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Rapid and sensitive high-performance liquid chromatographic determination of nicotine and cotinine in nonsmoker human and rat urines

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Abstract

A simple reversed-phase high-performance liquid chromatographic method with paired-ion and UV detection has been developed for the rapid quantification of urinary nicotine and cotinine. A one-step solid–liquid extraction on Extrelut® was used. Separation from endogenous substances was achieved with a decreasing flow-rate. With 20 ml of urine for extraction, the limit of quantification was 0.5 ng/ml for cotinine and 5 ng/ml for nicotine; linearity was obtained from 50 to 5000 ng/ml. The intra- and inter-day coefficients of variation were less than 9% for cotinine and 30% for nicotine. Average recoveries for cotinine were 92–100% and 47–86% for nicotine. The present method was applied to the urine analysis of smokers, nonsmoker children, and experimental animals. © 1998 Elsevier Science B.V.

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1. Introduction

The determination of nicotine and one of its major metabolites, cotinine, in biological fluids has aroused particular interest during the last decades. Indeed these biochemical markers have been used to estimate active smoking behaviour, to validate abstinence smoking, to evaluate exposure to environmental tobacco smoke (ETS) [1] and its effects on health, and to supplement information for epidemiological studies.

Many analytical methods have been described for the analysis of nicotine and cotinine: gas chromatography (GC) [2,3] on capillary or packed columns with a nitrogen-sensitive detector [4] or an alkali flame ionization detector, but the low long-term stability of this latter detector limits its routine application. GC coupled with a mass spectrometer [5], a highly specific gas detector, is very expensive for routine analysis. High-performance liquid chromatography (HPLC) is the preferred technique [6–11], sometimes involving precolumn derivatization with diethylthiobarbituric acid [12], but these are not suitable for routine assays because the coloured complexes are unstable. Enzyme-linked immunosorbent assay (ELISA) [13] and radio-immunoassay (RIA) have also been described [14,15] but the former lack sensitivity and the latter is very expensive.

Progress in analytical methodology has improved the understanding of nicotine metabolism [1]. Metabolites such as *trans*-3′-hydroxycotinine and glucuro-

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nide conjugates of nicotine, cotinine, and *trans*-3′-hydroxycotinine have been studied [16–23] but unconjugated *trans*-3′-hydroxycotinine is difficult to quantify in urine since its hydrophilicity hinders extraction [18], and otherwise the assay of glucuronide metabolites requires longer pretreatment. All these methods include liquid–liquid or solid–liquid extractions, and all the procedures reported are fairly complicated, time consuming, and not suitable for application to large numbers of samples with low concentrations.

In this paper, a simple, fast and sensitive reversed-phase ion-paired liquid chromatographic procedure using ultra-violet detection was developed with a single-step solid-phase extraction. The aim of this study was to evaluate this procedure in routine and simultaneous analysis of nicotine and cotinine in urine of smokers, nonsmokers exposed to ETS, and animals, in order to evaluate the pharmacological effects of this tobacco alkaloid.

2. Experimental

2.1. Reagents and standards

Nicotine and cotinine were purchased commercially from Sigma (St. Louis, MO, USA). Methanol, acetonitrile, chloroform, 2-phenylimidazole, citric acid, disodium hydrogenphosphate dodecahydrate, triethylamine and sodium octanesulphonate were from Aldrich (Strasbourg, France). All solvents were analytical grade.

Stock solutions of nicotine, cotinine and 2-phenylimidazole, as internal standard (I.S.), were prepared separately for each compound at a concentration of 1 mg/ml in methanol. These stock solutions were stable for at least 3 months at 4°C. Calibration standards were prepared before use each day by diluting stock solution in methanol.

The pH buffer for the mobile phase was an aqueous phosphate buffer [24] containing 0.1 *M* citric acid–0.2 *M* disodium hydrogenphosphate dodecahydrate (56.4:43.6, v/v) at pH 4.4. For the extraction, Extrelut®-20 columns for urine samples (Merck, Darmstadt, Germany) were used. These prepacked columns are filled with granular support material able to retain hydrophilic fluids. The column was eluted using organic solvents. Lipophilic compounds were extracted by the solvent from the aqueous phase. The eluate did not contain emulsions.

2.2. Instrumentation

The HPLC system consisted of a Beckman Gold System with a programmable model 126 pump, a programmable 20-μl loop injector, and a programmable model 166 multiwavelength detector monitored at 260 nm. The stationary phase was a μBondapack C_{18} column Inertsil ODS-3 (250×4.6 mm, particle size 5 μm; GL Sciences). The system was monitored by IBM GOLD software.

