. %Delta is the relative difference in the measured amount and the prepared amount after correcting for the dilution.

Table V. Accuracy and Precision Data for Rat and Human Serum Samples Prepared at 2.5 × LOQ and 50% and 95% of the Calibrated Range*

	Nicotine				Cotinine			
	Prepared (ng/mL)	Measured mean ± SD	RSD	%Delta	Prepared (ng/mL)	Measured mean ± SD	RSD	%Delta
Rat serum	5	5.3 ± 0.2	3.5%	6.7%	50	50.9 ± 2.8	5.4%	1.7%
	50	50.9 ± 0.7	1.3%	1.8%	500	504 ± 14	2.9%	0.8%
	95	96.6 ± 1.8	1.9%	1.6%	950	943 ± 21	2.3%	–0.8%
Human serum	5	5.7 ± 0.1	1.4%	13.3%	50	46.2 ± 0.9	2.0%	–7.6%
	50	49.0 ± 1.3	2.6%	–2.0%	500	478 ± 11	2.3%	–4.4%
	95	94.4 ± 1.1	1.1%	–0.7%	950	913 ± 16	1.7%	–3.9%

* %Delta is the relative difference in the measured and prepared concentrations. Both sample sets were measured using a calibration curve prepared in blank rat serum.

Table VI. Accuracy and Precision Data for Saliva Samples (N = 6) Prepared with Cotinine at 2.5 × LOQ and 50% and 95% of the Calibrated Range*

Prepared (ng/mL)	Measured Mean ± SD	RSD	%Delta
50	51 ± 3	6.3%	1.8%
500	555 ± 11	1.9%	11.0%
950	1038 ± 20	1.9%	9.2%

* %Delta is the relative difference in the measured and prepared concentrations.

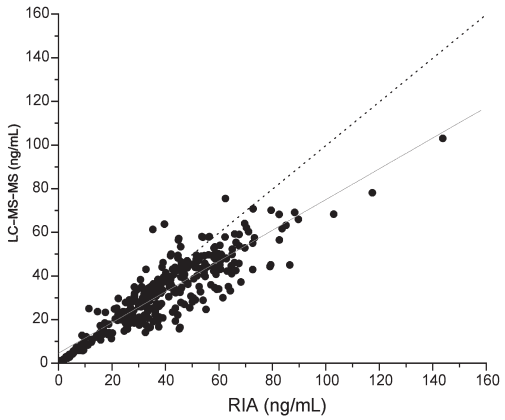

Figure 6. Plot of RIA versus LC–MS–MS data for nicotine in 322 human serum samples. The regression equation is $Y = 0.705X + 4.5$, $R = 0.881$. The line of unity (i.e., where $X = Y$) is shown as a dashed line in the plot. A paired *t*-test showed the RIA data were significantly higher than the LC–MS–MS data ($P = 5.5 \times 10^{-6}$).

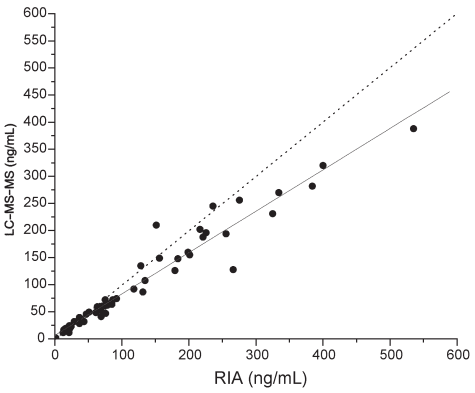

Figure 7. Plot of RIA versus LC–MS–MS data for nicotine in 63 rat serum samples. The regression equation is $Y = 0.763X + 6.7$, $R = 0.974$. The line of unity (i.e., where $X = Y$) is shown as a dashed line in the plot. A paired *t*-test showed the RIA data were significantly higher than the LC–MS–MS data ($P = 3.6 \times 10^{-5}$).

smokers. Sixty-three rat serum samples were also analyzed. Cotinine was compared in 21 saliva samples representing 7 different subjects. In addition, a set of freshly prepared samples spiked with nicotine at different levels in both serum and buffer solution were analyzed for nicotine by both methods. Data reported for the RIA determinations were based on agreement of duplicate samples (standard practice); if the duplicates had a RSD > 10%, then the data were discarded and the sample repeated.

Figure 6 is a plot of nicotine concentrations for the set of human serum samples of the RIA against the LC–MS–MS data. The line of unity in this plot (dashed line) gives the line where data would fall if both methods gave identical determinations for the same samples (i.e., $X = Y$). For the entire set ($N = 322$), the data were statistically different ($P < 0.05$) using a paired *t*-test with the mean RIA data 25% higher than the mean LC–MS–MS data. From the plot in Figure 6, most of the deviation in the two methods occurred at higher concentrations. In fact, from 0–10 ng/mL, the regression equation is $Y = 0.968X - 0.35$ ($N = 32$, $R = 0.896$) with the slope approaching unity and the sets are not statistically different ($P > 0.05$). Cross-reactivity of the assay, as has been noted with other nicotine metabolites (7), may be a factor at higher levels of exposure.

