Journal of Chromatography, 580 (1992) 347–353
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6345

Gas chromatographic analysis of nicotine and cotinine in hair☆

P. Kintz

Institut de Médecine Légale, 11 Rue Humann, 67000 Strasbourg (France)

(First received October 22nd, 1991; revised manuscript received January 22nd, 1992)

ABSTRACT

Non-invasive validation of cigarette- or cigar-smoking behaviour is necessary for large population studies. Urine or saliva samples can be used for confirmation of recent nicotine intake by analysis of cotinine, the major metabolite of nicotine. However, this test is not suitable for validation of survey data, since the quantification of cotinine in saliva only reflects nicotine exposure during the preceding week. To validate information on tobacco use, we investigated hair samples for quantifying nicotine and cotinine by gas chromatography–mass spectrometry. Hair (about 50–100 mg) was incubated in 1 *M* sodium hydroxide at 100°C for 10 min. After cooling, samples were extracted by diethyl ether, using ketamine as an internal standard. Drugs were separated on a 12-m BP-5 capillary column, and detected using selected-ion monitoring (*m/z* 84, 98 and 180 for nicotine, cotinine and ketamine, respectively). Hair from non-smokers and smokers contained nicotine and cotinine. Although it is difficult to determine an absolute cut-off concentration, more than 2 ng of nicotine per milligram of hair can be used to differentiate smokers from non-smokers. Some applications of this technique are developed to determine the status of passive smokers, the gestational exposure in babies and the pattern of an individual's nicotine use by cutting strands of hair into sections of one-month intervals.

INTRODUCTION

Nicotine is a highly toxic alkaloid that causes stimulation of autonomic ganglia and the central nervous system. The compound was first isolated in 1828 from tobacco, in which it is present in amounts of 0.5–8.0% by mass. Of the available nicotine, 10–50% is absorbed during mouth puffing and 80–100% during deep lung inhalation. Nicotine and its metabolites, cotinine and nicotine-1′-N-oxide, are excreted in urine. Nicotine and cotinine can be found in the urine of smokers and most non-smokers [1].

Numerous gas chromatographic (GC) methods for assaying nicotine and cotinine have been reported [2–5]. All the described procedures are proposed for plasma, urine and saliva, and can be used for confirmation of recent nicotine intake. However, these methods are for validation of survey data, since the quantification of nicotine and cotinine only reflects nicotine intake during the preceding week and does not indicate the frequency of smoking in subjects who might deliberately abstain for several days before annual biomedical screenings.

It is therefore important to have a method of evaluating longer-term use of tobacco products. In this report, we present the usefulness of hair samples for quantifying nicotine and cotinine. While analysis of urine specimens cannot distinguish between chronic use or single exposures, hair analysis can make this distinction.

Correspondence to: Dr. P. Kintz, Institut de Médecine Légale, 11 Rue Humann, 67000 Strasbourg, France.

☆ Part of this work was presented at the *43rd Annual Meeting of the American Association of Forensic Sciences, Feb. 18 23, 1991, Anaheim, CA.*

0378-4347/92/$05.00

EXPERIMENTAL

Chemicals

Methanol, dichloromethane and diethyl ether were high-performance liquid chromatography (HPLC) grade (Merck, Darmstadt, Germany). All other chemicals were analytical grade and provided by Merck. Nicotine and cotinine were purchased from Sigma (St. Louis, MO, USA). Ketamine base was provided by Parke Davis (Courbevoie, France).

Subjects

Hair was collected from smokers and non-smokers among laboratory personnel. Hair from neonates with a confirmed maternal history of cigarette smoking was collected at the Hospital of Strasbourg. To be eligible as a non-smoker, the subject had to attest to the absence of cigarette smoking for one year.

The studied population was as follows. Non-smokers: thirteen men, eighteen women, aged from 7 to 64 years; smokers: 35 men, 21 women, aged from 17 to 69 years; neonates: five boys, four girls.

All the subjects were Caucasian with natural-coloured hair. For each one, about 50–100 mg (but for neonates about 20 mg) of freshly shampooed hair was cut close to the scalp at the back of the head.

Analytical methods

Since the influence of environmental smoke exposure was being investigated, the hair samples were not decontaminated. The protein matrix of the hair was destroyed by incubation in 1 ml of 1 *M* sodium hydroxide for 10 min at 100°C.

