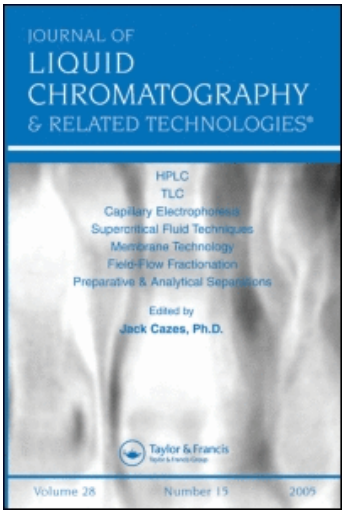

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CLINICAL ASSAY OF NICOTINE AND ITS METABOLITE, COTININE, IN BODY FLUIDS BY HPLC FOLLOWING SOLID PHASE EXTRACTION

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ABSTRACT

A rapid and accurate reversed phase high performance liquid chromatographic (RP-HPLC) method, is described for the simultaneous determination of nicotine and cotinine in body fluids. The analytical column, an MZ Kromasil C_8, 250×4 mm, 5 μm, was operated at ambient temperature with backpressure values of 290 kg/cm^2. The mobile phase consisted of A: 0.05 M ammonium acetate and phase B: CH_3OH at a volume ratio 60 : 40, delivered at a flow rate of 1.4 mL/min. Scopolamine was used as internal standard at a concentration of 85 ng/μL. Detection was performed with a variable wavelength UV-visible detector at 262 nm, resulting in a detection limit of 0.2 ng per 20 μL injection and a quantitation limit of 1.0 ng, while linearity held up to 20 ng/μL for nicotine and 30 ng/μL for cotinine. The statistical evaluation of the method was examined by performing

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intra-day ($n = 8$) and inter-day calibration ($n = 8$) and was found to be satisfactory, with high accuracy, and precision results. High relative extraction recoveries from biological matrices: blood serum and urine, ranging from 93.3% to 106.4% for cotinine and from 94.2% to 115.5% for nicotine, were achieved using Lichrolut RP-select B (Merck) SPE cartridges with tetrahydrofuran as eluent, requiring small volumes, 200 μL of blood serum, and 200 μL of urine.

Key Words: Nicotine; Cotinine; HPLC; SPE; Body fluids; Pharmacokinetics

INTRODUCTION

Nicotine is a natural alkaloid extracted from the leaves of the tobacco plant, *Nicotiana Tabacum*. People are generally exposed to nicotine through smoking or through the inhalation of environmental tobacco smoke (ETS). Nicotine is metabolised in the human liver by cytochrome P4502A6 (CYP2A6) with an average efficiency of about 70 to 80%, while 10 to 15% is excreted in urine in an unmetabolised form. Metabolites are mostly derived from the oxidation of the pyrrolidine ring. Among them cotinine has been shown to be the principal metabolite of nicotine in blood and urine. However, there is a high correlation between the clearance of nicotine and cotinine within individuals. Cotinine is eliminated primarily by oxidation to *trans*-3′-hydroxycotinine, but it also undergoes *N*-oxidation and glucuronidation before being excreted unchanged in urine. Smokers with deleted homozygous allele of the CYP2A6 gene tend to have a high nicotine plasma concentration and/or a negligible cotinine plasma concentration. The chemical structure of nicotine and cotinine, as well as the mechanism of the metabolic process, are shown in Fig. 1.[1–5]

Nicotine and cotinine concentration levels in body fluids may indicate the true smoking status of the subjects, though the precise cut-off points applied in order to distinguish smokers from non-smokers and the distribution of metabolites in body fluids, are dependent on several factors such as gender, age, diet, weight, and level of physical activity. For a given level of nicotine and cotinine concentration, intake is affected by the aforementioned factors, as well as the corresponding inhalation rate at the time of exposure. Consequently, individual differences in uptake, distribution, and metabolism will affect the biomarker concentration in body fluids. Biomarkers can be used to categorize individuals as exposed or unexposed to nicotine, identify deceivers (individuals misreporting their smoking status), or generally identify and estimate the degree of active smoking behaviour and the relative degree of exposure.

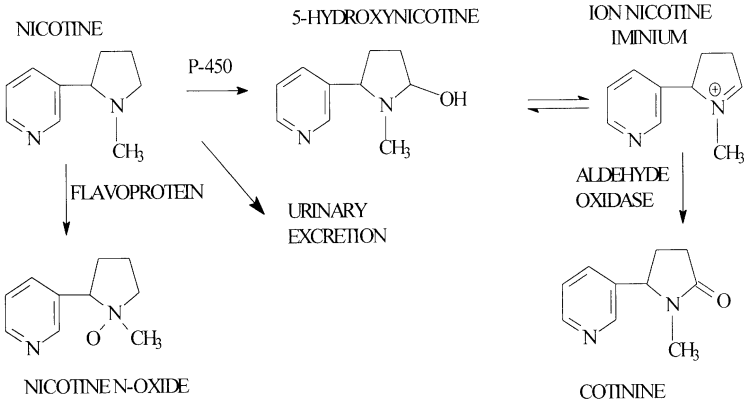

Figure 1. Metabolic pathways of nicotine and cotinine.

