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Short Communication

Determination of nicotine and four metabolites in the serum of smokers by high-performance liquid chromatography with ultraviolet detection

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ABSTRACT

A simple and reliable reversed-phase high-performance liquid chromatographic method with ultraviolet detection is described for the quantitation of nicotine and its metabolites cotinine, *trans*-3′-hydroxycotinine, norcotinine and cotinine N-oxide in human serum. The analytes and the internal standard, N-ethylnorcotinine, were extracted by solid-phase extraction before chromatography. Two different columns and mobile phases with gradient systems were used. The detection limit of the assay was 10 ng/ml for nicotine, 3 ng/ml for cotinine N-oxide and 5 ng/ml for cotinine, *trans*-3′-hydroxycotinine and norcotinine. The concentrations of nicotine and its metabolites in the serum of 12 cigarette smokers are reported.

INTRODUCTION

The complete characterization of nicotine metabolism in humans and the assessment of possible secondary metabolic pathways requires the measurement of the majority of nicotine metabolites [1]. At the moment, only radiometric high-performance liquid chromatographic (HPLC) assays enable the simultaneous quantification of several nicotine metabolites in biological fluids, but they appear to be of low applicability for routine determination in smokers [2–4]. Furthermore, assays have mostly been performed on urine samples, but rarely on blood samples [2,5,6]. A thermospray liquid chromatographic–mass spectrometric method has been described for the determination of a large number of metabolites [7,8]. However, the sensitivity achieved in this case allows quantification only in urine.

This paper describes an HPLC assay for the simultaneous determination of nicotine and four of its metabolites in human serum (Fig. 1), using UV detection. Data on the serum levels of the metabolites in 12 cigarette smokers are reported.

EXPERIMENTAL

Materials

Cotinine (COT) and nicotine (NIC) were purchased from Sigma (St. Louis, MO, USA). *trans*-3′-Hydroxycotinine (THOC), cotinine N-

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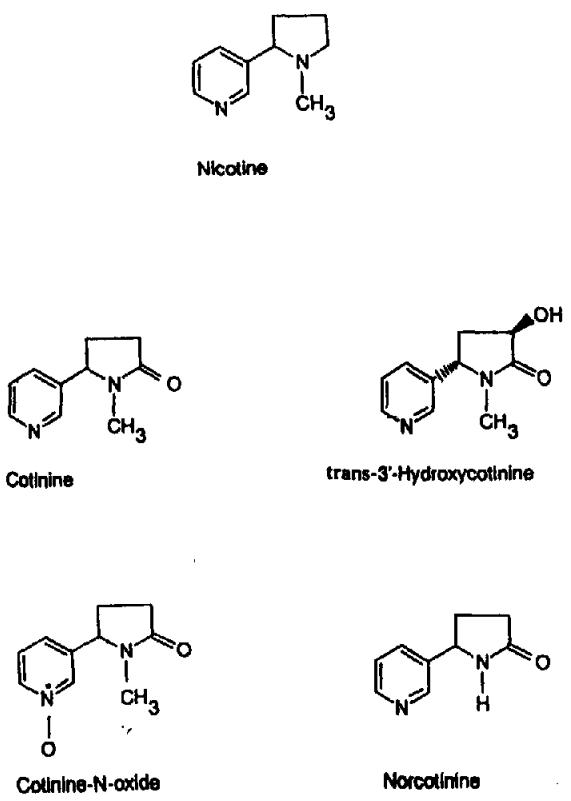

Fig. 1. Molecular structures of nicotine and four of its metabolites.

oxide (CNO), norcotinine (NCOT) and N-ethylnorcotinine (NENC) were obtained from Dr. Georg B. Neurath (Hamburg, Germany). Extrelut-3 extraction columns were from Merck (Bracco, Milan, Italy). All solvents were analytical grade.

Chromatography

The HPLC system consisted of a Waters 600 pump and system controller (Waters Chromatography Division, Rome, Italy), a Perkin-Elmer LC90J UV detector (Perkin-Elmer, Rome, Italy), set to 254 nm, and a Perkin-Elmer CC12 computing integrator (Perkin-Elmer).

Two different columns and mobile phase systems were used.

A Supelcosil LC_8DB column (5 μm particle size, 25 cm × 4.6 mm I.D.; Supelchem, Rome, Italy) was used with a binary gradient mobile phase of two solvents (A and B). Solvent A was water–acetonitrile (96.4:3.6, v/v) containing 2 ml/l of triethylamine and 0.012 *M* each of sodium heptanesulphonate, K_2HPO_4 and citric acid. The pH of the final solution was adjusted to 4.7 with citric acid. Solvent B was water–acetonitrile (80.3:19.7, v/v) containing 2 ml/l of triethylamine and 0.012 *M* each of sodium heptanesulphonate, K_2HPO_4 and citric acid, adjusted to pH 5.2 with citric acid. The flow-rate was 1.5 ml/min. The solvent programme consisted of a concave gradient with an initial 15-min step of 100% solvent A and a 20-min step from 100% solvent A to 50% solvent A and 50% solvent B. The initial conditions were re-established by equilibrating the column for 15 min at a flow-rate of 1.5 ml/min, or for 10 min at flow-rate of 1.8 ml/min, using a flow gradient during the analysis.

