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Gas chromatographic–mass spectrometric method for determination of anabasine, anatabine and other tobacco alkaloids in urine of smokers and smokeless tobacco users

Peyton Jacob III*, Lisa Yu, Gang Liang, Alexander T. Shulgin and Neal L. Benowitz

Division of Clinical Pharmacology and Departments of Medicine and Psychiatry, University of California, San Francisco, San Francisco General Hospital Medical Center, Building 100, Room 235, 1001 Potrero Avenue, San Francisco, CA 94110 (USA)

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ABSTRACT

A selected ion monitoring method for determination of the tobacco alkaloids anabasine, anatabine, nornicotine, metanicotine, dihydrometanicotine, and 2,3′-bipyridyl in urine of smokers and smokeless tobacco users is described. The method involves conversion of the secondary amine alkaloids to tertiary amine derivatives by reductive alkylation using an aldehyde and sodium borohydride, and chromatography on a 5% phenylmethylsilicone capillary column. These derivatives have good chromatographic properties, allowing determination of concentrations as low as 1 ng/ml. The alkaloid 2,3′-bipyridyl is unaffected by the derivatization procedure and may be determined simultaneously with the other alkaloids. The structural analogues 2-(3-pyridyl)hexahydroazepine, 5-methyldihydrometanicotine, and 6-methyl-2,3′-bipyridyl were synthesized for use as internal standards. Using the method, concentrations and 24 h excretion of anabasine, anatabine, and nornicotine in urine of twenty-two smokers, eight chewing tobacco users, and six oral snuff users were determined and compared with concentrations and excretion of nicotine and its metabolite cotinine. Excretion of nicotine and cotinine was similar in all tobacco users, but excretion of anabasine, anatabine, and nornicotine was substantially greater in urine of smokeless tobacco users, presumably due to absence of pyrolysis of these alkaloids in smokeless tobacco products.

INTRODUCTION

Tobacco and tobacco smoke contain a number of structurally related alkaloids (Fig. 1). In commercial tobaccos, the major alkaloid is nicotine, accounting for about 95% of the total alkaloid content. Nornicotine and anatabine are the two most abundant minor alkaloids, present in roughly equal amounts, each accounting for about 2–3% [1]. Anabasine is generally present in concentrations of about 0.3% of the total alkaloids. Anabasine is present in numerous *Nicotiana* as well as other families [1]. In some, notably *Nicotiana glauca*, anabasine is the principal alkaloid. Tobacco and tobacco smoke contain smaller amounts of 2,3′-bipyridyl, metanicotine (N-methyl-4-(3-pyridinyl)-3-buten-1-amine), dihydrometanicotine (3-[4-(methylamino)-1-butenyl]pyridine), and trace amounts of numerous other nitrogen-containing substances [2].

During the past several years, considerable information on the amount of nicotine absorbed by tobacco users has been obtained [3–5]. In contrast, nothing is known about the amounts of the other nicotine-related alkaloids absorbed during tobacco use. Nornicotine concentrations in urine of cigarette smokers has been reported [6,7] but, since it is also a nicotine metabolite, nornicotine in urine results both from direct absorption from

* Corresponding author.