Nicotine and cotinine are the most widely used biomarkers of ETS exposure. They are typically measured in blood, saliva, or urine, blood being largely recommended as the fluid of choice. Cotinine levels in saliva and plasma tend to be similar, whereas the ratio of urinary to plasma levels is generally a factor of 5 to 6. Urinary cotinine excretion varies across and within individuals, depending on renal function, urinary flow rate, and urinary pH. Corrected values of urinary results can be obtained, expressed as ng of cotinine per mg of creatinine, in order to avoid differences attributable to dilution effects. The average half-life of cotinine in different body fluids is approximately 15–20 hours, making it a good indicator of the level of ETS exposure over the previous 2–3 days. Nicotine has a shorter average half-life of 1–2 hours and is, therefore, a more accurate indicator of exposures occurring within the previous few hours. Thus, the determination of nicotine and cotinine in human plasma or urine is of particular interest in evaluating exposure to environmental tobacco smoke (ETS) and its effects on health.[3–6]

Besides the high toxicity of nicotine, its medical importance is expanded to therapeutic applications in a variety of disorders including Alzheimer's disease, Parkinson's disease, obesity, depression, anxiety, ulcerative colitis, Tourette's syndrome, and attention deficit disorder. Cotinine has also been reported to have a pharmacological action. It affects the release of brain neurotransmitters and enzymes involved in the synthesis of estrogen and testosterone. It also reduces vascular resistance and blood pressure in animals and has been reported to modify nicotine withdrawal symptoms in abstinent smokers. Therefore, a simple, fast, and sensitive method for the measurement of the levels of nicotine and of its major metabolite, cotinine, in biological samples is also essential for the development and clinical evaluation of nicotine treatment.[4,5,7]

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Analytical methods reported in literature include GC, HPLC, immunoassays, LC-MS, GC-MS, and TLC. Gas chromatographic and radioimmunoassay (RIA) techniques provide reliable results of nicotine and cotinine quantification, and are capable of discriminating accurately between smokers and non-smokers. However, cotinine levels in urine measured by the RIA method are 60% higher than the levels measured by the GC method. This is because immunoassays are less selective and can also react with other metabolites of nicotine. Chromatographic methods using HPLC and UV detection are used but sometimes they are time consuming, especially when sample preparation time is taken into account and they need a few millilitres of biological fluids either serum or urine.[1,7–14]

In the present paper, a simple, fast, and sensitive HPLC method is described for nicotine and cotinine levels in body fluids. Small sample volumes, only 200 mL, are adequate for analysis. The procedure that has been developed can be readily applied in clinical laboratories for the simultaneous determination of nicotine and cotinine in routine analysis of body fluids, serum, and urine, in order to evaluate the impact of active or passive smoking on health, as well as the pharmacological activity of these compounds.

EXPERIMENTAL

Reagents and Materials

Nicotine, cotinine, caffeine, and scopolamine hydrochloride used as internal standard, were purchased from Sigma (St. Louis, MO. USA). Paracetamol, ammonium acetate, and tetrahydrofuran were purchased from Merck (Darmstadt, Germany). All chemicals, studied for their convenience to be used as internal standards, were of the highest analytical grade.

HPLC grade methanol was obtained from Panreac (Barcelona, Spain), while HPLC grade isopropanol and acetonitrile were supplied from Riedel-de-Haen (AG, Seelze, Germany). Sodium hydroxide 1 M was prepared from Titrisol NaOH solution (Merck, Darmstadt, Germany). Urine samples were obtained from healthy volunteers: smokers and non-smokers. Serum samples were kindly provided from the Blood Donation Unity of a State Hospital.

Water used throughout analysis was de-ionised and filtered through a 0.2 μm filter.

HPLC Instrumentation

The chromatographic system operating in isocratic mode, consisted of the commercial components: a Shimadzu (Kyoto, Japan) LC-10AD pump, an SSI 500

variable UV/VIS detector (SSI, State College, PA, USA) operating at 262 nm with a sensitivity setting of 0.002 (AUFS), a 7125 Rheodyne (California, USA) injection valve with a 20 μL loop, and a HP 3396 II integrator (Hewlett-Packard, Avondale, PA, USA). The analytical column, a Kromasil C_8, 250 mm × 4 mm ID, 5 μm, was purchased from MZ Analysentechnik (Mainz, Germany).

Lichrolut RP-select B, Lichrolut RP-18 endcapped, and RP-8 Adsorbex SPE minicolumns, supplied from Merck, were used in this study, as well as C_8 Bond Elut and NEXUS ABSELUT by Varian (Harbor City, USA), Oasis HLB by Waters (Corporation, Massachusetts, USA) and DSC-18 by Supelco (Bellefonte, PA, USA).

