Analysis of [3′,3′-d₂]-nicotine and [3′,3′-d₂]-cotinine by capillary liquid chromatography–electrospray tandem mass spectrometry

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Abstract

A selective and sensitive LC/MS/MS assay was developed for the quantification of d_2-nicotine and d_2-cotinine in plasma of current and past smokers administered d_2-nicotine. After solid phase extraction and liquid–liquid extraction, HPLC separation was achieved on a capillary hydrophilic interaction chromatography phase column. The analytes were monitored by tandem mass spectrometry with electrospray positive ionization. Linear calibration curves were generated for d_2-nicotine (0.03–6.0 ng/ml plasma) and d_2-cotinine (0.15–25 ng/ml plasma). The lower limits of quantitation were 0.15 ng/ml and 0.25 ng/ml for d_2-nicotine and d_2-cotinine, respectively. The coefficient of variation was 3.7% for d_2-nicotine and 2.5% for d_2-cotinine. The method was applied to two ongoing studies of d_2-nicotine metabolism in prior and current smokers. Preliminary analysis of a subset of subjects from these studies detected a significantly lower rate of nicotine conversion to cotinine by past smokers compared to current smokers.
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1. Introduction

Nicotine is the main addictive agent in tobacco [1,2]. Smokers typically adjust their nicotine intake to maintain plasma nicotine levels. However, plasma nicotine concentrations vary among smokers due to both the extent of smoking and to individual differences in nicotine metabolism [3]. In smokers, nicotine is extensively metabolized, primarily by the hepatic enzyme P450 2A6, although P450 2A13, expressed in the respiratory tract, may also contribute [3,4]. P450 2A6 and P450 2A13 catalyze nicotine 5′-oxidation to the corresponding iminium ion that is then converted, by either aldehyde oxidase or P450 enzymes, to cotinine [5]. In both tobacco users and individuals using nicotine replacement therapies greater than 70% of the nicotine dose is converted to cotinine, which is subsequently oxidized to *trans*-3′-hydroxycotinine [3]. Plasma cotinine concentrations are routinely used to assess both tobacco use and environmental tobacco smoke (ETS) exposure [6–8]. However, plasma cotinine levels vary as much as two-fold due to individual differences in nicotine metabolism [3]. There are many studies being carried out to assess the role of nicotine metabolism in smoking behavior and nicotine addiction [9–11].

To study nicotine metabolism in both smokers and non-smokers, Benowitz and co-workers have carried out a number of elegant studies with [3′,3′-D₂]-nicotine (d_2-nicotine). The majority of these studies have analyzed d_2-nicotine and d_2-cotinine by GC/MS [12]. However, more recently the liquid chromatography-atmospheric pressure ionization–tandem mass spectrometry (LC-APCI–MS/MS) method developed by Bernert and co-workers for the determination of serum cotinine concentrations [13] has been applied to the analysis of d_2-cotinine [14–16]. Two of these studies also quantified urine concentrations of d_2-nicotine by LC/MS/MS [15,16], but GC/MS analysis was used to quantify plasma d_2-nicotine, suggesting that the LC/MS/MS method used was not sufficiently sensitive for the analysis of the lower d_2-nicotine concentrations in plasma.

A number of LC/MS/MS methods have been developed for the measurement of nicotine and cotinine in biological fluids. These methods were recently reviewed by Heavner et al. [17].

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The limit of quantification ranged from 1 to 10 ng/ml for nicotine and from 0.1 to 10 ng/ml for cotinine. A lower limit of quantification for cotinine, 0.05 ng/ml plasma, was reported by Bernert et al. [13]. Their method used both liquid–liquid extraction and solid phase extraction for sample clean-up and analyzed cotinine by reverse phase HPLC linked to atmospheric pressure (APCI) MS/MS. This method was sensitive, selective and robust. Only a handful of studies have used electrospray ionization (ESI) MS/MS for the analysis of either nicotine or cotinine [18–21]. For compounds with a high proton affinity, APCI is reported to be the more efficient ionization process [17]. In addition, APCI is much less sensitive than is ESI to interference from matrix on ionization, specifically salts and ion pairing agents present in the mobile phase used for reverse phase HPLC analyses. However, the use of silica-based columns operated under hydrophilic interaction chromatography mode (HILIC) has resulted in an increase in the sensitivity of LC–ESI/MS/MS detection of basic compounds [20–22].

In the study reported here, we extend the use of HILIC–ESI/MS/MS analysis to capillary chromatography to further lower the limit of detection for nicotine and cotinine in plasma. We apply this method to the determination of plasma d_2-cotinine and d_2-nicotine levels in smokers and non-smokers who were administered $[3',3'-d_2]$-nicotine. Unlike earlier reports [13,14], this assay quantifies both deuterated and non-deuterated nicotine in plasma by LC/MS/MS analysis.

2. Experimental

2.1. Chemicals and reagents

(−)-Nicotine (98%) was obtained from Sigma (St. Louis, MO). (−)-Cotinine, (d_3-methyl)-nicotine (99%), and (d_3-methyl)-cotinine (99%) was purchased from Toronto Research Chemicals (New York, ON, Canada). $[3',3'-D_2]$-nicotine and $[5',5'-D_2]$-cotinine was synthesized as previously described [23]. Both di-deuterated compounds were >99.4% pure as determined by GC/MS, LC/MS and LC/MS/MS analysis; less than 0.1% of the non-deuterated compounds were detected. The location of the label was confirmed by NMR analysis. All other solvents and reagents were of highest analytical grade and purchased from Sigma.

