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Short Communication

Determination of nicotine and its main metabolites in urine by high-performance liquid chromatography

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ABSTRACT

Nicotine and its main metabolites (cotinine, *trans*-3′-hydroxycotinine, *trans*-3′-hydroxycotinine glucuronide, nicotine-1′-N-oxide and 3-pyridylcarbinol) were analysed in urine after liquid–liquid extraction by high-performance liquid chromatography using norephedrine as internal standard, ultraviolet detection at 260 nm and scanning ultraviolet spectra with a photodiode-array detector. The conjugated *trans*-3′-hydroxycotinine was determined after enzymatic hydrolysis. Specific determination of 3-pyridylcarbinol was also carried out. Owing to its good selectivity, sensitivity and reproducibility, the method was applied to the analysis of urine samples from smokers and non-smokers. The results obtained suggest that the urinary markers used to assess active smoking or exposure to environmental tobacco smoke must be not only nicotine and cotinine, but also their main free and conjugated metabolites.

INTRODUCTION

The determination of nicotine and its metabolites in biological fluids provides a useful assessment of exposure to environmental tobacco smoke (ETS) for studying the possible associated health risks. A review of the present data on the metabolism of nicotine was recently published by Kyerematen and Vesell [1]. Furthermore, numerous methods have been developed during recent years for determining biological markers of active and passive smoking, especially in urine. They include gas chromatography [2–4], sometimes coupled with mass spectrometry [5–7], radioimmunoassay [8,9] and enzyme-linked immunoassay (ELISA) [10]. High-performance liquid chromatography (HPLC) has also been used for the same purpose after liquid–liquid extraction [7,11–14], solid–liquid extraction [15–19] or other methodological variations [20]. The present study was undertaken to develop an HPLC procedure for routine analysis of nicotine, cotinine, *trans*-3′-hydroxycotinine and its glucuronide, nicotine-1′-N-oxide and 3-pyridylcarbinol, which seem to be among the major compounds encountered in the urine of smokers and non-smokers exposed to ETS.

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EXPERIMENTAL

Reagent, glassware and standards

All reagents were of analytical grade. Sodium hydroxide (RPE = Reagente Puro Erba), glacial acetic acid (RPE), phosphoric acid (RPE), chloroform (RPE), diethyl ether (RPE), methanol (RS = Reagente Speciale, for HPLC) and acetonitrile (RS for HPLC) were from Carlo Erba (Milan, Italy). Potassium dihydrogenphosphate (pro analysi) was from Prolabo (Paris, France). Octanesulphonic acid (sodium salt), β-glucuronidase (*Escherichia coli*, type IX A), pH 6.8 phosphate buffer, nicotine, cotinine and 3-pyridylcarbinol were from Sigma (Saint-Quentin Fallavier, France). *trans*-3-'-Hydroxycotinine and nicotine-1'-N-oxide were kindly supplied by Dr. G. B. Neurath (Hamburg, Germany). Norephedrine (internal standard, I.S.) was obtained from Fournier Labs. (Dijon, France).

All glassware was first washed with a 3% RBS 25 biodegradable alkaline solution from Biolyon (Dardilly, France), containing a mixture of anionic and non-ionic detergents, and then rinsed with distilled water and dried before use.

Stock solutions of tested compounds were prepared in methanol at 1 $\mu g\ \mu l^{-1}$ and stored at 4°C. The quality remained good for at least one month. Stock solutions were diluted with methanol before use for preparation of calibration standards.

Procedure

Determination of nicotine, cotinine, free trans-3'-hydroxycotinine, nicotine-1'-N-oxide and 3-pyridylcarbinol. Urine (1–10 ml) was pipetted into a glass centrifuge tube. A 50-μl volume of norephedrine (internal standard, I.S.) at 1 $\mu g\ \mu l^{-1}$ in methanol, 1 ml of 3 *M* sodium hydroxide and 10 ml of chloroform–methanol (9.5:0.5, v/v) were added. The mixture was shaken for 10 min and centrifuged at 2800 *g*. The extraction was repeated twice. The organic layers were collected on 10 μl of glacial acetic acid to avoid volatilization leakage [21], and evaporated under a stream of nitrogen at 25°C. The residue was dissolved in 100 μl of 0.0033 *M* phosphoric acid. After vortex-mixing for 30 s, the acid solution was washed by shaking with 4 ml of diethyl ether for 30 s. After centrifugation at 2800 *g* for 5 min, the ether layer was discarded. A 20-μl aliquot of the residual acid solution was injected into the chromatograph.

Determination of conjugated trans-3'-hydroxycotinine. In urine *trans*-3'-hydroxycotinine is only conjugated with glucuronic acid (no sulphate conjugation [22]). Total *trans*-3'-hydroxycotinine was determined as follows. The tested urine sample was incubated at 37°C for 24 h with 1 ml of phosphate buffer (pH 6.8) containing 7600 U of β-glucuronidase, and then treated as described for the non-conjugated fraction. The amount of the conjugated *trans*-3'-hydroxycotinine was obtained by calculating the difference between the total metabolite and its free fraction.

Apparatus and chromatographic parameters

Chromatographic analysis was performed on a Waters-Millipore system (Saint Quentin-en-Yvelines, France) consisting of an M510 pump, a μBondapak C_{18} column (30 cm × 3.9 mm I.D., particle size 10 μm, ambient temperature), a Rheodyne M7125 20-μl high-pressure loop injector and an M490 multiwavelength detector monitored at 260 nm. The UV spectrum of each separate compound was also scanned between 200 and 350 nm with an M990 photodiode-array detector coupled to an NEC APC IV computer.

Mobile phase

For the determination of the free compounds and the *trans*-3'-hydroxycotinine released from its conjugate the mobile phase was a mixture of acetonitrile–methanol–0.025 *M* potassium dihydrogenphosphate (9:3:88, v/v/v). A 0.43-ml volume of 0.01 *M* sodium octanesulphonate solution was then added to the mixture. The flow-rate was 0.7 ml min^{-1}.

As 3-pyridylcarbinol is poorly separated from endogenous substances of urine under these operating conditions, a gradient elution was useful for its selective determination. Using a two-pump system the profile consisted of an initial 6-min linear gradient from 100 to 88% (2% per min) of

the aqueous phase (0.025 *M* potassium dihydrogenphosphate plus 0.01 *M* sodium octanesulphonate, 88:0.43, v/v) distributed by the first pump, and from 0 to 12% of the organic mixture (acetonitrile–methanol, 9:3, v/v) distributed by the second pump. The flow-rate was 0.7 ml min^{-1} for both solutions. The concentrations of the two solvents were then isocratically maintained at 88 and 12%, respectively, with the same flow-rate.

Calculation

The ratios between the peak areas of the compounds and that of the internal standard were calculated for the analysed urine and plotted against the concentrations of the compounds tested after analysis of blank samples spiked with increasing concentrations (100–2000 ng ml^{-1}) of nicotine, cotinine, *trans*-3′-hydroxycotinine, nicotine-1′-N-oxide and 3-pyridylcarbinol, and a constant amount of internal standard (50 μg of norephedrine).The blank was a urine sample from a non-smoker not exposed to environmental tobacco smoke, and analysed as a control before spiking.

RESULTS AND DISCUSSION

Separation

Fig. 1 shows chromatograms of a blank urine sample (A) and of the same urine spiked with the tested compounds (B) with the corresponding UV spectra. The maximum absorption was found at 260 nm, except for norephedrine (258 nm) and caffeine (273 nm). The retention times (relative to internal standard) and the response intensity at 260 nm are shown in Table I.

The μBondapak C_{18} reversed-phase column, under the described chromatographic conditions, exhibited good efficiency and short and symmetrical peaks were obtained. The high potassium dihydrogenphosphate content of the mobile phase (88% of 0.025 *M* solution) improved the separation of nicotine from its metabolites and the separation of the metabolites themselves. However, tailing of cotinine and nicotine-1′-N-oxide peaks was observed when the concentration of the potassium dihydrogenphosphate solution was lower than 0.02 *M*. So 0.025 *M* concentration was chosen in the eluent. The organic part of the mobile phase consisted of a mixture of acetonitrile and methanol. In the absence of methanol *trans*-3′-hydroxycotinine and nicotine-1′-N-oxide were eluted as a single peak, and this was also the case for cotinine and caffeine. The addition of methanol to acetonitrile (1:3) resolved the problem. The addition of a small volume of 0.01 *M* sodium octanesulphonate solution to the mobile phase ensured the reproducibility of the separation and improved the peak sharpness of the analysed compounds.

Using the above-described gradient elution procedure for the selective determination of 3-pyridylcarbinol, the polar urinary compounds, weakly retained, were eluted before the metabolite (Fig. 1C). Unfortunately, the column needed several minutes to again reach a good stability for the determination of the other compounds: nicotine and cotinine-1′-N-oxide (also cotinine and caffeine) were then eluted as single peak (Fig. 1C). Thus the separate determination of 3-pyridylcarbinol seems appropriate and the whole procedure is preferably carried out on three samples (1–10 ml) of the same urine to determine the concentrations of nicotine and all of its considered metabolites.

TABLE I

RELATIVE RETENTION TIME AND RESPONSE AT 260 nm OF TESTED AND POSSIBLE ENCOUNTERED COMPOUNDS IN URINE

Compound	Retention time (relative to I.S.)	Response at 260 nm[a]
Endogenous substances	0.48–0.50	+
Nicotinamide	0.50	+
3-Pyridylcarbinol	0.53	+
Theobromine	0.61	±
Nicotine	0.65	+
trans-3′-Hydroxycotinine	0.76	+
Nicotine-1′-N-oxide	0.85	+
Theophylline	0.93	±
Norephedrine (I.S.)	1	±
Cotinine	1.43	+
Caffeine	1.59	±

[a] +, good response; ±, medium response.

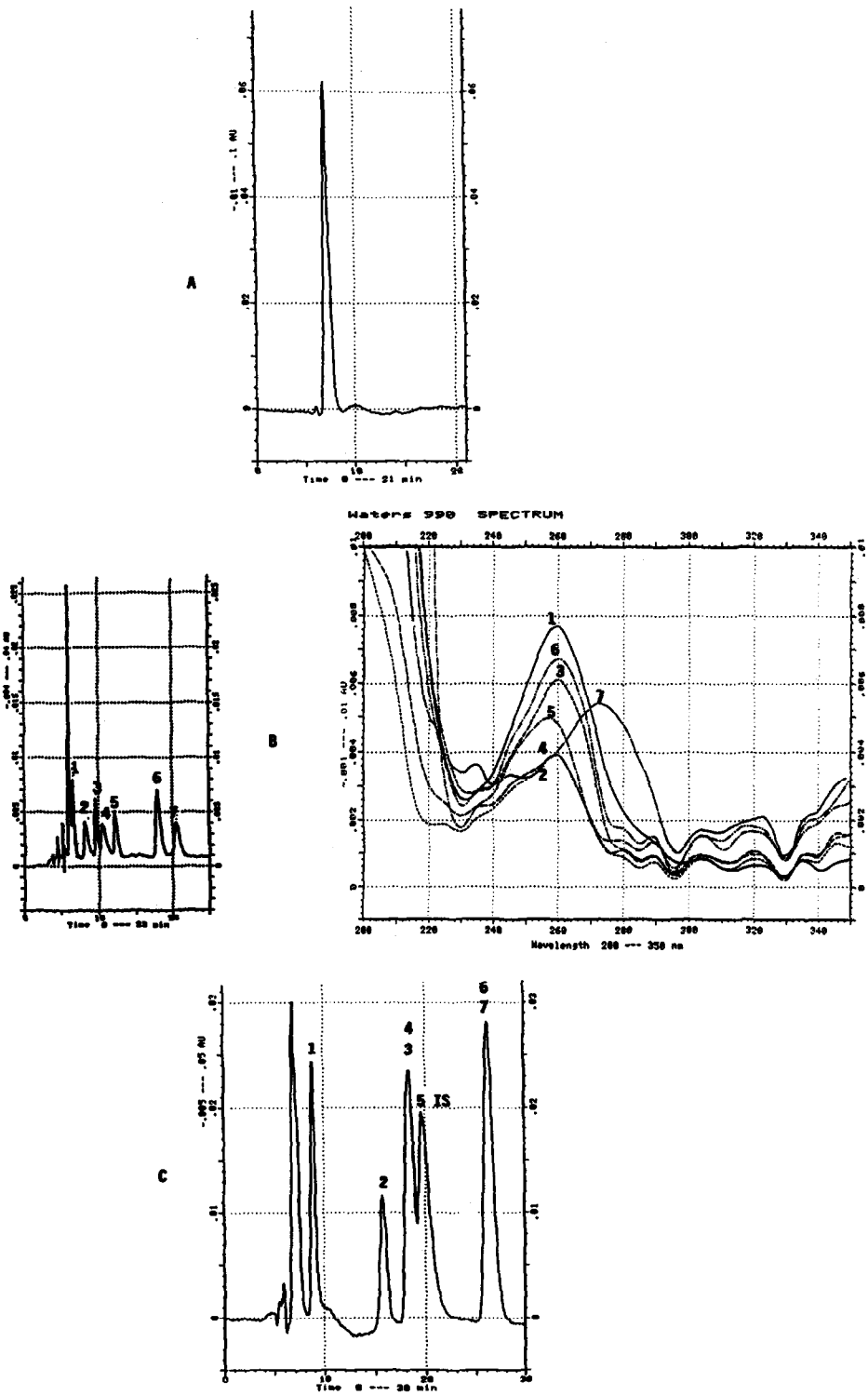

Fig. 1. Chromatograms and corresponding UV spectra. (A) Blank urine from a non-exposed non-smoker. (B) Blank urine (isocratic procedure) spiked with the tested compounds: 1 = 3-pyridylcarbinol, 50 ng ml^{-1}; 2 = nicotine, 60 ng ml^{-1}; 3 = *trans*-3′-hydroxycotinine, 60 ng ml^{-1}; 4 = nicotine-1′-N-oxide, 80 ng ml^{-1}; 5 = norephedrine (internal standard), 50 μg added; 6 = cotinine, 80 ng ml^{-1}; 7 = caffeine, 80 ng ml^{-1} (spectrum overwritten). (C) Blank urine (gradient procedure) spiked with the tested compounds: 1 = 3-pyridylcarbinol, 500 ng ml^{-1}; 2 = *trans*-3′-hydroxycotinine, 1000 ng ml^{-1}; 3 and 4 = nicotine, 1000 ng ml^{-1} and nicotine-1′-N-oxide, 1000 ng ml^{-1}; 5 = norephedrine (I.S.), 50 μg added; 6 and 7 = cotinine, 1000 ng ml^{-1} and caffeine 1000 ng ml^{-1}.

TABLE II

REPRODUCIBILITY

Values are mean ± S.D. (n = 8).

Amount added to blank urine (ng ml^{-1})	Nicotine		Cotinine		*trans*-3′-Hydroxycotinine				Nicotine-1′-N-oxide		3-Pyridylcarbinol	
					Free		Conjugated					
	Found (ng ml^{-1})	C.V. (%)	Found (ng ml^{-1})	C.V. (%)	Found (ng ml^{-1})	C.V. (%)	Found[a] (ng ml^{-1})	C.V (%)	Found (ng ml^{-1})	C.V. (%)	Found (ng ml^{-1})	C.V. (%)
100	95 ± 14	14.7	104 ± 10	9.6	102 ± 12	12.8			96 ± 14	14.5	101 ± 13	12.8
200	195 ± 18	9.2	197 ± 16	8	204 ± 20	9.8			188 ± 20	10.6	210 ± 22	10.5
400	401 ± 42	10.5	395 ± 37	9.4	410 ± 39	9.5			420 ± 43	10.2	407 ± 37	9.1
800	812 ± 60	7.4	795 ± 55	6.9	820 ± 70	8.5			808 ± 65	8	840 ± 70	8.3
1000	967 ± 58	6	1020 ± 49	4.8	1010 ± 75	7.4			956 ± 71	7.4	1024 ± 77	7.5
2000	1990 ± 60	3	2080 ± 58	4.8	2005 ± 69	3.4			1980 ± 68	3.4	2020 ± 80	3.9
Smoker's urine												
1							280 ± 32	12.3				
2							1700 ± 86	5				
3							405 ± 39	9.6				
4							121 ± 12	10				

[a] n = 5.

Release of trans-3′-hydroxycotinine from its conjugate

To confirm the complete release of *trans*-3′-hydroxycotinine from its glucuronic conjugate, several samples of the same urine from a smoker were studied. Each sample (1 ml) was incubated at 37°C with 1 ml of phosphate buffer (pH 6.8) containing 7600 U of β-glucuronidase. Every 4 h one of the samples was treated as described for determining the non-conjugated compounds. The amount of *trans*-3′-hydroxycotinine reached a plateau at an incubation time of 20 h, indicating the release was then complete. Thus a 24-h incubation time was retained for the enzymatic hydrolysis.

Recovery

The addition of methanol (5%) to the extraction solvent clearly improved the recovery, especially of nicotine-1′-N-oxide and *trans*-3′-hydroxycotinine. The recovery of each compound (100 ng ml^{-1}, $n = 8$) was measured under the extraction conditions described above. Peak-area ratios of the extracts were compared with those obtained by direct injection of the residue of the methanolic standard solutions after evaporation and dissolution of each residue in 100 μl of 0.0033 *M* phosphoric acid. The recoveries were 65% for nicotine and *trans*-3′-hydroxycotinine, 70% for cotinine, 60% for nicotine-1′-N-oxide and 55% for 3-pyridylcarbinol.

Linearity

Within the above concentration range, the relations were linear. The equations of the regression curves and their correlation coefficients (r) were as follows: nicotine, $y = 0.0012x - 0.015$ ($r = 0.996$); cotinine, $y = 0.0017x - 0.0030$ ($r = 0.998$); *trans*-3′-hydroxycotinine, $y = 0.0020x + 0.015$ ($r = 0.998$); nicotine-1′-N-oxide, $y = 0.0010x - 0.080$ ($r = 0.995$); 3-pyridylcarbinol, $y = 0.0020x - 0.006$ ($r = 0.995$).

Reproducibility

Only the within-day reproducibility was tested, using blank urine samples spiked with 100, 200, 400, 800, 1000 and 2000 ng ml^{-1} of each compound. Coefficients of variation obtained on the same day for all compounds were less than 14.7% (Table II). For the *trans*-3′-hydroxycotinine glucuronide, the tests were carried out on urine samples from smokers using the procedure described for the analysis of conjugated compounds (five determinations for the same sample): coefficients of variation were less than 12.3% (Table II).

Limit of determination

With concentrations less than 50 ng ml^{-1} nicotine, cotinine, *trans*-3′-hydroxycotinine and less than 100 ng ml^{-1} 3-pyridylcarbinol, the analysis must be carried out with a higher volume of urine. From 10 ml of urine, the limit of determination (signal-to-noise ratio = 3) was about 5 ng ml^{-1} for nicotine, cotinine and *trans*-3′-hydroxycotinine and about 10 ng ml^{-1} for nicotine-1′-N-oxide and 3-pyridylcarbinol.

Selectivity

Using the described procedure no interference with caffeine and its metabolites (theophylline and theobromine) was observed, but endogenous constituents of urine and nicotinamide were eluted in about the same time as 3-pyridylcarbinol (Table I), requiring a special gradient procedure to quantify this substance.

Applications

The proposed methods were applied to 24-h urine samples from several smokers and non-smokers. The results obtained are illustrated in Fig. 2A–D. Nicotine-1′-N-oxide was only identified in the urine of smokers. *trans*-3′-Hydroxycotinine was found to be the major metabolite among the majority of smokers and non-smokers exposed to ETS; the free form was about 64% of the total amount and the glucurono-conjugated form about 36% (mean values). Cotinine was quantitatively the second metabolite. Nicotine and/or 3-pyridylcarbinol took the third position.

Metabolic variations were noticed among the subjects investigated. Among the non-smokers exposed to ETS the concentrations found were low. Neither nicotine nor its metabolites were de-

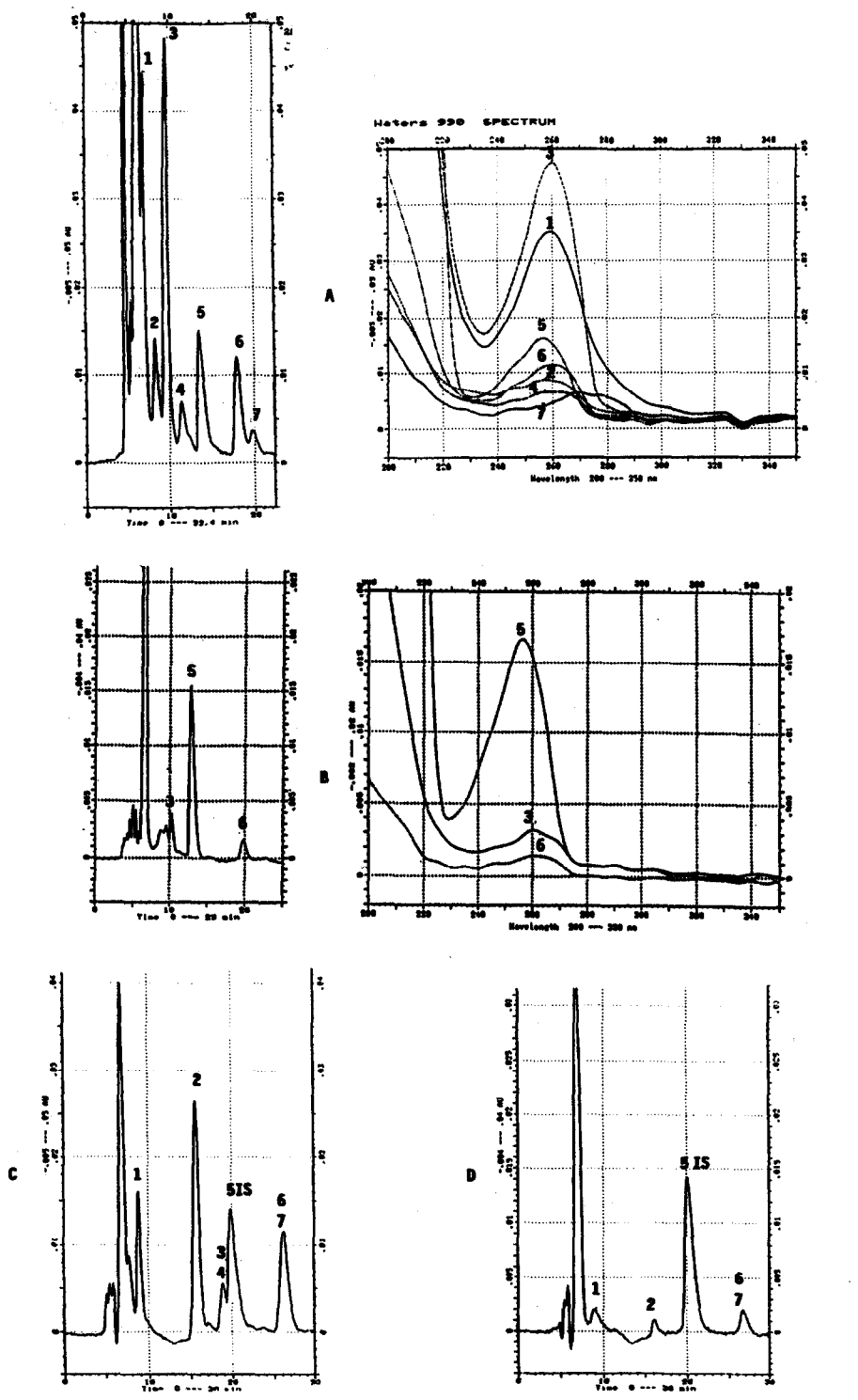

Fig. 2. Chromatograms and corresponding UV spectra. (A) Urine sample (isocratic procedure) from a smoker and coffee drinker: 1 = 3-pyridylcarbinol (interference); 2 = nicotine, 530 ng ml^{-1}; 3 = *trans*-3′-hydroxycotinine, 1900 ng ml^{-1}; 4 = nicotine-1′-N-oxide, 215 ng ml^{-1}; 5 = norephedrine (I.S.), 50 μg added; 6 = cotinine, 370 ng ml^{-1}; 7 = caffeine, traces (spectrum overwritten). (B) Urine sample (isocratic procedure) from a non-smoker exposed to ETS: 3 = *trans*-3′-hydroxycotinine, 10 ng ml^{-1}; 5 = norephedrine (I.S.), 50 μg added; 6 = cotinine, 6 ng ml^{-1}. (C) Urine sample (gradient procedure) from a smoker: 1 = 3-pyridylcarbinol, 370 ng ml^{-1}; 2 = *trans*-3′-hydroxycotinine; 3 and 4 = nicotine and/or nicotine-1′-N-oxide; 5 = I.S.; 6 and 7 = cotinine and/or caffeine. (D) Urine sample (gradient procedure) from a non-smoker exposed to ETS: 1 = 3-pyridylcarbinol, traces; 2 = *trans*-3′-hydroxycotinine; 5 = I.S.; 6 and 7 = cotinine and/or caffeine.

tected among the non-exposed non-smokers. These results obviously show that not only nicotine and cotinine themselves, but also their main free and conjugated metabolites, must be taken into account when assessing active smoking or exposure to environmental tobacco smoke by urine analysis.

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