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Note

Capillary gas chromatographic determination of *trans*-3′-hydroxycotinine simultaneously with nicotine and cotinine in urine and blood samples

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Almost three decades ago, McKennis and co-workers isolated hydroxycotinine, a nicotine metabolite, from the urine of dogs[1], rats[2] and humans[3] treated with nicotine or cotinine. Metabolically produced hydroxycotinine, isolated from human urine, was found to be identical with a synthesized product which was thougth to be 3- or 4-hydroxycotinine[4]. The exact structure of the metabolically produced hydroxycotinine isolated from the urine of cotinine-treated monkeys was shown by Dagne and Castagnoli[5] to be *trans*-1-methyl-3-(*R*)-hydroxy-5-(*S*)-3-pyridyl-2-pyrrolidone (*trans*-3′-hydroxycotinine, THOC). Recently, THOC has been identified as a major metabolite of nicotine in the urine of cigarette smokers[6–9].

Only a few chromatographic methods for the determination of THOC in urine or blood have been published[6,10–12]. Three methods used high-performance liquid chromatography (HPLC)[10–12]. Neurath and Pein[6] described a gas chromatographic (GC) method for the determination of THOC in human urine and plasma: after extraction at alkaline pH using dichloromethane, an aliquot of the extract is derivatized with heptafluorobutyric anhydride; after a solvent exchange, the derivative is analyzed by packed column GC by means of electron-capture detection. This method was recently used to investigate the pharmacokinetics of THOC in cigarette smokers[9].

A new GC assay for the determination of THOC without derivatization simultaneously with nicotine and cotinine in urine and blood has been developed.

EXPERIMENTAL

Materials

trans-3′-Hydroxycotinine, N-ethylnornicotine and N-ethylnorcotinine were obtained from the Institut für Biopharmazeutische Mikroanalytik, G. Neurath (Hamburg, F.R.G.). Nicotine (research grade) was supplied by Serva (Heidelberg, F.R.G.), and cotinine (purity >99%) was obtained from Roth (Karlsruhe, F.R.G.). Anhydrous potassium carbonate (purity >99%) was obtained from Aldrich (Steinheim, F.R.G.). The solvent *n*-butyl acetate (Aldrich) was of HPLC grade and dichloromethane (Merck, Darmstadt, F.R.G.) was specified as residue analysis grade.

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Urine and blood samples were obtained from an heavy smoker (approximate daily consumption 50 cigarettes, nicotine delivery in smoke 1 mg per cigarette). The samples were collected at 13:30 p.m. By this time the individual had already smoked 20 cigarettes, the last one 10 min prior to sample collection. Blank samples of urine and plasma were obtained from non-smokers who were not exposed to environmental tobacco smoke for at least 1 week before the samples were taken. All samples were stored at −20°C until used.

Extraction

An 0.5-ml volume of *n*-butyl acetate and 2 ml saturated aqueous potassium carbonate solution were added to 0.5 ml urine in a 5-ml sample tube with a PTFE-sealed screw cap (Macherey-Nagel, Düren, F.R.G.). The extraction solvent *n*-butyl acetate contained the internal standards N-ethylnornicotine (400 μg/l) for nicotine and N-ethylnorcotinine (440 μg/l) for cotinine and THOC. The mixture was shaken for 10 min on a rotary mixer (Cenco, Breda, The Netherlands) at 40 rpm and centrifuged for 5 min at 1000 *g*.

The extraction of 1 ml of plasma or serum was performed using 1 ml dichloromethane and 2 ml saturated aqueous potassium carbonate solution. The extraction solvent dichloromethane contained the internal standards N-ethylnornicotine (250 μg/l) and N-ethylnorcotinine (275 μg/l).

Gas chromatography

Detection was performed using either a nitrogen–phosphorus detector to determine THOC alone in urine or a mass-selective detector for the simultaneous determination of THOC, nicotine and cotinine in urine and blood.

