A Single-Step Extraction Method for the Determination of Nicotine and Cotinine in Jordanian Smokers' Blood and Urine Samples by RP-HPLC and GC–MS

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Abstract

A simple, rapid, reliable, and low cost one-step extraction method is developed and validated for the determination of nicotine and cotinine in human plasma and urine in smokers using reversed-phase high-performance liquid chromatography (RP-HPLC) and gas chromatography–mass spectrometry (GC–MS). The run times are 16 and 10 min for HPLC and GC–MS, respectively. The method is validated over a wide linear range of 1–5000 ng/mL with correlation coefficients being consistently greater than 0.9985. The criteria considered for validation are: limit of quantitation, linearity, accuracy, precision, recovery, specificity, and selectivity. This study is aimed to estimate the nicotine and cotinine in Jordanian smokers' blood and urine samples; to study the relationship between the concentration of nicotine in urine and plasma samples; and to investigate the effect of pH on the extraction of nicotine and cotinine in urine samples. In the presented study, one hundred blood and urine samples are collected from eighty smokers and twenty nonsmokers. Samples are taken from the same volunteer at the same time after each volunteer fills in a questionnaire. Results of nicotine concentrations in smokers' plasma are in the range of 181–3702 ng/mL with an average of 1263.1 ng/mL, whereas nicotine in urine samples is in the range of 1364–1972 ng/mL, with an average of 1618 ng/mL. Cotinine concentrations in smokers' plasma are in the range of 21–4420 ng/mL with an average of 379.4 ng/mL, whereas cotinine in urine is in the range of 6–3946 ng/mL with an average of 865 ng/mL. Statistical analysis indicates highly significant differences in nicotine and cotinine concentrations in smoker samples compared with nonsmoker samples ($p < 0.05$).

Introduction

Tobacco smoke consists of a heterogeneous mixture of gases, uncondensed vapors, tar, and particulate phase, which contains approximately 4000 compounds (1) such as nicotine (2), carbon monoxide (3), polycyclic aromatic hydrocarbons (4–6) [e.g., benzo(a)pyrene (4–5)], and heavy metals (7–8). Nicotine is a natural alkaloid which occurs in the leaves of *Nicotiana tubacum* as a tertiary amine composed of a pyridine and a pyrrolidine rings. The primary metabolite of nicotine is cotinine (9). The enzymes responsible for the metabolism of nicotine in human liver to cotinine are cytochrome P4502A6 (CYP2A6) and cytosolic aldehyde oxidase (3,10). Nicotine has a relatively short half-life, approximately 2 h, and cotinine has a half-life of approximately 20 h (11). The metabolism of nicotine is shown in Figure 1.

The true smoking status is based on cotinine and nicotine levels in the body fluids (12) that are dependent on sex, age, diet, racial and ethnic differences, as well as many other factors (1). Smoking is a major risk factor for periodontal disease, causing bone and tooth loss (13). Cigarette smoking increases the risk of heart diseases, damage to the lungs, and lung cancer. Moreover, many other diseases such as malignant tumors of the respiratory and digestive tracts, the bladder, and renal pelvis and pancreas may be caused by cigarette smoking (5–6,14–16).

Assessments of nicotine and cotinine in biological fluids such as blood, urine, and other biological markers have become an important component of direct or passive exposure to tobacco smoke (17). Nicotine and cotinine in biological samples can be detected using different instrumental techniques. For example, many laboratory methods have been developed to measure nicotine and its metabolites including high-performance liquid chromatography (HPLC) using UV detector (18) or mass spectrometry detector (MS)

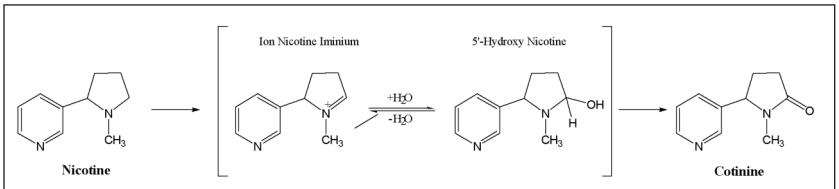

Figure 1. Metabolism of nicotine.

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(9,19). Moreover, other researchers have determined nicotine and/or cotinine concentrations in smokers' and non-smokers' blood by HPLC and/or gas chromatography (GC)–MS (20–23).

