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Determination of nicotine and cotinine in human plasma by liquid chromatography–tandem mass spectrometry with atmospheric-pressure chemical ionization interface

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Abstract

Here we report a sensitive liquid chromatographic–tandem mass spectrometric (LC–MS–MS) method capable of quantifying nicotine down to 1 ng/ml and cotinine to 10 ng/ml from 1.0 ml of human plasma. The method was validated over linear ranges of 1.0–50.0 ng/ml for nicotine and 10.0–500.0 ng/ml for cotinine, using deuterated internal standards. Compounds were simply extracted from alkalinized human heparinized plasma with methylene chloride, reconstituted into a solution of acetonitrile, methanol and 10 m*M* ammonium acetate (53:32:15, v/v) after the organic phase was dried down, and analyzed on the LC–MS–MS, which is a PE Sciex API III system equipped with a Keystone BDS Hypersil C_{18} column and atmospheric pressure chemical ionization (APCI) interface. The between-run precision and accuracy of the calibration standards were ≤6.42% relative standard deviation (R.S.D.) and ≤11.8% relative error (R.E.) for both nicotine and cotinine. The between-run and within-run precision and accuracy of quality controls, (2.5, 15.0, 37.5 ng/ml for nicotine and 25.0, 150.0, 375.0 ng/ml for cotinine), were ≤6.34% R.S.D. and ≤7.62% R.E. for both analytes. Sample stabilities in chromatography, in processing and in biological matrix were also investigated. This method has been applied to pharmacokinetic analysis of nicotine and cotinine in human plasma.

Keywords: Nicotine; Cotinine

1. Introduction

Cigarette smoking is the leading cause of preventable disease and death in the United States today. In the last few years there has been a dramatic increase in the determination of nicotine (Fig. 1A) and its metabolites in biological fluids for the evaluation of smoking cessation therapies [1–6]. The purpose of such smoking replacement treatment is to achieve a sufficient plasma concentration of nicotine to counter-act the craving for cigarettes. Cotinine (Fig. 1B), on the other hand, is a major nicotine metabolite. It has a longer elimination half-life (about 20 h compared with 2 h for nicotine), and is therefore an ideal measure of average nicotine intake [7]. It is a sensitive and specific biochemical marker of smoke intake for use in clinical trials.

Numerous methods have been introduced during

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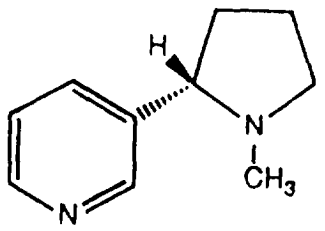
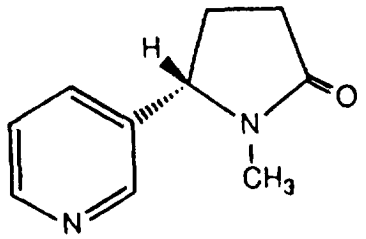


A



B

Fig. 1. Structure of (A) nicotine; (B) cotinine.

recent years for determination of nicotine and its metabolites. They include gas chromatography (GC) [8–11], sometimes coupled with mass spectrometry (GC–MS) [12–15], high-performance liquid chromatography (HPLC) [16–19], radioimmunoassay (RIA) [20], and enzyme-linked immunoassay (ELISA) [21]. However, these methods appear to have either a complicated extraction procedure, low sensitivity, or a long chromatographic run-time, prederivatization, or to be vulnerable to low recovery and contamination.

Here we present a liquid chromatographic–tandem mass spectrometric method (LC–MS–MS) for simultaneous quantitation of nicotine and cotinine in human plasma. By applying liquid chromatography equipped with a triple quadrupole mass spectrometer, this method is highly sensitive, specific, and with a high throughput. It is practical for processing a large number of clinical samples.