2.3. Mobile phase

The mobile phase was a mixture of acetonitrile–methanol–pH buffer (90:30:880, v/v) containing 0.3 m*M* of sodium octane sulfonate as ion pair. The pH of the mobile phase was adjusted to 4.8 with triethylamine [25], to improve resolution and thus prevent the coelution of caffeine with cotinine. Before use, the mobile phase was degassed by filtration under reduced pressure through a 0.45-μm HA filter (Millipore, Bedford, MA, USA). The flow-rate was 1 ml/min, and 0.6 ml/min between 3 and 7 min of retention time, to separate nicotine from endogenous urine compounds which elute close to it.

2.4. Biological sample collection

Urine samples were collected in sterile bottles and stored at −20°C until analysis. They were thawed immediately prior to use.

2.5. Procedure

All glass tubes were soaked overnight in sulfochromic acid, rinsed with distilled water, and dried in an oven at 100°C.

2.6. Urine samples

A 10-ml sample of smoker urine (20 ml for nonsmoker urine) was mixed with 100 μl of a 0.01 mg/ml 2-phenylimidazole solution in methanol and 0.5 ml of 0.5 *M* sodium hydroxide. The mixture was

shaken and immediately transferred to an Extrelut-20 column. After a minimum of 30 min, the analytes were eluted with 40 ml of chloroform in a conical vial containing 100 μl of glacial acetic acid. The acid organic phase was evaporated to dryness at 40°C under a nitrogen stream, and the residue was reconstituted in 100 μl of mobile phase (1000 μl of mobile phase for heavy smoker urine). A 20-μl sample was introduced into the injector loop. This procedure was applied to smoker urine samples, nonsmoker urines (children exposed to parental ETS), and rat urine samples (exposed to experimental ETS).

3. Results

Fig. 1 shows typical results obtained with a blank nonsmoker's urine and the same blank urine spiked with nicotine, cotinine and I.S. solutions; and a low exposure urine sample from a child.

3.1. Linearity

The urine of a nonexposed nonsmoker was used for the standard addition studies. Various amounts of nicotine and cotinine (10–500 ng/ml of urine) were added to 10-ml aliquots of the blank urine to prepare the standard curve. To each blank urine sample, 100 μl of 0.01 mg/ml 2-phenylimidazole was added as I.S. Extractions were carried out six times for each spiked urine. The urine samples were processed as described above, and the evaporated residue was dissolved with either 100 μl or 1000 μl of mobile phase. A 20-μl volume was injected and chromatographed. The peak-surface ratios (nicotine and cotinine areas to 2-phenylimidazole area) were plotted versus the original concentrations and evaluated by linear least-squares regression analysis. The equations of the regression curves and their correlation coefficients (r) are shown in Table 1.

The calibration graph was linear from 50 to 500 ng/ml and from 100 to 5000 ng/ml.

To increase the sensitivity (for non-heavy-smokers) we used 20-ml aliquots of urine instead of 10 ml, and we reconstituted the evaporated extract in 100 μl.

3.2. Precision

The within-day and between-day variations, using blank urine samples spiked with aliquots of a methanolic solution of nicotine, cotinine, and I.S. were calculated to determine the precision of the method. Table 2 shows the averaged peak-surface ratios of the compounds with their variation coefficients.

3.3. Recovery

The recovery of each compound was measured under the extraction conditions described above. The peak area-ratios of the spiked urine samples were compared with those of standard solutions directly injected, without extraction and evaporation. The recoveries were excellent for cotinine, 92–100%, but only 47–86% for nicotine, (Table 3).

3.4. Limit of determination

The limit of detection (LOD) is defined as the amount of analyte giving a peak surface three times the maximum noise peak of a blank biological sample observed at the retention time of each analyte. The LOD values were 0.5 ng/ml for cotinine and 5.0 ng/ml for nicotine (signal-to-noise ratio=3) (Table 4). These results are based on the fact that up to 20 ml of urine may be used for extraction (factor of concentration=20), and the evaporated extract may be reconstituted with 100 μl of mobile phase for analysis (concentration factor=10, total concentration factor=200).

