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IMPROVED METHOD FOR ROUTINE DETERMINATION OF NICOTINE AND ITS MAIN METABOLITES IN BIOLOGICAL FLUIDS

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SUMMARY

Extrelut^{R} extraction and glass capillary gas chromatography were applied to the routine determination of nicotine and its metabolites cotinine, nicotine-1′-N-oxide and cotinine-1-N-oxide in urine and plasma. After extraction of nicotine and cotinine both N-oxides and phendimetrazine-N-oxide (used as internal standard) were reduced to their bases by SO_2 on-column and eluted by a mixture of diethyl ether and dichloromethane. The minimum detectable concentrations are 0.03 μg/ml for urinary nicotine and cotinine and 0.1 μg/ml for the N-oxides. In plasma samples the corresponding values are 5 ng/ml and 15 ng/ml, respectively, with sample values as small as 2 ml. The advantage of the direct determination of all four compounds of interest in one sample reduced the amount of plasma required. The straightforward and rapid extraction and reduction procedure as well as the long-term stability of the gas chromatographic separation system make the method suitable for routine application.

INTRODUCTION

The determination of nicotine and its metabolites in biological fluids is of particular interest to investigators studying correlations between the biological effects of tobacco smoking and smoking habits (active, passive, frequency, type of cigarette, etc.).

Nicotine taken up from cigarette smoke is primarily metabolized in the human organism to cotinine and nicotine-1′-N-oxide [1]. Both compounds are formed by independent pathways [2]. Which of them predominates depends on the age of the test person and the urinary pH [3]. Cotinine undergoes further oxidation to cotinine-1-N-oxide [4, 5]. All these metabolites are excreted in urine together with the non-metabolized nicotine.

The expected concentrations for nicotine, cotinine and nicotine-1′-N-oxide are below 1 μg/ml in most urine samples [3]. Disregarding heavy cigarette

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smokers, plasma contains nicotine below 60 ng/ml and cotinine below 200 ng/ml. The low concentration as well as the minute amounts of plasma available demand a sensitive method permitting the determination of all compounds in one sample. The analysis of nicotine and cotinine in urine described by Beckett and Triggs [6] has been modified and completed by several workers [3, 4—11]. All these methods are time consuming and therefore not suitable for a large number of samples. As a rule they include liquid—liquid extraction, determination of nicotine-1′-N-oxide after reduction by $TiCl_3$, and gas chromatographic (GC) analysis. However, ether extraction of nicotine and successive extraction of cotinine with dichloromethane [7] results in unavoidable losses of cotinine [12]. Furthermore, the experimental error is increased by the determination of the N-oxide as the difference between the total nicotine content after reduction of the oxide and the original nicotine amount. Finally, the analysis of nicotine-1′-N-oxide in plasma proved to be rather difficult by this method.

If the GC analysis of the extracts is carried out on packed columns, the high elution temperature of cotinine considerably limits the lifetime of the column. For that reason, as well as for their high separation power and inertness, glass capillary columns proved to be superior [13].

The advantages of the nitrogen-sensitive alkali flame ionisation detector have been reported [10, 14], but its low long-term stability limits its routine application. The mass spectrometer, used as a highly specific GC detector [13, 15] is too expensive for routine analysis.

In our view a method for routine analyses should fulfil the following criteria: rapid and straightforward extraction and clean-up procedure; simultaneous analysis of nicotine and cotinine; determination of N-oxides independent of nicotine and cotinine in the same sample, not only for urine but also for plasma; efficient and stable separation and detection. These requirements were met by the application of Extrelut^{R} columns for the extraction step, the on-column reduction of the N-oxides in the presence of phendimetrazine-N-oxide as internal standard, and the use of glass capillary columns for separation.