2. Experimental

2.1. Materials and reagents

Nicotine and cotinine were obtained from Sigma (St. Louis, MO, USA). The internal standards (I.S.s), nicotine-methyl-d_3 and cotinine-methyl-d_3, were from Cambridge Isotope Laboratories (Andover, MD, USA). Ammonium acetate and concentrated ammonium hydroxide were from Mallinckrodt (purchased from Midland Scientific, Omaha, NE, USA). HPLC grade methanol, acetonitrile and methylene chloride were obtained from Fisher (St. Louis, MO, USA). Heparinized control plasma was collected in-house from nonsmokers.

2.2. Sample preparation

Primary stock solutions of nicotine and cotinine were prepared from separate weighings for standards and quality control samples (QCs). The primary and subsequent working stocks were prepared in methanol and stored at −20°C during the validation.

Working standards were prepared by spiking an appropriate amount of concentrated stock solutions into blank control plasma. The calibration range were 1.0–50.0 ng/ml for nicotine and 10.0–500.0 ng/ml for cotinine. Three levels of QC samples were prepared at 2.5, 15.0 and 37.5 ng/ml for nicotine and 25.0, 150.0, and 375.0 ng/ml for cotinine. These QC concentrations were chosen near the low, medium, and high working standard concentrations being prepared. QCs were stored at −20°C with clinical samples to be analyzed.

2.3. Instrumentation

The HPLC system consisted of a Waters 501 pump and a Waters 717 autosampler (Milford, MA, USA). A BDS C_{18} guard column (10×2.0 mm I.D.) and an analytical column, BDS Hypersil C_{18} (100×3.0 mm I.D., 3 μm) were purchased from Keystone (Bellefonate, PA, USA). The HPLC system was operated isocratically at a flow-rate of 1.4 ml/min. The mobile phase consisted of acetonitrile–methanol–10 m*M* ammonium acetate (53:32:15, v/v).

The mass spectrometer was a Perkin-Elmer Sciex API-III plus triple quadrupole mass spectrometer