Alternatively, a Suplex pKb-100 column (5 μm particle size, 25 cm × 2.1 mm I.D.; Supelchem) was used with a gradient system consisting of 95% solvent C (0.014 *M* KH_2PO_4–water; 1:1, v/v) and 5% solvent D (0.014 *M* KH_2PO_4–acetonitrile; 1:1, v/v) at the beginning and changed to 40% solvent C and 60% solvent D in 30 min. Column re-equilibration was carried out in 10 min. The flow-rate was 0.3 ml/min. The injection volume was 20 μl, and UV detection wavelength was set at 254 nm.

Standards and controls

Solutions of stock reference standards (1 mg/ml, 10 μg/ml, 1 μg/ml) were prepared in methanol and stored below 0°C. Dilutions were made fresh daily for each analysis.

Serum standards were prepared daily by adding known amounts of the stock standards to drug-free human serum; these standards were used to create HPLC calibration curves as a control.

Biological sample collection

Blood samples (5 ml) from 12 smokers were obtained by venipuncture with silicone-coated vacutainers. Six samples were collected at 8:00 a.m. after *ca.* 8 h abstinence from smoking (group 1), and six samples after consumption of two or three cigarettes, the last one 10 min prior

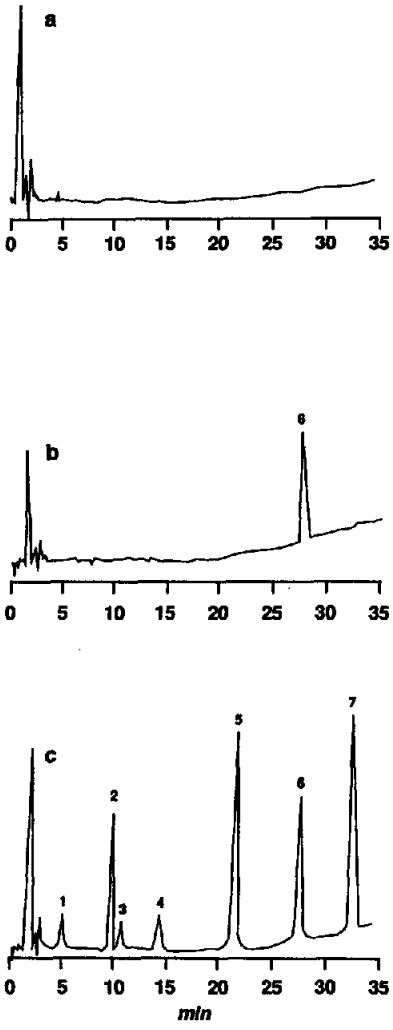

Fig. 2. LC_8DB column chromatograms of (a) reagent blank, (b) extract of 1.5 ml of serum blank sample from a non-smoker, and (c) extract of 1.5 ml of smoker's serum. Peaks:1 = cotinine-N-oxide (4.1 ng/ml); 2 = *trans*-3'-hydroxycotinine (70 ng/ml); 3 = norcotinine (8.1 ng/ml); 4 = nicotine (50.5 ng/ml); 5 = cotinine (310 ng/ml); 6 = caffeine (276 ng/ml); 7 = N-ethylnorcotinine (3 μg/ml).

to collection (group 2). Blank sera were obtained from non-smokers, who were not exposed to environmental tobacco smoke for at least 1 week. All samples were immediately centrifuged at 1000 *g* for 5 min. Serum was collected and stored at −20°C until analysis.

Extraction of biological samples

A 1.5-ml aliquot of serum, with 100 μl of

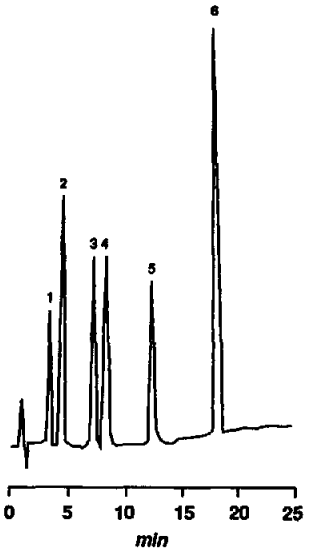

Fig. 3. Suplex pKb-100 column chromatogram of an extract of 1.5 ml of serum sample spiked with 500 ng/ml of nicotine (1), 100 ng/ml of cotinine-N-oxide (2), 500 ng/ml of *trans*-3'-hydroxycotinine (3), 500 ng/ml of norcotinine (4), 500 ng/ml of cotinine (5), and 3 μg/ml of N-ethylnorcotinine (6).