In this study, a simple, rapid, reliable, low cost, and one-step extraction method is developed, based on other methods recommended by other researchers with some modifications, in order to isolate and determine nicotine and cotinine in human plasma and urine in smokers, constituting liquid–liquid extraction with binary solvents (16–19) to get better detection limit, linearity over high range, recovery, and no interference peaks. The extraction method used is more rapid and simple compared with other extraction methods (24), and compared with others, the run time is higher (25). Another advantage of this method is that it utilizes a single extraction step with 3 mL of a solvent mixture, compared with other extraction methods with double extraction steps (12). The analyses were developed and validated using HPLC and GC–MS. The effects of pH of urine on the extraction of nicotine and cotinine were investigated in order to optimize the proper pH, by which better recovery could be achieved for both nicotine and cotinine in urine.

The developed method was used to determine the nicotine and cotinine in Jordanian smokers' blood and urine samples, because there is little data from that population group in the area of smoking and health; to study the relationship between the concentration of nicotine in urine and plasma samples; and to investigate the effect of pH on urine on the extraction of nicotine and cotinine in urine and plasma samples. This project may increase awareness of people to get rid of the smoking habit on the basis of the obtained results.

Experimental

Chemicals and reagents

All chemicals, analytical standards, reagents, and solvents used throughout this study were analytical grade and highly pure. Nicotine was purchased from Fluka Feinbiochemica (Buchs, Switzerland) with purity of ≥ 99% (for research and development use only); cotinine from Sigma, St. Louis, MO, purity of 98%; acetanilide (Fluka Feinbiochemica, purity of ≥ 99%) was used as internal standard in HPLC method; purified diphenylamine (Sigma, product of Japan) was used as internal standard in GC–MS method. Methanol (HPLC/SPECTRO) was purchased from TEDIA Company, Inc. (Fairfield, OH). Also, other chemicals and solvents were used including dichlormethane (TEDIA); water (HPLC, GFS Chemicals, Germany); potassium dihydrogen phosphate (KH_2PO_4) (Riedel-deHaën, Germany, 99% minimum assay); ortho-phosphoric acid (H_3PO_4) (Panreac Quimica, Spain, 85% assay); ethyl ether (Medical Export Co. Ltd., England); methylene chloride (Normapur, EEC, 99.5% minimum by GC); sodium hydroxide (NaOH) (Schrlau Chemie, Barcelona, Spain, made in the European Union); hydrochloric acid (HCl) (Schrlau Chemie, Barcelona, Spain, made in the European Union); glacial acetic acid (Schrlau Chemie); and *n*-hexane (LAB-SCAN, Ireland, 95% minimum by GLC).

Subjects and sample collection

One hundred samples were collected from Jerusalem Medical Laboratory–Amman. Eighty of these samples were collected from smokers and twenty from non-smokers. Blood and urine samples were taken from the same person at the same time after each volunteer filled out a questionnaire.

The questionnaire included, the age, the time of the sample collection, and the number of cigarettes smoked. Volunteers were divided into groups according to a range of cigarettes smoked per day (1–5, 6–10, 11–15, 16–20, 21–25, 26–30, 31–35, 36–40, 40–45, 45–50, 50–55, and 55–60 cigarettes per day).

Blood samples

The blood samples (4 mL of each sample) were collected in EDTA-tubes (Vacuette EDTA K2/gel tube, Griner Bio-One GmbH, Austria) and centrifuged immediately at 2800 rpm for 5 min (Centrifuge Instrument Type: Minor 35, M.S.E. Ltd., England). The plasma supernatant was then collected in eppendorf tubes in the laboratory at Jordan University of Science and Technology (JUST) and frozen at –70°C until analysis.

Urine samples

The urine samples were collected in washed high-density polyethylene plastic containers with distilled water, and then samples were transferred immediately to the laboratory at JUST and frozen at –20°C until analysis.

Extraction procedure of human plasma and urine

Extraction for HPLC analysis

A 0.5 mL aliquot of plasma or urine was placed into a screw-capped glass test tube 15 × 100-mm with 100 µL of 11 ppm acetanilide in 50% methanol (internal standard). Each sample was alkalinized with 100 µL of 2.5M NaOH for plasma samples and 100 µL of 5.0M NaOH for urine samples, then vortex mixed at 2800 rpm for 30 s. A 3 mL aliquot of dichloromethane–diethylether (1:1 v/v) was used for one-step single extraction, then vortex mixed at 2800 rpm for 2 min. The organic layer, after being centrifuged at 3500 rpm for 3 min, was transferred to a new glass tube containing 20 µL of 0.25M HCl. The organic phase was then evaporated under a stream of nitrogen at 35ºC until dryness and reconstituted to 250 µL with mobile phase consisting of a mixture of 0.272 g of KH_2PO_4, 0.184 g of sodium 1-heptane sulfonate (Sigma-Aldrich), 820 mL of water (HPLC-grade), and 180 mL of methanol (HPLC-grade). A 100 µL aliquot was injected automatically into the HPLC and analyzed.