2.2. Stock solutions and calibration standards

Primary stock solutions of d_0, d_2, and d_3-nicotine and cotinine (5 µg/ml) were prepared in both water and methanol and stored at −20 °C. The concentrations of the primary stock solutions were determined by UV spectrophotometry. A working solution of the internal standard (IS) was prepared in methanol and contained 0.082 ng/µl of both (d_3-methyl)-nicotine and (d_3-methyl)-cotinine. The primary stock solutions of d_0, d_2, and d_3-nicotine and cotinine were diluted with methanol to working stock solutions of 0.5 ng/µl, 0.05 ng/µl, and 0.005 ng/µl and used to optimize the MS/MS response. Working stock solutions that were analyzed directly by LC/MS/MS without going through the sample preparation protocol described below

were prepared from the primary stock solutions prepared in methanol. All other working stock solutions were prepared in water. Aliquots of these solutions were kept frozen at −20 °C prior to use.

Quantification was based on the addition of a known amount of IS. Calibration standards were prepared by adding decreasing amounts of the d_2-nicotine and d_2-cotinine working stock solutions to water, plasma from a non-smoker and plasma from a smoker. IS (0.82 ng) was added to each calibration standard and the samples prepared as described in the "sample preparation" section. d_2-nicotine and d_2-cotinine (0.005–5 ng) was also added directly to 100 µl methanol containing IS (8.2 pg/µl) to determine the instrument response.

2.3. Sample preparation

The plasma samples were stored at −20 °C until workup. The samples have currently been stored for up to 2 years without any noticeable degradation of the deuterated nicotine or cotinine. For analysis, plasma was thawed, mixed thoroughly and 100 µl or 200 µl added to 4 ml silanized vials containing 900 µl water and 2 ml Dulbecco's 10 mM phosphate buffered saline (pH 7.4, PBS). For method validation, the amount of plasma used ranged from 20 µl to 1 ml. IS (10 µl) was added to each sample and the samples were vortexed gently. Samples were loaded on Oasis MCX columns (60 mg sorbent material with an average particle diameter of 53 µm and 6 ml cartridge volume (Waters Corporation, Milford, MA), that had been prepared according to the manufacturer's recommendations (the columns were activated with 3 ml methanol and equilibrated with 3 ml of water followed by 3 ml PBS). The columns were then washed with 3 ml each of water, 0.1 N HCl, and methanol, and the samples eluted with 3 ml of methylene chloride/isopropanol/ammonium hydroxide (78:20:2). The eluted cotinine and nicotine were further purified with a series of acid–base liquid–liquid extractions. Water (1–2 ml) was added to the eluted samples, which were then extracted and the aqueous layer discarded. The organic layer was then extracted with an equal volume of 1 N hydrochloric acid. The aqueous layer was removed, an equal volume of 50% potassium carbonate added to it and cotinine and nicotine extracted into methylene chloride. The methylene chloride layer was transferred to a 4-ml silanized glass vial and 200 µl of methanol was added. The samples were then dried under nitrogen gas to a volume of 50–100 µl methanol.

2.4. Liquid chromatography–tandem mass spectrometry analysis

LC/MS/MS analysis was performed on an Agilent 1100 series capillary HPLC system (Agilent Technologies, Palo Alto, California) interfaced to a Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer (Thermo Electron, San Jose, California). The samples (0.2–3 µl) were injected onto a 300 µm × 100 mm Atlantis HILIC (5 µm particle size), silica-based, capillary column (Waters Corporation). Nicotine and cotinine were analyzed independently by injecting each

sample twice. d_0-, d_2-, and d_3-Nicotine were eluted isocratically with 7.5 mM ammonium acetate–acetonitrile (23:77, v/v) containing 0.4% acetic acid, at a flow rate of 15 µl/min. d_0-, d_2-, and d_3-cotinine were eluted isocratically with water–acetonitrile–formic acid (15:85:0.2, v/v/v) at a flow rate of 15 µl/min. The column was operated at 20 °C. For the first minute, the eluant was diverted to waste before directing the flow into the ESI source of the mass spectrometer. The void volume of the system at the 15 µl/min flow rate was 0.84 min. Alternatively, nicotine and cotinine were analyzed simultaneously with a mobile phase gradient from 99.8% acetonitrile (0.2% formic acid) to 59.8% water:40% acetonitrile:0.2% formic acid.