A Vac-Elut vacuum manifold column processor used for SPE was purchased from Analytichem International, a division of Varian. All evaporations were performed with a 9-port Reacti-Vap evaporator (Pierce, Rockford, IL, USA).

UV spectra for selecting the monitoring wavelength of detection were provided by a Varian DMS 100S UV/Vis double-beam spectrophotometer prior to chromatographic method development.

A glass vacuum-filtration apparatus obtained from Alltech Associates was employed for the filtration of mobile phase, using 0.2 μm membrane filters, obtained from Schleicher and Schuell (Dassel, Germany). Degassing of solvents was achieved by sonication in a Transonic 460/H Ultrasonic bath (Elma, Germany) or by vigorous Helium sparging prior to use. A Glass-col, Terre Haute 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the sample pre-treatment.

Preparation of Standard Solutions

Solutions of stock reference standards (100 ng/μL) were prepared in methanol and found to be stable for at least three months when stored below 0°C until utilized. Working standards were prepared in methanol by appropriate dilutions containing the internal standard. These solutions were found to be stable throughout experimental analyses. Quantitation was based on linear regression analysis of analyte to internal standard peak area ratio versus analyte concentration in ng/μL.

Serum and urine standards were prepared daily by adding known amounts of the stock standards to pooled samples. These standards were used to create calibration curves as a control.

Serum samples were kindly provided by the Blood Donation Unit of the University Hospital of Thessaloniki. Samples could not be classified, as no information was available to discriminate blood donors to smokers and non-smokers. The pooled samples were stored below 0°C and left to thaw at room temperature prior to SPE.

Classified Urine samples from 18 volunteers, smokers and non-smokers, were collected in sterile bottles and stored below 0°C until analysis. Prior to SPE pre-treatment these samples were thawed and their pH value was adjusted to 10 with sodium hydroxide 1 M.

Chromatographic Conditions

Two Kromasil analytical columns C_8, and C_{18} 250 mm × 4 mm ID, 5 μm, were tested at ambient temperature 22°C. The former was found more efficient. Various mobile phases were tested with different methanol content. The final eluting system was selected in terms of optimum resolution and peak shapes of investigated compounds with a short retention time. The elution system was comprised of two phases: phase A: 0.05 M ammonium acetate and phase B: CH_3OH at a volume ratio 60 : 40. This was isocratically pumped at a flow rate of 1.4 mL/min. Inlet pressure observed was 290 kg/cm^2. Eluent was monitored at 262 nm. The sensitivity setting of the UV-VIS detector was adjusted at 0.002 AUFS.

A wide variety of compounds such as xanthine derivatives, paracetamol, quinine, bamifylline, hydrochlorothiazide, anthraquinone derivatives, anthranilic acid derivatives, lamotrigine, benzoic acid derivatives, and scopolamine were assayed towards their application as internal standard. Scopolamine was selected due to its elution properties, spectra characteristics, and its absence in biological fluids. The concentration of internal standard was 85 ng/μL.

Resolution factors were 1.96 for Cotinine and Nicotine and 1.79 for Nicotine and Scopolamine. A typical chromatogram is illustrated in Fig. 2.

Calibration Graphs

Calibration graphs were constructed by measuring the working standards at concentrations: 0.05, 0.1, 0.5, 1.0, 3.0, 5.0, 10.0, 15.0, 20.0, and 30.0 ng/mL. Internal standard scopolamine was added in each solution at a final concentration 85 ng/mL. The calibration graphs were obtained by plotting the peak area ratios of nicotine and cotinine to the internal standard versus the absolute amount of nicotine and cotinine by regression analysis. Each point represents an average of five determinations. For each calibration graph, the correlation coefficient, the intercept, and the slope were calculated. The detector response was checked daily at a minimum of three concentrations.

Calibration graphs for serum and urine were constructed by transferring aliquots of the standard solutions and internal standard into blank serum and blank urine, to give final concentrations in the range of 0.1–20.0 ng/mL. These calibration standards were extracted as described under SPE paragraph. Calibration data are shown in Table 1.

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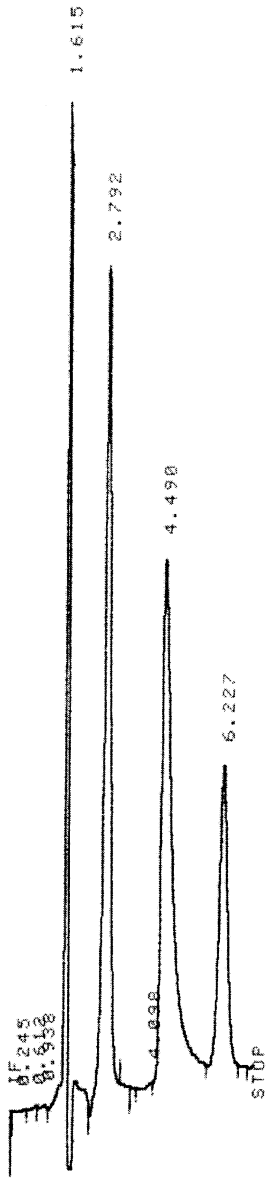

Figure 2. High performance liquid chromatogram of cotinine 2.792 min, nicotine 4.490 min, with 85 ng/μL scopolamine 6.227 min, as internal standard. Chromatographic conditions are described in text.