A gas chromatograph (Carlo Erba, 5300 Mega Series) equipped with a nitrogen–phosphorus detector and connected to a laboratory data system (Multichrom; VG Instruments, Wiesbaden, F.R.G.) was used. GC was performed with a fused-silica capillary column (10 m × 0.32 mm) coated with free fatty acid phase (FFAP) (Macherey-Nagel). A 1-μl volume of the *n*-butyl acetate extract was splitlessly injected. The split valve was closed for 0.5 min. The temperature of the injection port and the detector was 350°C. The oven temperature was programmed to increase from 100 to 240°C at a rate of 40°C/min. The temperature was kept at 240°C for 20 min. Helium was used as the carrier gas at a flow-rate of 6.5 ml/min at 240°C. The flow-rates of hydrogen, air and make-up helium were 30, 300 and 40 ml/min, respectively. Under these conditions THOC, nicotine and cotinine had retention times of *ca.* 6.5, 1.3 and 4.4 min, respectively (see Fig. 1). The observed variation in the retention times of cotinine and THOC is due to the steep increase in oven temperature (40°C/min) which could not be completely controlled before the final temperature of 240°C was reached.

For GC–mass spectrometric (MS) analysis a gas chromatograph (Hewlett-Packard, Model 5890) coupled to a computer-controlled mass spectrometer (Hewlett-Packard, Model 5990B) was used. A DB5 fused-silica capillary column (30 m × 0.32 mm; J & W via Carlo Erba, F.R.G.) was directly coupled to the ion source (ionization voltage: 70 eV). A 1-μl volume of the extract was splitlessly injected. The split valve was closed for 0.5 min. The splitless injection port temperature was 280°C. The oven temperature was kept at 100°C for 2.5 min and then raised at 20°C/min to 240°C.

Helium was used as the carrier gas at a flow-rate of 2.4 ml/min at 240°C. Under these conditions the retention times of THOC, nicotine and cotinine were 8.3, 5.1 and 7.8 min, respectively (see Fig. 2).

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 and 161; N-ethylnornicotine, *m/z* 98, 130, 161 and 176; cotinine, *m/z* 98, 118 and 176; N-ethylnorcotinine, *m/z* 112, 118, 146 and 190; THOC *m/z* 106, 135 and 192.

Calibration

Standard solutions of THOC in *n*-butyl acetate were added to blank urine samples in order to obtain five different concentrations ranging from 100 to 3400 μg/l. For each concentration the samples were extracted in duplicate as described and the analysis was performed by GC using nitrogen–phosphorus detection (NPD). Calibration graphs were obtained from linear regressions of the THOC concentrations of the five duplicate extracts *versus* the peak areas.

Standard solutions containing nicotine, cotinine and THOC at five different concentrations and the internal standards at constant concentrations were prepared in *n*-butyl acetate as well as in dichloromethane. In order to obtain calibration samples, blank urine samples were spiked with the *n*-butyl acetate standard solutions and blank plasma samples with the dichloromethane standard solutions. The resulting concentrations ranged from 10 to 1320 μg/l for nicotine, from 10 to 900 μg/l for cotinine and from 20 to 3400 μg/l for THOC. The samples were extracted in duplicate as described and nicotine, cotinine and THOC determined simultaneously in a single GC analysis. Calibration graphs were obtained from the linear regressions of the concentrations of the five duplicate extracts *versus* the peak area ratios.

Recovery

The overall recovery was determined by spiking blank samples with nicotine, cotinine and THOC. The samples were extracted as described without the addition of the internal standards. After separation of the organic layer, the internal standard solution was added yielding equal internal standard concentrations as mentioned in the extraction procedure. The recoveries were calculated by comparing the peak area ratios of the samples with that of the non-extracted standard solution.

RESULTS

The THOC determination in urine by capillary GC with NPD was performed using the external standard method. The linear regression analysis obtained from five different concentrations in duplicate *versus* the peak areas yielded a correlation coefficient r^2 of 0.999 for THOC.