The ESI source was operated in the positive ion mode with a collision energy of 25 V, the tube lens offset set to 60 V, the collision gas pressure set to 1.4 mTorr and the source CID collision energy set to 10 V. Peak area obtained from selected reaction monitoring (SRM) of the mass transitions for the various analytes were used for quantification. The scan width was 0.35 (m/z) and the scan time 0.2 s and the peak widths for both Q1 and Q3 were 0.30. The mass transitions were as follows: d_0-nicotine (m/z $163 \rightarrow 130$ and m/z $163 \rightarrow 117$), d_2-nicotine (m/z $165 \rightarrow 131$ and m/z $165 \rightarrow 118$), d_3-nicotine (m/z $166 \rightarrow 130$ and m/z $166 \rightarrow 117$), d_0-cotinine (m/z $177 \rightarrow 98$ and m/z $177 \rightarrow 80$), d_2-cotinine (m/z $179 \rightarrow 100$ and m/z $179 \rightarrow 80$), d_3-cotinine (m/z $180 \rightarrow 101$ and m/z $180 \rightarrow 80$).

The presence of common product ions for some of the SRM transitions theoretically could result in "cross-talk" between these transitions due to incomplete clearance of the collision cell resulting in quantitation errors. The acquisition time for the SRM transitions of 0.2 s is long enough such as that the effect should be negligible. The injection of each analyte and internal standard independently of the others while monitoring all SRM transitions confirmed this to be the case.

2.5. Application

A smoker who had abstained from smoking overnight was administered d_2-nicotine intravenously (2 µg/min/kg) and blood samples were collected in plasma separator tubes (Vacutainer PST gel tube with green marble top, Fisher Scientific Corp.). Plasma was frozen at −20 °C until analysis. No loss of cotinine or nicotine in the PST gel was detected when blood from non-smoking individuals was supplemented with either cotinine or nicotine. In a second on-going study, subjects, current smokers and prior smokers (abstinent for more than 1 year), were administered 2 mg d_2-nicotine orally, blood samples were collected at 30 min, 90 min and 240 min. Samples from the 30 min collection were analyzed by the method described above. Samples from both studies were analyzed in batches of 12–16 with a positive control (a pooled plasma sample from a non-smoking subject administered d_2-nicotine) and a water blank. For each batch all samples and controls were analyzed in duplicate. Both protocols were approved by the University of Minnesota Human Subjects review board.

3. Results and discussion

3.1. Chromatography

Recently, Shou and Naidong [21] described a HILIC–ESI-tandem mass spectrometry (MS/MS) method for the analysis of eight basic compounds. Nicotine was one of the eight analyzed, however, the response for nicotine was significantly less than for many of the other compounds. We report here the development of a sensitive new method for the analysis of nicotine and cotinine in plasma that utilizes capillary HILIC–ESI-MS/MS. This method has been applied to the characterization of d_2-nicotine metabolism in smokers. Both undeuterated and deuterated nicotine and cotinine were quantified in plasma.

In our initial experiments, we analyzed cotinine and nicotine in a single LC analysis using a gradient of water and acetonitrile containing 0.2% formic acid. However, the use of an elution gradient required significant time to re-equilibrate the capillary column between injections. In addition, the conditions for optimal peak shape were different for cotinine and nicotine. Therefore, the independent analysis of nicotine and cotinine by two isocratic LC systems was both more time efficient and more reproducible than a gradient system. We began our analysis using conditions similar to those of Shou and Naidong [21] in which the mobile phase contained a small amount of trifluoroacetic acid (TFA) with either formic or acetic acid, and acetonitrile. However, we obtained good sensitivity and reproducible peak shape for nicotine with a mobile phase that was 23% 7.5 mM ammonium acetate, 76.7% acetonitrile and 0.3% acetic acid. The inclusion of ammonium acetate in the mobile phase was essential for optimum nicotine peak shape. Cotinine peak shape was best when the mobile phase did not contain ammonium acetate. In both cases, a low pH (<3) was critical for good peak shape (Fig. 1 A–F). Cotinine peak shape was very sensitive to the presence of water in the sample. Water (<0.5%) caused significant peak broadening and an increase in retention time (data not shown). Therefore, during sample preparation, it was important not to carry any water into the final methylene chloride extraction. The liquid–liquid extractions following the solid phase extraction of the samples were necessary to obtain reproducible chromatography on the capillary column and to maintain the sensitivity of the method.