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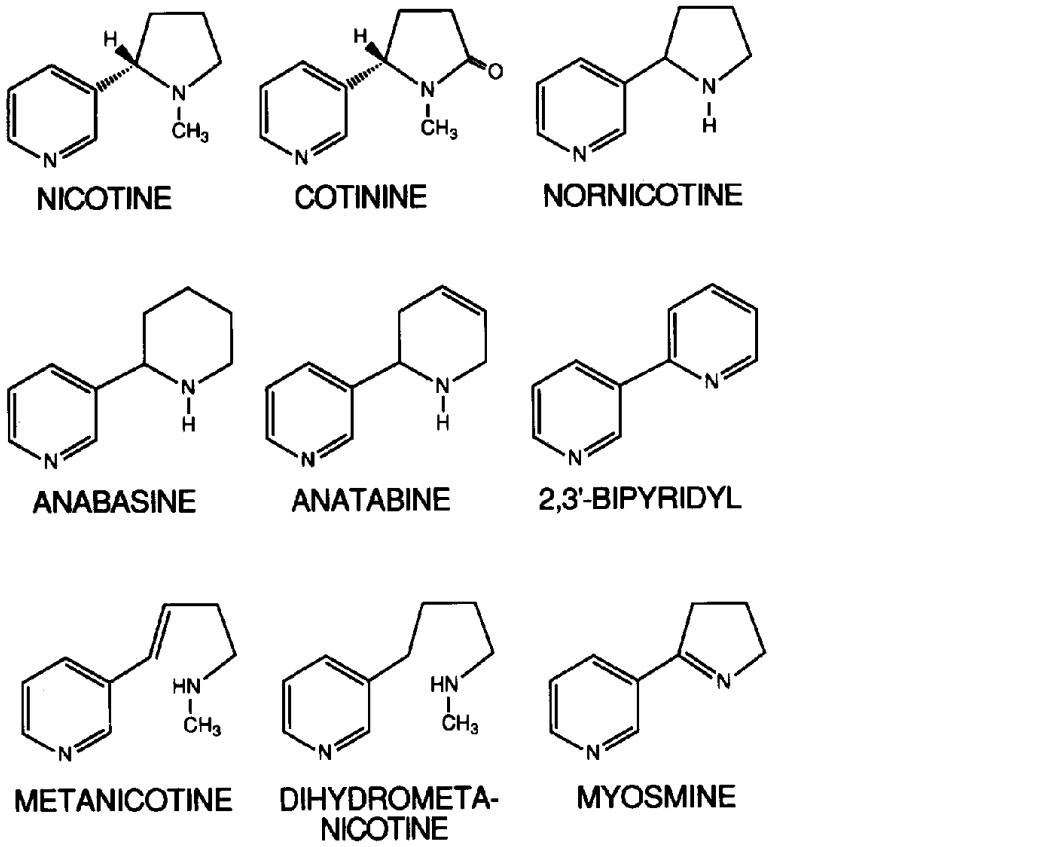

Fig. 1. Structures of tobacco alkaloids.

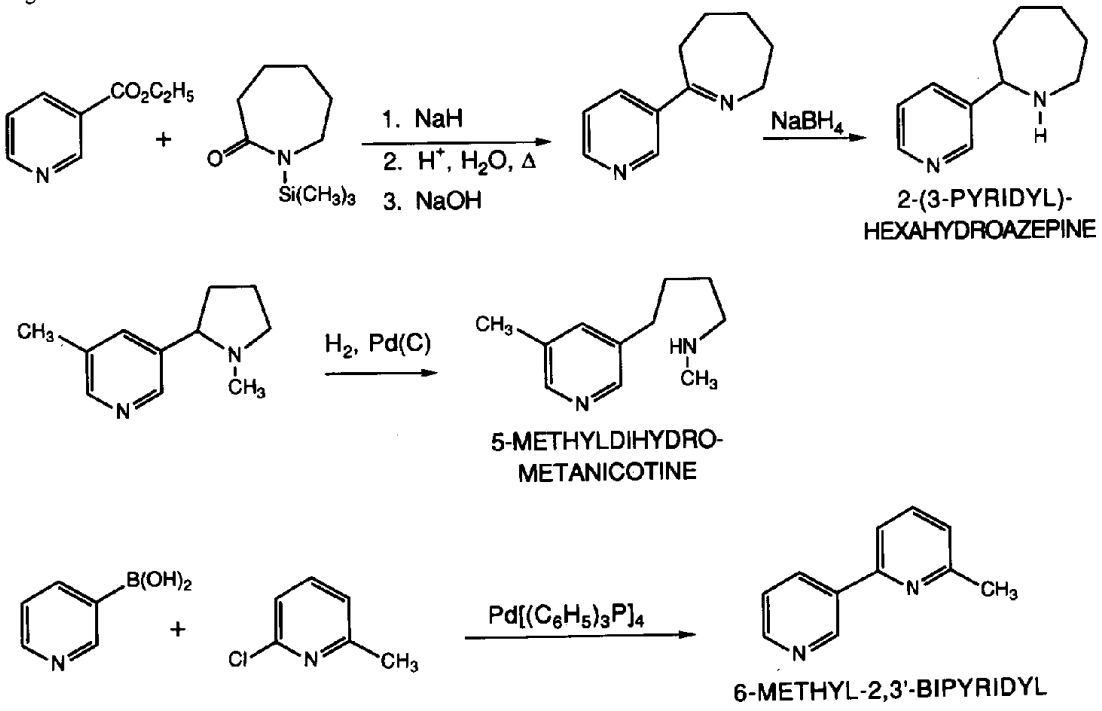

Fig. 2. Synthesis of internal standards.

tobacco and by metabolic demethylation of nicotine [8]. Anabasine concentrations in urine have been reported following oral administration of the alkaloid to human subjects [9], but to our knowledge nornicotine is the only minor tobacco alkaloid whose concentrations have been determined in biologic fluids of humans following tobacco use.

