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IMPROVED GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF NICOTINE AND COTININE IN BIOLOGIC FLUIDS

PEYTON JACOB, III*, MARGARET WILSON and NEAL L. BENOWITZ

Division of Clinical Pharmacology, Department of Medicine, School of Medicine, University of California, San Francisco, and San Francisco General Hospital Medical Center, 1001 Potrero Avenue, San Francisco, CA 94110 (U.S.A.)

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SUMMARY

Improved methods have been developed for the determination of nicotine and its major metabolite, cotinine, in blood, plasma, and urine samples. These methods utilize gas chromatography with alkali flame ionization (nitrogen—phosphorus) detection and structural analogs of nicotine and cotinine as internal standards.

INTRODUCTION

A variety of methods for the determination of nicotine in biologic specimens have been reported in the literature, including methods based on radioimmunoassay [1, 2], liquid chromatography [3, 4], and gas chromatography using electron-capture [5], flame ionization [6—8], or alkali flame ionization [9—12] detectors or combined gas chromatography—mass spectrometry [13, 14]. In attempting to use published gas chromatographic methods, we encountered difficulty with reproducibility. This paper describes improved gas chromatographic methods for nicotine and cotinine determination with structural analogs of both substances utilized as internal standards. Using these methods, concentrations of nicotine as low as 1 ng/ml, and concentrations of cotinine as low as 5 ng/ml may be reliably measured in 1-ml samples of blood, plasma, or urine.

MATERIALS AND METHODS

Reagents and chemicals

Commercial nicotine base was converted to the bitartrate with (+)-tartaric

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acid, and purified by three recrystallizations from 80% aqueous ethanol. Cotinine was synthesized by the method of Bowman and McKennis [15] and converted to the crystalline fumarate (2:1) salt. Myosmene was synthesized by the procedure of Brandänge and Lindblom [16], and γ-3-pyridyl-γ-oxobutyric acid was synthesized by the method of McKennis et al. [17]. Other chemicals were obtained from commercial sources. All solvents were reagent grade.

Synthesis of the internal standard for nicotine, N-ethylnornicotine

Sodium cyanoborohydride (1 g) was added to a solution of myosmene [16] (3.0 g) in 50 ml of 50% aqueous ethanol. The pH of the vigorously stirred solution was adjusted to 2.2 by the dropwise addition of concentrated hydrochloric acid. For a period of 1.5 h, the solution was stirred and the pH was maintained in the range of 1.8—2.2 by periodic addition of concentrated hydrochloric acid. Analysis of an aliquot by gas chromatography indicated that the reduction to nornicotine was largely complete. The pH was adjusted to 6.0 by the addition of concentrated aqueous sodium acetate, then acetaldehyde (4 g) and fresh sodium cyanoborohydride (0.2 g) were added. The solution was stirred for 30 min, made alkaline (pH 12) with sodium hydroxide, and extracted with two 50-ml portions of methylene chloride. Concentration of the extract on a rotary evaporator followed by distillation provided 2.6 g of a colorless liquid, boiling point 96—99°C (2.5 mmHg). The 60 MHz NMR spectrum was consistent with the structure of N-ethylnornicotine. The free base (2.6 g) was converted to the bis-oxalate by addition of a solution of oxalic acid dihydrate (3.7 g) in 50 ml of methanol. The methanol was evaporated, and the residue was recrystallized twice from absolute ethanol to give, after vacuum drying, 4.0 g (64% yield from myosmene) of white crystalline powder, melting point 130.5—132°C. Microanalytical data for carbon, hydrogen, and nitrogen were within accepted limits.