Extraction for GC–MS analysis

A 0.5 mL aliquot of plasma or urine was added into a screw-capped glass test tube 15 × 100-mm with 100 µL of 2 ppm diphenylamine in 50% methanol (internal standard). Each sample was alkalinized with 100 µL of 2.5M NaOH for plasma samples and 100 µL of 5.0M NaOH for urine samples, then vortex mixed at 2800 rpm for 30 s. A 2.5 mL aliquot of dichloromethane–hexane (1:1 v/v) used for one-step single extraction, then vortex mixed at 2800 rpm for 2 min. The organic layer was transferred to a new glass tube containing

10 µL of glacial acetic acid after being centrifuged at 3500 rpm for 3 min. The organic phase was evaporated under a stream of nitrogen at 35ºC until dryness, then reconstituted to 100 µL with hexane. A 2 µL aliquot was injected manually into the GC–MS and analyzed.

Instrumentation

HPLC

The HPLC system (Merck Hitachi, Darmstadt, Germany) consisted of L-7000 interface, L-7455 photodiode array detector (PAD), L-7200 auto sampler, L-7150 pump, L-7612 solvent degasser, and 100 µL loop injector. The stationary phase represents the analytical column was a LiChroCART, Purospher STAR RP-18 endcapped (125 cm × 4 mm i.d., 5 µm particle size) (Merck).

HPLC operating conditions. The isocratic mobile phase was a mixture of 0.272 g of KH_2PO_4, 0.184 g of sodium *n*-heptane sulfonate in order to form a less polar ion pair with a charged solute to get better reproducibility and reliability together with improved accuracy, 820 mL of water (HPLC-grade), and 180 mL of methanol (HPLC-grade) to reduce analysis time without overlapping with other peaks. The pH of the mobile phase was adjusted by drop wise addition of ortho-phosphoric acid (pH ≈ 3.2). The flow rate used was 1.0 mL/min, and the wavelength was fixed at 254 nm.

GC–MS

A Varian/Chrompack Model CP-3800 GC with a 1079 universal capillary injector was used. The oven has a temperature range from ambient to 490ºC with a temperature stability of 0.1ºC and a programming rate from 0.1–50ºC/min, equipped with a Saturn 2000 ion-trap MS with *m/z* ranging from 10 to 650 (Varian Instruments, Sunnyvale, CA). The temperature program was started at 120ºC with slower temperature gradient (20ºC/min). The carrier gas was 99.999% (grade-5) helium with water, oxygen, and nitrogen concentrations less than 2 ppm.

GC–MS operating conditions. Separation was achieved with a SPB-5 fused silica capillary column coated with a 0.25 µm film thickness of non-polar poly (5 % diphenyl, 95% dimethylsiloxane), length of 30 m, and internal diameter of 0.25 mm. The flow rate of helium was 1.0 mL/min. The operating parameters were as the following: injector temperature 300ºC; transfer line temperature 300ºC; oven temperature was programmed from 120ºC to 220ºC (20ºC/min), then held for 2 min. The electron-impact mode was used. The scan mode was from *m/z* 155 to *m/z* 175 to ensure unwanted lower mass ion are ejected from the trap and therefore cannot limit the ionization time of AGC mode. The ions selected in this study were as the following: *m/z* 162 for nicotine, 167 and 168 for diphenylamine, and 176 for cotinine.

Results and Discussion

Results in smokers' plasma for cotinine were in the range of 21–4420 ng/mL with an average of 379.4 ng/mL, whereas cotinine in urine was in the range of 36–3946 ng/mL with an average of 865 ng/mL. Results for nicotine in plasma were in the range of 181–3702 ng/mL with an average of 1263.1 ng/mL, whereas nicotine in urine ranged from 1364–1872 ng/mL with an average of 1618 ng/mL. Figures 2A and 2B show the distribution of cotinine and nicotine concentrations in plasma and urine samples for smokers, respectively.

For non-smokers, cotinine in plasma was in the range of 34.9–65.19 with an average of 50.0 ng/mL, whereas cotinine in urine was in the range of 37–223 ng/mL with an average of 84.7 ng/L. Results for nicotine in plasma were in the range of 76–700 ng/mL with an average of 331 ng/mL, whereas nicotine in urine was in the range of 36–890 ng/ mL with an average of 350.5 ng/mL. Figures 3A and 3B show the distribution of cotinine and nicotine concentrations in the plasma and urine samples for non-smokers, respectively.