In order to assess human exposure to minor tobacco alkaloids, we required methods for determination of the alkaloids in biologic fluids of smokers and users of smokeless tobacco. This paper describes a selected ion monitoring method for determination of several minor tobacco alkaloids in urine and reports urinary excretion of the alkaloids in tobacco users.

EXPERIMENTAL

Materials

Solvents used in extractions were HPLC grade and inorganic chemicals were analytical reagent grade from Fisher Scientific (Pittsburgh, PA, USA), unless otherwise noted. The dipicrate salts of standards and internal standards were prepared by combining the base with a slight excess of picric acid (Aldrich, Milwaukee, WI, USA) in ethanol and recrystallizing the product from 80% aqueous ethanol. Nornicotine dipicrate was synthesized as previously described [10]. Anabasine (racemic) was obtained from Sigma (St. Louis, MO, USA) and converted to the dipicrate salt, fine yellow crystals, m.p. 214–215°C (lit. m.p. 214°C) [11]. Anatabine was synthesized by the method of Quan *et al.* [12] and converted to the dipicrate, yellow plates, m.p. 200–201°C (lit. m.p. 201–201.5°C) [13]. Metanicotine (N-methyl-4-(3-pyridinyl)-3-buten-1-amine; 3-[4-(methylamino)-1-butenyl]pyridine) was synthesized by the method of Späth and Bobengerger [14] and converted to the dipicrate, fluffy yellow crystals, m.p. 165–166°C (lit. m.p. 164°C). Dihydrometanicotine (N-methyl-3-pyridinebutanamine; 3-[4-(methylamino)butyl]pyridine) was synthesized by hydrogenation of nicotine according to the method of Hromatka [15]; the picrate was obtained as fine yellow needles, m.p. 165–166°C (lit. m.p. 167°C). 2,3′-Bipyridyl was obtained from Aldrich and converted to the dipicrate, m.p. 163.5–165°C (lit. m.p. 163–165°) [16]. The internal standards, 2-(3-pyridyl)azepine, 5-methyldihydrometanicotine, and 6-methyl-2,3′-dipyridyl, were synthesized as described below (Fig. 2).

Synthesis of 2-(3-pyridyl)hexahydroazepine dipicrate

N-Trimethylsilylcaprolactam. To a stirred suspension of 56 g (500 mmol) of ε-caprolactam (Aldrich) in 500 ml of anhydrous diethyl ether were added 64 ml (54 g, 500 mmol) of chlorotrimethylsilane (Aldrich), followed by 70 ml (500 mmol) of triethylamine, portionwise. A heavy white precipitate formed. The mixture was heated under reflux for 2 h, cooled, and filtered through Celite. The precipitate was washed well with diethyl ether, and the filtrate was concentrated on a rotary evaporator and distilled (2.7 kPa) to give 45 g (49% yield) of the trimethylsilyl derivative as a colorless liquid, b.p. 125–128°C.

7-(3-Pyridyl)-3,4,5,6-tetrahydro-2H-azepine. To a suspension of sodium hydride [from 7.2 g of a 50% mineral oil dispersion (150 mmol), washed free of oil with petroleum ether] in 100 ml of toluene was added a solution of the above N-trimethylsilylcaprolactam (20 g, 108 mmol) and ethyl nicotinate (15 g, 100 mmol, Aldrich) in 50 ml of toluene. The mixture was heated cautiously, and an exothermic reaction commenced with gas evolution. The solution, which had turned yellow-brown, was heated under reflux for 1.5 h. After cooling to room temperature, 50 ml of water were added cautiously (gas evolution), followed by addition of hydrochloric acid to bring the pH of the aqueous phase to 1. The pH was then adjusted to 5 with ammonium hydroxide, the organic layer was separated, and the aqueous layer was extracted with two 100-ml portions of 3:2 chloroform–ethanol. The combined organic layers were evaporated using a rotary evaporator to give a dark oil. This was combined with 100 ml of concentrated hydrochloric acid and heated under reflux for 4 h. The mixture was cooled, brought to pH 9 with ammonium hydroxide, and extracted with two 50-ml portions of methylene chloride.