NENC (3 μg/ml aqueous solution) added, was mixed with 1.4 ml of 0.5 *M* sodium hydroxide and transferred to an Extrelut-3 glass column, which was preconditioned with 12 ml of dichloromethane the day before. After 15 min, the analytes were eluted under gravity with 10 ml of dichloromethane–isopropyl alcohol (9:1, v/v). The organic phase, with 300 μl of methanolic HCl (25 m*M*) added, was evaporated to dryness under nitrogen and redissolved in 100 μl of water. A 20-μl volume was injected into the HPLC column.

RESULTS AND DISCUSSION

The separation of nicotine and its metabolites by HPLC on the LC_8DB column is shown in Fig. 2. To shorten the length of the chromatographic run, a pKb 100 column for basic compounds was used (Fig. 3). This latter assay permitted the elution of caffeine at the end of the chromatographic run; but when the LC_8DB column was used a lengthy gradient programme was necessary to elute caffeine between cotinine and NENC. Caffeine, which was present in 90% of our samples,

TABLE I

DETECTION LIMITS AND RETENTION TIMES

Compound	Retention time (min)		Detection limit (ng/ml)
	LC_8DB column	pKb-100 column	
Cotinine N-oxide	4.90	4.58	3
trans-3'-Hydroxycotinine	9.45	7.55	5
Norcotinine	10.66	8.78	5
Nicotine	14.75	3.17	10
Cotinine	21.41	13.55	5

represented a possible interference in the HPLC determination of nicotine and its metabolites [9], as it eluted together with the other analytes when our solid-phase extraction procedure was used.

Calibration curves of the peak area *versus* amount of analytes (μg/ml) were prepared and checked daily from sera spiked with five different concentrations of the analytes and carried through the entire procedure. They were linear over the ranges 10–500 ng/ml for NIC ($y = 0.14x + 0.06$; $r = 0.99$), 5–500 ng/ml for COT ($y = 0.01x + 0.14$; $r = 0.90$) and THOC ($y = 0.016x$

TABLE II

RECOVERY AND VARIABILITY

Concentration (ng/ml)	Recovery, mean ± S.D., $n = 5$ (%)	Variability, mean, $n = 5$ (%)	
		Intra-day	Inter-day
NIC			
20	77.4 ± 3.1	4.1	6.2
100	79.1 ± 3.1	3.9	5.9
500	81.0 ± 2.8	3.6	5.4
COT			
20	89.0 ± 0.9	1.1	1.7
100	90.0 ± 2.0	2.2	3.3
500	90.0 ± 2.3	2.5	3.7
THOC			
20	72.8 ± 2.0	2.6	4.0
100	73.0 ± 2.0	2.7	4.0
500	73.3 ± 2.1	2.8	4.1
CNO			
20	60.5 ± 2.5	4.1	6.0
100	62.1 ± 2.6	4.2	6.4
500	63.0 ± 3.0	4.6	6.9
NCOT			
20	93.7 ± 3.5	3.8	5.5
100	93.8 ± 3.4	3.6	5.0
500	93.9 ± 3.5	3.7	4.7

TABLE III

CONCENTRATIONS IN SERUM SAMPLES OF SMOKERS

Compound	Concentration, mean ± S.D. (ng/ml)	
	Group 1[a]	Group 2[b]
NIC	0	43.7 ± 38
COT	286.5 ± 94	315.0 ± 84
THOC	68.3 ± 30	93.2 ± 40
CNO	3.6 ± 1.1	3.1 ± 1.3
NCOT	8.3 ± 2.8	6.5 ± 4.2

[a] 8 h abstinence from smoking.
[b] Two or three cigarettes prior to blood collection.

+ 0.10; $r = 0.93$) and NCOT ($y = 0.03x - 0.13$; $r = 0.99$), and 3–500 ng/ml for CNO ($y = 0.05x + 0.4$, $r = 0.99$). The detection limit of the assays at a signal-to-noise ratio of 3 and the retention times of the analytes are shown in Table I.

The analytical recoveries of these compounds, and the intra-day and inter-day variabilities proved to be almost identical using the two different assays. The values obtained with the LC_8DB column are shown in Table II. Using the LC_8DB column procedure, we have analysed sera of two groups of smokers (Table III).

Group 1 consisted of six smokers who had not smoked during the night and in the early morning, and Group 2 consisted of six smokers, who had smoked two or three cigarettes just prior to blood collection. Cotinine appeared as the major serum metabolite in both groups, followed by *trans*-3′-hydroxycotinine. Cotinine N-oxide and norcotinine were detectable in only trace amounts.

Our findings indicate that this HPLC method can be useful for pharmacokinetic studies of minor nicotine metabolites in humans, and for the characterization of sub-populations of smokers with an altered nicotine metabolism leading to different ratios between metabolites.

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