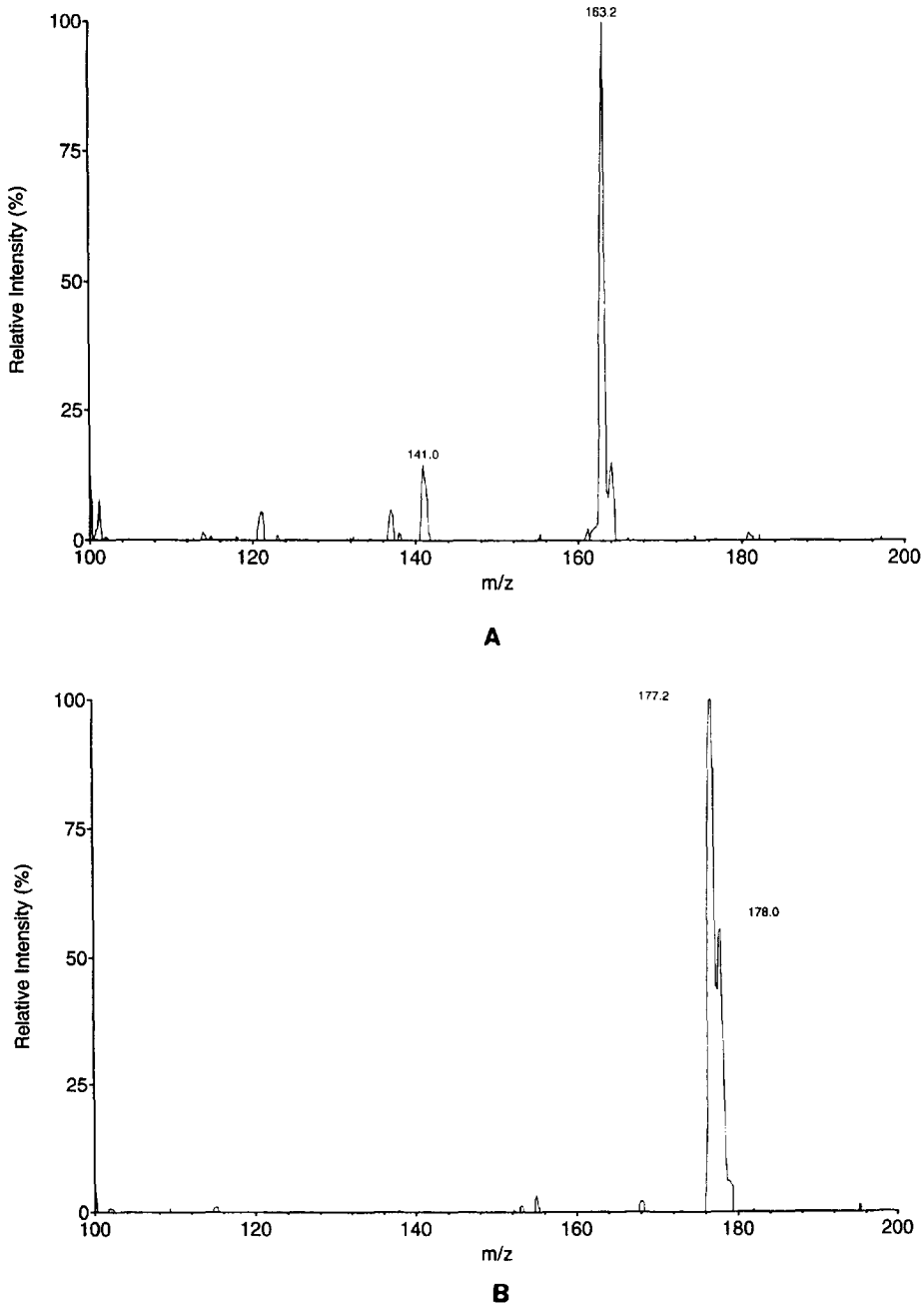


Fig. 2. Full scan Q1 mass spectra of (A) nicotine and (B) cotinine. The molecular ion (MH^+) of each analyte was used for the daughter ion scan.