The extract was dried over anhydrous potassium carbonate, concentrated on the rotary evaporator, and short-path distilled to give 0.75 g (4.3%) of light yellow liquid, b.p. 88–90°C (0.0027 kPa).

2-(3-Pyridyl)hexahydroazepine dipicrate. A solution of the above tetrahydro-2*H*-azepine (0.1 g) in 5 ml of 80:20 methanol–acetic acid was cooled to −78°C with a dry ice–acetone bath. With vigorous stirring, 0.2 *g* of sodium borohydride (Aldrich) was added, and the mixture was stirred while allowing to warm to room temperature. After standing overnight, the mixture was poured into 100 ml of water, made basic with sodium hydroxide, and then extracted with two 50-ml portions of methylene chloride. The combined extracts were concentrated with a rotary evaporator and distilled bulb-to-bulb (Kugelrohr) to give a colorless oil, b.p. 110–120°C (0.0133 kPa). This was converted to the dipicrate by combining with 120 mg of picric acid in 5 ml of ethanol. Recrystallization of the resulting solids provided 94 mg of sparkling yellow plates, m.p. 184–185°C. Analysis Calculated for $C_{23}H_{22}N_8O_{14}$: C, 43.54; H, 3.50; N, 17.66; found: C, 43.45; H, 3.42; N, 17.68.

Synthesis of 5-methyldihydrometanicotine

Hydrogenolysis of 0.25 g of 5-methylnicotine [17] in 10 ml of water was carried out using 0.2 g of 5% palladium on charcoal catalyst (Matheson, Coleman and Bell, East Rutherford, NJ, USA) at 70°C for 16 h, analogous to the synthesis of dihydrometanicotine described by Hromatka [15]. The catalyst was removed by filtration through Celite, which was then washed with 10 ml of 10% ammonium hydroxide in methanol. The combined filtrate and wash was extracted with two 25-ml portions of methylene chloride, and the combined extracts were concentrated with a rotary evaporator. Bulb-to-bulb distillation (Kugelrohr) at 3.3 kPa provided 100 mg of a colorless liquid, b.p. 175–180°C. This was converted to the dipicrate, which was recrystallized to give 0.2 *g* of fine yellow crystals, m.p. 154–155°C. Analysis Calculated for $C_{23}H_{24}N_8O_{14}$: C, 43.40; H, 3.80; N, 17.61; found: C, 43.58; H, 3.75; N, 17.59.

Synthesis of 6-methyl-2,3'-bipyridyl

The method used by Ishikura *et al.* [16] for the synthesis of related compounds was used. To a solution of 1 g (6.8 mmol) of diethyl-3-pyridylborane (Aldrich) in 25 ml of dry tetrahydrofuran under argon were added 1.3 g (10.3 mmol) of 2-chloro-6-methylpyridine (Aldrich), 1.5 g (27 mmol) of powdered KOH, 1.1 g (3.4 mmol) of tetrabutylammonium bromide (Aldrich), and 0.3 g (0.3 mmol) tetrakis(triphenylphosphine)palladium[0] (Aldrich). The mixture was heated under reflux for 8 h, then cooled, poured into 100 ml of water, and extracted with two 50-ml portions of ethyl acetate. The combined extracts were back-extracted with two 50-ml portions of dilute sulfuric acid. The combined aqueous acid phases were made basic with sodium hydroxide, and extracted with two 50-ml portions of methylene chloride. The methylene chloride extracts were combined and concentrated to give an oil which was distilled bulb-to-bulb (Kugelrohr) to give 1.1 g of colorless liquid, b.p. 140–150°C at 4.7 kPa. An 0.3-g (2 mmol) portion of the base in 5 ml of 80% aqueous ethanol was combined with 1.1 g (5 mmol) of picric acid in 25 ml of boiling 80% aqueous ethanol. The solid that crystallized on cooling was recrystallized from 35 ml of 80% aqueous ethanol to give 0.8 g of fine yellow crystals. This was recrystallized twice from 80% aqueous ethanol to give 0.5 g, m.p. 181–183°C.