Synthesis of the internal standard for cotinine, N-(2-methoxyethyl)-norcotinine

Sodium hydroxide (0.3 g) was added to a solution of γ-(3-pyridyl)-γ-oxobutyric acid [17] (1 g) in 5 ml of 2-methoxyethylamine, and the solution was stirred and warmed until the solid had dissolved. Toluene (25 ml) was added, and the solution was heated to about 60°C while the solvent was removed under reduced pressure using a rotary evaporator. The residue was taken up in a solution of anhydrous methanol (15 ml), and 2-methoxyethylamine (5 ml) was added followed by sodium borohydride (0.5 g). An exothermic reaction with gas evolution resulted. After stirring overnight, excess hydride was decomposed by addition of methanolic HCl (to pH 1.0), and solvent was removed using a rotary evaporator. Triethylamine (5 ml) was added to the residue, which was then distilled bulb to bulb (130—150°C at 0.1 mmHg) using a Kugelrohr distillation oven. The distillate was dissolved in dilute aqueous HCl (10 ml) and extracted with two 10-ml portions of methylene chloride. The aqueous layer was made basic with sodium hydroxide, and the product was extracted with methylene chloride (10 ml twice). The extract was concentrated in a rotary evaporator, and then distilled bulb to bulb (150°C oven temperature at 0.1 mmHg) to give 0.43 g of a yellow oil. The 80 MHz NMR spectrum was consistent with the structure of N-(2-methoxyethyl)-norcotinine. The succinate salt was prepared from 102 mg (0.44 mmol) of free base by combining with a solu-

tion of succinic acid (47 mg, 0.40 mmol) in methanol. Evaporation of the solvent under reduced pressure gave a light brown oil that solidified on standing. The product was recrystallized twice from ethyl acetate to give a white crystalline solid, melting point 92—93°C. Microanalytical data for carbon, hydrogen, and nitrogen were within accepted limits.

Gas chromatography

Gas chromatographic analyses were performed using a Hewlett-Packard Model 5711A instrument equipped with dual alkali flame (nitrogen—phosphorus) detectors and a Varian Model 9176 strip-chart recorder. The nitrogen (carrier gas), air, and hydrogen flow-rates were 30, 50, and 5 ml/min, respectively. Columns (1.8 m for nicotine, 1.2 m for cotinine) were 2 mm I.D. glass, configured for on-column injection, packed with 2% Carbowax 20M + 2% KOH on Gas-Chrom P (100—120 mesh) (Applied Science Labs., State College, PA, U.S.A.) or 3% SP-2250 DB on Supelcoport (100—120 mesh) (Supelco, Bellefonte, PA, U.S.A.). The injection port and detector temperatures were 250°C and 300°C, respectively.

Assay of nicotine

Preparation of reagents and tubes for blood collection. Diethyl ether (anhydrous, reagent grade) was distilled from sodium benzophenone ketyl before use. Solutions of sodium hydroxide and hydrochloric acid were prepared from reagent grade chemicals using tap distilled water that had been redistilled from dilute chromic acid. All manipulations of samples in open tubes were carried out under an Edgegard^{R} Model EGB-4252 laminar flow hood (Baker, Sanford, ME, U.S.A.) in a laboratory in which smoking was prohibited. Nicotine bitartrate and the internal standard, N-ethyl-nornicotine oxalate, were stored as aqueous solutions. These solutions were found to be stable for at least one month when refrigerated. Meticulous preparation and storage of tubes was found to be necessary to avoid contamination with environmental nicotine. Screw-top glass tubes were soaked overnight in 30% nitric acid, rinsed thoroughly and dried at 80°C. Tubes were stored in the oven until just before use when they were transferred to the laminar flow hood, where anticoagulant was added. The PTFE-lined tube caps were soaked overnight in dilute HCl, rinsed, dried in the oven, and stored in tightly closed containers.