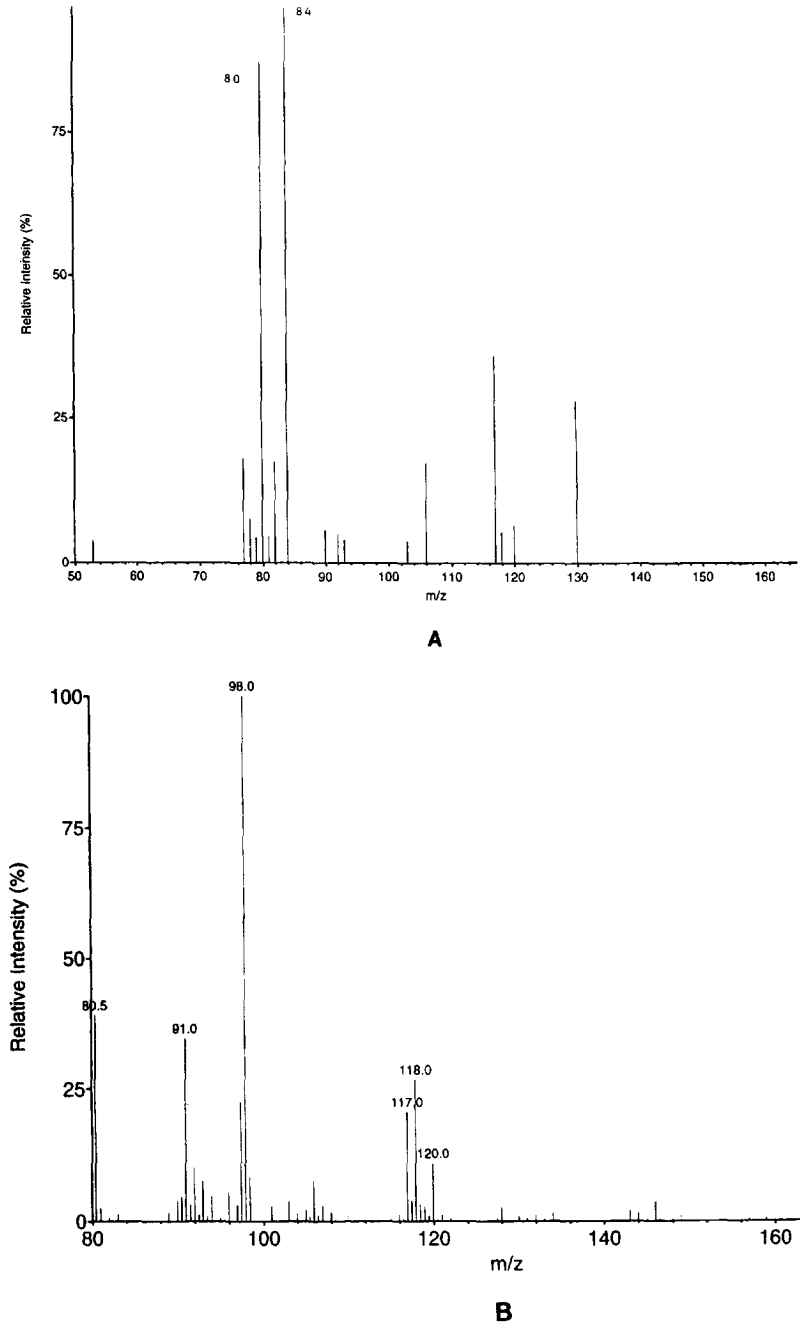


Fig. 3. Daughter ion mass spectra of (A) nicotine and (B) cotinine.

Table 1
Ions monitored under MRM mode on LC–MS–MS

	Q1 *m/z*	Q3 *m/z*
Nicotine	163.2	84.0
Nicotine-d_3	166.2	87.0
Cotinine	177.2	98.0
Cotinine-d_3	180.2	101.0

(Thornhill, Canada) equipped with an atmospheric pressure chemical ionization (APCI) interface. The heated nebulizer was set at 480°C and pressure of 551 kPa; the flow-rates of auxiliary nitrogen gas and curtain gas were both set at 1.2 l/min; the interface heater temperature was 55°C. Ions monitored in the multiple reaction monitoring (MRM) mode were *m/z* 163.2 (parent ion) to *m/z* 84.0 (daughter ion) for nicotine, *m/z* 166.2 (parent ion) to *m/z* 87.0 (daughter ion) for nicotine-methyl-d_3 (I.S.), *m/z* 177.2 (parent ion) to *m/z* 98.0 (daughter ion) for cotinine, and *m/z* 180.2 (parent ion) to *m/z* 101.0 (daughter ion) for cotinine-methyl-d_3 (I.S.). Argon was used as the collision gas and the electron multiplier was set at 4000 V.

2.4. Data treatment

A weighted $1/y$ linear regression was used to determine slopes, intercepts and correlation coefficients, where y=ratio of the compound peak area to the I.S. peak area. The resulting ratios were used to calculate nicotine and its metabolite from the following equation:

$$\text{concentration} = (y - \text{intercept})/\text{slope}$$

2.5. Extraction procedure

To 1.0 ml heparinized plasma samples, 100 μl of I.S. in methanol (100 ng/ml nicotine I.S. and 1000 ng/ml cotinine I.S.) was added. After vortex-mixing briefly, 0.1 ml of 5 *M* ammonium hydroxide was added, followed by adding 8 ml of methylene chloride and shaking for 5 min on a horizontal shaker to extract nicotine, cotinine, and their I.S.s from the plasma. The organic phase was then separated from the aqueous phase by centrifugation at 2500 rev/min for 5 min. The aqueous layer was aspirated off and the organic phase was transferred to