Phenylacetaldehyde derivatizing agent

Phenylacetaldehyde dimethylacetal, 1 ml (Aldrich), was added to 9 ml of an 8:1:1 (v/v/v) mixture of isopropyl alcohol–acetic acid–water. To this was added one drop of concentrated hydrochloric acid, HCl, and the solution was heated at 50°C for 1 h prior to use. The solution was made fresh daily.

Propionaldehyde derivatizing agent

Propionaldehyde, 1 ml (Aldrich 99%), was added to 9 ml of an 8:1:1 (v/v/v) mixture of isopropyl alcohol–acetic acid–water. The solution was made fresh daily.

Standards and controls

The dipicrate salts of the alkaloids were dis-

solved in 1% concentrated hydrochloric acid–methanol to make 100 μg/ml (as the bases) stock solutions. The stock solutions were diluted with 0.2 *M* aqueous hydrochloric acid and used to spike non-smokers' (confirmed by measurement of cotinine concentrations, which were less than 6 ng/ml) urine to concentrations spanning the range 0–500 ng/ml. Spiked urine samples were stable for at least four months stored frozen at −20°C.

Extraction procedure

Internal standards (100 μl of a solution of 5 μg/ml each of 2-(3-pyridyl)hexahydroazepine, 5-methyldihydrometanicotine, and 6-methyl-2,3'-bipyridyl) were added to 5-ml urine samples, standards, or controls in 100 × 16 mm glass culture tubes. To each tube was added 1 ml of 2 *M* sodium hydroxide, and the tubes were vortex-mixed briefly. The samples were added to C_{18} extraction columns (Fisher Prep-Sep, 300 mg absorbent, 12 ml capacity from Fisher Scientific) which had previously been conditioned by washing sequentially with 3 ml of methanol and 3 ml of distilled water. Vacuum was applied to the columns to draw the samples through in ca. 2 min. The columns were washed with 3 ml of distilled water, and the samples were eluted with 3 ml of methanol, drawn through the columns in ca. 2 min. To the eluates, in 100 × 16 mm culture tubes, were added 100 μl of a 10% solution of *p*-toluenesulfonic acid (Eastman Organic Chemicals, Rochester, NY, USA) in methanol, and the methanol was evaporated using a vortex vacuum evaporator (Haake-Buchler, Saddle Brook, NJ, USA) at about 60°C. To the residues was added 0.5 ml of the propionaldehyde derivatizing agent or phenylacetaldehyde derivatizing agent (see above) and, while vortex-mixing the open tubes on a multitube vortex mixer (Kraft Apparatus, Minneola, NY, USA), 100 μl of 2 *M* sodium borohydride (Sigma) in 0.5 *M* sodium hydroxide were added to each tube. While vortex-mixing, a second 100-μl aliquot of sodium borohydride solution was added to each tube, and then the tubes were let stand for 5 min. The derivatization procedure was carried out in a fume hood, since propionaldehyde is irritating to the eyes and mucous membranes. To each tube was added 1 ml of 2 *M* sodium hydroxide in 0.2 *M* aqueous ammonia and 3 ml of 70:30 (v/v) toluene–1-butanol. The tubes were vortex-mixed for 5 min, centrifuged at 2 500 *g* for 10 min to break the emulsions, and then placed in a dry ice–acetone bath to freeze the aqueous layers. The organic layers were poured into 100 × 13 mm culture tubes containing 0.5 ml of 1 *M* sulfuric acid. The tubes were vortex-mixed for 5 min, centrifuged at 2 500 *g* for 10 min, and placed in a dry ice–acetone bath to freeze the aqueous layers. The organic layers were poured off and discarded, and 0.5 ml of 50% potassium carbonate in 0.2 *M* aqueous ammonia and 0.12 ml of 90:10 toluene–1-butanol (v/v) were added to each tube. The tubes were vortex-mixed for 5 min, centrifuged at 2 500 *g* for 10 min, and placed in a dry ice–acetone bath to freeze the aqueous layers. The organic layers were poured into micro autosampler vials (300-μl conical Hewlett-Packard 5080-8779 or 200-μl 5181-1269) and evaporated to a volume of about 20–30 μl. An aliquot (2 μl) was injected into the GC–MS system.