The HPLC chromatogram for cotinine, acetanilide, and nicotine extracted from plasma is shown in Figure 4A, and the GC–MS chromatogram for nicotine, diphenylamine, and cotinine is shown in Figure 4B.

To investigate the effect of pH on extraction efficiency for HPLC method, urine samples were extracted under different pH conditions over the range of 5.0–13.0, using phthalate buffer for pH 5, dihydrogen phosphate buffer for pH 6–8, borate buffer for pH 9 and 10, hydrogen phosphate buffer for pH 11 and 12, and NaOH/KCl for pH 13 (26). The nicotine and cotinine concentrations were 4000 ng/mL plus internal standard spiked at each pH level then extracted using extraction method without alkalization step. The accuracy was calculated at each pH level.

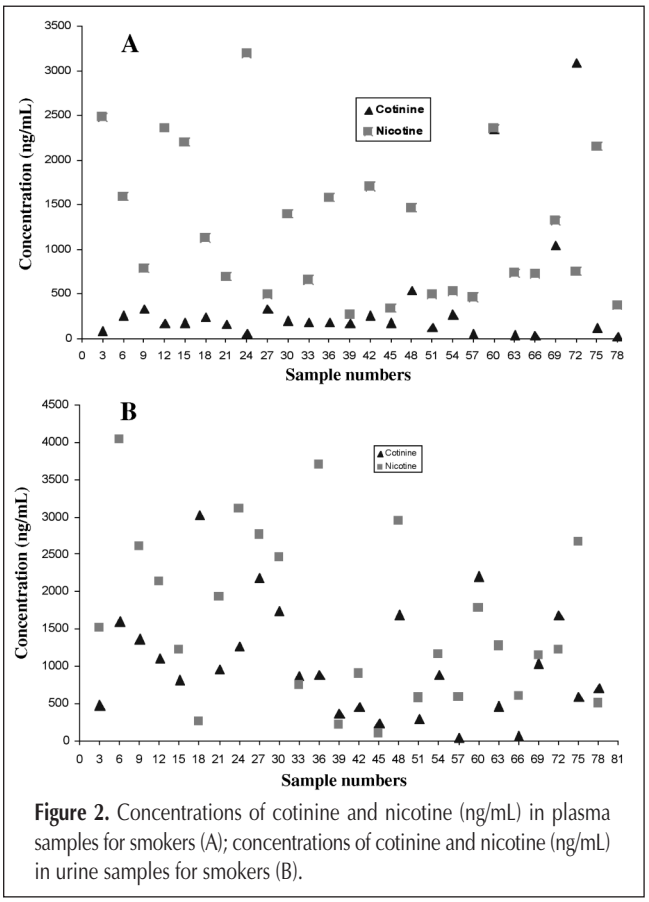

Figure 2. Concentrations of cotinine and nicotine (ng/mL) in plasma samples for smokers (A); concentrations of cotinine and nicotine (ng/mL) in urine samples for smokers (B).

According to the results obtained, plasma and urine samples were made basic using NaOH (pH > 13) to improve the extraction of cotinine and nicotine, because when increasing the pH of urine samples, the % relative error (the difference between spiked and detected concentration divided by spiked concentration times 100) decreases as shown in Figure 5, and the recovery increases as shown in Figure 6. Moreover, the best efficiency of extraction for both nicotine and cotinine was achieved at pH 13, because pK_b values of nicotine and cotinine are 4.9 and 9.7, respectively; nicotine is more basic than cotinine. At high pH value, the analytes are in the basic form (unionized state) and can be easily extracted by the organic solvents, but at low pH value the analytes are in the acidic form (ionized stable) and cannot be easily extracted by the organic solvents.