EXPERIMENTAL

Materials

Cotinine (70% aqueous solution) and nicotine-1′-N-oxide (purity ca. 80%) were purchased from R. Hallermayer, Augsburg, G.F.R. Lidocaine, lidocaine hydrochloride and phendimetrazine hydrochloride were kindly supplied by Gerot Pharmazeutika, Vienna, Austria. Quinoline (purity 99%) purchased from Fluka (Buchs, Switzerland) and nicotine (synthesis grade, 98%) purchased from Merck-Schuchardt (Munich, G.F.R.) were distilled shortly before use.

Phendimetrazine-N-oxide was prepared from phendimetrazine by H_2O_2 oxidation. Phendimetrazine was obtained by adding 1 ml of 5 *N* NaOH to 0.54 g of phendimetrazine hydrochloride, extracted three times with 3 ml of ethyl ether and dried over Na_2SO_4. After evaporation of the solvent the resulting 0.43 g of phendimetrazine were oxidized with 4 ml of 5% H_2O_2 at room temperature for 18 h and at 40°C for a further 20 h. Finally, the unreacted phendimetrazine was removed by extraction with diethyl ether and the sample con-

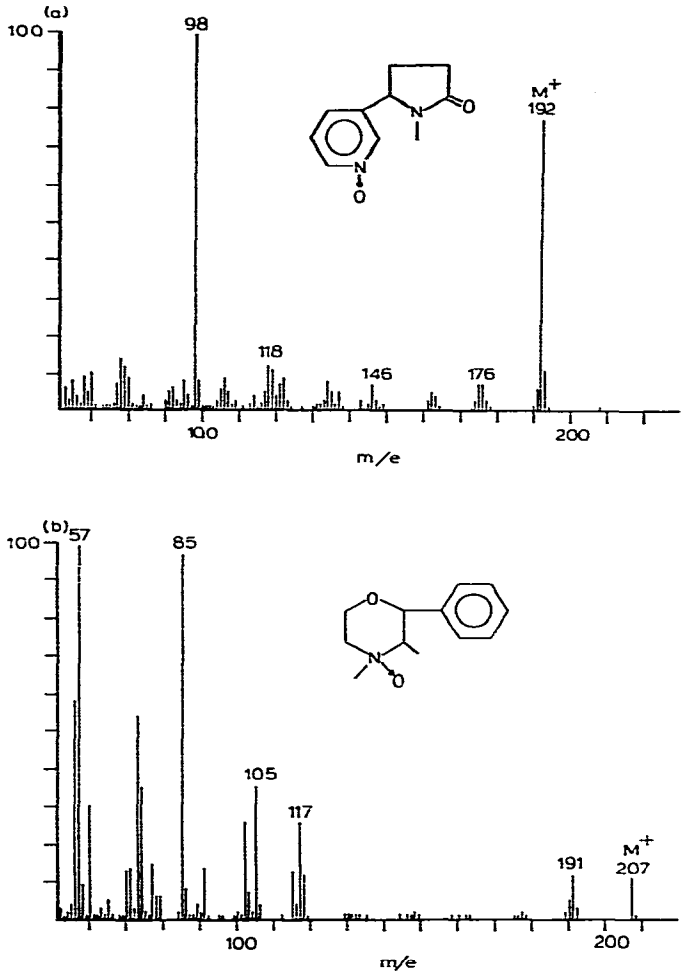

Fig. 1. Mass spectra of cotinine-1-N-oxide (a) and phendimetrazine-N-oxide (b). Varian CH 5 DF, 70 eV, direct probe 120°C.

taining the N-oxide was freeze-dried. The procedure yielded 0.44 g of phendimetrazine-N-oxide. The structure was verified by mass spectrometry (Fig. 1).

Cotinine-1-N-oxide was synthesized in a similar way. The oxidation was carried out with a 10% solution of H_2O_2 at 60°C for 72 h. The residual H_2O_2 was degraded by a short heat treatment and the unreacted material removed by repeated extraction with dichloromethane. The mass spectrum is shown in Fig. 1.

For Extrelut extraction two types of columns (dimensions 23 mm × 93 mm and 7 mm × 10 mm) were filled at a packing density of 0.30 g/cm^3 with different loadings of Extrelut (Merck) according to the sample size [16].