GC–MS Analysis

GC–MS analyses were performed using electron ionization at 70 eV on a Hewlett-Packard (Hewlett-Packard, Avondale, PA, USA) 5890 GC with a capillary direct interface to an HP 5970 quadrupole mass-selective detector (Hewlett-Packard, Palo Alto, CA, USA). The injection port temperature was 250°C, and the transfer line temperature was 280°C. Prior to analyses, the mass spectrometer was calibrated with perfluorotributylamine (PFTBA) using the HP software Autotune program. Samples were injected in the splitless mode using an HP 7673A automatic sampler, into injection port liners that were deactivated as previously described [18]. The septum purge on-time was 0.8 min. An HP 12 m × 0.2 mm I.D. fused-silica capillary column was used, with a stationary phase of crosslinked 0.33 μm thickness 5% phenylmethylsilicone. Helium was used as the carrier gas at a flow rate of 1.0 ml/min at 70°C. The column oven temperature was pro-

TABLE I

TYPICAL RETENTION TIMES FOR TOBACCO ALKALOIDS AND DERIVATIVES

Alkaloid	Retention time (min)	
	Propyl derivative	Phenethyl derivative
Nornicotine	8.66	12.08
Anabasine	9.34	12.57
Anatabine	9.65	12.86
Dihydrometanicotine	9.63	12.80
Metanicotine	10.10	13.16
2,3′-Bipyridyl	8.89[a]	
2-(3-Pyridyl)hexahydroazepine (internal standard)	10.42	13.51
5-Methyldihydrometanicotine (internal standard)	10.40	13.38
6-Methyl-2,3′-bipyridyl (internal standard)	9.38[a]	

[a] Not converted to a derivative.

grammed from 70°C (after a 2.3-min hold) to 250°C at 17°C/min.

Analyses were carried out by monitoring the following ions: *m/z* 156 (2,3′-bipyridyl), *m/z* 161 (nornicotine derivative), *m/z* 173 (anatabine derivative), *m/z* 175 (anabasine derivative and metanicotine derivative), *m/z* 177 (dihydrometanicotine derivative), *m/z* 170 (6-methyl-2,3′-bipyridyl, internal standard), *m/z* 189 (2-(3-pyridyl)-azepine derivative, internal standard), and *m/z* 191 (5-methyldihydrometanicotine derivative, internal standard). Typical retention times are given in Table I. A microcomputer-based workstation, HP 59970, was used for controlling the instrument and data reduction.

Calibration procedure

Quantification was achieved by integration of

TABLE II

EQUATIONS FOR STANDARD CURVES

Equations were determined by linear regression: response ratio = slope × amount ratio + intercept.

Analyte	Derivative	Slope	Intercept	Correlation coefficient[a] (r^2)
Anabasine	Propyl	0.309	−0.00383	0.998
	Phenethyl	0.117	−0.00159	0.992
Anatabine	Propyl	0.482	0.000007	0.999
	Phenethyl	0.286	−0.00299	0.998
Nornicotine	Propyl	1.16	0.00416	0.997
	Phenethyl	1.13	−0.000839	1.000
Metanicotine	Propyl	0.0383	0.0125	0.909
	Phenethyl	0.263	0.00496	0.997
Dihydrometanicotine	Propyl	1.20	−0.00246	0.998
	Phenethyl	2.05	−0.0103	0.991
2,3′-Bipyridyl[b]		0.705	−0.00579	0.999
2,3′-Bipyridyl[c]		0.958	−0.00384	0.999

[a] Calculated to three significant figures.

[b] Determined simultaneously with other analytes converted to propyl derivatives.

[c] Determined simultaneously with other analytes converted to phenethyl derivatives.

the ion chromatograms and constructing standard curves of peak-area ratio of analyte/internal standard *versus* concentration by linear regression. Standard curves were obtained by analysis of non-smokers' urine spiked with known amounts of the alkaloids to achieve final concentrations of 1, 2, 5, 10, 50, and 100 ng/ml. Standard curves were linear over the entire concentration range (Table II).

Experimental subjects

Paid volunteers were maintained on a research ward and allowed to smoke or use smokeless tobacco *ad libitum*. Urine samples were collected during two successive 24-h periods. The samples were acidified to pH 2–3 and immediately frozen.

RESULTS AND DISCUSSION

Recently we reported a gas chromatographic method for determination of nornicotine in smokers' urine [6]. The method involves reductive alkylation of nornicotine to the N'-propyl derivative, which has much better chromatographic properties than the parent secondary amine, and which allows determination of low nanogram per milliliter concentrations. Since the most abundant of the minor tobacco alkaloids are secondary amines closely related to nornicotine, we anticipated that the reductive alkylation procedure would be applicable to these alkaloids as well.

