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Determination of nicotine and two major metabolites in serum by solid-phase extraction and high-performance liquid chromatography, and high-performance liquid chromatography–particle beam mass spectrometry

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ABSTRACT

A rapid and selective assay of nicotine, cotinine and *trans*-3′-hydroxycotinine in human serum, based on high-performance liquid chromatography with UV detection has been developed. The compounds were subjected to solid-phase extraction, using Extrelut 1 cartridges. Recoveries were *ca.* 95% for nicotine, 90% for cotinine and 50–55% for *trans*-3′-hydroxycotinine. The limit of quantitation observed with this method was 10 ng/ml for nicotine and 5 ng/ml for each of the metabolites. The compounds were also identified using high-performance liquid chromatography with particle beam mass spectrometry, to confirm their presence in human serum.

INTRODUCTION

The major tobacco alkaloid (−)-nicotine (NIC) is metabolized by humans to give a number of products, most of which are derived by oxidation of the pyrrolidine ring. Cotinine (COT) and *trans*-3′-hydroxycotinine (THOC) have been shown to be the principal metabolites of nicotine in blood and urine [1,2]. The simultaneous quantitation of these analytes in several biological fluids (blood, urine, saliva, cervical fluid, etc.) can be of importance to investigate the toxic effects associated with the presence of nicotine and its metabolites in a particular compartment [3].

For many years, nicotine and its metabolites have been assayed by specific and sensitive methods. These include gas chromatography [4,5] and high-performance liquid chromatography (HPLC) [6–11]. The method presented here is for the assay of NIC, COT and THOC in serum samples by reversed-phase HPLC using a UV detector, operating at 254 mm.

The feature of this method is that solid-phase extraction is used to obtain the compounds from serum samples [12,13]. Previous methods have always involved liquid–liquid extraction. HPLC with particle beam mass spectrometry (MS) was also used as a rapid confirmation of the analytes in serum.

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EXPERIMENTAL

Chemicals

Nicotine, cotinine, sodium heptanesulphonate, and reagent-grade triethylamine were purchased from Sigma (St. Louis, MO, USA). *trans*-3′-Hydroxycotinine and N-ethylnorcotinine (NENC) were obtained from Dr. Georg B. Neurath (Hamburg, Germany). Extrelut-1 extraction columns were from Merck (Bracco, Milan, Italy). All solvents were analytical grade.

HPLC analysis

The HPLC system consisted of a Merck-Hitachi L6200 intelligent pump, a Merck-Hitachi L4200 UV–VIS detector set at 254 nm, and a Merck-Hitachi D2000 chromato-integrator (Bracco). The column was a LC_8DB steel column (5 μm particle size, 25 cm × 4.6 mm I.D.; Supelchem, Rome, Italy). The mobile phase was water–acetonitrile (80:9, v/v), containing 5 ml of triethylamine, 670 mg/l sodium heptanesulphonate, and 0.034 *M* each of K_2HPO_4 and citric acid. The pH of the final solution was adjusted to 4.4 with citric acid. The flow-rate was 1.6 ml/min.

HPLC–MS analysis

Liquid chromatography was performed with a Waters 600 MS multisolvent delivery system (Waters Chromatography Division, Rome, Italy) equipped with a U6K universal liquid chromatography injector. The column was a μBondapak C_{18} steel column (10 μm particle size, 30 cm × 2.0 mm I.D.; Waters Chromatography Division). The elution was carried out with 0.1 *M* ammonium acetate–methanol (70:30) containing 0.4% triethylamine (pH 4.4 with acetic acid) at a flow-rate of 0.5 ml/min.

The HPLC system was connected to a LINC VG particle beam interface (Fisons Instruments, Milan, Italy). The HPLC effluent was converted into an aerosol in a nebulizer, in which helium was introduced coaxially, and sprayed into a desolvation chamber maintained at a low pressure

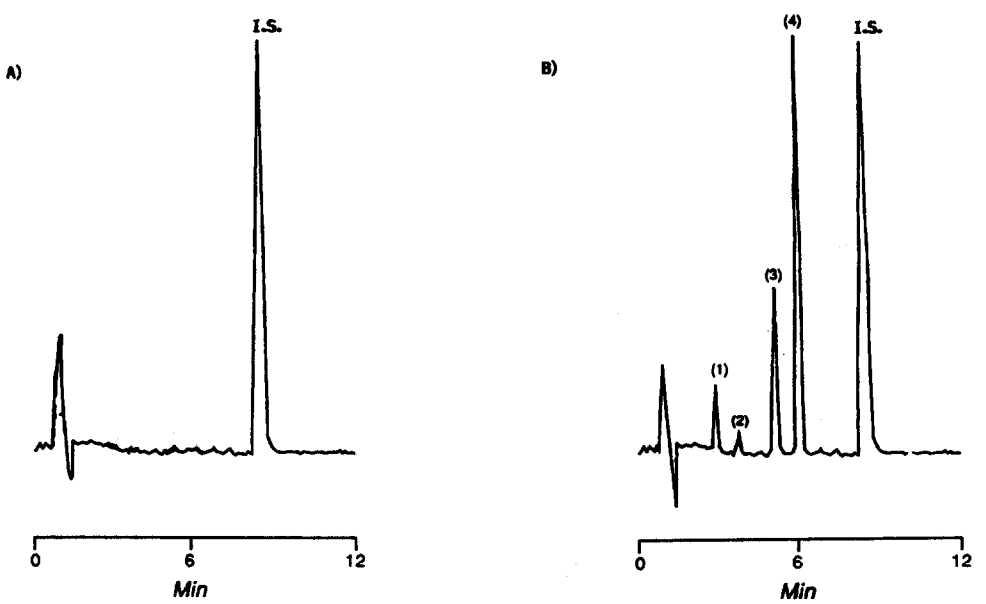

Fig. 1. Chromatograms of (A) extract of a 0.5-ml serum sample from a non-smokers, spiked with 3 μg/ml N-ethylnorcotinine (I.S.), (B) extract of a 0.5-ml serum sample from a non-smoker, spiked with 28 ng/ml THOC (1), 20 ng/ml nicotine (2), 68 ng/ml cotinine (3), 146 ng/ml caffeine (4), and 3 μg/ml N-ethylnorcotinine (I.S.).

and at a temperature of 40°C. Finally, the beam of solvent-free particles was introduced into the ion source of a VG TRIO 2 mass spectrometer (Fisons Instruments). The MS system was operated in electron impact (EI) mode with an electron energy of 100 eV, an emission current of 500 μA and an ion source temperature of 250°C. Prior to the analysis, the instrument was tuned in EI mode using the fragments *m/z* 69, 219, 220, 502, 503, 614 and 615 from the perfluorotributylamine. The mass spectrometer was operated either in the full scan or selected-ion monitoring (SIM) mode. For SIM the following ions were used: nicotine, *m/z* 84, 133, 162; cotinine, *m/z* 98 and 176; THOC, *m/z* 106, 192; N-ethylnorcotinine, *m/z* 112, 146, 190.

Standards and controls

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

TABLE I

RECOVERY AND VARIABILITY

Concentration (ng/ml)	Recovery (mean ± S.D.) (%)[a]	Variability[a] (%)	
		Intraday	Interday
Nicotine			
20	95.66 ± 1.15	1.11	2.75
100	96.10 ± 1.11	1.14	2.86
500	96.88 ± 1.17	1.19	2.98
Cotinine			
20	89.32 ± 1.53	1.70	4.00
100	90.66 ± 2.40	2.34	4.15
500	91.38 ± 1.80	1.95	3.98
trans-3'-Hydroxycotinine			
20	51.32 ± 1.50	2.80	4.85
100	52.32 ± 1.50	2.75	4.60
500	54.06 ± 1.33	2.46	4.33

[a] $n = 5$.

TABLE II

DETECTION LIMIT AND LINEARITY

Compound	Retention time (min)	Detection limit (ng/ml)	Linearity
Nicotine	3.80	10	$y = 0.08x + 0.09$
Cotinine	4.86	5	$y = 0.2x + 2.50$
trans-3'-Hydroxy-cotinine	3.04	5	$y = 0.5x + 2.45$

Biological samples collection

Blood samples (5 ml) from 20 smokers were obtained by venipuncture with silicone-coated vacutainers. Ten samples were collected at 8.00 a.m., after *ca.* 8 h of abstinence from smoking (Group 1) and ten samples after consumption of

TABLE III

CONCENTRATIONS IN SERUM SAMPLES OF SMOKERS

Subject No.	NIC (ng/ml)	COT (ng/ml)	THOC (ng/ml)
Group 1			
1	0.0	77.4	10.6
2	0.0	69.0	40.0
3	0.0	295.1	61.6
4	0.0	112.0	10.0
5	0.0	135.6	36.6
6	0.0	134.1	30.6
7	0.0	65.0	8.3
8	0.0	194.2	41.6
9	0.0	191.1	15.0
10	0.0	61.7	3.3
Mean ± S.D.	0.0	133.5 ± 75.0	25.7 ± 19.1
Group 2			
1	48.8	325.5	20.6
2	36.4	496.8	81.0
3	38.2	563.3	121.0
4	19.4	252.1	24.3
5	36.0	393.4	135.0
6	19.9	358.1	33.3
7	28.1	250.0	66.6
8	22.0	300.0	50.0
9	18.1	199.9	42.3
10	23.9	271.1	29.0
Mean ± S.D.	29.1 ± 10.2	341.0 ± 115.2	60.3 ± 40.4

two or three cigarettes, the last one 10 min prior to collection (Group 2). Blank samples 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 0.5 ml aliquot of serum with 100 μl of NENC (3 μg/ml) added was mixed with 0.4 ml of 0.5 *M* NaOH and transferred to an Extrelut 1 glass column, which was preconditioned with 8 ml of dichloromethane the day before. After 10 min, the analytes were eluted under gravity with 5 ml of dichloromethane. The organic phase, with 25 m*M* methanolic HCl added, was evaporated to dryness under nitrogen and redissolved in 100 μl of HPLC mobile phase. A 20-μl volume was injected into the HPLC column.

For HPLC–MS analysis ten serum samples of both groups were pooled together. A 5-ml aliquot was extracted as described above, redissolved in 100 μl of HPLC–MS mobile phase and injected into the HPLC–MS column.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatographic separation of NIC and its metabolites COT and THOC in a spiked serum from a non-smoker. There were no interfering peaks in blank samples.

The analytical recoveries of these compounds, and the intra-day and inter-day variabilities, are shown in Table I. The detection limit (signal-to-noise ratio of 3) and linearity of the method are

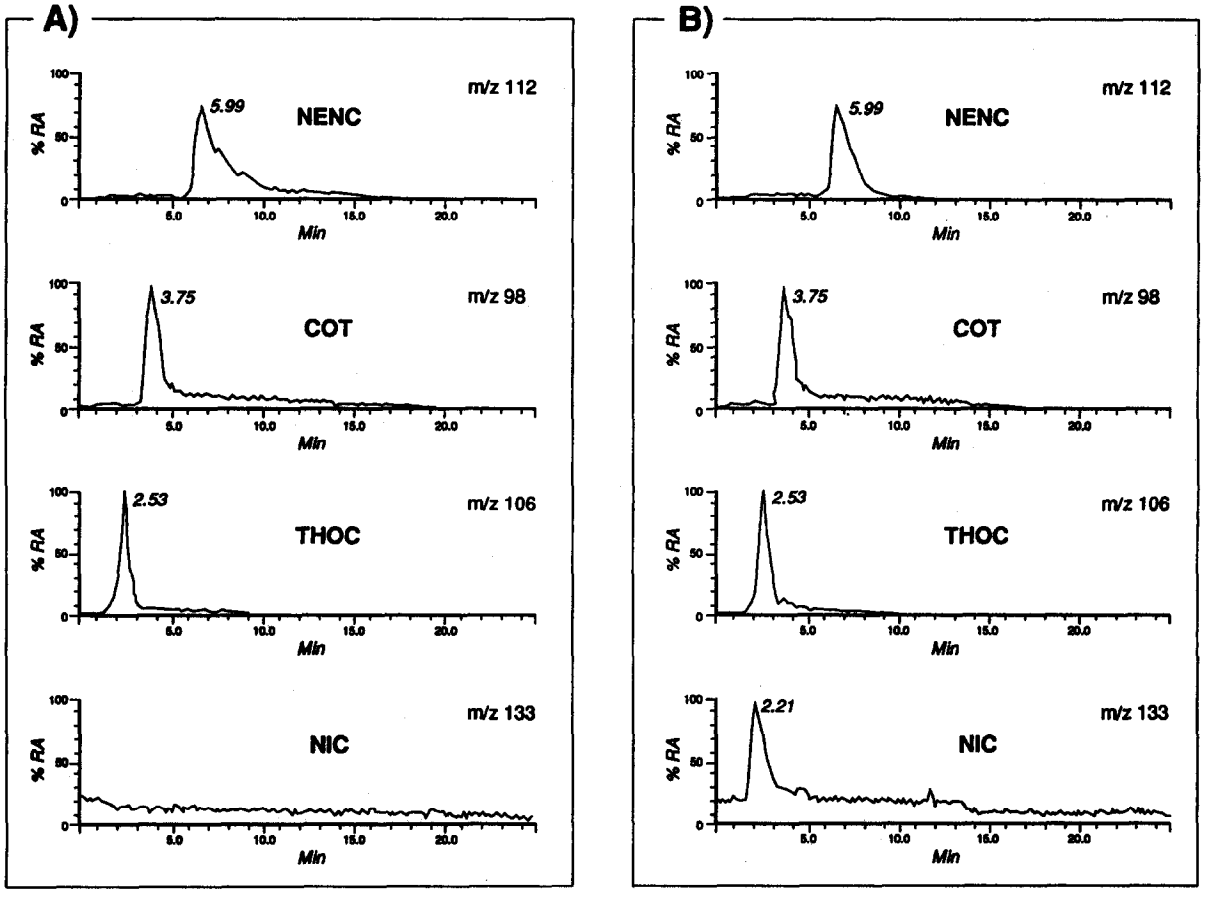

Fig. 2. SIM chromatograms of the pool of serum samples obtained from (A) Group 1 and (B) Group 2.

shown in Table II. The calibration curves were linear over the range 10–500 ng/ml for NIC, and 5–500 ng/ml for COT and THOC.

An example of the method's application was the determination of the analytes in the serum from 20 smokers. Under the condition of 8 h abstinence nicotine was absent in all the samples, whereas it was present after the consumption of two or three cigarettes; COT was always the major metabolite (Table III). These results were confirmed using HPLC–MS analysis. Fig. 2 shows SIM chromatograms of the pool of serum samples obtained in both groups.

Our results demonstrate that this HPLC method permits quick and simple extraction and simultaneous determination of NIC, COT and THOC in serum, with suitable resolution, sensitivity, recovery and reproducibility. Furthermore, HPLC with particle beam MS is a useful tool for rapid confirmation of the results obtained, with the advantage of the simultaneous analysis of compounds of different polarity.

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