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DETERMINATION OF NORNICOTINE IN SMOKERS' URINE BY GAS CHROMATOGRAPHY FOLLOWING REDUCTIVE ALKYLATION TO N'-PROPYLNORNICOTINE

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SUMMARY

A sensitive gas chromatographic assay has been developed to measure concentrations of nornicotine in human urine. A structural analogue, 5-methylnornicotine, is used as an internal standard. Both nornicotine and the internal standard were converted to the corresponding N'-propyl derivatives using a novel reductive alkylation procedure The N'-propyl derivatives have good chromatographic properties, which allows quantitative measurement in the low nanograms per milliliter range. Concentrations of nornicotine in smokers' urine were quantitated and compared with the concentrations of nicotine and its metabolites, cotinine, nicotine-N-oxide and cotinine-N-oxide.

INTRODUCTION

Nornicotine is a major tobacco alkaloid [1] and may also be a metabolite of nicotine [2]. Nornicotine comprises 15–20% of the total alkaloids in cigarette tobacco [3] and has pharmacological [4,5] and toxicological [3] actions similar to those of nicotine. However, very little data on concentrations of nornicotine in biological fluids of tobacco users have been reported. Some reports have indicated in vivo and in vitro formation of nornicotine from nicotine [6–9], but there is still some controversy as to whether nornicotine is truly a metabolite of nicotine in humans [10,11]. To investigate the pharmacokinetics and metabolic origin of nornicotine in humans, a rapid and sensitive assay was

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needed. Radiolabeled nornicotine has been measured by high-performance liquid chromatography for studies in guinea pigs [12] and rats [13]. Measurements of nornicotine in urine by gas chromatography (GC) has been referred to in a recent publication, but details of the method were not presented [14]. This paper describes a novel method for determination of nornicotine in human urine based on the conversion to an N′-alkyl derivative, which can then be quantitated by GC.

EXPERIMENTAL

Instrumentation

GC analyses were carried out using a Hewlett-Packard Model 5890A chromatograph with a nitrogen–phosphorus detector, a Hewlett-Packard Model 3393 integrator, Model 7673A automatic liquid sampler and a split–splitless capillary injection port. A 25 m×0.31 mm cross-linked 5% phenylmethylsilicone, 0.52 m film thickness, fused-silica column (Hewlett-Packard) was used for the determination. The carrier gas (helium) flow-rate was 2 ml/min; detector air and hydrogen flow-rates were 50 and 5 ml/min, respectively. The nitrogen–phosphorus detector bead voltage was adjusted to give a background current of 12–20 pA.

A Hewlett-Packard 5890A gas chromatograph with a capillary direct interface to a Hewlett-Packard 5970B quadrupole mass-selective detector was used for the gas chromatographic–mass spectrometric (GC–MS) analyses. A 12 m×0.21 mm I.D. fused-silica capillary column coated with cross-linked 5% phenylmethylsilicone, 0.33 μm film thickness, was used with helium carrier gas at a flow-rate of 1 ml/min. Injections of 1 μl were made in the splitless mode at an injection port temperature of 250°C. The mass spectrometer was calibrated with perfluorotributylamine at masses of 69, 219 and 502 using the Hewlett-Packard software Autotune program. Ionization was carried out by electron impact at 70 eV. Spectra were obtained scanning the mass range 35–250 a.m.u. at a scan rate of 400 a.m.u./s.

Chemicals and reagents

All solvents and chemicals were reagent grade. Nornicotine, 5-methylnornicotine and N′-propylnornicotine were synthesized as described below. Microanalyses were performed by Galbraith Labs. (Knoxville, TN, U.S.A.).