After cooling, the drugs were extracted using 5 ml of diethyl ether in the presence of 20 μl of ketamine (1 mg/l) as an internal standard (I.S.). After agitation and centrifugation, the organic phase was removed. A 20-μl aliquot of octanol was then added to ensure non-volatility of nicotine. After evaporation of the diethyl ether, the residue was dissolved in 15 μl of dichloromethane and 1 μl of the solution was injected in the chromatographic column.

A Model 8500 (Perkin-Elmer, Norwalk, CT, USA) gas chromatograph with an ion-trap detector (ITD), a capillary column and a splitless inlet injection system was employed. The data system used was an Epson PC AX computer. Data acquisition and manipulation were performed using standard software. The ITD was operated in electron-impact mode at 70 eV with an ion source temperature of 200–220°C. The electron multiplier voltage of the detector was set at 1350 V. A fused-silica capillary column (SGE, Austin, TE, USA), BP-5 (methylmethylsiloxane), 12 m × 0.22 mm I.D., was used. The flow-rate of carrier gas (helium purity N 55) through the column was 3.2 ml/min and the head pressure on the column was maintained at 124 kPa. The injector port temperature was 250°C. The column oven temperature was programmed from an initial temperature of 60°C (held for 0.9 min) to 280°C at 30°C/min and held at 280°C for the final 1 min. Splitless injection with a split valve off-time of 0.9 min was employed.

Quantification was made for drugs by plotting peak-area ratios of the selected ions (drug/I.S.) against the concentration of standards to produce standard curves and by comparing the results for the case samples with the curve. Selected-ion monitoring was used as follows: nicotine, m/z 84; cotinine, m/z 98; and ketamine, m/z 180. Calibration curves and analytical parameters were realized with homogenates of hair of guinea-pig spiked with nicotine and cotinine.

RESULTS AND DISCUSSION

Chromatography and peak separation

The internal standard, ketamine, was chosen because it could be well separated from the other drugs, is well extracted in the described procedure and has some chemical similarities with nicotine. Under the chromatographic conditions used, there was no interference with the drugs or the I.S. by any extractable endogenous materials present in hair.

Typical GC tracings of extracts from a smoker's hair (A) and selected-ion monitoring of the same smoker (B) are shown in Fig. 1. Under these