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Table 1. Calibration Data, Sensitivity, and Linearity Range of Nicotine and Cotinine for Standards, Serum, and Urine Samples

Analyte	Calibration Data	
Nicotine	Slope	0.022995 ± 0.048315
	Intercept	0.165535 ± 0.184376
	R	0.9993
	LOD (ng)	0.2
	LOQ (ng)	1
	Upper Limit (ng/μL)	20
Cotinine	Slope	0.024902 ± 0.000107
	Intercept	0.083737 ± 0.015108
	R	0.99997
	LOD (ng)	0.2
	LOQ (ng)	1
	Upper Limit (ng/μL)	30
	Serum	
Nicotine	$y = (0.014117 \pm 0.000182)\ x + (0.623042 \pm 0.02434)$	
	$R = 0.9990$	
Cotinine	$y = (0.019405 \pm 0.000271)\ x + (0.148154 \pm 0.041114)$	
	$R = 0.9990$	
	Urine	
Nicotine	$y = (0.0157387 \pm 0.000485)$	
	$x + (0.013265 \pm 0.038943)$	
	$R = 0.997$	
Cotinine	$y = (0.019389 \pm 0.000364)\ x + (0.316279 \pm 0.045766)$	
	$R = 0.9990$	
	$y =$ peak area ratio $x =$ ng	

Reproducibility and Accuracy

Accuracy was estimated by the following equation:

$$\text{Accuracy}(\%) = \frac{[\text{Mean determined value} - \text{theory (added amount)}]}{\text{theory}} \times 100$$

The intra-day repeatability was determined by analyzing three standards of eight replicates on the same day. The inter-day precision (defined as the RSD of

triplicate analyses) was obtained by analyzing the three standards on eight consecutive days. The results obtained are reported in Table 2.

Solid Phase Extraction Protocols

In order to investigate the recovery efficiency of several SPE sorbents from different manufacturers, the following adsorbents were studied: Lichrolut RP-select B, Lichrolut RP-18 endcapped, and RP-8 Adsorbex SPE minicolumns, supplied from Merck, C_8 Bond Elut and NEXUS ABSELUT by Varian, Oasis HLB by Waters, and DSC-18 by Supelco.

SPE procedure optimization was performed in several steps in order to obtain the higher recovery values. Wash and elution solvents of different strength were tested.

Recovery was calculated by comparing the peak area ratio of the analytes to the internal standard from the processed sample to the corresponding peak area ratio of the non-processed sample. The protocols under assay gave various recovery results, which are tabulated in Table 3. Low absolute recoveries were achieved with

Table 2. Intra-Day and Inter-Day Accuracy and Precision Data for Nicotine and Cotinine Determination

Analyte	Added Amount (ng)	Measured ± SD (ng)	Accuracy %	RSD
		Intra-day ($n = 8$)		
Nicotine	10	10.9 ± 0.5	9.0	4.7
	100	96.9 ± 1.8	−3.1	1.9
	200	209.1 ± 9.0	4.6	4.3
Cotinine	10	10.6 ± 0.6	6.0	5.7
	100	96.8 ± 5.1	−3.2	5.3
	200	217.6 ± 3.2	8.8	1.5
		Inter-day[a] ($n = 8$)		
Nicotine	10	10.9 ± 1.1	9.0	9.9
	100	98.5 ± 0.9	1.5	0.9
	200	205.8 ± 14.5	2.0	7.0
Cotinine	10	9.9 ± 0.9	−1.0	9.5
	100	97.7 ± 9.4	−2.3	9.6
	200	182.9 ± 15.1	−8.6	8.3

[a]Mean value of three measurements over a period of eight days.

Table 3. Nicotine and Cotinine Recovery After SPE with Different Extraction Protocols