3.5. Applications

The method was applied to determine nicotine and cotinine in the urine of heavy smokers consuming between 20 and 40 cigarettes per day. Each assay was performed three times. The results are reported in Table 5. However, nicotine was not always measurable because its retention time was very close to that of endogenous urinary compounds. We also observed a high variability in urinary nicotine concentration among the different smokers, about 20-fold (170–4654 ng/ml) instead of 7-fold (342–2350 ng/ml) for urinary cotinine. This variability of

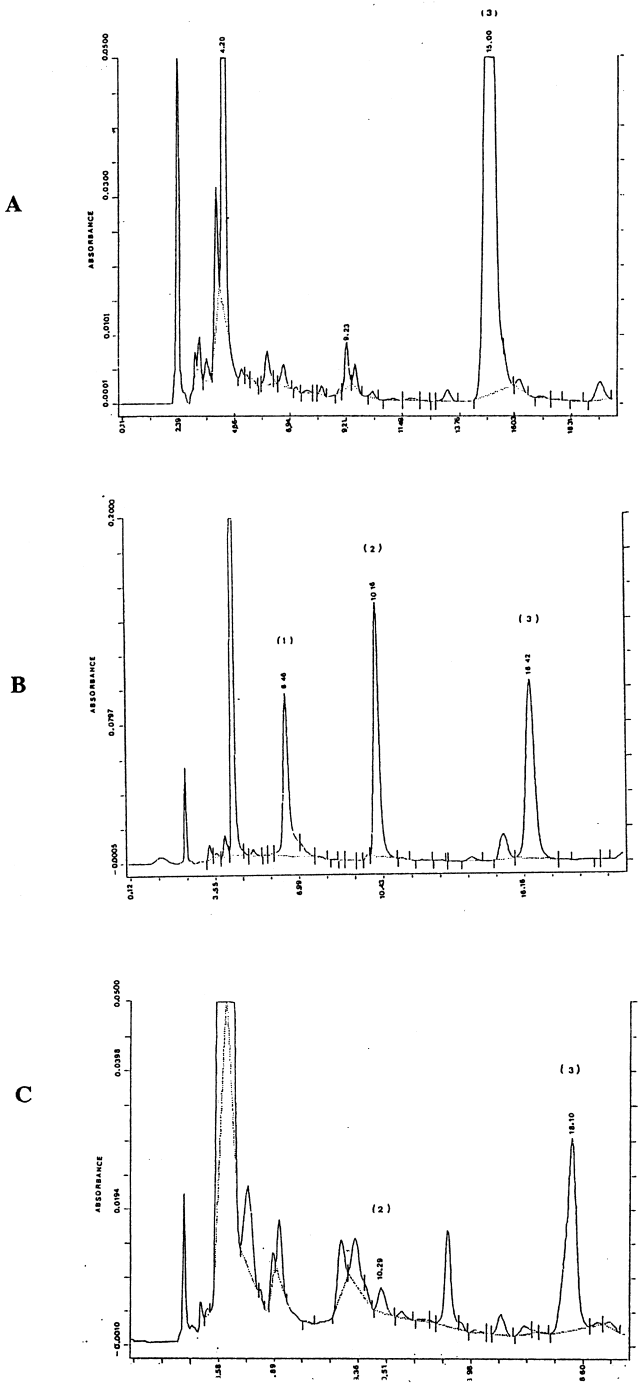

Fig. 1. (A) Blank urine from a nonexposed nonsmoker spiked with (3) 2-phenylimidazole (I.S.). (B) Blank urine spiked with the tested compounds (conc. 500 ng/ml): (1) nicotine, (2) cotinine, (3) 2-phenylimidazole. (C) Urine sample of a nonsmoker (child) exposed to ETS: (2) cotinine, (3) 2-phenylimidazole.

Table 1
Linearity

Compound	Concentrations (ng/ml)	*n*	Regression analysis	Correlation coefficient (*r*)
Nicotine	50–500	11	$y=0.0011x-0.0190$	0.984
	100–5000	15	$y=0.00085x+0.0325$	0.976
Cotinine	50–500	13	$y=0.0019x-0.0003$	0.998
	100–5000	27	$y=0.0023x-0.1148$	0.998

Table 2
Estimated variation of the HPLC method for urinary compounds

Compound	Concentration (ng/ml)	*n*	C.V. (%)
Within-day variation			
Nicotine			
	100	4	13.1
	200	4	31.4
	500	5	29.5
	5000	4	20.1
Cotinine			
	100	4	9.1
	200	5	4.6
	500	8	7.3
	2000	5	2.9
	5000	6	5.3
Between-day variation			
Nicotine			
	100	4	7.4
	200	4	23.7
	1000	4	8.7
Cotinine			
	100	4	12.1
	200	4	5.8
	1000	6	4.9
2-Phenylimidazole			
	100	6	4.6

nicotine probably resulted from different nonsmoking periods before urine collection.