Chromatograms of blank and spiked urine samples from a non-smoker and the chromatogram of a urine sample from a heavy smoker are presented in Fig. 1. As indicated in the chromatogram of the smoker's urine, not only THOC but also nicotine and cotinine are present in the same extract. This led to a modification of the method to enable the determination of THOC simultaneously with nicotine and cotinine. The modifications involved changes of the chromatographic conditions as well as the use of a mass-selective detector in the SIM mode and internal standards. The

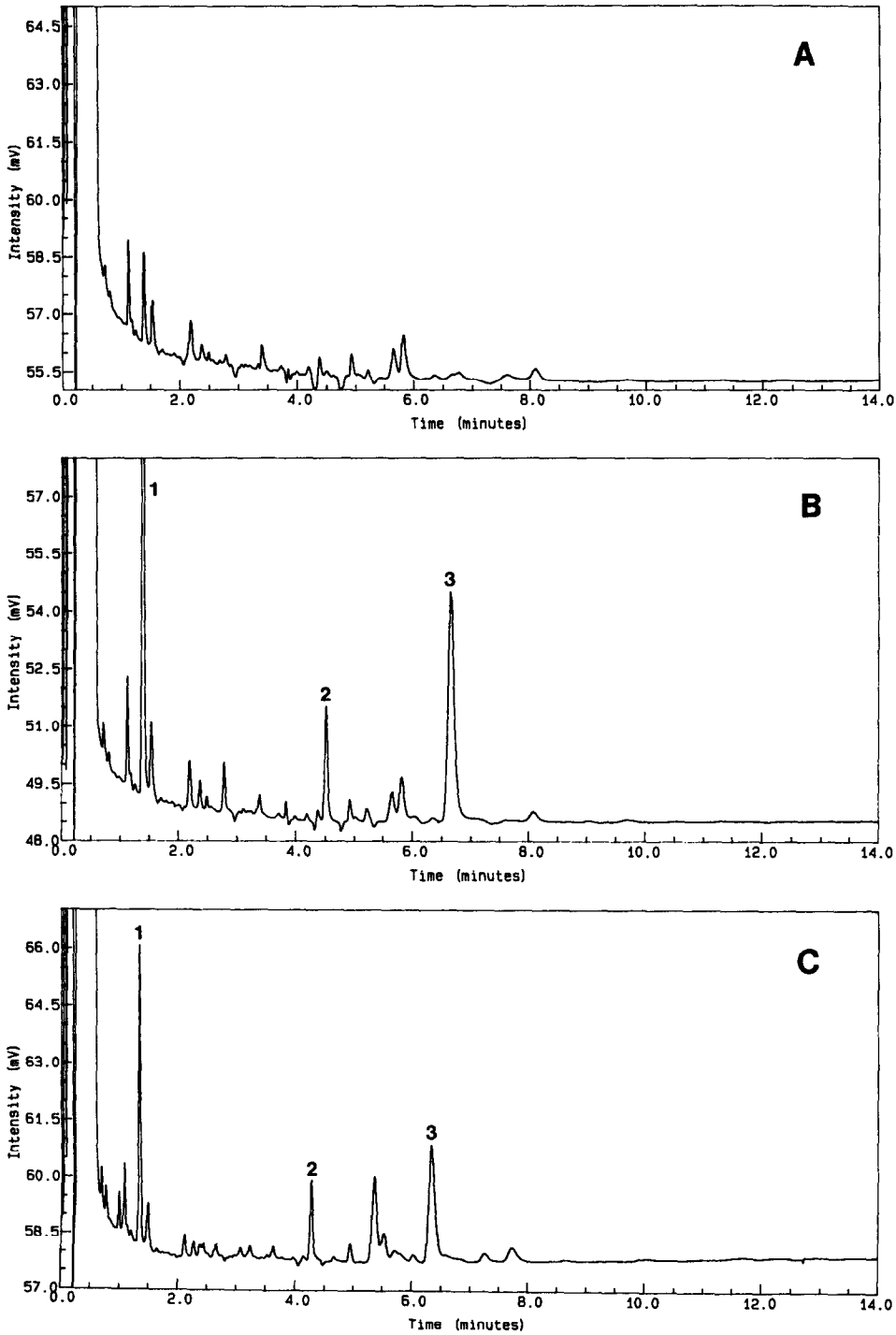

Fig. 1. Capillary gas chromatograms: blank and spiked urine samples from a non-smoker (A,B); urine sample from a heavy smoker (C). Nitrogen–phosphorus detection. Peaks: 1 = nicotine; 2 = cotinine; 3 = *trans*-3′-hydroxycotinine.

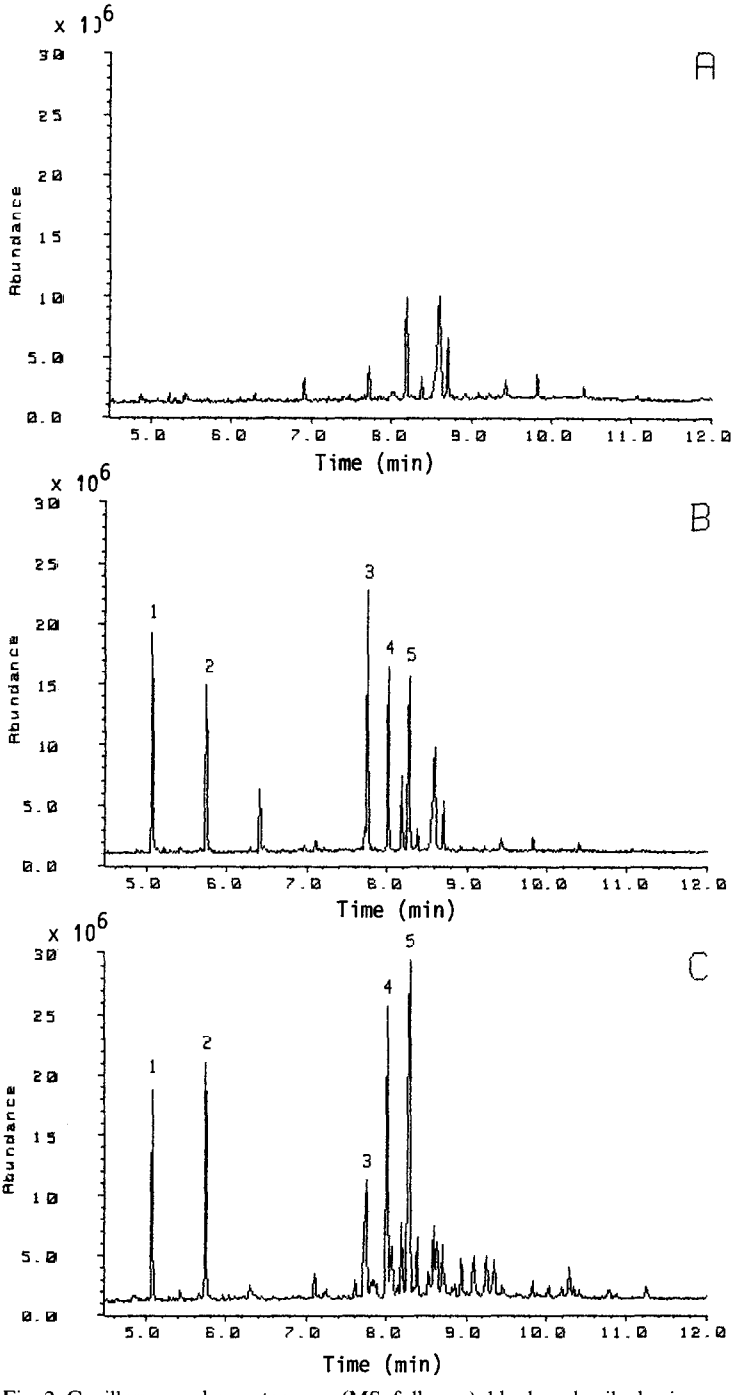

Fig. 2. Capillary gas chromatograms (MS, full scan): blank and spiked urine samples from a non-smoker (A,B); urine sample from a heavy smoker (C). Peaks: 1 = nicotine; 2 = N-ethylnornicotine; 3 = cotinine; 4 = N-ethylnorcotinine; 5 = *trans*-3′-hydroxycotinine.