SPE Cartridge	Eluting Solvent	Added, ng	Found Nicotine, ng	Recovery Nicotine, %	Found Cotinine, ng	Recovery Cotinine, %
Merck RP-18 (endcapped)	CH_3OH	10	2.41 ± 0.08	24.1	5.88 ± 0.42	58.8
		20	8.08 ± 0.63	40.4	13.22 ± 1.04	66.1
		100	30.4 ± 1.88	30.4	52.7 ± 1.56	52.7
Merck RP-18 (endcapped)	CH_3CN	10	1.24 ± 0.06	12.4	2.15 ± 0.08	21.5
		20	1.94 ± 0.13	9.7	4.98 ± 0.38	24.9
		100	6.5 ± 0.67	6.5	33.0 ± 1.56	33.0
Varian Bond Elut C8	CH_3OH	10	0.76 ± 0.10	7.6	8.17 ± 0.65	81.7
		20	0.48 ± 0.08	2.4	15.36 ± 1.15	76.8
		100	2.8 ± 0.29	2.8	62.7 ± 1.67	62.7
LC 18 Discovery	CH_3OH	10	2.73 ± 0.27	27.3	5.39 ± 0.34	53.9
		20	4.14 ± 0.23	20.7	9.10 ± 0.52	45.5
		100	29.5 ± 0.62	29.5	30.3 ± 0.99	30.3
Oasis HLB	CH_3OH	10	2.79 ± 0.31	27.9	5.85 ± 0.64	58.5
		20	7.64 ± 0.45	38.2	9.12 ± 0.61	45.6
		100	12.6 ± 1.01	12.6	23.8 ± 1.14	23.8
RP-8 Adsorbex	CH_3OH	10	2.64 ± 0.29	26.4	5.11 ± 0.43	51.1
		20	7.58 ± 0.37	37.9	8.22 ± 0.86	41.1
		100	30.2 ± 1.15	30.2	53.8 ± 2.42	53.8
Varian Bond Elut C8	CH_3CN	10	3.95 ± 0.43	39.5	9.56 ± 0.78	95.6
		20	7.72 ± 0.54	38.6	16.34 ± 0.84	81.7
		100	4.4 ± 0.51	4.4	56.1 ± 3.12	56.1

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Varian Nexus	CH_3OH	10	4.15 ± 0.36	41.5	5.53 ± 0.29	55.3
		20	8.64 ± 0.77	43.2	14.78 ± 1.32	73.9
		100	34.8 ± 1.59	34.8	51.0 ± 2.01	51.0
Varian Nexus	CH_3CN	10	4.28 ± 0.39	42.8	4.85 ± 0.40	48.5
		20	10.4 ± 0.73	52.0	8.70 ± 0.57	43.5
		100	26.7 ± 1.79	26.7	29.6 ± 1.66	29.6
Varian Nexus	THF	10	4.58 ± 0.35	45.8	5.62 ± 0.37	56.2
		20	13.7 ± 1.49	68.5	7.80 ± 0.76	39.0
		100	57.4 ± 1.16	57.4	71.3 ± 3.49	71.3
Varian Nexus	2-Propanol	10	3.75 ± 0.44	37.5	4.56 ± 0.58	45.6
		20	7.80 ± 0.63	39.0	9.62 ± 1.03	48.1
		100	33.9 ± 2.00	33.9	30.2 ± 1.69	30.2
Varian Nexus	$CH_3COONH_4-CH_3OH$ 60:40)	100	45.4 ± 1.26	45.4	48.2 ± 2.34	48.2
	$CH_3COONH_4-CH_3OH$ 20:80)	100	23.2 ± 0.66	23.2	34.5 ± 1.35	34.5
RP-8 Adsorbex	$CH_3COONH_4-CH_3OH$ 60:40)	100	30.8 ± 1.16	30.8	28.7 ± 1.77	28.7
Lichrolut RP-select B Merck	CH_3OH	10	4.96 ± 0.43	49.6	5.67 ± 0.51	56.7
		20	16.7 ± 0.78	83.5	24.60 ± 1.56	123.0
		100	42.5 ± 2.18	42.5	43.8 ± 2.15	43.8
Lichrolut RP-select B Merck	THF	10	6.5 ± 0.32	65.0	7.78 ± 0.40	77.8
		20	13.7 ± 0.49	68.5	15.38 ± 0.39	76.9
		100	64.8 ± 1.98	64.8	79.7 ± 2.71	79.7

most sorbents and eluent solvents used. The highest recovery rates 66% for nicotine and 78% for cotinine were obtained using Lichrolut RP select B cartridges by Merck, especially designed for basic compounds, and THF as elution solvent.

Wash solvent optimization was performed to establish the optimum clean-up procedure with as little analytes losses as possible. All SPE experiments were made at room temperature. Results are shown in Table 4.

Evaporation of eluting solvent under gentle nitrogen stream was performed by two ways: in a water bath at 45°C and at room temperature. The latter gave significantly higher nicotine recovery. Optimum setup parameters are summarized in Fig. 3.

Application to Biological Fluids: Blood Serum–Urine

Human Blood Serum

Aliquots of 200 μL pooled human blood serum were spiked with 200 μL of standard solution, at concentration levels of 2.0, 3.0, 5.0, 8.0, and 10.0 ng/mL. Each sample was treated with 400 μL of CH_3CN in order to precipitate proteins. After vortex mixing for two minutes, the sample was centrifuged at 3500 rpm for 10 min and the excess of acetonitrile was removed by evaporation at room temperature. Alternatively, the supernatant could be diluted with 1.5 mL water. Subsequently, the sample was slowly applied to the solid-phase cartridge (Lichrolut Select B) and treated according to the protocol as shown in Fig. 3. After elution with tetrahydrofuran, each sample was evaporated to dryness at room temperature under a gentle stream of nitrogen. The resultant residual was then reconstituted in 200 μL of 85 ng/μL internal standard solution and injected on to the analytical column. No interference from endogenous compounds was observed. The precision and the recovery, defined as the percentage of the added concentrations that was found, were evaluated on spiked serum samples (quality control samples) at concentrations of 3.0, 5.0, and 8.0 ng/mL.