Procedures

Urine samples. To 4.0 ml of urine 0.5 ml of 0.5 *N* NaOH and the internal standard solution (quinoline, lidocaine, phendimetrazine and phendimetrazine-

N-oxide, 5.0 μg of each dissolved in 0.1 ml of acetone) were added. The sample was applied to a column filled with 3.0 g of Extrelut. After 30 min, 13 ml of the solvent mixture (diethyl ether—dichloromethane, 1:4, v/v) were poured into the column. The eluate (ca. 10 ml), containing nicotine, cotinine, quinoline, lidocaine and phendimetrazine, was concentrated in a water bath at room temperature under a stream of nitrogen to a volume of 1 ml and analyzed by GC.

Afterwards, the N-oxides of nicotine, cotinine and phendimetrazine were reduced by passing a stream of SO_2 through the column; 30 min after saturation of the column with SO_2 the sample was made alkaline by passing a stream of NH_3-saturated air through the column. The pH of the gaseous effluents in both steps was monitored by an indicator strip. The neutralization with NH_3 was carried out slowly to avoid warming up beyond 40°C. The

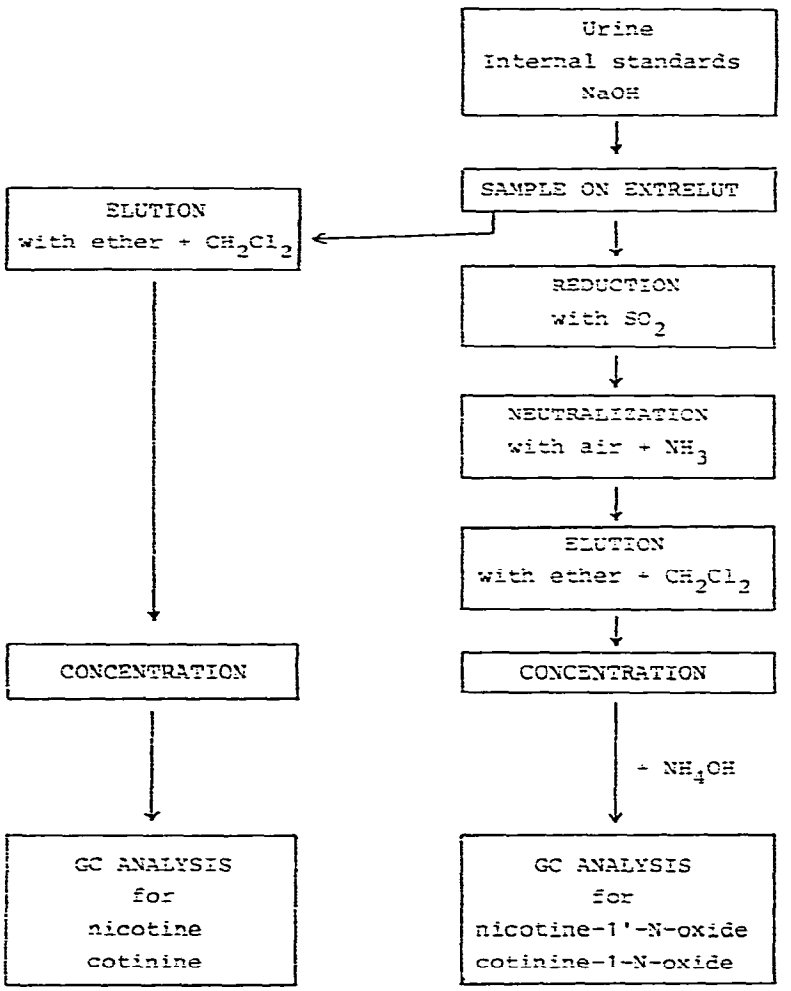

Fig. 2. Flow diagram of urine processing for the determination of nicotine, cotinine and their N-oxides.

resulting bases were eluted and concentrated as described above. Prior to GC analysis 5 μl of concentrated NH_4OH were added to each sample. A flow diagram of the procedure is shown in Fig. 2.