Synthesis of (±)-nornicotine di-(±)-camsylate

To a solution of 2.5 g myosmine [15] in 50 ml of methanol–acetic acid (80:20) cooled in a dry ice–acetone bath was added 0.75 g of sodium borohydride portionwise over 5 min. After evaporating the solvents, the mixture was made basic with dilute sodium hydroxide and extracted with two 50-ml portions of methylene chloride. The methylene chloride was evaporated with a rotary

evaporator and the residue was distilled bulb-to-bulb (Kugelrohr oven, 85–100°C, 0.1 mmHg), giving 2.2 g (87% yield) of colorless liquid. To the nornicotine base (15 mmol) in 20 ml of methanol were added 7.5 g (30 mmol) racemic camphor-10-sulfuric acid (Aldrich, Milwaukee, WI, U.S.A.) in 50 ml of methanol. Evaporation of the methanol yielded a waxy solid. The crude product was purified by dissolving in 50 ml warm isopropyl alcohol, and then cooling in ice until crystallization began. The cold mixture was diluted with 200 ml of anhydrous diethyl ether added portionwise with stirring over 15 min. The product was collected by vacuum filtration, washed with 50 ml of ether–isopropyl alcohol (90:10) and dried under vacuum, yielding 8.3 g (91.2%) of white crystalline powder, m.p. 195–197°C. Anal. calcd. for $C_{29}H_{44}N_2O_8S_2$: C, 56.84; H, 7.24; N, 4.57; S, 10.46. Found, C, 56.80; H, 7.05; N, 4.64; S, 10.60.

Synthesis of 5-methylnornicotine bis(picrate)

To a solution of 0.25 g of 5-methylmyosmine [16] in 25 ml of water (pH 2) was added 0.08 g of sodium cyanoborohydride. The solution was stirred for 30 min, during which the pH of the solution was kept between 1.8 and 2.5 by periodic addition of a few drops of concentrated hydrochloric acid. Additional sodium cyanoborohydride (0.08 g) was added, and the solution was stirred for 10 min after which the pH was lowered to 1.5. The solution was made basic with sodium hydroxide and extracted twice with 50-ml portions of methylene chloride. The combined organic phase was back-extracted with 1 *M* sulfuric acid (2×50 ml). The acidic aqueous phase containing the product was made basic with sodium hydroxide and then extracted twice with 50-ml portions of methylene chloride. Evaporation of the methylene chloride using a rotary evaporator followed by bulb-to-bulb distillation (Kugelrohr oven, 110–120°C, 0.1 mmHg) provided 100 mg (40% yield) of colorless liquid. The picrate was prepared by combining 50 mg of the base with excess of picric acid in ethanol, which led to immediate precipitation of the product. Filtering the yellow precipitate followed by recrystallization from 80% aqueous ethanol provided lustrous yellow plates, m.p. 174–175°C. Anal. calcd. for $C_{22}H_{20}N_8O_{14}$: C, 42.59; H, 3.25; N, 18.06. Found: C, 42.34; H, 3.41; N, 17.89.

Synthesis of N′-propylnornicotine

To 0.5 g nornicotine base in 10 ml of methanol were added 4 ml of propionaldehyde and 8 ml of acetic acid. While stirring, 1.5 g of sodium borohydride were added portionwise over 20 min, followed by stirring for another 20 min. After evaporating most of the solvent using a rotary evaporator, the mixture was made basic with aqueous ammonia and extracted with two 20-ml portions of methylene chloride. The combined methylene chloride layer was evaporated, made acidic with dilute sulfuric acid and washed twice with 25 ml methylene chloride. The aqueous layer was made basic with ammonia and extracted twice with 25-ml portions of methylene chloride. Evaporation of the methylene

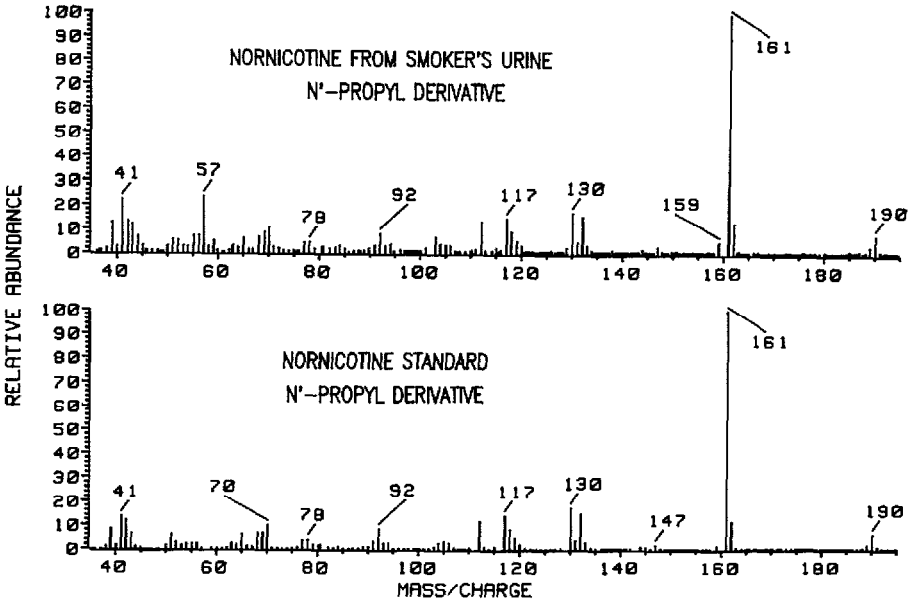

Fig 1 Electron-impact mass spectra of the propyl derivative of nornicotine extracted from a smoker's urine and N'-propylnornicotine standard.

chloride followed by bulb-to-bulb distillation (Kugelrohr oven, 70–80°C, 0.1 mmHg) provided 0.45 g (70% yield) of light yellow liquid. Thin-layer chromatography (TLC) and GC–MS analysis indicated that the product was pure N'-propylnornicotine (Fig. 1).

Standards and controls

(±)-Nornicotine di-(±)-camsylate was dissolved in water to make a stock solution containing 1.0 mg/ml nornicotine base. The stock solution was diluted with water to prepare aqueous working standards or to spike non-smokers' urine to concentrations spanning the range 2–1500 ng/ml.

Sample preparation and gas chromatographic determination

Urine samples were acidified to a pH of 3 with hydrochloric acid and frozen until analysis. To 1-ml urine samples, standards or controls in 100 mm×13 mm Pyrex culture tubes were added 100 μl of internal standard (2 μg/ml aqueous 5-methylnornicotine). A 0.3-ml volume of 30% sodium hydroxide and 3 ml of methyl *tert.*-butyl ether–isopropyl alcohol (90:10) were added, and the tubes were vortexed for 5 min and then centrifuged. The phases were separated by placing the tubes in a dry ice–acetone bath to freeze the aqueous layer, and then pouring the organic phase into a new tube. To the organic phases was added 2% concentrated hydrochloric acid in methanol (50 μl) and the solvent

was evaporated to dryness at room temperature with a current of nitrogen. The residues were dissolved in 1 ml of isopropyl alcohol–acetic acid–propionaldehyde (95:11:5). While vortex-mixing the open tubes in a multi-tube mixer (Kraft), two 100-μl portions of 2 *M* sodium borohydride in 0.5 *M* sodium hydroxide were added. Mixing was continued for 5 min. A 1-ml volume of 0.5 *M* sulfuric acid and 2.5 ml of toluene–butanol (70:30) were added to the tubes which were vortex-mixed and centrifuged. The aqueous layers were frozen in a dry ice–acetone bath, and the organic layers were discarded. The aqueous layers were washed with 2.5 ml of toluene–butanol (70:30). Aqueous 2 *M* sodium hydroxide (0.5 ml) and toluene–butanol (90:10, 0.3 ml) were added, and the tubes were vortex-mixed, centrifuged and frozen in dry ice–acetone bath. The organic layers were poured into autosampler vials for GC analysis. Injections (1 μl) were made in the splitless mode, and the oven temperature was programmed from 90 to 180°C at 35°C/min, from 180 to 220°C at 10°C/min and from 220 to 275°C at 30°C/min after a 0.5-min initial hold. Retention times for the N′-propyl derivatives of nornicotine and internal standard were 5.25 and 6.28 min, respectively (Fig. 2). The computing integrator was calibrated on a 100 ng/ml standard using the internal standard method and peak-area ratios. Standard curves were linear from 2 to 1500 ng/ml; $y = -0.4285 + 194.0x$, $r = 1.00$ for aqueous standards; $y = 0.8926 + 197.4x$, $r = 1.00$ for spiked urine standards.