A high performance liquid chromatogram of nicotine and cotinine extracted from human blood serum is shown in Fig. 4a.

Urine

Aliquots of 200 μL of pooled urine sample collected from non smokers who had not been exposed to environmental tobacco smoke recently (children with non smoking parents), were spiked with 200 μL of standard solution at concentration levels of 2.0, 3.0, 5.0, 8.0, and 10 ng/mL. Each sample was

Table 4. Wash Solvent Effect on Nicotine and Cotinine Recovery from Serum and Urine Samples Using Lichrolut RP-Select B (Merck) SPE Cartridges

Sample	Wash Solvent	Nicotine Recovery	Cotinine Recovery	Clean-Up
	Serum			
200 µL serum + 200 µL standard + 10 µL NaOH (1 M)	H_2O	—	+++	+++++
	H_2O—CH_3OH 85 : 15%v/v	+	—	++++
	H_2O—CH_3OH 80 : 20%v/v	—	—	++++
	H_2O—CH_3OH 95 : 5%v/v	++++	+++++	+++++
	Urine			
200 µL urine + 200 µL standard solution + 10 µL NaOH (1 M)	H_2O	++	+++	+++++
	H_2O—CH_3OH 95 : 5%v/v	+++	+++++	+++++
	H_2O—CH_3OH 90 : 10%v/v	++	++++	+++++
	H_2O—CH_3OH 85 : 15%v/v	—	+	+++++

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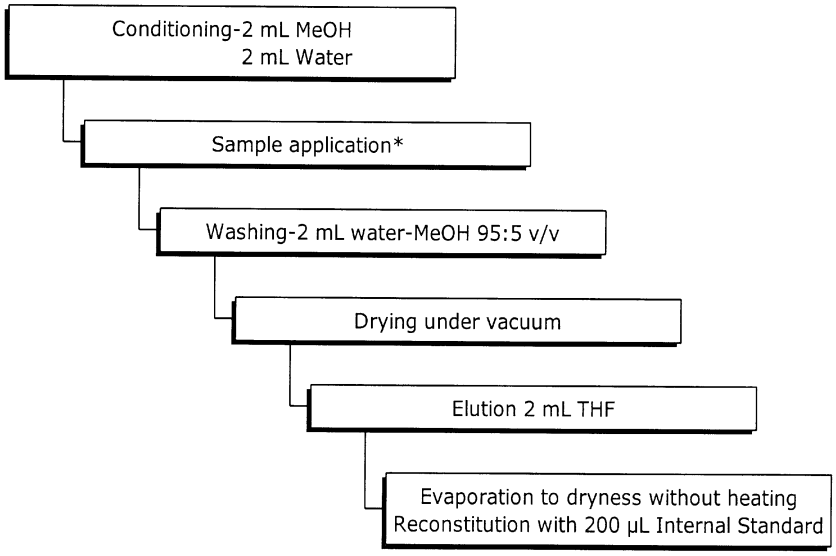

- 200 μL standard solution or
- 200 μL serum + NaOH (20:1)
- 200 μL serum +200 μL standard solution or
- 200 μL urine or
- 200 μL urine + 200 μL standard solution

Figure 3. Setup parameters for solid phase extraction optimization.

treated with 400 μL of CH_3CN. After vortex mixing for two minutes, the sample was centrifuged at 3500 rpm for 10 min and the supernatant was diluted with 1.5 mL water. Subsequently, the sample was slowly applied to the solid-phase cartridge and extracted according to the protocol described above. After elution with tetrahydrofuran, each sample was evaporated to dryness at room temperature under a gentle stream of nitrogen. Reconstitution to 200 μL was performed with an 85 ng/μL internal standard solution. The precision and the recovery defined as the percentage of the added concentrations that was found, were evaluated on spiked urine samples (quality control samples) at concentrations of 3.0, 5.0, and 8.0 ng/mL. Recovery results from both biological matrices are tabulated in Table 5.