linear regression analysis obtained from the five different concentrations of the three substances in blank urine, and all in duplicate, *versus* the peak area ratios yielded correlation coefficients, r^2, of 1.000 for THOC, 0.999 for nicotine and 1.000 for cotinine.

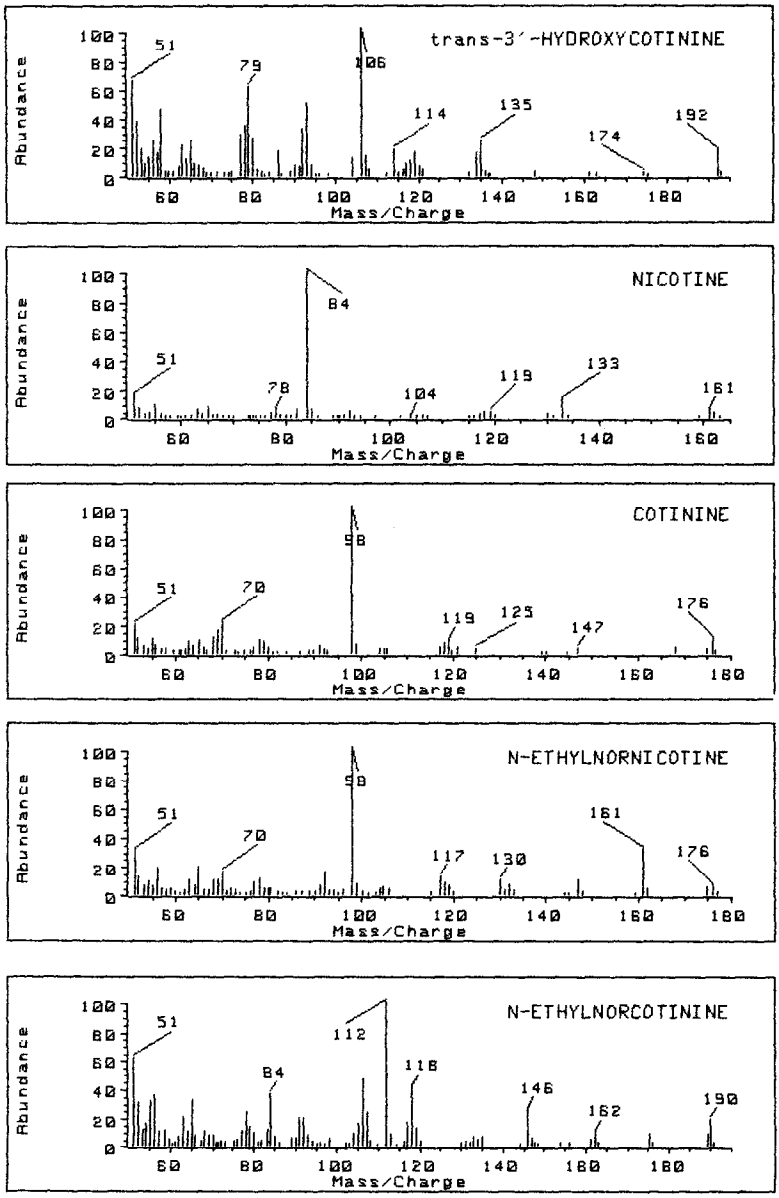

Fig. 3. Electron-impact mass spectra of *trans*-3'-hydroxycotinine, nicotine and cotinine, and of the internal standards N-ethylnornicotine and N-ethylnorcotinine. Mass spectra were obtained from the gas chromatogram of a urine sample from a heavy smoker (see Fig. 2).