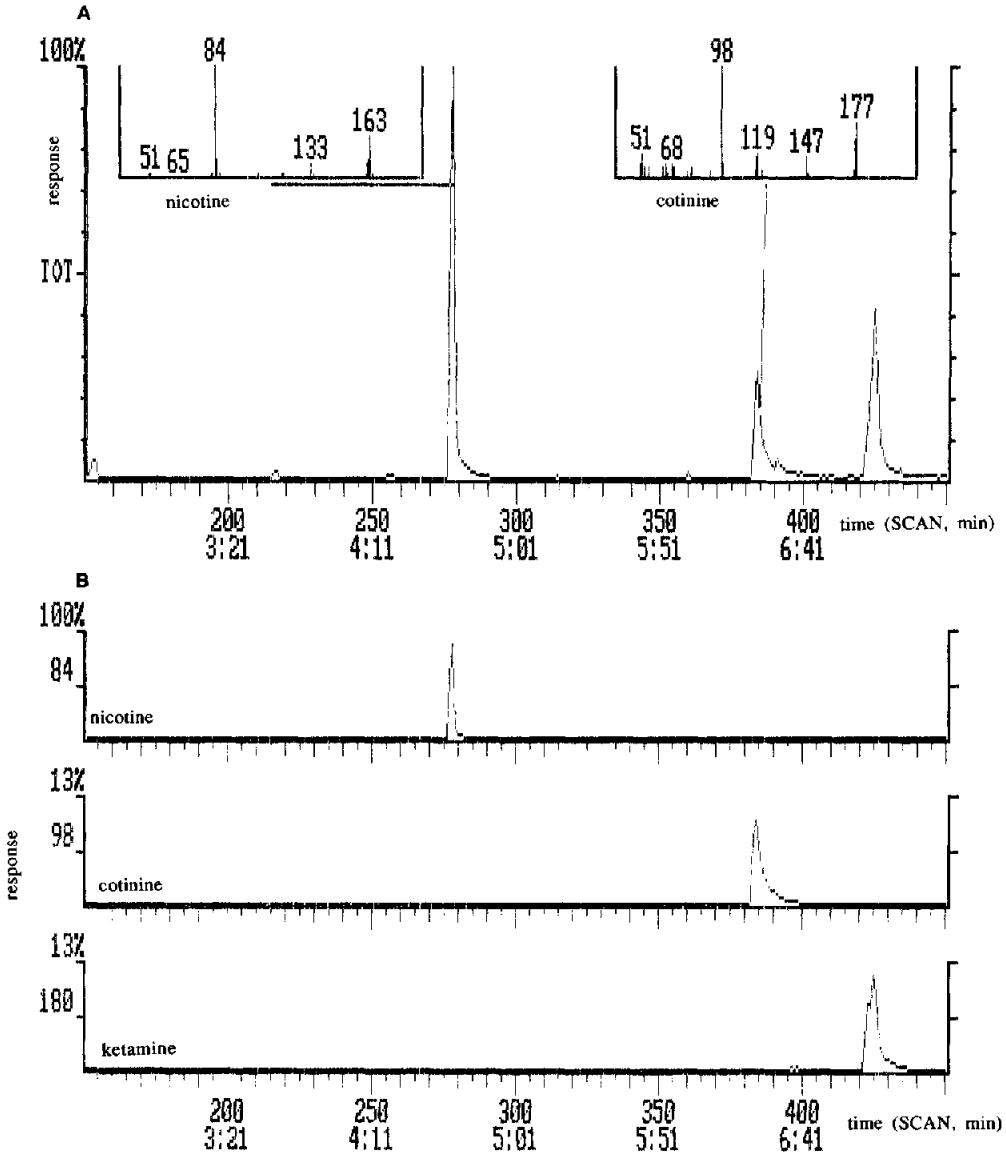

Fig. 1. Typical chromatograms obtained from (A) extract of a smoker's hair (nicotine, 2.41 ng/mg; cotinine, 0.59 ng/mg) and (B) display of selected-ion monitoring (same subject) (m/z 84, nicotine; m/z 98, cotinine; m/z 180, ketamine).

experimental conditions, the retention times were 4.39, 6.25 and 7.06 min for nicotine, cotinine and ketamine, respectively.

The detection and measurement not only of the parent drug but also of its major metabolite are of particular interest. Their simultaneous presence dramatically enhances the selectivity of the assay.

Linearity

A total of six single standards of a mixture of nicotine (0.1, 1.0, 2.0, 5.0, 20.0 and 50.0 ng/mg) and cotinine (0.01, 0.05, 0.10, 0.50, 1.00 and 5.00 ng/mg) were run daily. The mathematical expressions and the correlation coefficients of the drug were, respectively: nicotine, $y = 1.47x + 0.56$ ($r = 0.995$); and cotinine, $y = 3.37x - 3.21$

TABLE I

INTER-DAY PRECISION AND ACCURACY FOR DETERMINATION OF NICOTINE AND COTININE IN HAIR

Drug	Concentration (ng/mg)	Drug recovered after extraction (mean ± S.D.) (ng/mg)	R.S.D. (%)	Error (%)
Nicotine	0.50	0.54 ± 0.03	7.1	+ 8.0
	2.00	2.13 ± 0.11	6.4	+6.5
	20.00	21.54 ± 1.82	5.0	+7.7
Cotinine	0.10	0.09 ± 0.03	7.7	−9.0
	1.00	1.04 ± 0.06	5.9	+4.0
	2.00	1.93 + 0.14	4.1	3.5

($r = 0.991$). The results indicated a good linear proportionality between the ITD response (in selected-ion monitoring mode) and the concentration of the drugs in the range used.

Extraction recovery

Extraction recovery was determined for nicotine and cotinine by comparing the representative peak areas of extracted hair with the peak areas of methanolic standards at the same concentration. Diethyl ether was chosen because of its ability to achieve a suitable recovery: 83 and 73% for nicotine and cotinine, respectively.

Precision and accuracy

The inter-day precision and accuracy of the method were assessed by carrying six replicate samples of three concentrations of the two drugs through the entire procedure in one analysis day. The inter-day precision (expressed as the relative standard deviation, R.S.D.) and the accuracy (expressed as the error relative to the least-squares equation) are presented in Table I.

The intra-day precision, at the concentrations used above, was established by analysing these standards on six separate days (Table II). The response ratios from six separate days for each freshly prepared standard concentration were pooled and the mean ± standard deviation (S.D.) calculated.

TABLE II

INTRA-DAY PRECISION FOR DETERMINATION OF NICOTINE AND COTININE IN HAIR

Drug	Concentration (ng/ml)	Response ratio (mean ± S.D.) (ng/mg)	R.S.D. (%)
Nicotine	0.50	0.56 ± 0.05	7.9
	2.00	2.17 ± 0.14	6.9
	20.00	21.64 ± 1.90	5.5
Cotinine	0.10	0.09 ± 0.05	8.0
	1.00	1.07 ± 0.09	6.4
	2.00	1.95 ± 0.19	5.1

Limit of detection

By this method, concentrations as low as 0.005 ng/mg nicotine and 0.01 ng/mg cotinine could be quantified. These detection limits were evaluated with decreasing concentrations of the drugs, until a response equivalent to three times the background noise was observed.

Applications

Hair from the smokers and the non-smokers contained nicotine and cotinine (Table III). The distribution of nicotine and cotinine is shown by a histogram in Fig. 2. It was possible to determine a nicotine cut-off value of 2 ng/mg of hair for distinguishing smokers from non-smokers. This was not observed in previous studies [6–8]. Table IV shows the comparative hair distribution of nicotine and cotinine in the literature.

TABLE III

NICOTINE AND COTININE CONCENTRATIONS IN HAIR SAMPLES

Population	Concentration (ng/mg)	
	Nicotine	Cotinine
Smokers	0.91–38.27	0.09–4.99
Non-smokers	0.06–1.82	0.01–0.13

The great variation in nicotine and cotinine concentrations is still unclear. The present experimentation was undertaken with gas chromatography coupled to mass spectrometry, while others have used radioimmunoassay (RIA). Such an approach requires the rapid destruction of the organic protein matrix of hair under conditions that are sufficiently mild not to damage the protein antibodies subsequently added for the RIA assay.

In the non-smoker population, it was possible to distinguish passive smokers (Table V). The nicotine content was over 0.5 ng/mg in the group of passive smokers and lower in non-exposed non-smokers. The presence of varying amounts of nicotine in passive smokers' hair can be explained by atmospheric deposition and inhalation of smoke which is rich in nicotine [1]. As cotinine is only produced *in vivo*, it cannot be present in smoke and its detection in the hair of non-exposed non-smokers is not clearly explained. Nevertheless, the presence of cotinine within the hair shaft should validate exposure to, and body metabolism of, nicotine. Therefore, on-

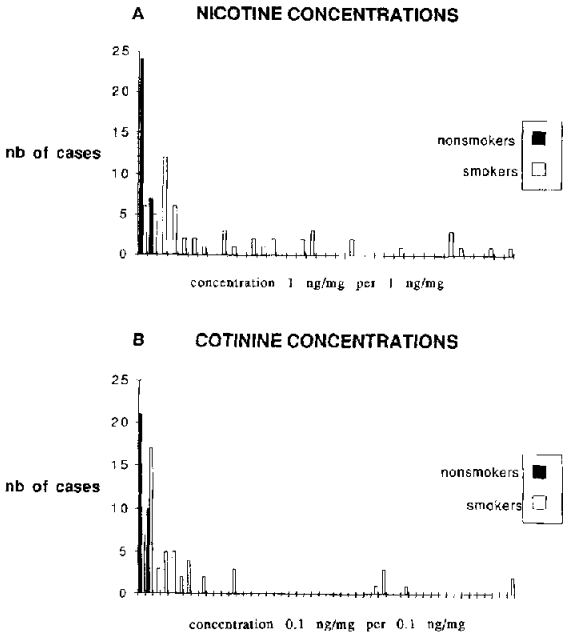

Fig. 2. Distribution in hair. (A) Nicotine, each interval represents 1 ng/mg; (B) cotinine, each interval represents 0.1 ng/mg.

TABLE IV

NICOTINE AND COTININE CONCENTRATIONS REPORTED IN THE LITERATURE

The number of cases is given in parentheses. N.D. = not detected.

Case	Concentration (ng/mg)	
	Nicotine	Cotinine
Present study		
Smokers (56)	0.91–38.27	0.09–4.99
Non-smokers (31)	0.06–1.82	0.01–0.13
Haley and Hoffmann [6]		
Smokers (10)	3.0–38.7	N.D.–1.4
Non-smokers (10)	N.D.–11.3	N.D.–1.0
Ischiyama et al. [7]		
Smokers and non-smokers	18–177.2	
Balabanova and Schneider[a] [8]		
Smokers (10)	0.9–11.1	
Non-smokers (5)	< 0.6	

[a] Concentrations are the sum of nicotine and cotinine.

TABLE V

NICOTINE AND COTININE CONCENTRATIONS ACCORDING TO ENVIRONMENTAL SMOKE EXPOSURE IN NON-SMOKERS' HAIR

Population	Concentration (ng/mg)	
	Nicotine	Cotinine
No environmental smoke exposure	0.06–0.33	0.01–0.13
Environmental smoke exposure	0.54–1.82	0.01–0.13

ly the nicotine concentration can be used to differentiate smokers from non-smokers and, in the latter population, passive smokers from other non-smokers. The high nicotine concentration in the hair of the passive smokers was confirmed by a questionnaire regarding their habits (the presence of smokers at home, at the workplace, during spare-time activities; the duration and the frequency of the exposure).

The analysis of nicotine in hair of non-smokers to validate information on environmental smoke exposure seems to be a good alternative to evaluating urine cotinine excretion [9] or saliva nicotine and cotinine concentrations [10] or the serum thiocyanate level [11]. This is of particular interest as several reports suggest that the risk of developing cancer increases as a result of passive inhalation of smoke. The estimated increase in risk of lung cancer among non-smokers living with smokers is 53% [1].

Because head hair grows at approximately 1.0 ± 0.3 cm per month, the window of detection for hair analysis can range over many months to years. Furthermore, if the strands of hair are cut into sections (for example one-month intervals, about 1 cm), one is able to obtain information on the pattern of an individual's drug use, that is whether use is decreasing, constant or increasing. Typical examples of sectional analysis are shown in Fig. 3. Case A illustrates the utility of hair analysis in a rehabilitation setting. The individual in question is seen to have stopped his cigarette habit four months previously. Case B illustrates a case of relatively constant cigarette use.

Determination of gestational nicotine exposure by hair analysis is another application of this technique. Pregnant smokers deliver babies who are lighter and smaller. The risk of spontaneous abortion, fetal death and neonatal death is increased. Sudden infant death syndrome is more common among infants of smoking mothers. The nicotine content of neonatal hair from nine infants whose mothers were known cigarette users ranged from 0.27 to 1.37 ng/mg (Table VI). Hair analysis may identify intrauterine exposure to the drug in babies when a maternal drug history is not available or of doubtful truthfulness.

In summary, it appears that the value of hair

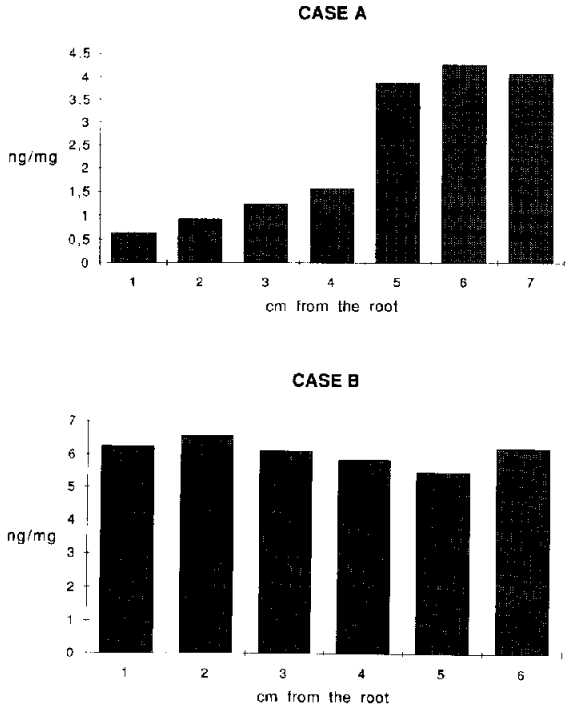

Fig. 3. Pattern of nicotine use by the analysis of hair strands cut into sections corresponding to one-month intervals. Case A: hair analysis corroborates a self-reported decrease in cigarette use over a seven month period. The subject was asked to stop cigarette smoking four months previously. Case B: hair analysis corroborates self-reported constant cigarette use of approximately 25 cigarettes per day over a six month period.

TABLE VI

RESULTS OF ANALYSIS OF HAIR FROM BABIES WITH KNOWN IN UTERINE EXPOSURE TO NICOTINE

Case	Nicotine (ng/mg)
1	0.37
2	0.40
3	1.12
4	1.37
5	0.88
6	0.36
7	0.42
8	0.27
9	1.15

analysis for the identification of cigarette or cigar users is steadily gaining recognition. Testing human hair for nicotine offers the possibility of revealing an individual's recent history of drug exposure, beginning at the sampling time and dating back over a period from months to years. In the medical arena, we see hair analysis increasingly applied in the identification of cigarette use, particularly for diagnosing pulmonary diseases and problems relating to prenatal drug exposure.

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