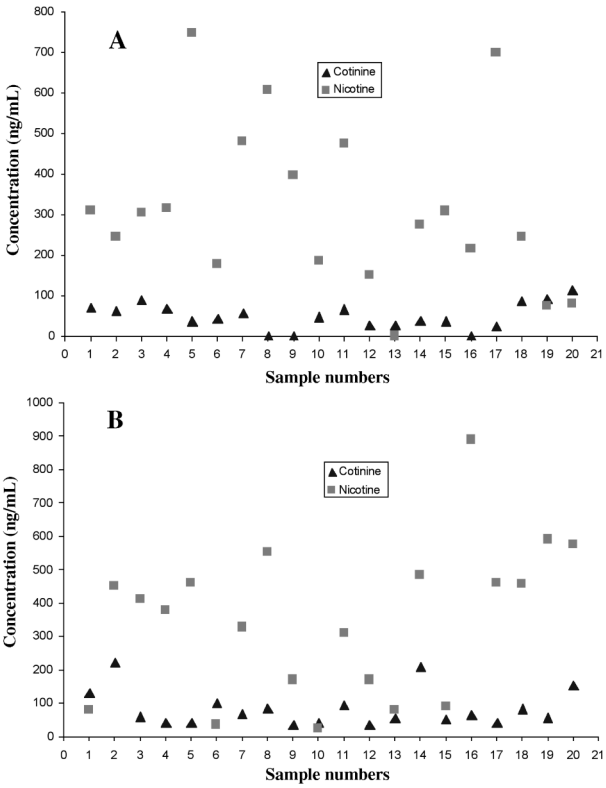

Figure 3. Concentrations of cotinine and nicotine (ng/mL) in plasma samples for non-smokers (A); concentrations of cotinine and nicotine (ng/mL) in urine samples for non-smokers (B).

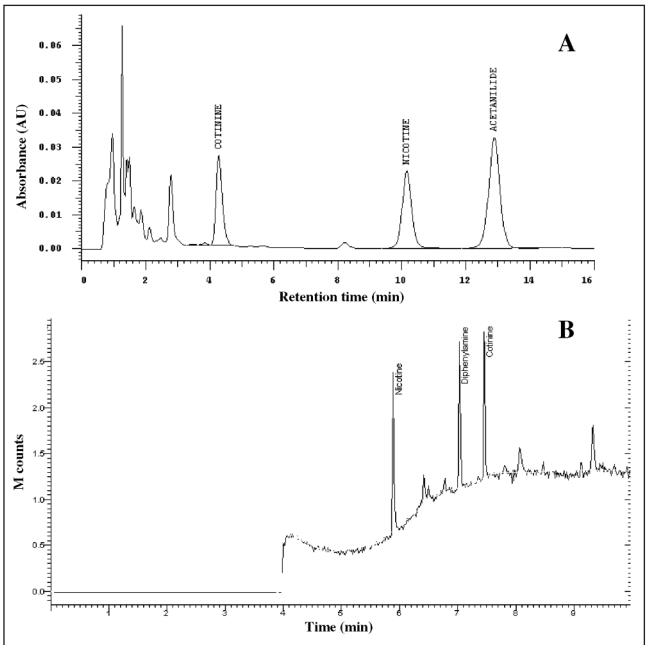

Figure 4. Chromatograms of the extracts from plasma. HPLC chromatogram of cotinine, nicotine, and acetanilide (A); GC chromatogram of nicotine, diphenylamine, and cotinine (B).

Linearity

Examination of calibration curves by computing a linear least-squares regression analysis on the plot of the peak area ratios of cotinine and nicotine to the internal standard versus concentrations demonstrated a linear relation over the range 1–500.0 ng/mL (using six concentration levels) with correlation coefficients (R^2) being consistently greater than 0.9985. Table I shows the best fit data for the analytes in HPLC and GC–MS, taking into consideration that the extractions were done at pH 13.

Moreover, in the presented study, the limit of detection (LOD) values for nicotine using HPLC were 0.32 and 0.15 ng/mL in plasma and urine samples, respectively; whereas LOD values for cotinine were 0.26 and 0.13 ng/mL in plasma and urine, respectively. A signal to noise (S/N) ratio of approximately 3:1 for LOD was observed, and concentration was

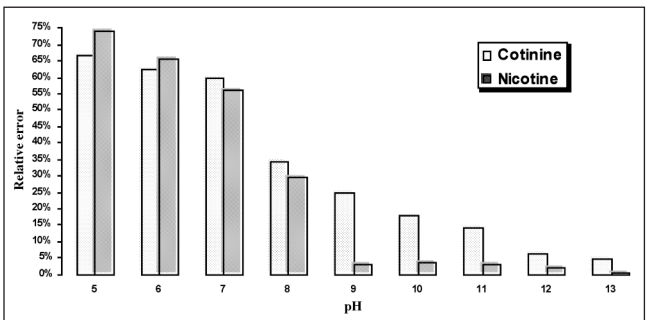

Figure 5. The effect of different pHs of urine on the %R.E. of cotinine and nicotine.