Plasma samples. To 2.0 ml of plasma 2.0 ml of 0.1 *N* NaOH and the internal standard solution (quinoline, lidocaine, phendimetrazine and phendimetrazine-N-oxide, 200 ng of each dissolved in 10 μl of acetone) were added. The sample was applied to a column filled with 2.5 g of Extrelut. After 30 min 13 ml of the solvent mixture were poured onto the column. The eluate (ca. 10 ml), containing nicotine, cotinine, quinoline, lidocaine and phendimetrazine, was collected into a calibrated conical tube. After addition of 0.5 ml of 0.2 *N* HCl, mixing and concentrating to a volume of about 6 ml, the sample was ultrasonicated for 15 min and centrifuged at 550 *g* for 5 min. The aqueous layer (ca. 0.45 ml) was quantitatively transferred with a syringe to a smaller vial and further purified by washing with 2 ml of diethyl ether. Then the aqueous sample was made alkaline by adding a drop of 6 *N* NaOH and applied to a column of the smaller type [16], filled with 0.25 g of Extrelut. After an equilibration time of 30 min, nicotine, cotinine and the corresponding standards were eluted with 1.5 ml of the solvent mixture. The eluate (ca. 1.1 ml) was concentrated to a volume of 100 μl, mixed with 5 μl of concentrated NH_4OH and analyzed by GC.

Following the elution of the bases the N-oxides remaining on the first column (2.5 g of Extrelut) were reduced, made alkaline and eluted as described for the urine sample. The next step followed was as described for the plasma sample, including re-extraction with 0.5 ml of 0.2 *N* HCl. A flow diagram of the procedure is shown in Fig. 3.

GC instrumentation and working conditions

For GC a Carlo Erba instrument Model 2150, equipped with a Dani automatic liquid sampler ALS 3641 (modified to hold syringes with 70 mm needle length) was used. The detector signals were quantitated by a Spectra-Physics Integrator System I. The gas chromatograph was equipped with a split/splitless injection port, containing a glass insert filled in a length of 30 mm with Carbowax 1000 deactivated glass beads (100—120 mesh). This precolumn kept the non-volatile sample components off the capillary column [17].

The raw capillaries were drawn from borosilicate glass to a length of 28 m with an inner diameter of 0.3 mm. After formation of an intermediate layer of $BaCO_3$ [18] they were coated with a 0.1 μm thick film of the polyglycol phase Pluronic F 68 (Fluka).

The analyses were carried out under the following conditions: injection port temperature 250°C; oven temperature program: starting isothermal 40°C for 1 min, first heating rate 30°C/min, intermediate isothermal 80°C for 2 min, second heating rate 10°C/min, final isothermal 230°C for 10 min; detector, FID, base temperature 250°C; carrier gas hydrogen, inlet pressure 0.55 bar (linear gas velocity 50 cm/sec at room temperature); injection volume (splitless injection) 1.0 μl.

The evaluation was carried out by the internal standard method. The standards were quinoline and lidocaine for nicotine and cotinine, respectively. In addition, phendimetrazine was used as an overall indicator of the adsorptivi-

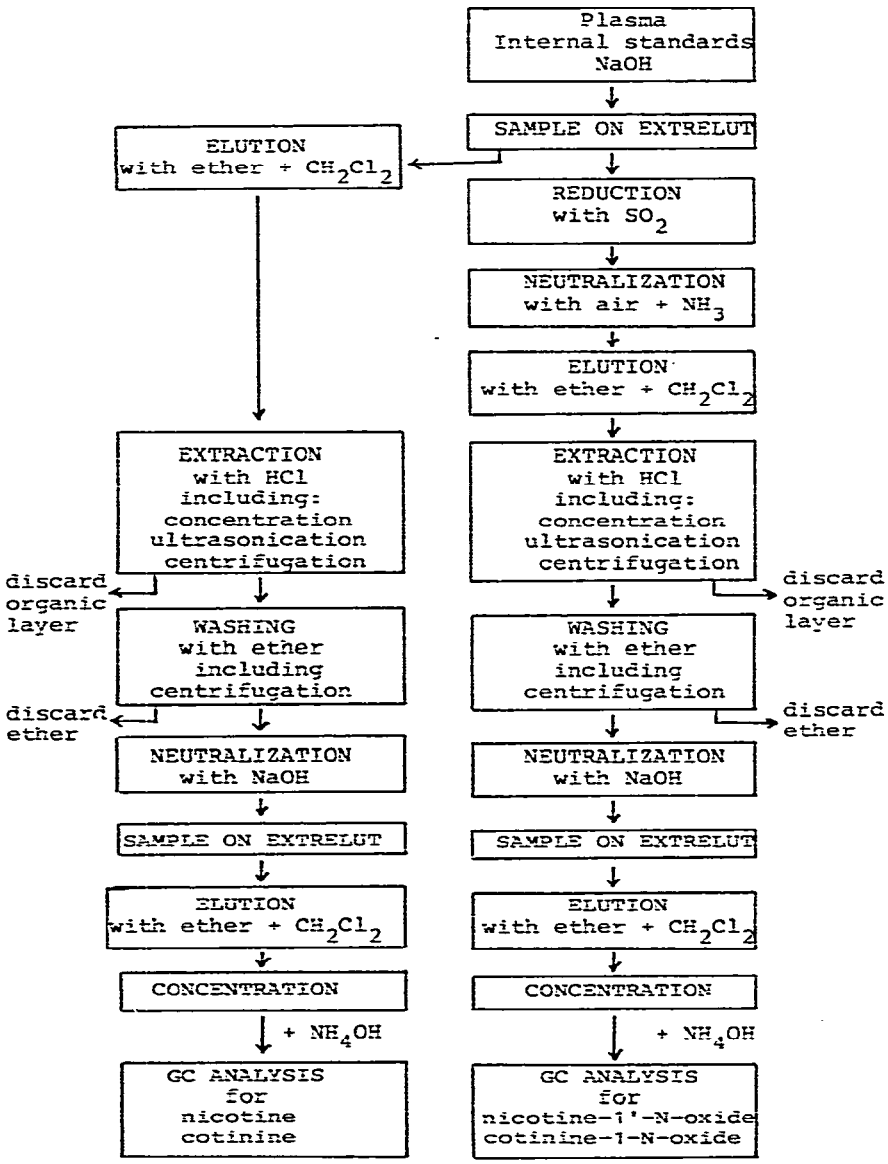

Fig. 3. Flow diagram of plasma processing.

ty of the system. If the phendimetrazine peak showed a broadening or a retardation the injection was repeated after addition of 5 μl of concentrated NH_4OH. The determination of the nicotine- and cotinine-N-oxides was based on phendimetrazine-N-oxide as internal standard.

RESULTS AND DISCUSSION

Figs. 4 and 5 show gas chromatograms of plasma samples processed as described above. The good separation of the peaks of interest from coextracted impurities and the adequate sensitivity of the flame ionisation detector

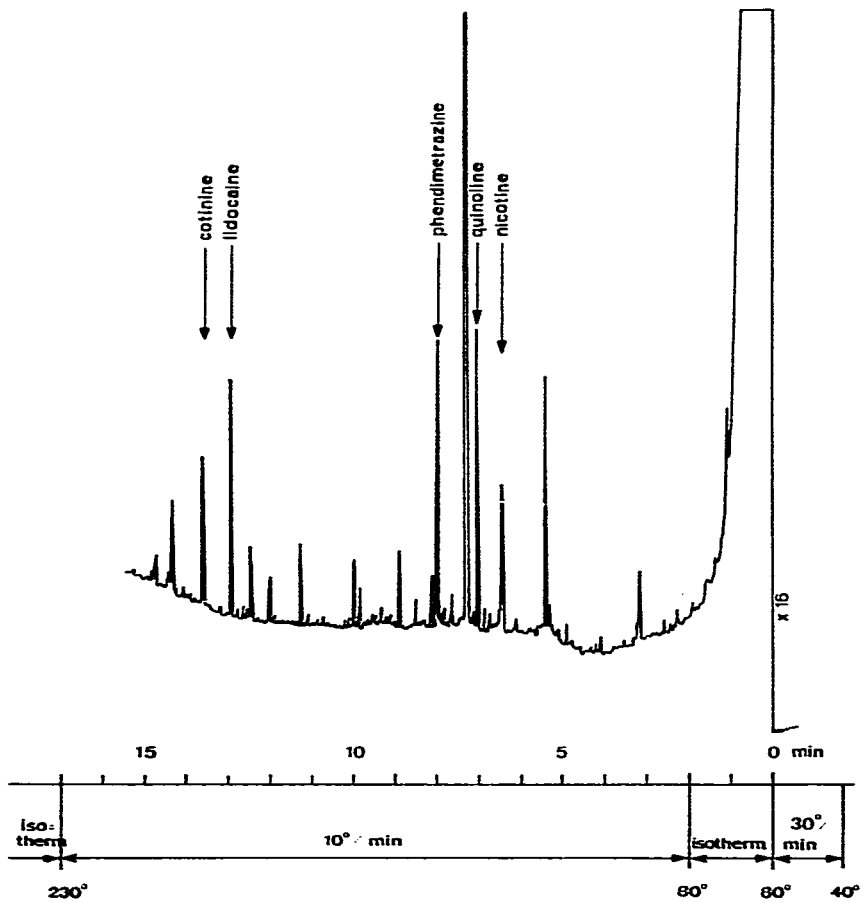

Fig. 4. Gas chromatogram of an extract of a 2-ml plasma sample containing nicotine and cotinine (each 60 ng/ml) and processed as described in the text.

rendered specific detectors like alkaline flame ionisation or mass spectrometry unnecessary. The use of a precolumn as well as the relatively mild working conditions guaranteed a satisfactory lifetime of the capillary columns. Thus the GC system exhibited the high long-term stability needed for routine application.

The minimum detectable concentrations using the urine procedure were 0.03 μg/ml for nicotine or cotinine and 1.0 μg/ml for nicotine-1′-N-oxide. The recovery was at least 85% for the bases and about 60% for the N-oxides. The plasma-processing procedure was more sensitive, permitting the detection of about 5 ng/ml nicotine or cotinine and 15 ng/ml N-oxides. In this case the recovery was at least 80% for the bases and about 30% for the N-oxides. Calibration curves for the overall procedure, represented by linear regression equations, are given in Table I.

During extraction, especially in the case of plasma samples, extreme care has to be taken to keep the laboratory air free from nicotine. There is ample experimental evidence that even trace amounts of cigarette smoke in the laboratory air significantly increase the nicotine values.

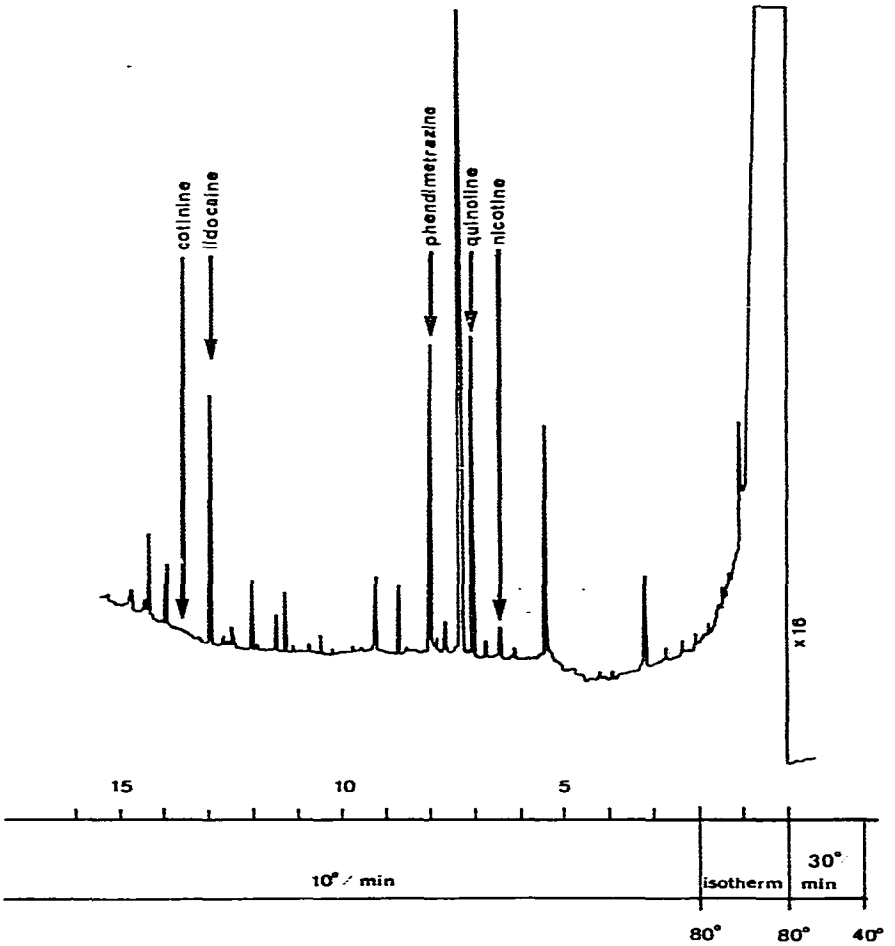

Fig. 5. Gas chromatogram of an extract of a 2-ml plasma blank taken from a non-smoker working in a smoking environment.

In samples reduced with SO_2 a broadening and/or retardation of the phendimetrazine peak and, to a lesser extent, of the nicotine peak was sometimes observed. This effect could be suppressed by the addition of 5 μl of concentrated NH_4OH to the 1-ml sample prior to GC analysis. NH_4OH prevents adsorption of alkaline compounds like nicotine on glass surfaces [19] and also neutralizes traces of SO_2 which might be present in the eluent.

The application of Extrelut considerably simplified the extraction procedure, thus substantially reducing the analysis time. The internal standard phendimetrazine-N-oxide, which is reduced and eluted under the same conditions as nicotine-1′-N-oxide or cotinine-1-N-oxide, allowed easy monitoring of the overall process. Moreover, only one internal standard mixture for both bases and N-oxides had to be added.

The solvent mixture used (diethyl ether—dichloromethane, 1:4, v/v) eluted nicotine as well as cotinine, thus excluding the uncontrolled cotinine losses which usually occur during solvent—solvent extraction. The Extrelut extraction

TABLE I

LINEAR REGRESSION OF COMPOUND/INTERNAL STANDARD PEAK HEIGHT RATIO AGAINST CONCENTRATION

Range: 0—15 μg/ml for urine, 0—250 ng/ml for plasma.

	Regression coefficient	Intercept on y-axis	Correlation coefficient
Urine			
Nicontine	0.6062	0.0075	0.9999
Cotinine	0.6660	—0.0070	0.0089
Nicotine-1'-N-oxide	0.3273	0.0020	0.9958
Cotinine-1-N-oxide	0.3450	0.0200	0.9980
Plasma			
Nicotine	0.0072	0.0233	0.9971
Cotinine	0.0101	—0.0154	0.9982
Nicotine-1'-N-oxide	0.0056	0.0240	0.9957
Cotinine-1-N-oxide	0.0064	0.0005	0.9957

allowed the determination of both the N-oxides independently from their bases in the same sample. The method is successful even for amounts of plasma as small as 2 ml.

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