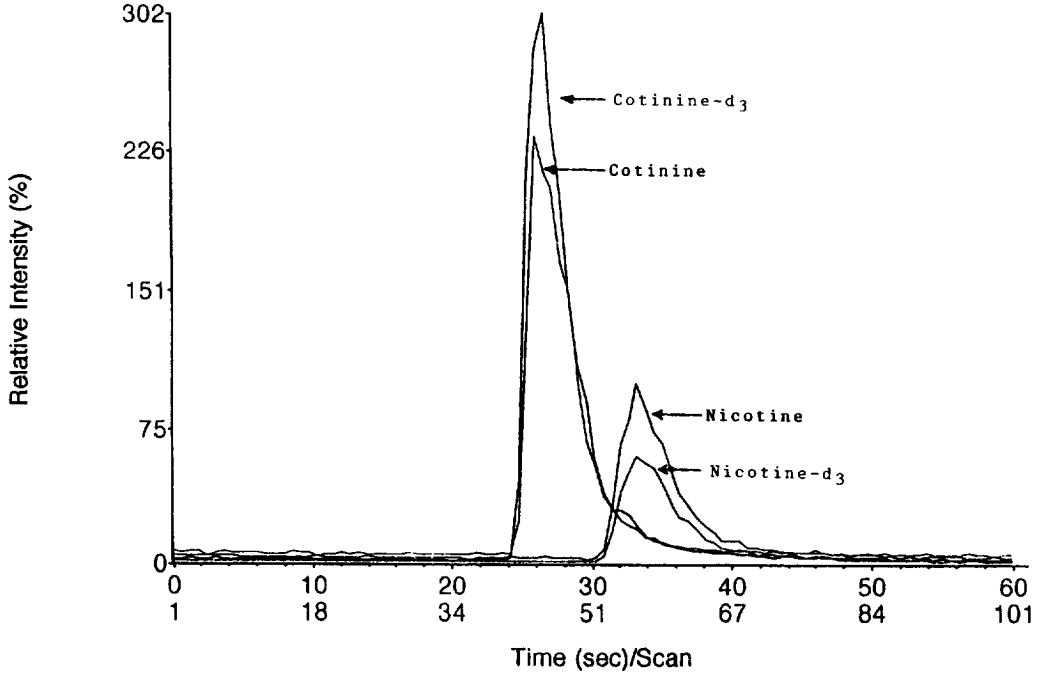


Fig. 4. Total ion chromatogram of nicotine, cotinine, nicotine-d_3 and cotinine-d_3.

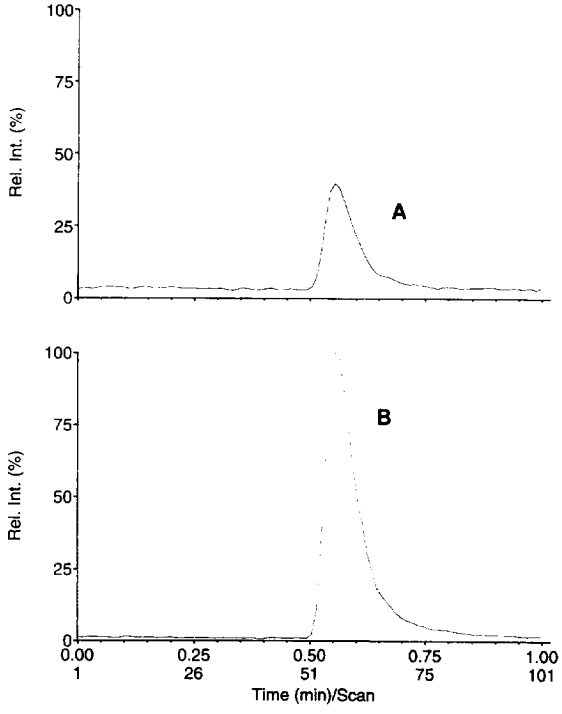


Fig. 5. Chromatogram of extracted 1.0 ng/ml standard: (A) nicotine; (B) I.S.

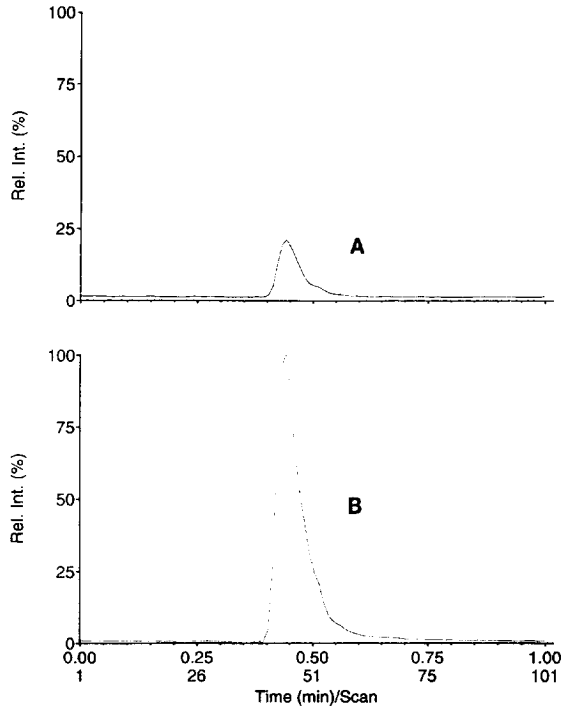


Fig. 6. Chromatogram of extracted 10.0 ng/ml standard: (A) cotinine; (B) I.S.

a clean conical tube. After evaporation to dryness under nitrogen gas in a 37°C water bath, the residue was reconstituted in 100 μl of mobile phase, and 10 μl was injected onto the LC–MS–MS system.

3. Results and discussion

3.1. LC–MS–MS separation

An LC–MS–MS method for the detection of nicotine and cotinine in human plasma was investigated. Fig. 2A and Fig. 2B show respectively the full scan Q1 mass spectra of nicotine and cotinine, where the molecular ion (MH^+) of nicotine was at m/z 163.2 and the one of cotinine was at m/z 177.2. The daughter ion spectra of nicotine and cotinine (Fig. 3A and Fig. 3B) illustrate respectively a major peak at m/z 84.0 and m/z 98.0, which are both due to a loss of the 3-pyridyl group from the molecular ion.

MRM ion chromatograms were used to determine nicotine, cotinine, and their I.S. levels in plasma. The ions monitored for these compounds were listed in Table 1. Fig. 4 shows a typical total ion chromatogram of nicotine, cotinine, and their I.S.s. The dwell time was 120 ms for each ion. Both nicotine and its I.S. eluted at 34.5 s, and both cotinine and its I.S. eluted at 27.8 s. The mobile phase with a high percentage of organics made the run time as short as 1 min.

A heated nebulizer probe with corona discharge chemical ionization (APCI) was used as the interface in this method. It has consistent response to the analytes under the MRM mode. By adjusting resolutions (RE1 and RE3) and peak width (Δ mass), the instrument selectivity and sensitivity were greatly enhanced.

During the method validation, nine lots of blank control plasma from in house draws were screened on the LC–MS–MS. Seven lots did not show interfering peaks at the retention times of the compounds of interest. Fig. 5 and Fig. 6 show, respectively, the extracted 1.0 ng/ml nicotine standard and 10.0 ng/ml cotinine standard. In Fig. 5, the I.S.