Five secondary amine alkaloids of interest to us (nornicotine, anabasine, anatabine, metanicotine, and dihydrometanicotine) underwent clean reductive alkylation with propionaldehyde and sodium borohydride (Fig. 3). We had previously found that electron-ionization mass spectrometry of N'-propylnornicotine produced as the major ion *m/z* 161, which results from loss of an ethyl radical from the propyl group. We anticipated that the propyl derivatives of the other alkaloids would produce analogous ions (Fig. 3) which would be suitable for quantitation by selected ion monitoring. The derivatives of anabasine and anatabine gave *m/z* 175 and 173, respectively, resulting from loss of an ethyl radical, as the ions of highest abundance (Table III). These ions proved

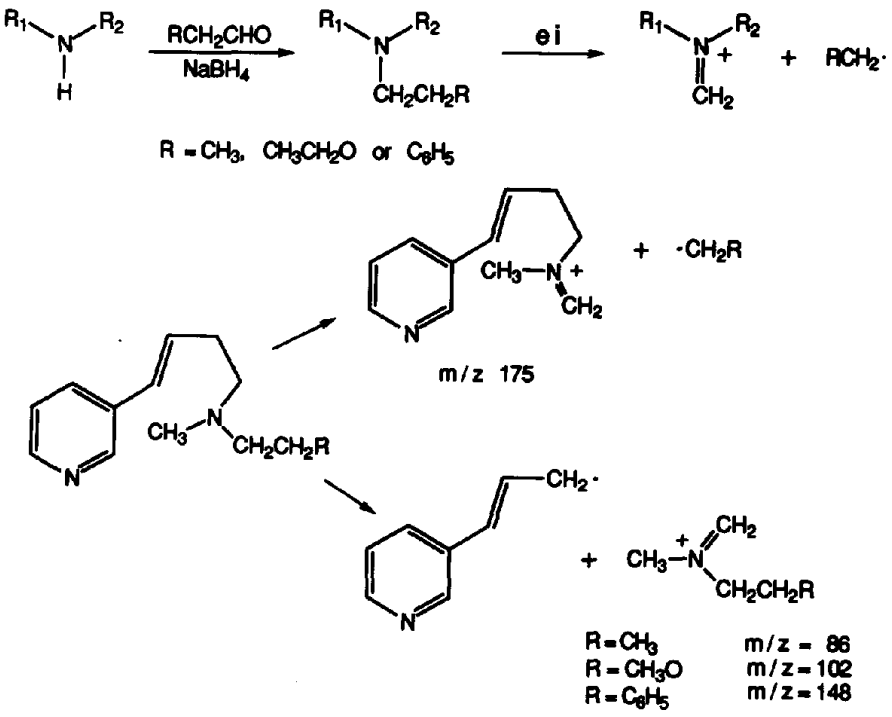

Fig. 3. Conversion of alkaloids to N'-alkyl derivatives and major ions formed on electron ionization.

TABLE III

MASS SPECTRAL DATA FOR TOBACCO ALKALOID DERIVATIVES

Molecular ions (M^+) and fragment ions of $m/z > 50$ with relative abundances of > 10% are presented.