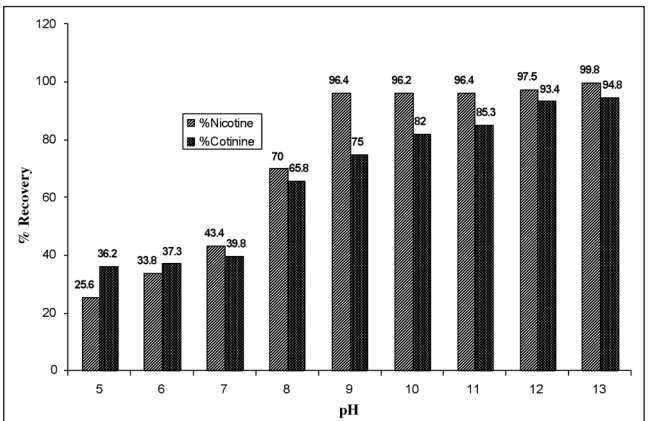

Figure 6. The effect of different pHs of urine on the recovery of cotinine and nicotine.

calculated with 12 spiked blank plasma and urine with internal standard and extracted as described. The LOD was calculated using the following equation: LOD = (3s/m), where s is the standard deviation of the 12 peak ratio (peak area of analyte/peak area of internal standard), and m is the slope of a calibration curve. The LOD values using GC–MS for nicotine and cotinine in plasma and urine were 0.25 and 0.2 ng/mL, respectively. Compared with other studies, LOD values reported for nicotine in urine using HPLC were 10 (27) ng/mL, and the reported values of LOD for cotinine in urine were 0.5 (20) and 5 ng/mL (24); whereas the LOD values reported for nicotine in plasma using GC–MS were 016 (2) and 0.2 ng/mL (12), and LOD values for cotinine in plasma were 0.16 (2) and 1.0 (12), and for cotinine in urine were 0.16 (4) and 0.2 ng/mL (12).

Limit of quantitation

The limit of quantitation (LOQ) values in HPLC were determined to be 0.9 ng/mL and 1.0 ng/mL for cotinine and nicotine in plasma, respectively. The LOQ was evaluated as the concentration equal to 10 times of the value S/N. The values for urine samples were 0.4 ng/mL and 0.5 ng/mL for cotinine and nicotine, respectively. The LOQ values for GC–MS method were determined to be 0.8 ng/mL for cotinine and nicotine in plasma, and 0.7 ng/mL for cotinine and nicotine in urine by GC–MS method. Compared with previous assays, LOQs achieved in this study were much better than those obtained in previous studies.

The LOQs of nicotine and cotinine in the presented study were determined by HPLC to be 1.06 and 0.86 in plasma, and 0.5 and 0.43 ng/mL in urine, respectively. The LOQ of nicotine in plasma has been previously reported to be 10 ng/mL (27), for cotinine in plasma it was 1 ng/mL (27), and in urine was 17 (24); whereas the LOQs of nicotine and cotinine in the presented study determined by GC were 0.83 ng/mL in plasma, and 0.66 ng/mL in urine, respectively. The LOQ of nicotine in plasma was 1.25 ng/mL (2). The LOQ of cotinine in the plasma and urine was 1.25 ng/mL (2).

Specificity/selectivity

The terms selectivity and specificity are often used interchangeably. Specificity is the ability to assess unequivocally the analyte in the presence of endogenous compounds. Selectivity includes the ability to separate the analyte from degradation products, metabolites, and co-administered drugs (28).

In case of specificity of HPLC–PDA, there are no interferences due to common drugs (paracetamol, mefenamenic acid, salicylic acid, caffeine, indomethancin, and metronidazole) against cotinine, nicotine, and acetanilide. So the method is able to accurately measure the analyte response in the presence of potential sample components. The selectivity of HPLC–PDA, the purity check of peaks of interest for plasma and urine showed more than 95% in purity. So, the method is selective, and in GC–MS there are no interferences with peaks of interest.

Accuracy, precision, and recovery

Accuracy is expressed as percent relative error (%R.E.). Precision is expressed as percent relative standard deviation (%RSD). Recovery was calculated as the following: (peak area ratio of processed spiked plasma and urine standards / peak area ratio of pure standard which has not been subjected to

Table I. The Best Fit Lines and Correlation Coefficients (R^2) of Cotinine and Nicotine in Plasma and Urine for HPLC and GC–MS Methods*

	Regression curve equation ($y = ax + b$)	
	Cotinine (R^2)	Nicotine (R^2)
HPLC		
Plasma	$Y = 3.561 \times 10^{-4}x - 6.141 \times 10^{-3}$ (0.9997)	$Y = 3.009 \times 10^{-4}x - 1.795 \times 10^{-2}$ (0.9997)
Urine	$y = 3.225 \times 10^{-4}x - 5.348 \times 10^{-3}$ (0.9986)	$Y = 3.053 \times 10^{-4}x + 2.086 \times 10^{-3}$ (0.9996)
GC–MS		
Plasma	$y = 1.711 \times 10^{-4}x + 1.260 \times 10^{-2}$ (0.9995)	$Y = 1.413 \times 10^{-4}x - 3.980 \times 10^{-5}$ (0.9987)
Urine	$y = 1.827 \times 10^{-4}x - 1.559 \times 10^{-3}$ (0.9985)	$Y = 1.481 \times 10^{-4}x + 6.870 \times 10^{-3}$ (0.9992)

* Extractions were done at approximately pH 13.