No interference from matrix compounds was observed. Caffeine eluted at 3.080 min is sufficiently separated from nicotine and cotinine with a resolution

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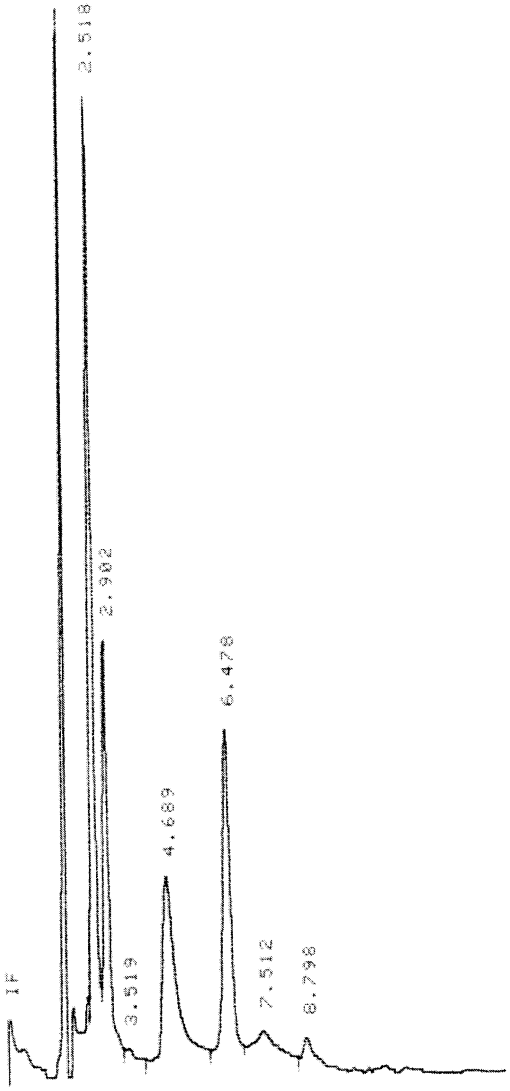

Figure 4a. High performance liquid chromatogram of determination of cotinine 2.902 min, nicotine 4.689 min, with 85 ng/μL scopolamine 6.478 min, as internal standard, in spiked human blood serum after SPE, using the conditions described in text.

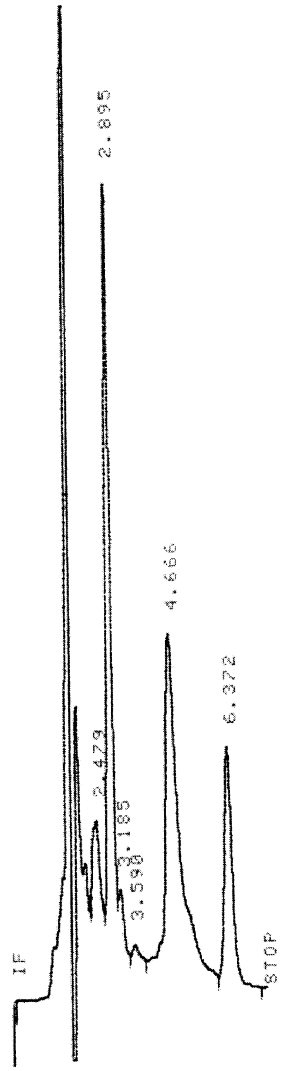

Figure 4b. High performance liquid chromatogram of determination of unknown endogenous compound 2.479 min, cotinine 2.895 min, nicotine 4.666 min, scopolamine 6.372 min (IS) in spiked urine sample after SPE, using the conditions described in text.

Table 5. Recovery of Nicotine and Cotinine from Biofluids: Serum and Urine After SPE

	Added (ng)	Found (ng)	Recovery %
		Serum	
Nicotine	60	58.1 ± 2.3	96.8
	100	115.5 ± 3.9	115.5
	160	156.3 ± 5.5	97.7
Cotinine	60	61.3 ± 1.4	102.2
	100	93.3 ± 5.9	93.3
	160	161.3 ± 8.1	100.8
		Urine	
Nicotine	60	57.3 ± 3.2	95.5
	100	94.2 ± 0.7	94.2
	160	155.5 ± 6.3	97.2
Cotinine	60	57.0 ± 2.9	95.0
	100	95.7 ± 4.2	95.7
	160	170.3 ± 8.1	106.4

factor of 0.9. A high performances liquid chromatogram of nicotine and cotinine, extracted from human urine, is shown in Fig. 4b.

Urine samples were collected from eighteen subjects classified as follows:

Group A: Unexposed Non-smokers.
Group B: Passive Smokers. ETS exposed Non-smokers.
Group C: Smokers (2–6 cigarettes daily).
Group D: Heavy Smokers (more than 20 cigarettes daily).

These samples were analyzed according to the developed protocol. Table 6 summarizes the results of these analyses. Figure 5 illustrates two representative chromatograms from class B and D.