Tobacco alkaloid derivative	Mass spectral data
Nornicotine propyl derivative	117 (12%), 130 (14%), 132 (13%), 161 (100%), 162 (11%), 190 (6%, M^+)
Nornicotine phenethyl derivative	117 (13%), 130 (14%), 132 (12%), 161 (100%), 162 (11%), 252 (< 1%, M^+)
Anatabine propyl derivative	92 (20%), 119 (31%), 144 (69%), 173 (100%), 174 (13%), 202 (15%, M^+)
Anatabine phenethyl derivative	102 (10%), 144 (34%), 173 (55%), 202 (13%), 230 (26%), 231 (100%), 232 (19%), 264 (< 1%, M^+)
Anabasine propyl derivative	92 (10%), 126 (15%), 132 (25%), 175 (100%), 176 (13%), 204 (6%, M^+)
Anabasine phenethyl derivative	65 (13%), 91 (17%), 92 (11%), 132 (28%), 175 (100%), 176 (13%), 266 (< 1%, M^+)
2-(3-Pyridyl)hexahydroazepine propyl derivative	92 (14%), 119 (10%), 161 (11%), 189 (100%), 190 (13%), 218 (9%, M^+)
2-(3-Pyridyl)hexahydroazepine phenethyl derivative	91 (12%), 92 (13%), 189 (100%), 190 (14%), 280 (< 1%, M^+)
Dihydrometanicotine propyl derivative	58 (39%), 65 (13%), 86 (100%), 92 (21%), 177 (39%), 206 (5.8%, M^+)
Dihydrometanicotine methoxyethyl derivative	57 (16%), 58 (100%), 59 (37%), 65 (30%), 92 (46%), 102 (26%), 134 (14%), 177 (64%), 222 (< 1%, M^+)
Dihydrometanicotine phenethyl derivative	51 (12%), 57 (14%), 58 (100%), 65 (37%), 77 (16%), 79 (11%), 91 (13%), 92 (43%), 134 (11%), 177 (66%), 268 (< 1%, M^+)
Metanicotine propyl derivative	58 (16%), 86 (100%), 175 (0.7%), 204 (< 1%, M^+)
Metanicotine methoxyethyl derivative	59 (58%), 72 (10%), 102 (100%), 175 (2%), 220 (< 1%, M^+)
Metanicotine phenethyl derivative	51 (18%), 57 (12%), 65 (23%), 77 (27%), 79 (21%), 91 (16%), 105 (100%), 117 (23%), 130 (17%), 132 (10%), 148 (90%), 175 (18%), 266 (< 1%, M^+)
5-Methyldihydrometanicotine propyl derivative	58 (39%), 77 (11%), 86 (100%), 106 (11%), 191 (14%), 220 (2.6%, M^+)
5-Methyldihydrometanicotine methoxyethyl derivative	53 (11%), 57 (16%), 58 (100%), 77 (23%), 79 (12%), 102 (33%), 106 (32%), 148 (17%), 191 (57%), 236 (< 1%, M^+)
5-Methyldihydrometanicotine phenethyl derivative	51 (10%), 57 (15%), 58 (100%), 65 (17%), 77 (33%), 79 (23%), 91 (16%), 105 (26%), 106 (38%), 148 (20%), 191 (83%), 192 (11%), 280 (< 1%, M^+)
2,3′-Bipyridyl	50 (14%), 51 (18%), 78 (14%), 128 (12%), 130 (21%), 155 (72%), 156 (100%, M^+), 157 (11%)
6-Methyl-2,3′-bipyridyl	130 (12%), 144 (15%), 169 (72%), 170 (100%, M^+), 171 (13%)

to be excellent for quantitation, providing high sensitivity. Electron ionization of the propyl derivatives of metanicotine and dihydrometanicotine also resulted in fragmentation by loss of an ethyl radical, producing ions of m/z 175 and 177, respectively, but these were minor fragmentation pathways (Table III). These derivatives underwent fragmentation primarily to m/z 86 which, due to its low mass, was more prone to interferences from other substances present in the extracts.

We synthesized three compounds structurally related to the analytes for use as internal standards (Fig. 2). The ring-expanded analogue of anabasine, 2-(3-pyridyl)hexahydroazepine, was used as the internal standard for anabasine and anatabine, 5-methyldihydrometanicotine was used as the internal standard for metanicotine and dihydrometanicotine, and 6-methyl-2,3′-bipyridyl was used as the internal standard for 2,3′-bipyridyl.

We evaluated both solid-phase and liquid–liquid extraction of the alkaloids from urine. Solid-phase extraction with C_{18}-modified silica gave better recoveries than extraction with mixtures of methyl *tert.*-butyl ether and isopropyl alcohol, which we had previously used for extraction of nornicotine [6], especially for anatabine and metanicotine. Following extraction, the columns were washed with water, and then eluted with methanol. The eluates were acidified with *p*-toluenesulfonic acid to convert the analytes to non-

volatile salts and then evaporated. The residues were dissolved in a solution containing isopropyl alcohol, acetic acid, water, and propionaldehyde and then treated with aqueous sodium borohydride to convert the analytes and internal standards to the N'-propyl derivatives. Following acid–base partitioning steps to clean up and concentrate the extracts, aliquots were analyzed by GC–MS (Fig. 4).

Quantitation was achieved by integration of the reconstructed ion chromatograms using the system software, with standard curves of response ratios (analyte/internal standard) plotted against amount ratios by linear regression (Table II). The use of both peak areas and peak heights was evaluated; generally, the results were similar (Table IV). