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SIMULTANEOUS DETERMINATION OF NICOTINE AND COTININE IN PLASMA USING CAPILLARY COLUMN GAS CHROMATOGRAPHY WITH NITROGEN-SENSITIVE DETECTION

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SUMMARY

A rapid and sensitive method is described for the simultaneous determination of nicotine and its principal metabolite, cotinine, in plasma. A one-step extraction procedure is employed and the quantitative analyses are performed by capillary column gas chromatography using a thermionic specific detector. Other special measures to avoid contamination from external sources such as atmosphere, solvents and laboratory equipment, which constitutes the major limiting factor of nicotine assay, were also undertaken. The structural analogues of nicotine and cotinine, N-methylanabasine and N-ethylnorcotinine, are used as internal standards. Moreover, a micromethod, which requires only 0.1 ml of plasma and found to be suitable for analysis of cotinine in finger-tip samples of blood, is described. Linearity over the concentration ranges 5—100 ng of nicotine per ml of plasma and 5—500 ng of cotinine per ml of plasma is demonstrated. The precision of the method has been investigated by determining the reproducibility at different levels of nicotine and cotinine within the working ranges, for both 1-ml and 0.1-ml samples of plasma.

INTRODUCTION

A large number of publications dealing with the quantitative determination of nicotine and/or cotinine in plasma have appeared in the literature during the past decade. Although some of the methods described are based on radioimmunoassay [1, 2] or liquid chromatography [3, 4], the vast majority are founded on gas chromatography using electron-capture [5], flame ionization [6, 7] or thermionic specific detectors [8—14], or a mass spectrometer in a low- or medium-resolution mode [15, 16].

The specificity and sensitivity of the gas chromatographic methods depend largely on the work-up procedures, the gas chromatographic separation and the

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detector characteristics. Since their accuracy and reproducibility are primarily influenced by the work-up techniques, most recent studies have been focused on this part of the analysis. Thus, Kogan et al. [14] have presented a method for the simultaneous extraction and determination of nicotine and cotinine, Feyerabend and Russell [10] have introduced a single-step micro-extraction procedure for the analysis of nicotine, and Jacob et al. [13] have demonstrated that the use of internal standards closely analogous to nicotine and cotinine is advantageous, and lowers the limits of detectability. The fact remains, however, that all methods presented so far for the determination of nicotine and cotinine in one sample involve several extraction steps and require at least 1 ml of blood. The main reason for this is the interference from extraneous nicotine, derived from the environment and from contaminated solvents and glassware. Thus, one-step rather than multi-step extraction procedures should be employed and the loss in separation thereby experienced compensated by more efficient gas chromatography and/or higher detector selectivity.

In view of this and the fact that all workers but Dow and Hall [16] have used packed columns for the gas-chromatographic separation, we have developed an assay for the simultaneous determination of nicotine and cotinine which is applicable to both 1-ml and 0.1-ml samples of plasma. It is based on a one-step single extraction of nicotine and cotinine from plasma, more suitable internal standards, and capillary column gas chromatography utilizing a thermionic specific detector.

MATERIALS AND METHODS

Glassware

All glassware for the extraction procedure was cleaned by overnight immersion in a Deconex 11 detergent solution, washed consecutively with hot tap water and ethanol in an ultrasonic bath. The glassware was kept in an oven at 100—200°C for some hours before use.

Chemicals and reagents

(−)-Nicotine, which was purchased from Fluka, Buchs, Switzerland, was purified by distillation under nitrogen before the preparation of standard solutions. N-Methylanabasine was a gift from Stockholm University, Sweden. (−)-Cotinine was synthesised by the method of Bowman and McKennis [17] and double distilled under nitrogen before use. N-Ethylnorcotinine was prepared using adopted methods of synthesis, i.e. condensation of N-vinylpyrrolidone with ethyl nicotinate to form myosmine by the method of Brandänge and Lindblom [18], reduction of myosmine to nornicotine [19], ethylation of nornicotine to N-ethylnornicotine [20] followed by oxidation to N-ethylnorcotinine according to the method of Bowman and McKennis [17]. N-Ethylnorcotinine was purified by liquid chromatography over silica gel. The structures and purities of the alkaloids used in the assay were confirmed by gas chromatography, proton nuclear magnetic resonance and mass spectrometry.

Dichloromethane (IR spectroscopic grade) was purchased from Fluka. The purity of every new bottle was checked by gas chromatography of the evapora-

tion residue from 10 ml of dichloromethane. Ethanol (99.9%) was of spectroscopic quality. The sodium hydroxide solution, which was prepared from pellets (Fluka) previously heated to 200°C, was extracted twice with dichloromethane just before use.

Instrumentation

The gas chromatographic analyses were performed on a Varian Model 3700 instrument, equipped with an all-glass capillary injector (Varian direct injector) and a thermionic specific detector containing an electrically heated ceramic-alkali bead. The detector was operated at a bias voltage of −4 V and the bead heating current potentiometer was set between 3.0 and 3.8 A, depending upon the age of the bead. Optimal sensitivity and specificity for nitrogenous compounds was achieved at flow-rates of hydrogen, air and make-up helium of 4.5, 175 and 35 ml/min, respectively. The flow-rate of helium carrier gas through the column was 40 cm/sec. A fused-silica capillary column (25 m × 0.32 mm I.D.) wall-coated with a free fatty acid phase, SP-1000 (film thickness 0.15 μm), and purchased from Orion Analytica, Finland, was used throughout these studies. Sharp peaks with minimal tailing were obtained by introducing the outlet of the column through the detector liner directly into the flame-tip of the detector. The column temperature was 135°C during the nicotine analyses and 200°C during the cotinine analyses. The injector and detector temperatures were 250°C and 290°C, respectively.

Using the chromatographic conditions described above, the retention times for nicotine and N-methylanabasine were 2.3 and 3.2 min, respectively, and for N-ethylnorcotinine and cotinine, 4.6 and 5.0 min, respectively.

The plotting of the chromatograms and the integration of the peak areas were carried out by a Hewlett-Packard Model 3388 A plotter/integrator or by a Perkin-Elmer Model 561 recorder coupled to a Varian Model CDS-111 integrator.

Extraction procedure

Plasma (1 ml) and internal standards (100 ng of N-methylanabasine and 100 ng of N-ethylnorcotinine in 100 μl of ethanol) were pipetted into a 10-ml tube fitted with a screw cap having a PTFE—faced rubber liner, and thoroughly mixed. The sample was made alkaline by addition of aqueous sodium hydroxide (5 *M*, 1 ml), extracted with dichloromethane (2 ml) for 5 min using a Vortex-Genie mixer and then centrifuged at 3000 *g* for 15 min in an MSE-GF-6 universal centrifuge to break the emulsion. Most of the aqueous layer was discarded and the organic and remaining aqueous layers were separated by freezing the tube in a dry-ice—acetone bath and subsequently transferring the unfrozen organic layer to a conical 2-ml septum-cap vial. The dichloromethane was evaporated under a gentle stream of nitrogen, keeping the tubes in an electrically heated sample concentrator (Tecam-Dri Block DB-3) at 40°C. The dichloromethane was exchanged with ethanol by addition of the latter solvent (100 μl) during the concentratiom step, which was continued until less than 20 μl of solution remained. Part (0.1—0.3 μl) of the remaining ethanolic solution was injected onto the chromatographic column.

Micro-extraction

Plasma (0.1 ml) was mixed with an ethanolic solution (10 μl) of internal standards (N-methylanabasine 1 ng/μl, N-ethylnorcotinine 1 ng/μl) in a conical 2-ml septum-cap vial. The sample was made alkaline by the addition of aqueous sodium hydroxide (5 *M*, 100 μl) and then extracted with dichloromethane (500 μl). The work-up procedure was the same as that described in the paragraph above.