Assay procedure

A flow diagram of the extraction procedure is shown in Fig. 1. To 1-ml aliquots of blood or plasma were added 20 ng of N-ethylnornicotine [and N-(2-methoxyethyl)-norcotinine as well, if cotinine was to be assayed] followed by 0.5 ml 2 *N* sodium hydroxide. Freshly distilled diethyl ether (2 ml) was added to each tube, and the tubes were agitated for 1 min using a vortex mixer. After centrifuging to break up emulsions, the ether layers were transferred to tubes containing 0.5 ml 1 *N* HCl. The tubes were vortex mixed for 1 min and then the ether layers were removed and discarded. Sodium hydroxide (0.5 ml of 2 *M*) and 0.5 ml of ether were added to the aqueous layer; the tubes were again agitated on the vortex mixer for 1 min. The ether layers were separated and dried over anhydrous potassium carbonate in small vials. The extracts (1—5-μl

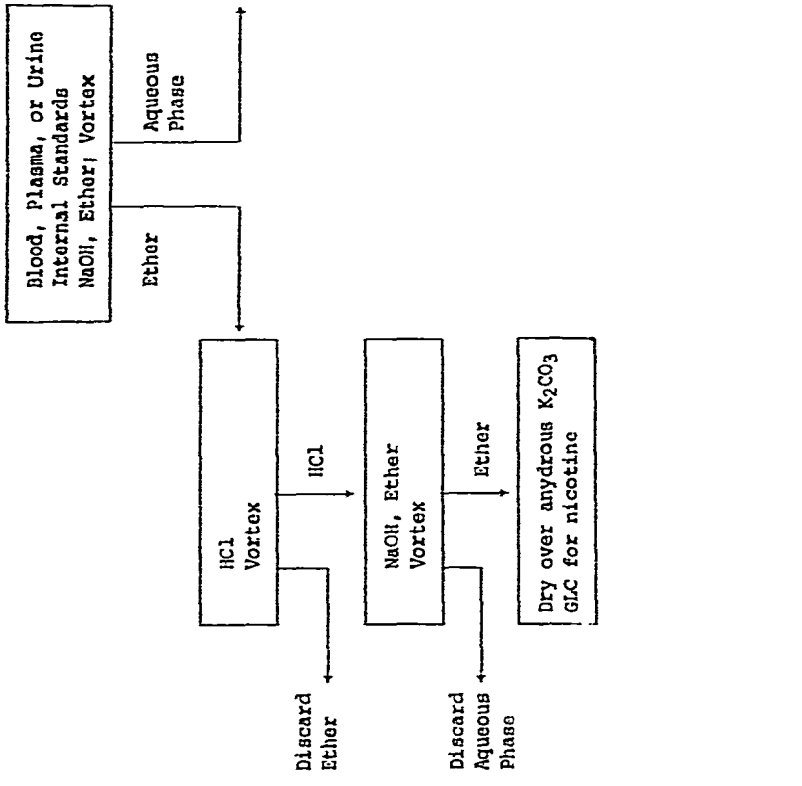


Fig. 1. Flow diagram of nicotine and cotinine extraction procedure.

aliquots) were analyzed by gas chromatography on 1.8 m × 2 mm I.D. Carbowax—KOH or SP-2250 DB columns at 145°C. Examples of chromatograms from plasma extracts are presented in Fig. 2. Standard curves, prepared from blank plasma samples to which nicotine and internal standard were added, were linear over the entire range studied, 0—100 ng/ml. Quantitation was achieved by calculating peak height ratios of nicotine to internal standard and referring to the standard curve. The assay for urine samples was identical, with the exception that larger amounts of internal standard (200 ng) were added.

Assay of cotinine

To 1 ml of blood or plasma were added internal standard, N-(2-methoxyethyl)-norcotinine (20 ng or 100 ng, depending on the anticipated cotinine levels), and 0.5 ml 2 *N* NaOH. (If the samples were to be assayed for nicotine as well, internal standards for both nicotine and cotinine were added. The

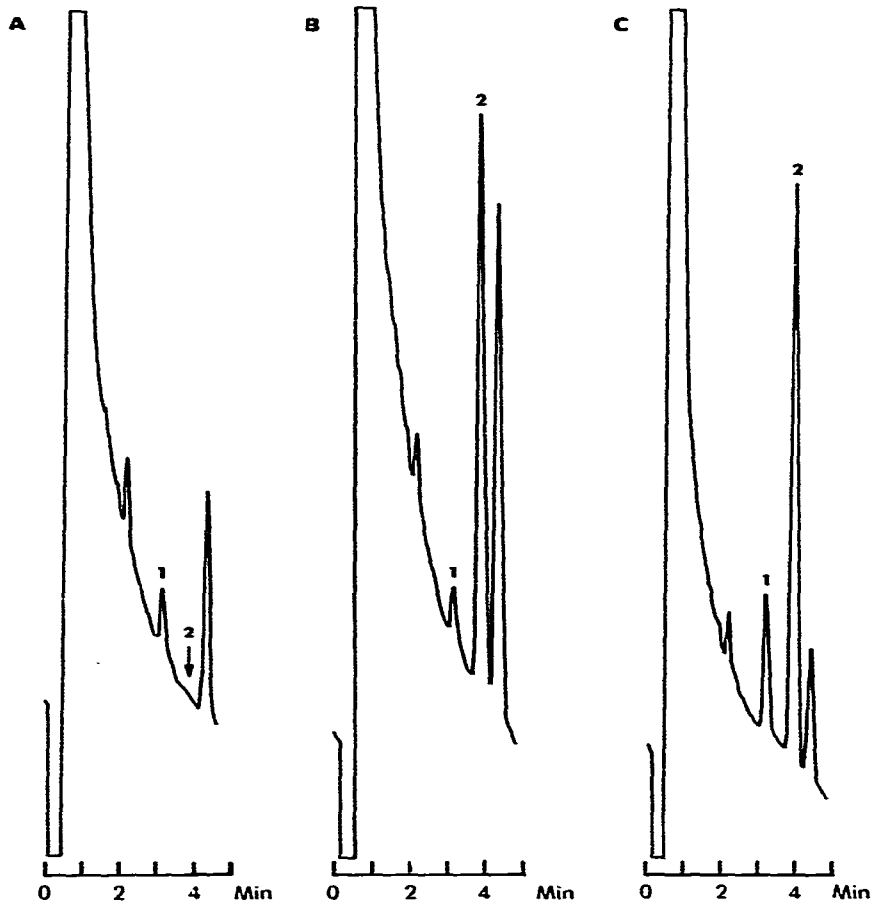


Fig. 2. Gas chromatograms of plasma extracts containing nicotine (1) and internal standard (2). (A) Plasma extract of nonsmoker; (B) extract of nonsmoker's plasma containing 20 ng/ml internal standard; (C) extract of plasma containing 5 ng/ml nicotine and 20 ng/ml internal standard.

cotinine analysis was carried out following the extraction of nicotine.) The samples were extracted with 2.5 ml 1-butanol by vortex mixing for 2 min, then cooled in a dry ice—acetone bath and centrifuged to break up emulsions. The butanol layers were transferred to tubes containing 0.5 ml 1 *N* sulfuric acid and vortex mixed for 1 min. After centrifuging to facilitate separation of layers, the butanol layers were separated and discarded. Sodium hydroxide (0.5 ml of 2 *N*) was added to the aqueous layers, which were then extracted with 1-ml aliquots of methylene chloride by vortex mixing for 1 min. The methylene chloride layers were transferred to small conical vials and evaporated under a current of nitrogen. Ethyl acetate (50 μl) and a small amount of anhydrous potassium carbonate were added; the tube was agitated for a few seconds on the vortex mixer, and an aliquot (2—5 μl) of the extract was analyzed by gas chromatography on a 1.2 m × 2 mm I.D. Carbowax—KOH column at 210°C. Standard curves were linear over the entire concentration range studied, 0—1000 ng/ml. Typical chromatograms are reproduced in Fig. 3. Calculations were performed as described for nicotine.