Table 6. Concentration Range of Nicotine and Cotinine in Urine Samples from Various Subjects

Subject Group	Nicotine Concentration (ng/μL)	Cotinine Concentration (ng/μL)
A	ND	ND
B	0.6–1.0	ND
C	0.7–2.4	0.2–2.3
D	3.7–12.9	2.6–5.5

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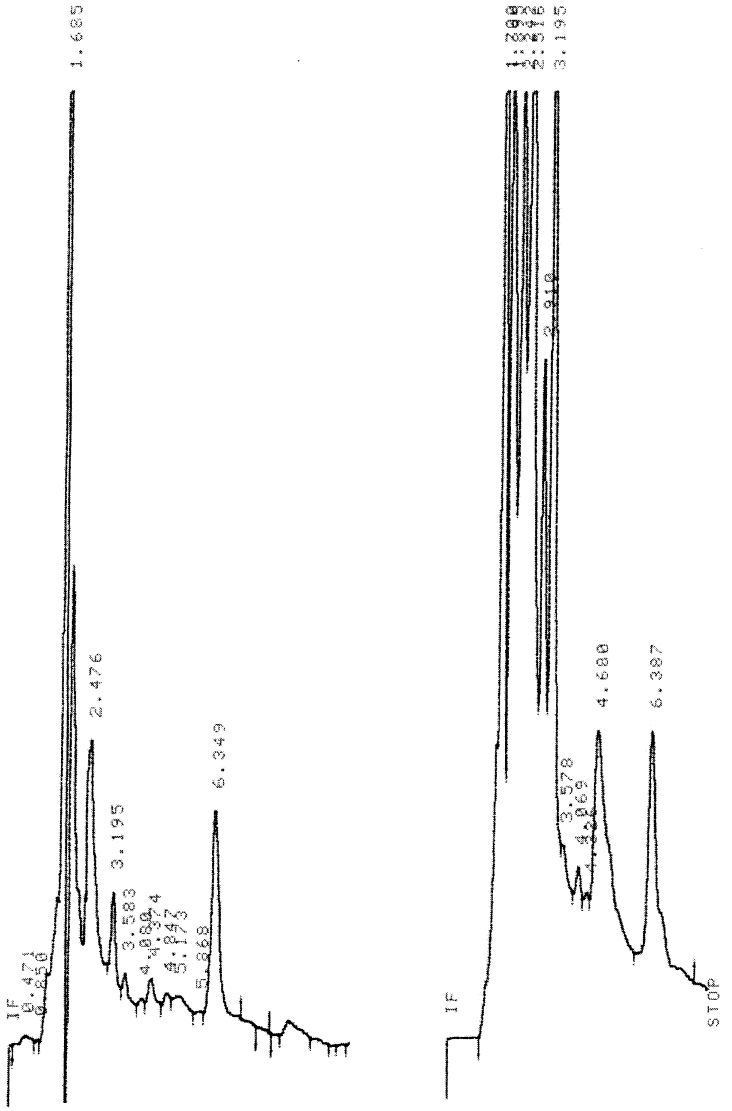

Figure 5. HPLC chromatograms of nicotine and cotinine determination in urine samples of passive and active smokers. Left—Class B: Passive smokers (exposed non-smokers) unknown endogenous compound 2.476 min, nicotine 4.374, caffeine 3.195 min, scopolamine 6.349 min. Right—Class D: Active smokers: unknown endogenous compound 2.516 min, cotinine 2.910 min, caffeine 3.195 min, nicotine 4.680 min, scopolamine 6.387 min.

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CONCLUSIONS

The determination of nicotine and cotinine, whose presence in body fluids is mainly attributed to exposure to tobacco smoke, is of great significance, as they are used as biomarkers to evaluate exposure to environmental tobacco smoke (ETS) and its effects on human health.

The method developed in the present study for the separation and quantitation of nicotine and its metabolite cotinine in serum and urine, is sufficiently selective, sensitive, accurate, and reproducible. Analysis time is approximately 6 min. Sample preparation requires 30–40 min., which can be reduced when multiple samples can be handled simultaneously.

A Kromasil C_8 column provided better resolution and performance than a Kromasil C_{18} of the same dimensions and same manufacturer. Linearity extends from 0.05 ng/mL to 20 ng/μL for nicotine and from 0.05 ng/mL to 30 ng/μL for cotinine. Limit of Detection is 0.2 ng for both compounds. The inter-day and intra-day variability was less than 8.3%. The mean value of all quality control standards deviated from the nominal concentration by less than 9%.

Low absolute recoveries were achieved with most sorbents and eluent solvents used. Lichrolut RP-select B Merck SPE cartridges were more suitable for sufficient and reproducible recovery of nicotine and cotinine 66% and 78%, respectively, towards other examined sorbents, while tetrahydrofuran was the most effective solvent. Wash solvent H_2O-CH_3OH at a volume ratio 95 : 5 yielded a cleaner sample as it removed endogenous interference, while it provided less analytes losses. Relative recovery evaluations showed that nicotine was recovered from serum at a mean rate of 103.3% and from urine 95.6%. Cotinine was recovered at a rate of 98.8% from serum and 99% from urine.

Caffeine was not interfering with the analysis of biofluids and this is a significant advantage of the method, since epidemiological studies have shown that smokers tend to drink more caffeinated beverages than non-smokers, indicating a positive association between cigarette smoking and coffee drinking.

This assay can be used in pharmacokinetics of nicotine and cotinine. Small sample volumes required, make the method suitable for clinical studies.

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