Table 2
Inter-day precision and accuracy of standards ($n=5$)

Standard concentration (ng/ml)	Mean concentration found (ng/ml)	R.S.D. (%)	R.E. (%)
Nicotine			
1.00	1.02	2.94	+2.00
2.00	2.08	3.37	+4.00
5.00	4.77	3.98	−4.60
10.00	9.88	3.85	−1.20
20.00	20.64	2.62	+3.20
25.00	24.43	1.76	−2.28
40.00	38.95	3.54	−2.62
50.00	51.43	1.79	+2.86
Cotinine			
10.0	8.8	1.93	−11.8
20.0	18.8	6.42	−5.75
50.0	51.2	2.69	+2.50
100.0	104.3	2.85	+4.36
200.0	211.1	2.97	+5.57
250.0	261.1	4.11	+4.46
400.0	394.0	3.75	−1.48
500.0	483.3	0.43	−3.34

concentration was 100 ng/ml, and in Fig. 6, the I.S. concentration was 1000 ng/ml. The chromatograms are clean and with good resolution.

3.2. Extraction

A simple one-step extraction was introduced to extract analytes from plasma. After alkalizing the samples by ammonium hydroxide, the analytes were extracted into methylene chloride. Using this low polar organic solvent resulted in high and consistent recoveries for all compounds. The recoveries were calculated by direct comparison of the peak area of extracted standards to unextracted test solutions prepared in an interference free matrix at the same concentration. Nicotine recovery was 106%, cotinine recovery was 86.2%, and their I.S. recoveries were comparable (nicotine-methyl-d_3 recovery was 109% and cotinine-methyl-d_3 recovery was 83.3%). All recoveries had R.S.D. better than 2.43% throughout the entire standard concentration ranges, showing great consistency.

3.3. Validation performance

The calibration curves were plotted as the peak-area ratio (nicotine/nicotine I.S. or cotinine/cotinine I.S.) versus analyte (nicotine or cotinine) concentration. The calibration range for nicotine was from 1.0 ng/ml to 50.0 ng/ml, and that for cotinine was from 10.0 ng/ml to 500.0 ng/ml, each with eight calibrators. The standard curve range and limit of quantitation (LOQ) were designed to meet the requirement of clinical analysis of each analyte in plasma. Five validation curves were run over a three-day period. Consistently good correlation coefficients ($r>0.997$) were achieved throughout the entire concentration ranges. Table 2 shows inter-day precision and accuracy for each standard concentration.

LOQ was defined as the lowest standard level which meets the acceptance criteria in accuracy and precision of $\leq 15\%$ bias and R.S.D. The LOQ for nicotine was 1.0 ng/ml and for cotinine was 10.0 ng/ml, with excellent signal-to-noise ratios (≥ 100).

Table 3 displays the inter-day and intra-day precision and accuracy of three quality control levels for each analyte. The data shows that this LC–MS–MS method is very consistent and reliable with overall low R.E. and good R.S.D.

3.4. Stability

Stabilities of chromatography (reinjection), processing (refrigeration), and sample in biological

Table 3
Precision and accuracy of quality control samples

Theoretical concentration (ng/ml)	Mean concentration found (ng/ml)	R.S.D. (%)	R.E. (%)
Nicotine			
Inter-day ($n=30$)			
2.5	2.53	5.93	+1.20
15.0	14.97	6.21	−0.20
37.5	36.38	4.34	−2.99
Intra-day ($n=6$)			
2.5	2.46	0.81	−1.60
15.0	15.27	1.44	+1.80
37.5	37.11	1.19	−1.04
Cotinine			
Inter-day ($n=30$)			
25.0	23.7	6.34	−5.36
150.0	160.9	3.56	+7.27
375.0	359.2	4.09	−4.21
Intra-day ($n=6$)			
25.0	24.2	4.71	−3.20
150.0	154.7	1.12	+3.15
375.0	346.4	2.09	−7.62

matrix (benchtop, freeze–thaw cycles, and long term storage) were established during the method validation. The data is presented in Table 4. To mimic the possible freezing and thawing conditions of clinical samples, QCs were subjected to multiple cycles of freezing and thawing and then analyzed. The values of QCs after three freeze–thaw cycles were comparable to those having undergone just one cycle. Benchtop stability test was done by comparing the data from QCs thawed and stored on the bench to data from ones thawed and immediately processed. Long term storage stability test was done by comparing the data from QCs stored in biological matrix in freezer conditions anticipated for clinical samples to data from initial acceptable analysis after QC preparation. All results of stability tests of benchtop, sample storage, refrigeration, and reinjection were comparable to the controls. The results show that no significant degradation occurred during chromatography, extraction and sample storage processes.