An example of the method's application was the determination of nicotine and its aforementioned metabolites in the urine from an heavy smoker. The total ion current (TIC) chromatograms of blank and spiked urine samples from a non-smoker and the chromatogram of an urine sample of an heavy smoker are presented in Fig. 2. The concentrations of THOC, nicotine and cotinine were sufficiently high to operate the instrument in the full scan mode in order to identify the substances. The mass spectra of the three substances as well as those of the internal standards were obtained from the TIC chromatogram of the smoker's urine sample (see Fig. 3). They were identical with those obtained from the TIC chromatogram of a standard solution of the pure reference substances. A check of the peak purity did not indicate the presence of coeluting urinary compounds. The THOC, nicotine and cotinine concentrations in the smoker's urine were determined by GC–MS (SIM) after a ten-fold dilution of the sample in water (see Table I). The method has also been successfully used to determine THOC in urine from rats and hamsters.

Another application was the determination of the concentrations of THOC, nicotine and cotinine in the blood of a heavy smoker (see Table I). *n*-Butyl acetate, which was well suited for the extraction of THOC from urine samples, yielded poor recoveries when used to extract THOC from plasma or serum samples. Therefore, dichloromethane was used for such samples.

The linear regression analysis obtained from five different concentrations of the three substances in blank plasma *versus* the peak area ratios yielded correlation coefficients, r^2, of 0.985 for THOC, 0.999 for nicotine and 0.997 for cotinine. The SIM chromatograms of blank and spiked plasma samples from a non-smoker and the chromatogram of a plasma sample of a heavy smoker are presented in Fig. 4. The determinations performed on the plasma and serum obtained from the same blood sample of a heavy smoker yielded almost the same concentrations of THOC, nicotine and cotinine.

For urine samples, the overall recovery was 82% for THOC, 86% for nicotine and 81% for cotinine. To calculate the detection limits, blank samples were spiked with decreasing concentrations of the three compounds. When using NPD the limit of detection for THOC was 50 μg/l at a signal-to-noise ratio of *ca.* 10:1. With MS/SIM the limits of detection were 10 μg/l for THOC and cotinine, and 2 μg/l for nicotine at signal-to-noise ratios of *ca.* 3:1.

For blood samples the overall recovery was 49% for THOC, 77% for nicotine and 69% for cotinine. The limit of detection (MS/SIM) was 50 μg/l for THOC, 3 μg/l for nicotine and 10 μg/l for cotinine at signal-to-noise ratios of *ca.* 3:1.

TABLE I

CONCENTRATIONS OF NICOTINE, COTININE AND *trans*-3'-HYDROXYCOTININE (THOC) IN URINE AND PLASMA FROM A HEAVY SMOKER

Sample	*Concentration (μg/l)*		
	Nicotine	*Cotinine*	*THOC*
Urine	5000	2700	22 400
Plasma	8.7	194	209