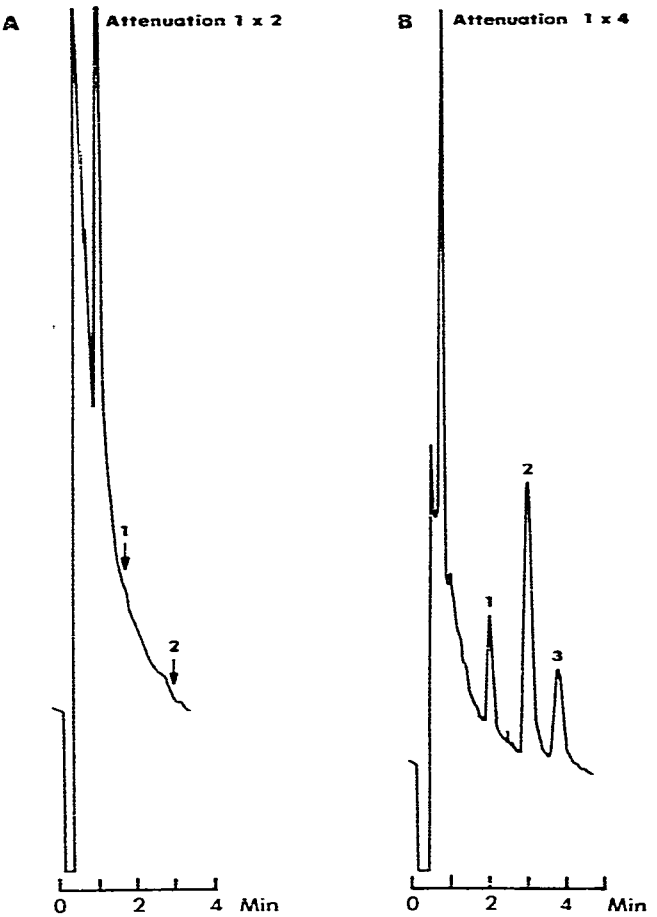


Fig. 3. Gas chromatograms of extracts containing cotinine (1) and internal standard (2). (A) Extract of control monkey blood; (B) extract of nonsmoker's plasma spiked with 5 ng/ml cotinine and 20 ng/ml internal standard. Peak 3 is caffeine.

Evaluation of extraction solvents for cotinine and internal standards

Stock solutions for all compounds listed in Table I were prepared in 1 *N* NaOH at concentrations for measuring UV absorbance [25 μg/ml and 261 nm for cotinine, N-(1-propyl)-norcotinine, and N-(2-methoxyethyl)-norcotinine; 200 μg/ml and 254 nm for lidocaine]. Aliquots of stock solutions (3 ml) were extracted with 3 ml of the appropriate solvent by vortex mixing for 1 min. The aqueous phase was separated, and the absorbance was measured using a Zeiss Model PMQ-II spectrophotometer. Concentrations were determined using a standard curve of each substance in 1 *N* NaOH, and were used to calculate the percent extraction into the organic solvents listed in Table I.

TABLE I

PERCENT EXTRACTION OF COTININE AND INTERNAL STANDARDS FROM AQUEOUS SOLUTION USING VARIOUS SOLVENTS

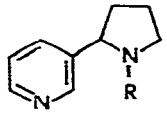
Solvent	Cotinine (%)	Lidocaine (%)	N-Propyl-norcotinine (%)	N-Methoxyethyl-norcotinine (%)
Heptane	4	93	7	3
Toluene	13			
Diethyl ether	8	>95	34	11
Ethyl acetate	33	>95	68	40
1-Octanol	80			
Methylene chloride	88	>95	92	93
1-Butanol	85		91	85
Chloroform	92			

RESULTS AND DISCUSSION

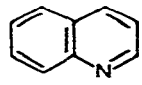
Nicotine analysis

Initially, we attempted to use previously described methods for analysis of nicotine using quinoline as an internal standard [7, 10, 12]. However, considerable variation occurred among identical samples, and occasionally we observed large variations in peak height ratios of nicotine to quinoline for the same ether extract injected repeatedly into the gas chromatograph. For example, repeated injection of one ether extract gave peak height ratios ranging from 1.6 to 5.5. It is likely that the variability was due to different relative losses of nicotine and quinoline during sample work-up and/or during gas chromatography. This problem might have been due to a poor choice of internal standard. Nicotine (pK_a 8.0) is considerably more basic than quinoline (pK_a 4.9). If an acidic site were present on glassware or in the gas chromatograph, a selective loss of nicotine would be expected, leading to variable results. (Subsequent to the development of our nicotine assay, a report appeared which described similar difficulties in the analysis of nicotine using quinoline as an internal standard [18].) Consequently, we have synthesized a new internal standard, N-ethylnornicotine (Fig. 4), a structural analog with chemical properties similar to nicotine.