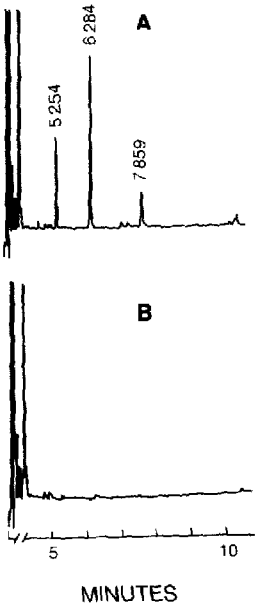

Fig. 2. Chromatograms of urine extracts. (A) Smoker, (B) non-smoker. Retention times: N′-propyl derivative of nornicotine, 5.254 min; internal standard, 6.254 min; cotinine, 7.859 min.

Overall recovery

Non-smokers' urine was spiked with nornicotine to a concentration of 1 μg/ml and analyzed as described above. The peak area of the derivative was compared to the area of synthetic N′-propylnornicotine diluted to the same concentration. In three replicate determinations, the yields ranged from 92 to 96%.

GC–MS confirmation of nornicotine in a smoker's urine

A smoker's urine specimen containing 240 ng/ml nornicotine, as determined by GC analysis, and 200 ng/ml 5-methylnornicotine was extracted and derivatized as described above. A 1-μl aliquot was analyzed by GC–MS. The GC separation was carried out with a temperature program of 70 to 225 °C at 25 °C/min after a 2.5-min initial hold. The mass spectrum of N′-propylnornicotine, which had a retention time of 8.29 min, displayed major ions at *m/z* 190 (6%, molecular ion), 161 (100%) and 147 (2%), which is in agreement with the mass spectral data from the synthetic N′-propylnornicotine described above (Fig. 1). The propyl derivative of the internal standard, 5-methylnornicotine, which had a retention time of 8.937 min, had a mass spectrum consistent with the structure of 5-methyl-N′-propylnornicotine: *m/z* 204 (6%, molecular ion), 175 (100%), 161 (2%) and 146 (15%). The base peaks in the mass spectra of the N′-propyl derivatives (*m/z* 161 and 175, respectively) presumably result from loss of an ethyl radical giving relatively stable iminium species.

RESULTS AND DISCUSSION

Initially, we attempted GC determination of nornicotine without derivatization, but poor peak symmetry and strong adsorption on both packed and capillary columns led to inadequate sensitivity and erratic results. In trace analyses, primary and secondary amines are frequently converted to acyl derivatives to improve chromatographic properties. We prepared pentafluoropropionyl and heptafluorobutyryl derivatives of nornicotine, which had good chromatographic properties but, unfortunately, urinary constituents interfered with the analysis at the relatively low nornicotine concentrations found in smokers' urine.

Due to success in GC determination of nicotine in a variety of biological matrices and concentrations as low as 1 ng/ml [17], we anticipated that N′-alkyl derivatives of nornicotine would be more suitable than acyl derivatives for nornicotine determination. The N′-alkyl derivatives of nornicotine are stable, have good chromatographic properties and can be carried through acid–base partitioning steps during sample clean-up. Secondary amines can be converted to tertiary amines by alkylation with an alkyl halide or by reductive alkylation with carbonyl compounds. The reductive alkylation would be expected to be more suitable for an analytical derivatization, since reaction with

an alkyl halide could yield non-volatile quaternary compounds as well as the desired tertiary amine.

We have found that nornicotine is readily converted to primary N′-alkyl derivatives by reaction with an aldehyde and sodium borohydride. The reaction, which is carried out by adding an aqueous solution of sodium borohydride to nornicotine and an excess of the aldehyde in isopropyl alcohol–acetic acid, is rapid and is highly suitable as an analytical derivatization reaction. Using this method, nanogram quantities of nornicotine extracted from urine have been converted to N′-alkyl derivatives ranging in size from ethyl to pentyl. The reaction appears to be quite general, and in principle should be applicable for conversion of a variety of primary and secondary amines to tertiary amine derivatives on an analytical scale. We also tried the reaction with ketones (acetone, 3-pentanone), but in these cases alkylation was incomplete, although with acetone we did observe significant conversion to N′-isopropylnornicotine.