The method was also used to evaluate ETS exposure. The concentration (mean of three assays each time) of urinary cotinine was measured in ten children (aged 5–14 years) who had household exposure to tobacco smoke. Using a 10-ml sample, we reconstituted the evaporated extract with 100 μl of mobile phase before chromatography. The results are shown in Table 6. The cotinine concentration depended on the number of cigarettes the parents smoked at home, on the child's age, on whether one or both parents smoked and even on which parent smoked. Urinary cotinine is a good reflection of smoke exposure because of its long half-life.

The method was also shown to be suitable for studying the effects of ETS on an experimental animal model. In the first experiment, five rats were exposed to side stream smoke from a smoking machine (Borgwald, Hamburg, Germany) from Gitane filterless cigarettes, for 4 days. Before the beginning of the experiment and at the end of the exposure, 24-h urines were collected and urinary cotinine was determined. The urine volume was less than 5 ml, thus the sample volume for assay was 2

Table 3
Recovery

Compound	Concentration (ng/ml)	Reference area ratios	After extraction area ratios	Recovery (%)
Nicotine	20	0.0748	0.0600	80
	100	1.0780	0.9255	86
	200	2.1792	1.4626	67
	1000	8.0827	4.5343	47
Cotinine	10	0.0605	0.0777	>100
	100	0.6270	0.5770	92
	200	1.7178	1.6957	99
	1000	9.1273	8.4890	92

Table 4
Limit of detection

Compound	Blank urine area		Spiked urine area	Observations
	A	$3A$	A'	
Nicotine	0.3567	1.0702	1.4761	>3S
Cotinine	0.0740	0.2221	0.7060	>3S

Table 5
Concentrations in smoker urines

Smokers	Nicotine (ng/ml)	Cotinine (ng/ml)
1	1006	342
2	1553	923
3	183	462
4	882	1392
5	2674	925
6	–	2350
7	–	1376
8	4654	1325
9	557	575
10	170	1140
Mean	1460	1080

ml. The results are reported in Table 7. No nicotine or cotinine was found in urine blanks (before experiment).

4. Discussion

The number of cigarettes smoked by parents inside and outside the house is a very subjective indicator of passive smoking in children, because smoking status is difficult to establish, and several other sources could introduce a bias, e.g., visitors at home, public transport, public buildings. Thus it was necessary to validate a method of evaluation of such exposure, one suitable for the processing of large

Table 6
Children exposed to environmental tobacco smoke

Child no.	Age (years)	Smoker at home	Number of cigarettes /day	Cotinine in urine (ng/ml)
1	14	Father	20	26.8
2	7	Father+mother	40	90.3
3	11	Mother	20	53.6
4	5	Father+mother	15	81.4
5	5	Mother	10	36.0
6	13	Father+mother	10	7.1
7	7	Father+mother	60	12.4
8	10	Mother	20	6.7
9	13	Father+mother	40	6.3
10	12	Father+mother	20	8.5

Table 7
Rats exposed to environmental tobacco smoke for 4 days

Rat no.	Urine volume (ml)	Sample volume (ng/ml)	Area ratio	Cotinine (ng/ml)
1	4.6	2	0.218	568
2	3.6	2	0.656	1587
3	4.0	2	0.272	675
4	3.5	2	0.218	525
5	4.5	2	0.273	675

numbers of samples for routine application and also for experimental animal models.

The present method improves a reliable procedure for the determination of cotinine levels for smokers and nonsmokers exposed to ETS, in term of speed and facility for routine analysis, involving no derivatization [11,16], no long liquid–liquid extraction with several steps [6], no need for special detectors such as photodiode array detector [22], and no special preparation to eliminate caffeine from the extract [25]. The Extrelut simplified the extraction procedure considerably, thus substantially reducing the analysis time, and improving the reproducibility of analysis. Use of an ion pairing column at pH 4.8 delayed the elution of the I.S., and improved the resolution of cotinine from caffeine. 2-Phenylimidazole is a good I.S. because of its efficient extraction, its good absorbtion at 260 nm, and its retention time (t_R=16–18 min) on the analytical column which permits rapid analysis. To optimise the separation of nicotine from endogenous urinary pigments (t_R=3–4 min), we propose a gradient of flow: we slowed the flow-rate between 3 and 7 min from 1 ml/min to 0.6 ml/min. But unlike cotinine, nicotine is not an efficient biochemical marker: its within-day and between-day reproducibility is higher than that of cotinine, its limit of detection is not as good, and its recovery is not satisfactory because of its high volatility, particularly during the extraction. Lastly, the nicotine level is a reflection of recent exposure because of it has a short half-life.

This method using cotinine as the biochemical marker of exposure to passive smoking, presents good sensitivity, recovery, reproducibility, and is suitable for a large number of applications.

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