3.2. LC/MS/MS Analysis

The product ion spectra obtained by ESI MS/MS analysis of the $(M+H)^+$ ion for d_0-, d_2-, and d_3-nicotine are illustrated in Fig. 2. The daughter spectra for d_0- and d_3-nicotine, m/z 163 and m/z 166, are identical; the most abundant ion is m/z 130 and the second most abundant ion is m/z 117 (Fig. 2A and C). The spectrum for d_2-nicotine is slightly different. The major fragment is m/z 131, due to the presence of one deuterium atom (Fig. 2B). The second most abundant ion is m/z 118; an ion, m/z 117 was present at a slightly lower abundance. The m/z 118 ion must contain one deuterium atom whereas the m/z 117 ion is likely the same fragment observed with d_0- and d_3-nicotine.
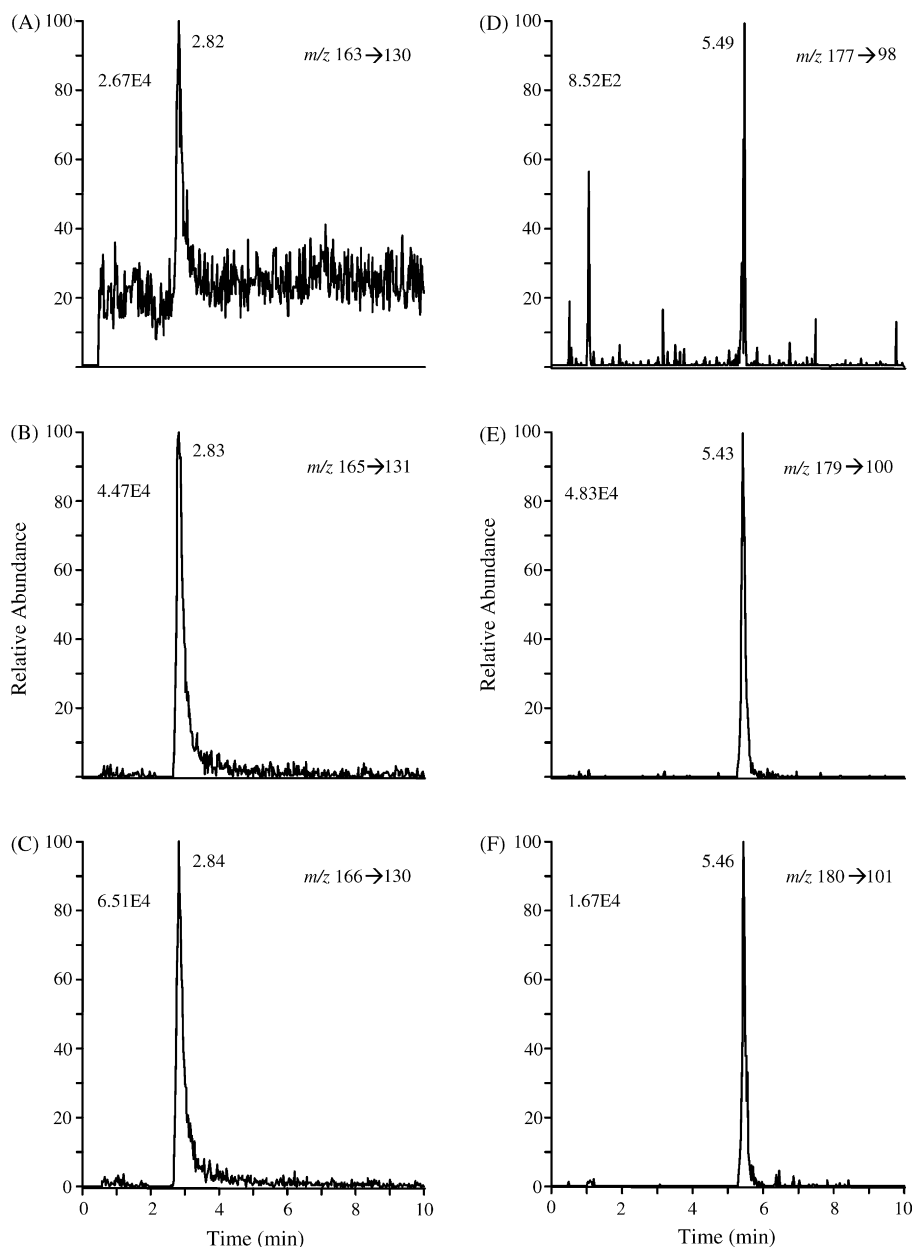
Fig. 1. Capillary HILIC-ESI SRM analysis of a plasma sample from a non-smoker administered d_2-nicotine orally. Six ion pair transitions were monitored d_0-nicotine, m/z $163 \rightarrow 130$ (A), d_2-nicotine, m/z $165 \rightarrow 131$ (B), d_3-nicotine, m/z $166 \rightarrow 130$ (C), d_0-cotinine, m/z $177 \rightarrow 98$ (D), d_2-cotinine, m/z $179 \rightarrow 100$ (E), d_3-cotinine, m/z $180 \rightarrow 101$ (F). Different chromatographic conditions were used for nicotine (A–C) and cotinine (D–F). Plasma (100 μl) was worked up for analysis and 1 μl of the 100 μl methanol preparation was injected.

The daughter ion spectra for d_0-, d_2-, and d_3-cotinine are similar to what has previously been reported for ESI-MS/MS analysis of d_0-cotinine [24]. The major fragment for all three cotinine isotopes is m/z 80. The second most abundant ions were m/z 98, 100, and 101 for d_0-, d_2-, and d_3-cotinine, respectively. These ions correspond to a d_0-, d_2-, and d_3-N-methylpyrrolidine fragment.

To quantify d_0- and d_2-nicotine and d_0- and d_2-cotinine plasma concentrations, SRM was used. Two ion transition pairs were monitored for each analyte. The ratio between the two transitions was used as confirmation of the identity of d_0- and d_2-nicotine or d_0- and d_2-cotinine. For d_2-cotinine the ratio of

100/80 was 0.54 ± 0.04 ($n = 32$) and for d_2-nicotine the ratio of 131/118 was 1.82 ± 0.13 ($n = 28$). As noted previously in the literature, the ratio of two progeny ion from multiple reaction ion monitoring (MRM) are not fundamental and may vary with analytical conditions, such as operation of Q2, the collision cell [13]. Therefore, the evaluation of these ratios in samples was based on standards analyzed at the same time under the same conditions.

To quantify d_0-, and d_2-nicotine the most abundant transitions, m/z $163 \rightarrow 130$, m/z $165 \rightarrow 131$, and m/z $166 \rightarrow 130$ for d_0-, d_2-, and d_3-nicotine, respectively, were used. However, to quantify d_0-,and d_2-cotinine the second most abundant
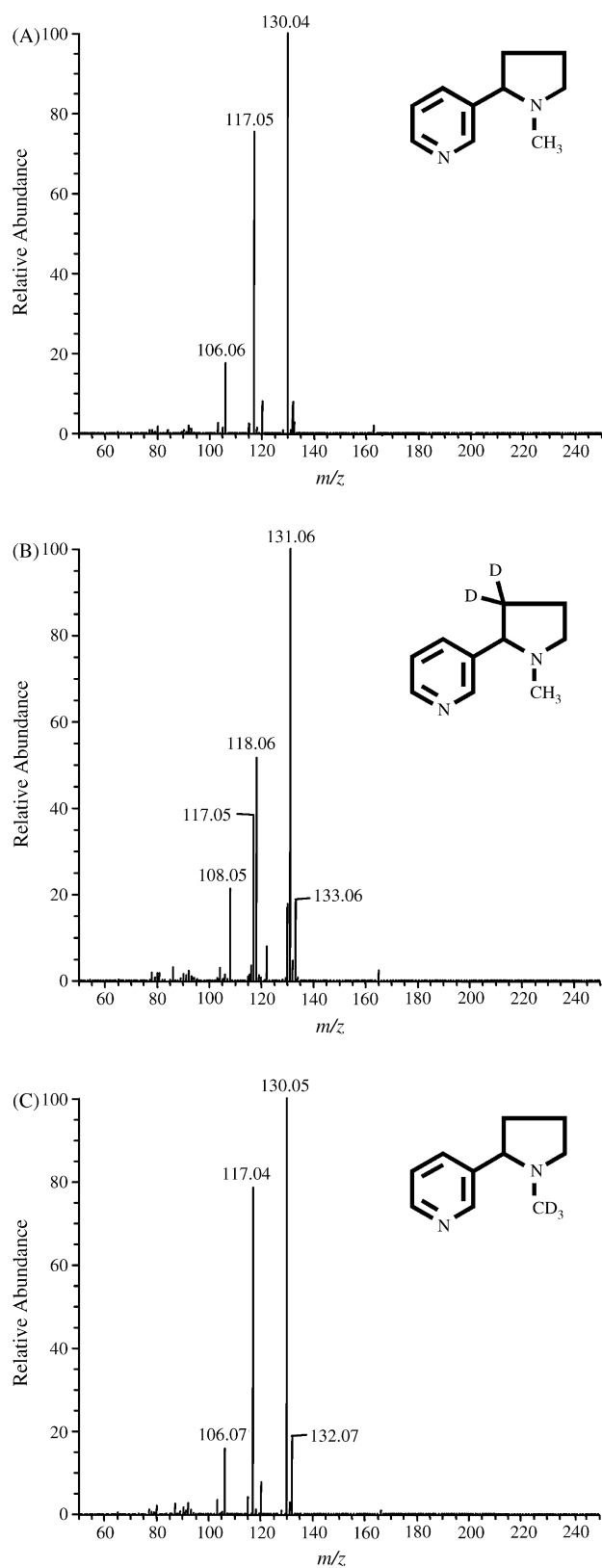
Fig. 2. ESI-MS/MS analysis of d_0-nicotine (A), d_2-nicotine (B) and d_3-nicotine (C). One ng of each of the three nicotine isotopes was injected onto the HILIC column and daughter ion spectra were collected at m/z 163, 165 and 166 for d_0-, d_2- and d_3-nicotine, respectively.
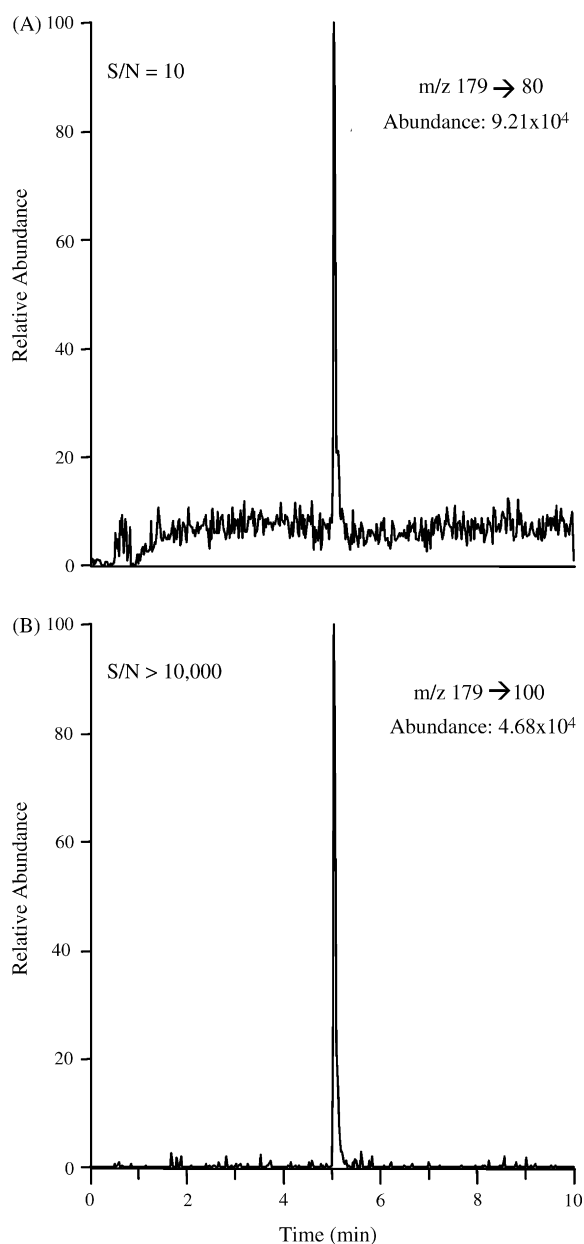

Fig. 3. Signal-to-noise comparison between the m/z 179 → 80 and 179 → 100 mass transitions for d_2-cotinine, obtained from HILIC–ESI-MS/MS analysis of plasma from a non-smoker administered d_2-nicotine orally.

ion transitions were used: m/z 177 → 98, m/z 179 → 100, m/z 180 → 101 for d_0-, d_2-, and d_3-cotinine, respectively. The signal-to-noise ratio for these transitions was significantly greater than for the most abundant transitions. The difference in signal to noise for the two transition ion pairs monitored for d_2-cotinine is illustrated in Fig. 3.

The presence of isotopomers of d_0-cotinine with the same nominal mass as d_2-cotinine (primarily due to the natural abundances of ^{13}C (1.11%), ^{15}N (0.37%), and ^{18}O (0.20%)) will result in a contribution to the d_2-cotinine SRM signal from d_0-cotinine present in the sample. The percent contribution from the d_0-cotinine is dependent upon the naturally occurring isotopic abundances, the molecular formula of the fragment ion, and any

Table 1
SRM analysis of d_0-, d_2-, d_3-nicotine and d_0-, d_2-, d_3-cotinine standards[a]

Analyzed compound	Mass transitions (percent of area)[b]		
	$163 \rightarrow 130$	$165 \rightarrow 131$	$166 \rightarrow 130$
d_0-Nicotine	99.8	0.16	–
d_2-Nicotine	–	99.6	0.35
d_3-Nicotine	–	–	100

Analyzed compound	Mass transitions (percent of area)[b]		
	$177 \rightarrow 98$	$179 \rightarrow 100$	$180 \rightarrow 101$
d_0-Cotinine	99.5	0.5	0.01
d_2-Cotinine	–	94.3	5.7
d_3-Cotinine	–	–	100

[a] One microliter of a 5 ng/μl standard solution of each cotinine and nicotine isotope was injected onto the HILIC column and analyzed by SRM, for the three transitions listed.

[b] The area % was calculated by dividing the area corresponding to each mass transition by the total area of the two or three mass transitions for a given standard solution. The value is the average of two independent determinations, which differed by <2%.

significant isotopic dependence of the fragmentation pathway. This was determined empirically using an authentic standard and is listed in Table 1. A similar contribution to the measured d_3-cotinine signal from d_2-cotinine is also in Table 1 as are similar analogous values for the nicotine analysis. These values were used to correct for the presence of d_0-cotinine and d_0-nicotine in the d_2-cotinine and d_2-nicotine analyses. The analysis of d_2-cotinine in the plasma of smokers administered d_2-nicotine is an example of when this correction was significant.

3.3. Characteristics of the method

The limit of detection of the SRM capillary LC/MS/MS method was determined by analyzing the response for injections of methanol containing 0.05–500 pg of d_0, d_2, d_3-nicotine and d_0-, d_2-, d_3-cotinine. The response was linear from 0.125 to 500 pg injected. The signal to noise ratio for 0.05 pg cotinine or nicotine injected was <50. Based on a SD of less than 10%, the limits of quantification of nicotine and cotinine standard injected on capillary LC were determined to be 0.125 and 0.24 pg, respectively. [The mean peak area ± SD ($n = 5$) for 0.24 pg d_2-cotinine was 3020 ± 261 (CV 9.0%). The mean ± SD ($n = 5$) for 0.125 pg d_2-nicotine was 49860 ± 3138 (CV, 6.0%)]. Therefore, if a 100 μl plasma was prepared for analysis and 1 μl of the resulting 100 μl methanol sample is injected for LC/MS/MS analysis, then theoretically the lowest limit of quantitation that may be obtained is 0.24 ng/ml d_2-cotinine and 0.12 ng/ml d_2-nicotine.

The limit of quantification of the assay was experimentally determined by analyzing non-smoker plasma samples (100 μl) supplemented with varying amounts of d_2-nicotine or d_2-cotinine. Both nicotine and cotinine were linear over the range of concentrations tested, 0.15–25 ng/ml for cotinine and 0.03–6 ng/ml for nicotine. The equations of the calibration curves obtained were $y = 1.09x - 0.05$ and $y = 1.02x - 0.01$ for d_2-nicotine and d_2-cotinine, respectively. The regression con-

Table 2
Calculated concentrations for LS/MS/MS determination of spiked plasma[a]

Compound	Expected (ng/ml)	Observed (mean ± SD) (ng/ml)	Observed/ expected (%)
d_2-Nicotine[b]	0.03	0.03[c]	
	0.15	0.14 ± 0.02	93
	0.3	0.29 ± 0.02	97
	0.6	0.62 ± 0.06	103
	3.0	3.0 ± 0.32	100
	6.0	6.6[c]	
d_2-Cotinine[d]	0.15	0.18 ± 0.041	128
	0.25	0.26 ± 0.042	104
	0.5	0.54 ± 0.090	108
	2.5	2.7 ± 0.08	112
	5.0	5.0 ± 0.46	100
	12.5	12.5 ± 1.07	100
	25.0	25.5 ± 1.11	102

[a] Either d_2-nicotine or d_2-cotinine was added to 100 μl plasma from a non-smoker at the indicated concentrations, and the samples prepared as described in Section 2.

[b] $n = 4$.

[c] $n = 2$.

[d] $n = 5$.

stants for both compounds were >0.99. The accuracy of the assay was good over the range tested and the limit of quantitation (based on a SD < 20%) was 0.25 ng/ml for d_2-cotinine and 0.15 ng/ml for d_2-nicotine (Table 2). Ion suppression of the analyte and internal standard signal due to coeluting material from the plasma matrix should be minimal due to the extensive sample clean up procedure employed. The isotopically labeled d_3-cotinine and d_3-nicotine internal standards coelute with the non-deuterated and di-deuterated cotinine and nicotine analytes, respectively, eliminating any effect ion suppression would have on the ability of the assay to properly quantify the analyte levels. This is confirmed by the linearity of the standard addition experiment. The variation in the assay with different amounts of plasma was assessed using plasma volumes as low as 20 μl and as high as 1 ml. Typically 100 or 200 μl of plasma was used for this assay, but when 20 μl plasma was used the results were identical (Table 3).

Intra- and inter-day variability was determined for a plasma sample spiked with d_2-nicotine and d_2-cotinine and a sample

Table 3
Determination of nicotine and cotinine concentration in varying plasma volumes[a]

Plasma volume (μl)	d_2-Nicotine[b] (ng/ml)	d_2-Cotinine[b] (ng/ml)
20	1.4 ± 0.16	24.8 ± 1.37
50	1.3 ± 0.09	23.5 ± 0.55
200	1.2 ± 0.10	23.3 ± 0.49

[a] Different volumes of plasma (a positive control sample prepared by pooling plasma from several individuals who were administered d_2-nicotine) were analyzed for d_2-nicotine and d_2-cotinine according to the protocol described in Section 2.

[b] The values are the means ± SD of three independent experiments performed in duplicate.

Table 4
Intra- and interday variability of the LC/MS/MS analysis

	Compound	Day 1 (n = 6)		Day 2 (n = 6)		Day 3 (n = 6)		Days 1–3	
		Mean (ng/ml) (range)	%CV	Mean (ng/ml) (range)	%CV	Mean (ng/ml) (range)	%CV	Mean (ng/ml)	%CV
Positive control[a]	d_2-Nicotine	0.84 (0.78–0.91)	5.4	0.85 (0.80–0.95)	5.9	0.86 (0.82–0.92)	5.0	0.85	0.4
	d_2-Cotinine	12.44 (12.14–12.84)	1.8	12.54 (12.09–13.11)	2.8	13.23 (12.2–13.99)	5.2	12.74	1.9
Standard (1 ng/ml)[b]	d_2-Nicotine	0.94 (0.90–0.98)	2.7	1.02 (0.92–1.06)	5.5	0.94 (0.92–1.00)	3.2	0.97	4.6
	d_2-Cotinine	0.94 (0.81–1.11)	12	1.06 (0.97–1.19)	7	0.94 (0.79–1.07)	12	0.98	7

[a] Plasma from subject administered d_2-nicotine.
[b] Plasma supplemented with 1 ng/ml d_2-nicotine and d_2-cotinine.

Table 5
Assay reproducibility[a]

Positive control sample[b]	Day 1 (n = 2)	Day 2 (n = 2)	Day 3 (n = 2)	Day 4 (n = 2)	Day 5 (n = 2)	Overall, days 1–5 (n = 10)		
	Average (ng/ml)					Average (ng/ml)	Standard deviation	%CV
Nicotine	2.44	2.26	2.46	2.45	2.46	2.41	0.09	3.7
Cotinine	25.9	25.5	26.8	26.2	27.2	26.3	0.67	2.5

[a] A plasma sample was worked up independently on 5 days and analyzed in duplicate.
[b] Plasma pooled from three individuals who had received d_2-nicotine.

from an individual who had been administered d_2-nicotine. Six injections from the same extracted sample were made on 3 days over a 1 month period (Table 4). The intra-day coefficients of variation (CV) ranged from 2.7 to 5.9% for d_2-nicotine and from 1.8 to 12% for d_2-cotinine. The higher CV at the lower cotinine concentration is primarily due to variable peak shape on the capillary column, and the difficulty in accurately integrating the peak. The inter-day CV was 0.4% for nicotine and 1.9% and 7%, respectively for low and high d_2-cotinine concentrations.

The analytical method reproducibility was determined from repeated analysis of a positive control sample consisting of plasma pooled from individuals who had been administered d_2-nicotine. Plasma samples were routinely analyzed in batches of 12–16. Each batch contained the positive control sample described above, and a water blank. The values obtained in the analysis of positive control from five consecutive analyses are presented in Table 5. The CV for d_2-nicotine was 3.7 % and for d_2-cotinine was 2.5%.

3.4. Application to plasma samples of subjects administered D_2-nicotine

The method described above was applied to two on-going studies. In one, d_2-nicotine was administered orally and plasma nicotine and cotinine concentrations were determined 30 min later. There were two groups of subjects in this study, current smokers and past smokers. The d_2-nicotine concentration was similar in both groups whereas d_2-cotinine levels were lower in past smokers (Table 6). The data are presented for only a subset of the 200 subjects in this study. However, it illustrates the usefulness of the method for simultaneously determining the concentrations of both deuterated and non-deuterated nicotine and cotinine in current smokers, who were not required to modify there normal smoking habits. In a second on-going

study d_2-nicotine was infused intravenously and its concentration and that of d_2-cotinine were monitored over time (Fig. 4). The peak plasma nicotine concentration occurred at 25 min and peak plasma cotinine concentrations at 120–150 min. At 23.5 h, d_2-nicotine was still detected in the plasma at a concentration of 0.5 ng/ml.
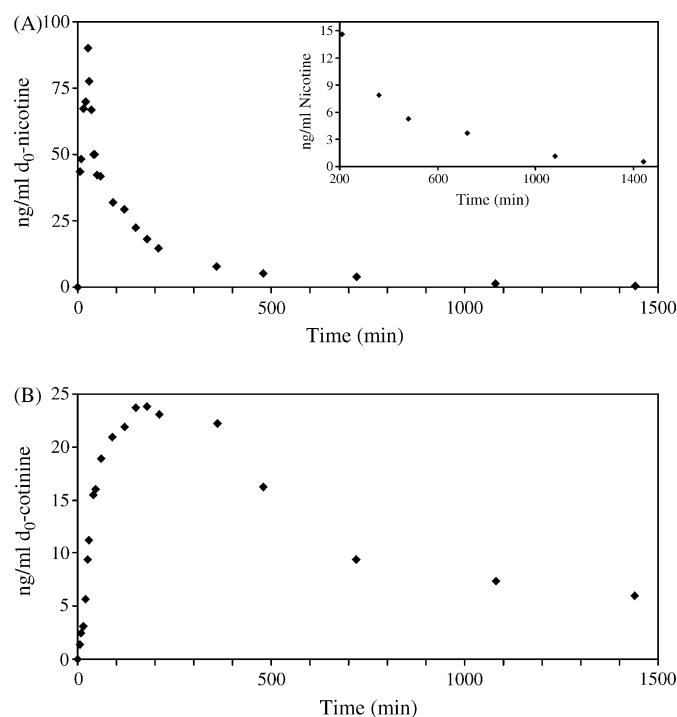

Fig. 4. Plasma d_2-nicotine (A) and d_2-cotinine concentrations (B) during and following the intravenous infusion of d_2-nicotine. d_2-Nicotine (2 μg/kg/min) was infused from 0 to 30 min. Insert shows plasma d_2-nicotine concentrations from 210 min to 23.5 h. Values are mean of duplicate determinations that differed by <10%.

Table 6
Application of HILIC–ESI-MS/MS method to the analysis of plasma d_2-nicotine and d_2-cotinine

	Plasma concentrations (ng/ml)			
	d_2-Nicotine	d_2-Cotinine	d_0-Nicotine	d_0-Cotinine
Smokers[a] ($n = 12$)	2.47 ± 1.02	17.8 ± 6.6	28.8 ± 8.1	440 ± 81
Past smokers[b] ($n = 10$)	2.7 ± 2.12	10.5 ± 6.4	ND[c]	0.6 ± 0.44

[a] Smokers and non-smokers were orally administered 2 mg d_2-nicotine and plasma collected for analysis at 30 min.

[b] Subjects have smoked a cigarette 20–90 min prior to d_2-nicotine administration.

[c] ND < 1.5 ng/ml, the limit of detection is due to contamination of the blanks with a mean of 1.5 ng/ml d_0-nicotine.

4. Conclusions

We report here the development of a LC-ESI/MS/MS method which uses capillary HILIC to quantify d_2-nicotine and d_2-cotinine in plasma of current smokers and non-smokers. Analysis may be carried out on as little as 20 μl of plasma and the limits of quantitation for d_2-cotinine and d_2-nicotine were 0.25 ng/ml and 0.15 ng/ml, respectively. These limits are similar to what has been reported previously for the analysis of d_2-cotinine in plasma by LC-APCI/MS/MS [14]. The method is being applied to on-going studies of nicotine metabolism in smokers and non-smokers administered d_2-nicotine. In smokers, non-deuterated nicotine and cotinine may be analyzed simultaneously with the deuterated isotopes.

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