When applied to urine samples, both propionaldehyde and butyraldehyde gave good results in terms of complete reaction and absence of interfering substances derived from urine. Propionaldehyde was chosen for the standard procedure, since vapors of butyraldehyde were found to be irritating to the eyes of some of our laboratory staff. For the analysis of biological samples, we used an internal standard, 5-methylnornicotine (Fig. 3), which is a structural analogue of nornicotine that undergoes an analogous reductive alkylation. The propyl derivatives of both nornicotine and the internal standard gave sharp, symmetrical peaks that were resolved from other substances extracted from urine (Fig. 2).

The extraction procedure is straightforward and can be readily used for extraction of samples in batches for metabolic studies. Conversion to the propyl derivatives is rapid, complete in less than 5 min. Recovery of nornicotine for extraction from urine and derivatization was >90%. Recovery from urine was virtually identical to recovery from aqueous solution, which allowed use of aqueous standards to construct calibration curves, which were linear from 2 to 1500 ng/ml. Precision and accuracy of the method were good. Within-run and between-run coefficients of variation (C.V.) were 0.9–4.0 and 1.1–4.6%, respectively, at concentrations found in smokers' urine. Accuracy (analytical

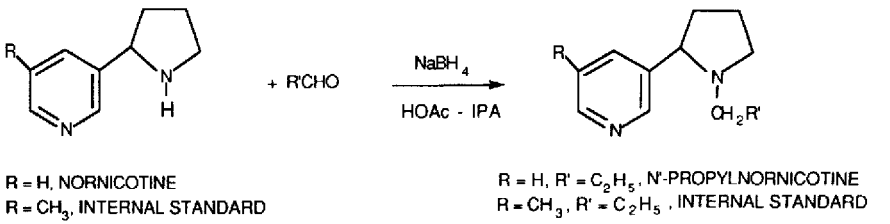

Fig. 3. Conversion of nornicotine and internal standards to N′-alkyl derivatives.

TABLE I

PRECISION AND ACCURACY OF THE METHOD FOR SPIKED URINE SAMPLES

Concentration (ng/ml)	Accuracy (%)	C V. (%)
Within-run (n=6)		
20	105.60	4.0
100	106.07	1.5
500	99.79	0.9
Between run (n=6)		
20	96.00	4.3
100	100.80	4.6
500	101.31	1.1

TABLE II

URINARY EXCRETION AND CONCENTRATIONS OF NORNICOTINE AND OTHER NICOTINE METABOLITES IN 25 CIGARETTE SMOKERS

Based on 24-h urine collections during ad libitum cigarette smoking.

Compound	Excretion (μg per 24 h)			Concentration (ng/ml)		
	Mean	Range	S D.	Mean	Range	S.D.
Nornicotine	157	12-361	100	85	6-207	62
Nicotine[a]	2618	212-5903	1882	1270	134-3132	919
Nicotine-1′-N-oxide[a]	1080	135-3192	687	574	63-1319	362
Cotinine[a]	2842	440-6196	1512	1368	200-2873	740
Cotinine-N-oxide[b]	703	45-1718	392	370	25-820	210

[a]Ref. 16.
[b]Ref. 18.

recovery) ranged from 96 to 106% (Table I). The limit of detection was about 2 ng/ml, with 3:1 signal-to-noise ratio.

Using this method, we measured concentrations of nornicotine in urine of 25 cigarette smokers. Concentrations ranged from 6 to 207 ng/ml, with a mean (±S.D.) of 85±62 ng/ml. Urinary excretion of nornicotine ranged from 12 to 361 μg and averaged 151 μg per 24 h. Concentrations and excretion rates are compared with excretion of nicotine and its metabolites (cotinine, nicotine-N′-oxide and cotinine-N-oxide) in Table II. Confirmation of the identity of nornicotine in a smoker's urine sample was obtained by GC–MS. Mass spectra of the propyl derivatives derived from urine and nornicotine standard were in good agreement (Fig. 1). Since nornicotine is present in tobacco, it is not known what percentage, if any, of the nornicotine present in smokers' urine is derived

from metabolic demethylation of nicotine. This question is currently being investigated in our laboratory using stable isotope-labeled nicotine administered intravenously to smokers.

In summary, a sensitive method for quantitation of nornicotine in smokers' urine has been developed. The method utilizes a novel reductive alkylation procedure to convert nornicotine to the N′-propyl derivative, which has excellent chromatographic properties. Analogous reductive alkylations should be useful for determination of a wide variety of primary and secondary amines.

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