Similar results were observed for the rat serum plot shown in Figure 7, with the LC–MS–MS mean data 15% lower than the RIA data and statistically different ($P < 0.05$). A plot of cotinine in human saliva is shown in Figure 8. Similar to the other data sets, the RIA concentrations averaged 28% higher than the mean LC–MS–MS concentrations and are statistically different ($P < 0.05$).

Additional comparisons were per-

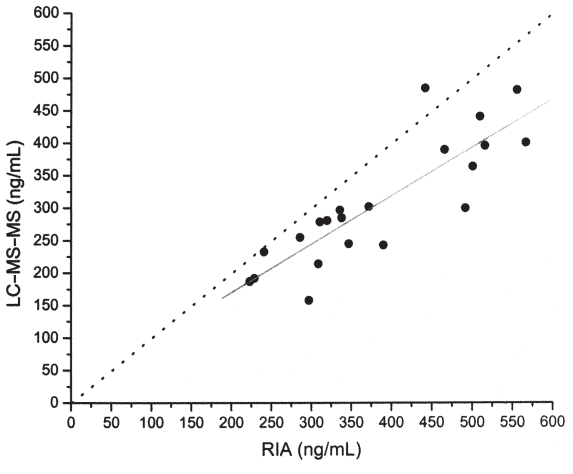

Figure 8. Plot of RIA versus LC–MS–MS data for cotinine in 21 saliva samples. The regression equation is $Y = 0.744X + 21$, $R = 0.851$. The line of unity (i.e., where $X = Y$) is shown as a dashed line in the plot. A paired *t*-test showed the RIA data were significantly higher than the LC–MS–MS data ($P = 5.5 \times 10^{-6}$).

Table VII. Comparison of Nicotine Determined in Spiked Samples of Blank Serum and Buffer by Two Methods

		LC–MS–MS			RIA		
	Prepared (ng/mL)	Measured mean ± SD	RSD	%Delta	Measured mean ± SD	RSD	%Delta
Serum	0	< LOQ			3.2 ± 1.8	56%	
	5	5.1 ± 0.4	7.0%	2.7%	8.4 ± 1.7	21%	67.4%
	20	20.7 ± 1.6	7.5%	3.5%	19.2 ± 2.5	13%	–4.1%
	40	40.3 ± 1.2	2.9%	0.8%	45.6 ± 3.0	6.6%	14.1%
Buffer	0	< LOQ			4.4 ± 1.9	43.3%	
	5	5.0 ± 0.6	11.3%	0.3%	6.8 ± 0.8	12.3%	36.3%
	20	20.4 ± 1.7	8.2%	2.2%	21.9 ± 3.5	16.1%	9.6%
	40	39.9 ± 1.3	3.2%	–0.3%	49.4 ± 4.8	9.7%	23.4%

formed by determining nicotine in spiked serum and phosphate buffered saline samples. The samples were analyzed blind and included a set of four blank human serum samples spiked with at 0, 5, 20, and 40 ng/mL nicotine (and 10 times these amounts of cotinine) and a similar series of spiked phosphate buffered saline samples. Six aliquots of each sample were analyzed and the results are listed in Table VII. For LC–MS–MS, all measured concentrations agreed within ± 15% of the prepared concentrations and all RSDs were < 15%. Except for the samples spiked at 20 ng/mL, the RIA data yielded somewhat higher measured concentrations than the prepared concentrations. None of the RIA samples were blank corrected. Because these samples were prepared and analyzed by both methods concurrently, the higher biases observed in the RIA data at higher concentrations seem genuine.

Conclusion

A simple, rapid method was developed and validated for nicotine and cotinine in human serum. Validation was successfully extended to rat serum for both nicotine and cotinine and to human saliva for cotinine. The method is linear in the range of 2–100 ng/mL for nicotine and 20–1,000 ng/mL cotinine. The single SPE step and specificity of the MRM monitoring of analyte and internal standard permit a rapid chromatography method with both analytes eluting in less than 1 min. Sample throughput exceeds that of the current RIA method, with routine loads of 500 samples per week performed. The requirement of only 100 µL of serum permits this method to be applied to rodent serum samples, which often are of limited volume.

Our approach is similar to that of Moyer (13) in using one SPE step; however, the 96-well format greatly simplifies sample preparation. The use of normal-phase chromatography permits elution from the extraction plate with an organic mixture (methanol–acetonitrile) similar to the mobile phase. Thus, the final collection plate is placed directly into the autosampler without evaporation or reconstitution.

Although the RIA method is rugged and requires less complicated and expensive instrumentation, the LC–MS–MS showed improved accuracy and precision on spiked serum and buffer samples. This likely results from reduced matrix effects in the LC–MS–MS method.

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