Plasma blanks

Human plasma was obtained from volunteers who had fasted overnight before donating blood. Bovine plasma has been used for calibration purposes, as problems were encountered in obtaining true blank human plasma. Extracts from bovine plasma and from the true blank of human plasma were found to give identical gas chromatograms.

Calibration procedure

Ethanolic nicotine standard solutions of different concentrations (0.05—1.0 ng/μl) containing the same concentration of the internal standard (1.0 ng/μl) were prepared for calibration purposes. These standard solutions (100 μl, 10 μl) were added to bovine plasma (1 ml, 0.1 ml) to provide samples containing 5, 10, 20, 50, 80 and 100 ng of nicotine and 100 ng of the internal standard, N-methylanabasine, per ml of plasma.

Similarly, bovine plasma (1 ml, 0.1 ml) was spiked with ethanolic standard solutions having different concentrations of cotinine and the same concentration of the internal standard, to provide samples containing 5, 10, 20, 50, 80, 100, 250 and 500 ng of cotinine and 100 ng of the internal standard, N-ethylnorcotinine, per ml of plasma.

The ethanolic standard solutions were stored in the absence of light at 4°C and were found to be stable for at least one year.

The samples were taken through the extraction procedure and analysed by gas chromatography. Calibration curves were constructed by plotting the amount of nicotine versus the peak area ratio of nicotine and N-methylanabasine and by plotting the amount of cotinine versus the peak area ratio of cotinine and N-ethylnorcotinine.

Recovery

Extraction efficiency. Human plasma (1 ml) was spiked with nicotine and cotinine (4 μg of each), and extracted at alkaline pH with dichloromethane (2 ml). A portion of the organic layer (0.2 ml) was transferred to a tube containing internal standards (0.4 μg of each) in ethanol (2 ml), and subsequently analysed as described below.

Overall recoveries. Human plasma (1 ml) was spiked with nicotine and cotinine (100 ng of each), and extracted with dichloromethane (2 ml) at alkaline pH. A portion of the organic layer (1 ml) was transferred to a conical tube and evaporated. The internal standards (50 ng of each) were added as ethanolic solutions at the end of the evaporation step.

Part of the ethanolic solutions were injected into the gas chromatograph. The peak area ratios of nicotine/N-methylanabasine and cotinine/N-ethyl-

norcotinine were estimated and the recoveries were calculated by comparison with the peak area ratios of non-extracted standard solutions. Similarly, the recoveries of N-methylanabasine and N-ethylnorcotinine were estimated by spiking human plasma with these compounds and using nicotine and cotinine as external standards.

RESULTS AND DISCUSSION

Contamination

A peak having the same retention time as nicotine has been observed by several authors during the analysis of plasma from non-smokers and has caused some confusion. We have also encountered this peak and have unambiguously identified the corresponding compound as nicotine by high-resolution mass spectrometry. It derives from extraneous nicotine present in the environment, which is incorporated into the plasma either by ingestion by the non-smoker or by contamination during sampling, sample work-up, or chromatographic analysis.

This interference, which originates from sources such as contaminated atmosphere, reagents and apparatus, has been minimized by rigorous care and control of all steps in the present assay. It has thus been possible, by undertaking the precautions discussed below, to reduce the nicotine concentration of "zero nicotine" plasma samples to between 0.3 and 0.6 ng/ml (Fig. 1A and B). Many of the contamination problems associated with the nicotine analysis do not occur in the cotinine analysis, since in tobacco smoke the concentration of the latter is much lower than that of nicotine.

In accordance with results of Feyerabend and Russell [21], we found that the atmosphere is the most serious source of contamination, and hence the entire analytical procedure was performed in a sealed laboratory, all air passing through an active carbon filter. All work-up of samples, i.e. extraction and evaporation, was carried out in a flow-hood. Smoking was strictly forbidden in the area surrounding this laboratory and the analyst had to be a non-smoker.