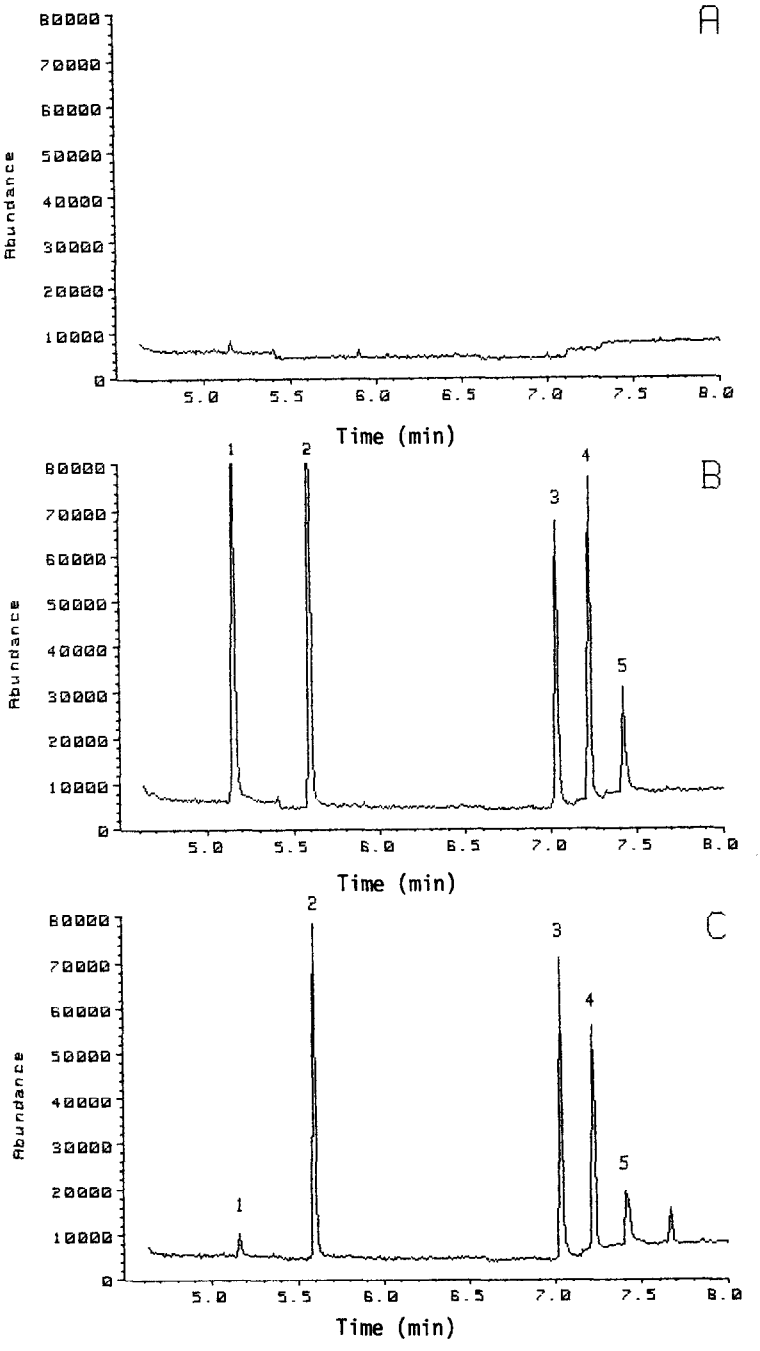

Fig. 4. Capillary gas chromatograms (MS, SIM): blank and spiked plasma samples from a non-smoker (A,B); plasma sample from a heavy smoker (C). Peaks: 1 = nicotine; 2 = N-ethylnornicotine; 3 = cotinine; 4 = N-ethylnorcotinine; 5 = *trans*-3′-hydroxycotinine.

The intra-assay coefficient of variation indicating the precision of the GC–MS method for urine samples was 7.3% for THOC at 280 μg/l, 4.0% for nicotine at 200 μg/l and 1.3% for cotinine at 550 μg/l. The intra-assay coefficient of variation indicating the accuracy of the method was 6.0% for THOC, 1.1% for nicotine and 0.7% for cotinine at the same concentrations as those used to calculate the precision. For blood samples, only the precision was checked. It was 3.0% for THOC at 200 μg/l, 9.5% for nicotine at 10 μg/l and 2.3% for cotinine at 200 μg/l.

The method described is simple and quick to perform. The main advantages are that only one extraction step is required and there is no need for derivatization and solvent exchange. The determination of THOC in urine using NPD was performed because an MS detector is not available in every laboratory. It has been demonstrated that nicotine and two of its major metabolites, cotinine and THOC, can be determined simultaneously from a single urine or blood extract in one GC analysis when using the MS detector.

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