Table II. Accuracy, Precision, and Recovery of Cotinine and Nicotine in Plasma and Urine using HPLC and GC–MS*

	Cotinine				Nicotine			
	True concentration level (ng/mL)	% Recovery	% RSD	% R.E.	True concentration level (ng/mL)	% Recovery	% RSD	% R.E.
HPLC plasma								
	4000	95.8	1.7	2.4	4000	96.0	2.5	4.4
	2000	98.0	1.0	1.9	2000	94.3	2.3	2.0
	500	93.6	6.0	1.4	500	90.5	7.4	1.8
HPLC urine								
	4000	101.5	1.6	0.8	4000	96.0	1.8	1.3
	2000	85.8	0.1	1.4	2000	91.6	3.9	3.2
	500	85.5	4.8	3.0	500	105.0	7.3	1.4
GC–MS plasma								
	4000	98.2	1.5	3.5	4000	101.2	1.1	0.5
	2000	93.0	0.7	1.5	2000	95.2	0.4	0.5
	500	85.7	6.3	4.4	500	94.5	0.3	1.0
GC–MS urine								
	4000	100.2	1.6	1.0	4000	103.8	3.8	1.7
	2000	95.3	0.4	1.4	2000	104.4	2.0	4.7
	500	80.1	0.1	2.0	500	109.6	1.2	1.0

* Extractions were done at approximately pH 13.

Table III. Correlation (R) and p-Values of Plasma-Urine Cotinine and Nicotine*

	Plasma		Urine	
Correlation (R)	Cotinine	Nicotine	Cotinine	Nicotine
Plasma				
Cotinine	–	–0.043 (0.000)	0.315 (0.000)	–0.111 (0.000)
Nicotine	–0.043 (0.000)	–	0.178 (0.015)	0.53 0 (0.023)
Urine				
Cotinine	0.315 (0.000)	0.178 (0.015)	–	0.282 (0.000)
Nicotine	–0.111 (0.000)	0.530 (0.023)	0.282 (0.000)	–

* p-Values are in parentheses.

Table IV. Concentrations (ng/mL) of Cotinine and Nicotine in Selected Plasma and Urine Samples Using HPLC–PAD and GC–MS

	Cotinine conc./plasma		Nicotine conc./plasma		Cotinine conc./urine		Nicotine conc./urine	
Sample #	HPLC–PDA	GC–MS	HPLC–PDA	GC–MS	HPLC–PDA	GC–MS	HPLC–PDA	GC–MS
6	259	288	1591	1347	1602	1775	4045	4411
20	1308	1193	2040	1945	1243	1269	2755	3124
38	218	236	1483	1307	886	862	955	865
55	134	119	760	784	1140	936	1607	1747

Table V. Comparison with Other Methods Using HPLC

Parameter	This study	Reference 20	Reference 27	Reference 28
Biological fluids	plasma/urine	urine	plasma	urine
Sample volume (mL)	0.5	10	1/nicotine 0.5/cotinine	5
Extraction solvent(s)	3 mL CH_2Cl_2–ether	40 mL $CHCl_3$	4 Ml CH_2Cl_2	5 mL CH_2Cl_2
LOQ (ng/mL)				
cotinine/plasma	0.9	—	1.0	—
nicotine/plasma	1.0	—	0.2	—
cotinine/urine	0.4	0.5	—	17
nicotine/urine	0.5	5	—	—
R^2				
cotinine/plasma	0.9997	—	0.994	—
nicotine/plasma	0.9997	—	0.995	—
cotinine/urine	0.9986	0.984	—	0.99
nicotine/urine	0.9996	0.998	—	—
%Recoveries				
cotinine/plasma	93.6–98.0	—	82.1–103.3	—
nicotine/plasma	90.5–96.0	—	97.6–102.0	—
cotinine/urine	85.5–101.5	92–100	—	88.8–97.5
nicotine/urine	91.6–105.0	47–86	—	—
Run time (min)	16	18	20	16

sample pretreatment) × 100%. The recoveries of nicotine using HPLC were in the range of 90.5–96% and 91.6–105.0% in plasma and urine, respectively. In GC–MS, the recoveries were 94.5–101.2% and 103.8–109.6% for nicotine in plasma and urine, respectively. The recoveries of cotinine using HPLC were in the range of 93.6–98.0% and 85.5–101.5% in plasma and urine, respectively. In GC–MS, they were 85.7–98.2% and 80.1–101.2% for nicotine in plasma and urine, respectively. The results obtained are displayed in Table II.