To avoid contamination from reagents, their purity was checked before use. Gas chromatographic examination of evaporation residues derived from different grades of solvents showed that dichloromethane (IR-spectroscopic grade from Fluka) and ethanol (Spectroscopic grade) could be employed without purification. However, the purity of every new batch of dichloromethane had to be checked as the quality of this solvent varied considerably. The sodium hydroxide solution, which is easily contaminated by nicotine, had to be prepared and stored with care and its purity examined before use. Pure sodium hydroxide solutions were prepared by dissolving pellets, heated beforehand to 200°C, in deionized water and extracting the resulting solution with dichloromethane just prior to use.

Cleaning of laboratory equipment, such as tubes, pipettes and syringes, was found to be of great importance since nicotine as well as the other bases used in the assay readily bind to glass and metal surfaces. All glassware was meticulously washed and subsequently kept in an oven at 200°C until required.

Besides extraneous nicotine, the plasma samples could also be contaminated by other compounds interfering with the chromatographic analysis. Thus blood

samples treated with heparin in the course of the preparation of plasma, were found to contain a component giving rise to a peak interfering with the nicotine peak in the chromatographic analysis. Therefore, this anticoagulant was replaced by sodium citrate. Interfering peaks were also encountered when plastic bags or tubes were used for storage of plasma.

We have also investigated a microtechnique for the analysis of nicotine and cotinine, the major advantage of which is a faster and simpler sampling procedure. In agreement with results of Feyerabend and Russell [10], we found this technique applicable to samples of blood obtained in the normal way but not to those obtained by finger-tip puncture, since these samples were heavily contaminated by nicotine derived from the skin. However, this technique was found to be suitable for the analysis of cotinine, where contamination is much less of a problem.

Extraction

Interference by extraneous nicotine is mainly due to incorporation during the extraction procedure. In order to minimize the exposure of the samples to the atmosphere, reagents and glassware, we have developed a simplified extraction technique, which involves a single extraction of nicotine and cotinine from plasma by dichloromethane at alkaline pH. Dichloromethane was found to be the most suitable organic solvent for a simultaneous extraction of nicotine and cotinine and their internal standards as the recoveries of these compounds from a single extraction were found to be between 95 and 100% (Table I). Purification of the samples by time-consuming back-extraction steps could be omitted as high-resolution gas chromatography was used in the analytical step. This direct extraction procedure, which involves high recoveries and low interference by extraneous nicotine, enabled accurate quantification of small amounts of nicotine and cotinine in plasma. Thus, this extraction procedure was a prerequisite for the analysis of 0.1-ml plasma samples.

TABLE I

RECOVERIES FROM HUMAN PLASMA

Compound	Extraction recovery (%)	Overall recovery (%)
Nicotine	96	83
N-Methylanabasine	99	100
Cotinine	97	96
N-Ethylnorcotinine	103	95

Gas chromatography

The sensitivity and selectivity of the thermionic specific detector for nitrogen-containing compounds allowed a relatively precise determination of small amounts of nicotine and cotinine. The detection limit at a signal-to-noise ratio of four was estimated to be 5 pg of nicotine and 20 pg of cotinine. The detector response was found to be linear and reproducible down to 5 pg of nicotine and 20 pg of cotinine, which corresponds to a minimal detectable amount of 0.1 ng of nicotine and 0.4 ng of cotinine per ml of plasma.

The use of the simplified extraction procedure described above, required a refined separation technique in the analytical step. This was achieved by using high-resolution capillary column gas chromatography. By optimization of critical parameters and instrumental configuration, excellent resolution and sensitivity were obtained; of particular importance were the use of a direct injector, insertion of the outlet of the fused-silica column directly into the flame-tip, and use of helium as carrier gas. A single injection of a plasma extract gave no significant interference by any endogenous material present in normal human plasma, but in the nicotine analysis interfering peaks appeared in the chromatograms after ten consecutive injections. These were readily removed by conditioning the column at 220°C for about 10 min. Typical gas chromatograms obtained after extraction of 1 ml of blank human plasma and of 1 ml of an authentic human plasma sample from a heavy smoker, containing 35 ng of nicotine and 350 ng of cotinine, are shown in Figs. 1 and 2.

As shown, the gas chromatographic analyses of nicotine and cotinine were performed isothermally at different column temperatures. The compounds were eluted at low k' values and sharp peaks with minimal broadening were obtained. Under these conditions the full resolution capability of the column was not utilized, but the chromatographic resolution was still sufficient to achieve good baseline separations of nicotine and cotinine from their internal standards. The sensitivity and reproducibility were also improved by this gas

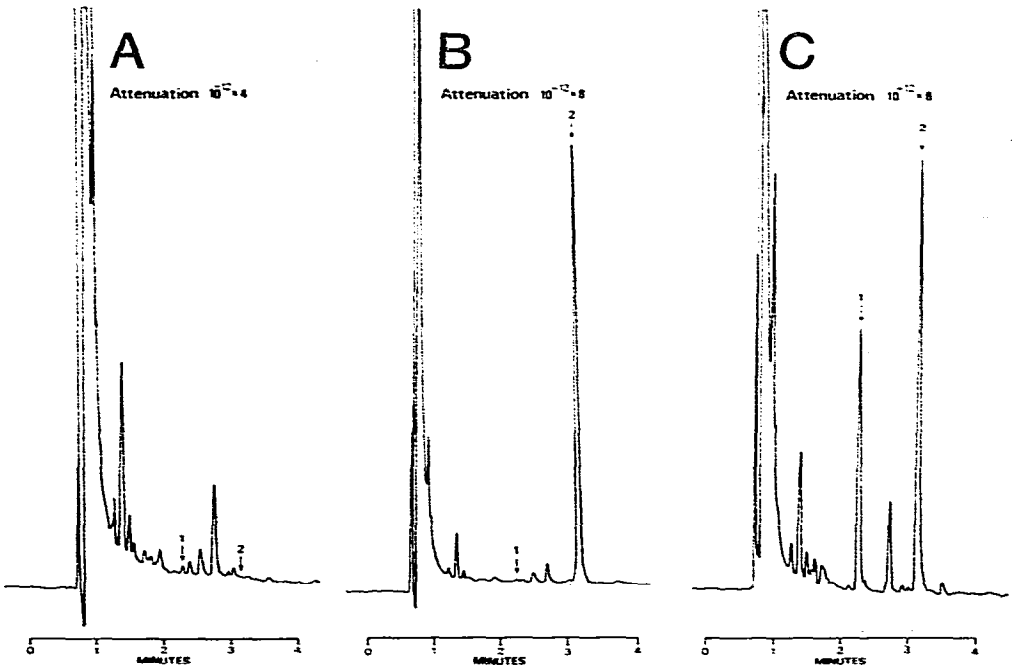

Fig. 1. Gas chromatograms obtained from extracts from human plasma samples (1 ml). (A) Extract of a non-smoker's plasma. (B) Extract of a non-smoker's plasma spiked with 100 ng/ml of the internal standard, N-methylanabasine. (C) Extract of an authentic plasma sample from a smoker containing 35 ng of nicotine and spiked with 100 ng of N-methylanabasine. Nicotine (1) and the internal standard, N-methylanabasine (2), were eluted isothermally at 135°C.

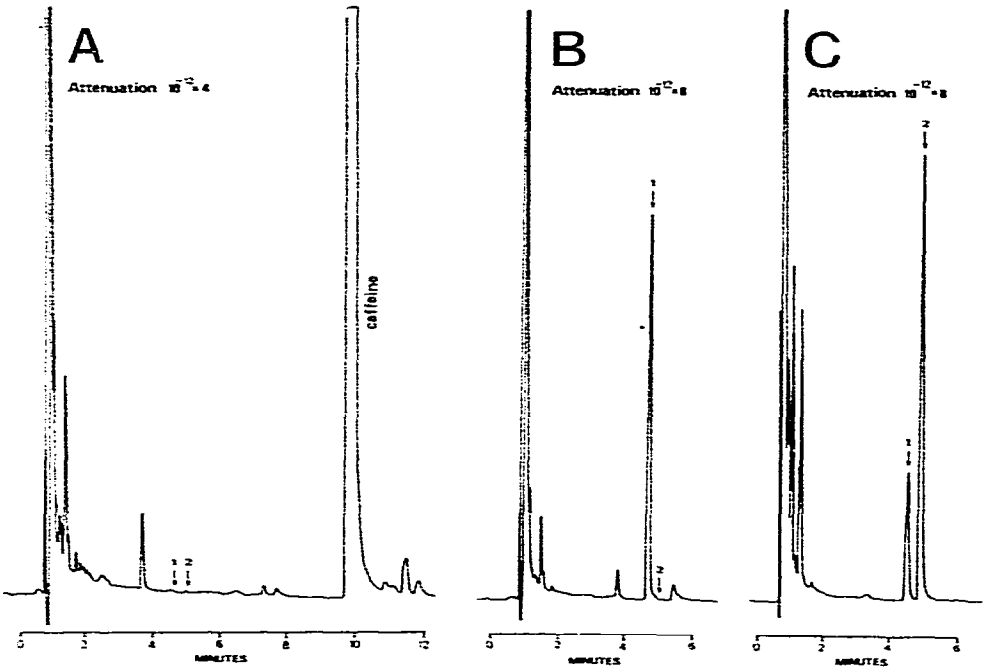

Fig. 2. Gas chromatograms obtained from extracts of human plasma samples (1 ml). (A) Extract of a non-smoker's plasma. (B) Extract of a non-smoker's plasma spiked with 100 ng/ml of the internal standard, N-ethylnorcotinine. (C) Extract of an authentic plasma sample from a smoker containing 350 ng of cotinine and spiked with 100 ng of N-ethylnorcotinine. N-Ethylnorcotinine (1) and cotinine (2) were eluted isothermally at 200°C.

chromatographic procedure and, moreover, the total analytical time was shortened, especially when the two analyses were performed on separate gas chromatographs.

A factor which limits the accuracy of quantification is the reduction in sensitivity which results from repeated injections of plasma extracts. However, the sensitivity could be restored by cleaning the injector insert and disposing of 10 cm of the column inlet to remove accumulated residues.

Internal standards

The choice of internal standards is important for the sensitivity and the precision of the method. Since there are great differences in solvent partitioning and chromatographic properties of nicotine and cotinine, separate internal standards have been used for the analysis of these compounds. Our initial attempts to use quinoline as standard for nicotine and lidocain for cotinine, as described in previous methods, failed. In accordance with Jacob et al. [13], we observed large variations in the peak area ratios of nicotine to quinoline and of cotinine to lidocain, results which are probably due to different relative losses during work-up and/or during gas chromatography.

These difficulties were circumvented by using the structural analogues N-methylanabasine and N-ethylnorcotinine as internal standards (Fig. 3). Their extractability into dichloromethane, volatility and affinity to glass and metal

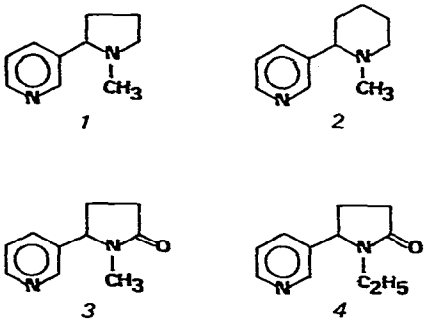

Fig. 3. Structures of nicotine (1), N-methylanabasine (2), cotinine (3) and N-ethylnorcotinine (4).

surfaces are similar to those of nicotine and cotinine, as demonstrated by their high overall recoveries (Table I). Moreover, the use of these analogues as internal standards also improved the precision of the chromatographic analysis as their detector responses and retention times are close to those of nicotine and cotinine.

Precision and calibration

Both 1-ml and 0.1-ml samples of blank bovine plasma were spiked with 100 ng of each of the internal standards and with nicotine and cotinine to give samples of different concentrations within the range 5—100 ng/ml nicotine and within the range 5—500 ng/ml cotinine. Ten samples at each concentration were prepared and taken through the extraction procedure. The precision of

TABLE II

PRECISION OF DETERMINATION OF NICOTINE AND COTININE APPLIED TO 1-ml SPIKED BOVINE PLASMA SAMPLES

	Amount added (ng/ml)	Mean amount found* (ng/ml, $n=10$)	Standard deviation (S.D.)	Coefficient of variation (C.V. %)
Nicotine	5	5.1	0.4	8.6
	10	9.7	0.5	4.8
	20	19.1	0.6	2.9
	50	47.9	2.0	4.6
	80	78.6	3.5	4.4
	100	99.3	2.6	2.7
Cotinine	5	5.4	0.7	13.6
	10	10.4	0.6	6.2
	20	20.5	1.3	6.4
	50	50.4	1.2	2.3
	80	80.0	2.0	2.5
	100	98.7	1.5	1.5
	250	257.2	1.0	0.4
	500	496.8	3.5	0.7

*The results of the nicotine analyses have been corrected for a background level of nicotine.

TABLE III

PRECISION OF DETERMINATION OF NICOTINE AND COTININE APPLIED TO 0.1-ml SPIKED BOVINE PLASMA SAMPLES

	Amount added (ng/ml)	Mean amount found* (ng/ml, $n=10$)	Standard deviation (S.D.)	Coefficient of variation (C.V.%)
Nicotine	5	5.4	0.6	11.2
	10	10.1	0.5	5.4
	20	20.4	1.2	5.8
	50	49.5	1.3	2.7
	80	78.6	2.5	3.2
	100	102.4	3.7	3.6
Cotinine	5	5.1	0.6	11.2
	10	11.2	0.7	6.4
	20	21.9	1.0	4.7
	50	52.1	1.5	3.0
	80	80.2	1.3	1.6
	100	98.6	1.7	1.7
	250	252.4	5.0	2.0
	500	489.9	10.3	2.1

*The results of the nicotine analyses have been corrected for a background level of nicotine.

the method was evaluated by calculating the coefficient of variation at each concentration of nicotine and cotinine. The mean values, standard deviations and coefficients of variation of each plasma level of nicotine and cotinine are given in Tables II and III. In the case of nicotine analysis, the background level obtained from blank plasma samples was subtracted (see Fig. 1). The results show that plasma levels could be accurately determined down to 5 ng of nicotine and cotinine per ml of plasma.

Ten calibration curves were constructed for each of the ranges 5—100 ng of nicotine and 5—500 ng of cotinine per ml of plasma with both 1-ml and 0.1-ml samples. The calibration curves were linear over the entire range investigated and the mean correlation coefficient of the ten curves were in all cases better than 0.9995. The calibration curves are reproducible from day to day, which is demonstrated by the small coefficients of variation (0.5—3.5%) of the slopes.

TABLE IV

CONCENTRATION RANGES AND MATHEMATICAL EXPRESSIONS OF THE NICOTINE AND COTININE CALIBRATION CURVES

Concentration ranges	Amount of plasma (ml)	Regression equation ($y = ax + b$)		Correlation coefficient (r)	n
		a	b		
Nicotine (5—100 ng/ml)	1.0	0.989	0.210	0.9997	10
	0.1	1.006	1.389	0.9995	10
Cotinine (5—500 ng/ml)	1.0	0.990	1.086	0.9996	10
	0.1	0.982	1.532	0.9998	10

The concentration ranges of nicotine and cotinine, the coefficients of correlation and the mathematical expression of the calibration curves are summarized in Table IV.

CONCLUSION

A rapid and sensitive method has been developed for the simultaneous determination of nicotine and cotinine at nanogram levels using as little as 0.1 ml of plasma. The high degree of precision achieved is attributable to the exclusion of extraneous contaminants, the use of a single-step extraction procedure, the choice of suitable internal standards, and the use of high-resolution capillary gas chromatography and thermionic specific detection. It is well suited for routine analysis of nicotine and cotinine in plasma not only from smokers and users of snuff, but also from "passive smokers".

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