Table 4
Stability of quality control samples

	Time period	As % of control	
		Nicotine	Cotinine
Reinjection	3 h	101–104	97.9–101
Refrigeration	72 h	98.4–99.9	95.4–106
Benchtop	5.5 h	100–106	100–102
Freeze–thaw (3×)	–	105–106	101–104
Sample storage (−20°C)	41 weeks	96.4–109	108–118

4. Clinical application

This LC–MS–MS method was used to study the pharmacokinetic profiles of nicotine and its metabolite in human plasma. Volunteers who had their last cigarette 24 h prior to study were given a single dose of nicotine 45 mg patch and subsequently sampled at specific time points. Drawn blood was heparinized, centrifuged, and plasma was collected and stored in freezer set at −20°C for later determination. A plot of nicotine concentrations in plasma versus time after treatment from a single subject is shown in Fig. 7.

5. Conclusion

An LC–MS–MS method with APCI interface was developed and validated for the quantitative determination of nicotine and its metabolite, cotinine, in

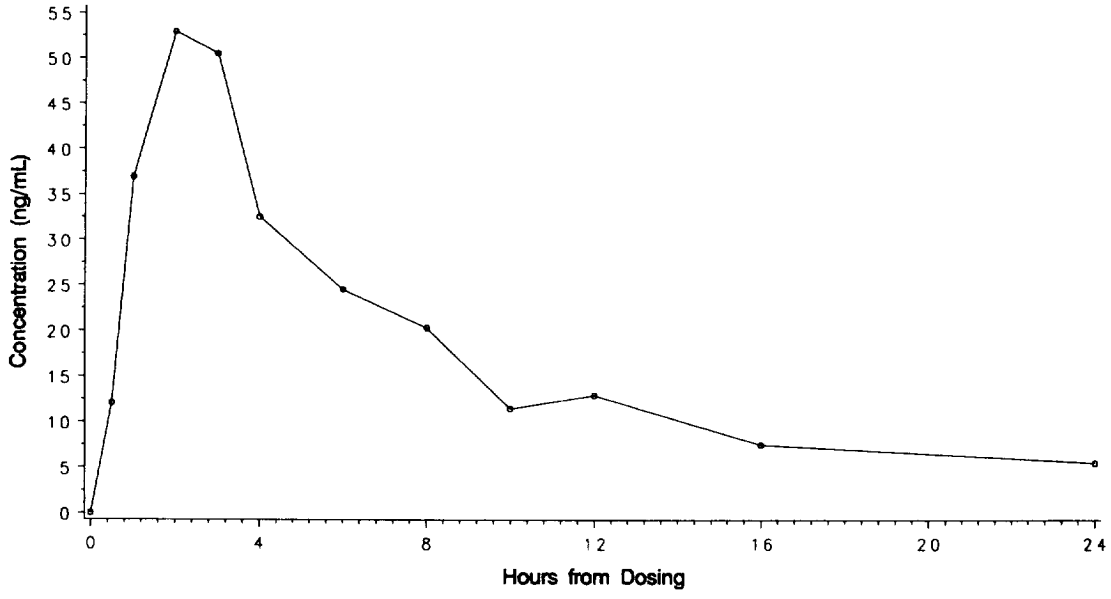


Fig. 7. Pharmacokinetic profile of a subject dosed with a 45-mg nicotine patch.

human plasma. The sensitivity of nicotine and cotinine was suitable for pharmacokinetic studies of nicotine formulations used to aid smoking cessation. This method was successfully applied to several pharmacokinetic studies with more than 6000 clinical samples. In these studies, more than 400 samples were analyzed per day. The method was found to be simple, rugged, and with high throughput.

Acknowledgments

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