Statistical Analysis

Minitab program was used for statistical analysis of data. The results obtained from HPLC–PDA and GC–MS show that there was no significant difference in cotinine concentrations in plasma p (0.96) > 0.05 using HPLC and GC at 95% confidence level. Also, there was no significant difference in nicotine concentrations plasma p (0.74) > 0.05 using HPLC and GC at 95% confidence level. Moreover, there is no significant difference in cotinine concentrations p (0.98) > 0.05 and nicotine concentrations p (0.86) > 0.05 in urine using HPLC and GC at 95% confidence level.

The relationships of plasma-urine cotinine and nicotine concentrations regarding correlation (R) and p-value, respectively, are shown in Table III, which showed that the cotinine in plasma and urine, and nicotine in plasma and urine had a positive correlation.

According to the results obtained, cotinine in urine and nicotine in plasma and urine were positively correlated to the number of smoked cigarettes per day (R = 0.267, 0.251, and 0.213, respectively), but the cotinine in plasma showed negative correlation with number of cigarettes smoked per day (R = –0.086). When volunteers were divided into groups according to a range of cigarettes smoked per day (1–5, 6–10, 11–15, 16–20, 21–25, 26–30, 31–35, 36–40, 40–45, 45–50, 50–55, and 55–60 cigarettes per day), the positive correlation between average concentrations and range of cigarettes smoked per day increased for cotinine in urine and nicotine in plasma and urine (R = 0.924, 0.712, and 0.713, respectively), but the cotinine in plasma showed a negative correlation with number of cigarettes smoked per day (R = –0.431).

The concentrations of cotinine and nicotine in plasma and urine were affected also by the type of tobacco smoked, whether high or low nicotine. Most of results obtained showed higher concentrations of cotinine and nicotine in plasma and urine for high nicotine tobacco smoke than low nicotine tobacco smoke for the same number of cigarettes smoked per day. Moreover, the results of nicotine and cotinine concentrations in plasma and urine using HPLC–PAD and GC–MS are in good agreement, as shown in Table IV.

Table VI. Comparison with Other Methods Using GC

Parameter	This study	Reference 2	Reference 12	Reference 21
Biological fluids	plasma/urine	serum/urine	plasma/urine /saliva	plasma
Sample volume (mL)	0.5	0.2/serum 2/uine	0.5/plasma 5/urine	1.0
Extraction solvent(s)	2.5 Ml CH_2Cl_2–hexane	Toxi-Lab A tube	6 mL ethyl ether–plasma 7 mL ethyl ether–urine	5 mL CH_2Cl_2
LOQ (ng/mL)				
cotinine/plasma	0.8	1.25	3.3	—
nicotine/plasma	0.8	1.25	3.3	0.5
cotinine/urine	0.7	1.25	0.66	—
nicotine/urine	0.7	1.25	0.66	—
R^2				
cotinine/plasma	0.9995	0.97	0.998	—
nicotine/plasma	0.9987	0.99	0.999	> 0.99
cotinine/urine	0.9985	1.0	0.998	—
nicotine/urine	0.9992	1.0	0.998	—
%Recoveries				
cotinine/plasma	85.7–98.2	> 95	94.8–96.4	—
nicotine/plasma	94.5–101.2	> 95	88.1–90.5	Maximum 79
cotinine/urine	80.1–100.2	> 95	96.3–98.2	—
nicotine/urine	103.8–109.6	> 95	92.1–97.7	—
Run time (min)	10	6	7.5	6

Conclusions

The modified methods used in this study are applicable and reliable for the determination of nicotine and cotinine in plasma and urine using HPLC and GC–MS. This method has good results regarding LOD, LOQ, correlation coefficient, %R.E., %RSD, and better recovery compared with other methods in the literature (16,24,27). Tables V and VI show comparisons between this method and other methods using HPLC and GC, respectively.

This method can be used in the processing and quantitation of a large series of plasma and urine samples in any study such as tobacco cessation drug delivery systems.

Moreover, nicotine and cotinine concentrations in urine samples were higher than their concentrations in plasma. Moreover, results obtained for analysis of cotinine and nicotine in plasma and urine samples using HPLC and GC–MS showed that there are no significant differences between these two chromatographic techniques using the *t*-test ($p > 0.05$).

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