Experiments have demonstrated that N-ethylnornicotine is a highly satisfac-

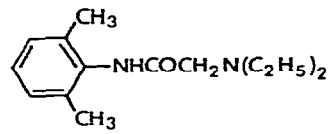


Nicotine, R=CH_3
N-Ethylnornicotine, R=C_2H_5



Quinoline

Cotinine, R=CH_3
N-n-Propylnorcotinine, R=n-C_3H_7
N-(2-Methoxyethyl)-norcotinine, R=$CH_2CH_2OCH_3$



Lidocaine

Fig. 4. Structures of nicotine, cotinine, and internal standards.

TABLE II

DAY-TO-DAY VARIATION OF IDENTICAL PLASMA SAMPLES

Results are mean of determinations performed on 6 different days.

Concentration (ng/ml)	Nicotine				Cotinine				
Given	5	10	20	50	5	20	50	150	600
Internal standard	20	20	20	20	20	20	20	150	150
Found, mean	5.3	9.9	19.3	52.2	4.78	19.4	49.4	147.5	598.7
Standard deviation	0.26	0.52	0.81	1.92	0.20	0.80	0.63	3.84	9.94
Coefficient of variation	4.9	5.3	4.2	3.7	4.2	4.1	1.3	2.6	1.7

tory internal standard for the gas chromatographic determination of nicotine. (The authors cited in ref. 18 found that a structural analog of nicotine, N-(1-propyl)-nornicotine, was a more satisfactory internal standard than quinoline, although they did not describe an assay procedure for biological samples.) Sharp peaks and baseline separation of nicotine from N-ethylnornicotine were obtained on both Carbowax—KOH (Fig. 2) and SP-2250 DB columns. Good reproducibility (Table II) was obtained under the same conditions that gave poor reproducibility using quinoline as an internal standard. A simple extraction scheme (Fig. 1) using diethyl ether as the extracting solvent was employed. To minimize contamination from environmental nicotine, extractions were carried out under a laminar flow hood located in a laboratory in which smoking was prohibited. All solvents had to be purified to minimize contamination by nicotine and other substances.

Cotinine analysis

Cotinine, the major metabolite of nicotine in humans, is of interest because of the relatively high concentrations found in the blood of smokers [19, 20] and its pharmacological activity [21—23]. Methods for cotinine determination

in plasma and urine specimens have been reported [6, 8, 11]. We have improved upon the published methods in two ways. Our extraction procedure (Fig. 1), which utilizes 1-butanol instead of methylene chloride [6, 11] to extract cotinine from blood or plasma, results in less emulsion formation and cleaner separation of layers. Furthermore, we have synthesized an internal standard, N-(2-methoxyethyl)-norcotinine (Fig. 4), which is a structural analog with solvent partitioning properties (Table I) and pK_a value similar to cotinine. Lidocaine, the internal standard utilized in published methods, is considerably more lipophilic and more basic than cotinine and, therefore, is a poor choice for an internal standard. Our internal standard may be added *prior* to the nicotine extraction procedure (unlike the published methods in which lidocaine must be added *after* extraction of nicotine) which corrects for losses of cotinine during nicotine extraction.

CONCLUSION

In summary, new methods have been developed for the determination of nicotine and cotinine concentrations in blood using gas chromatography with alkali flame ionization (nitrogen—phosphorus) detection. Simple solvent extraction procedures permit the use of the assays for large numbers of samples required for pharmacokinetic studies. Structural analogs were synthesized for use as internal standards and have resulted in assays that appear to be more reliable than those previously reported.

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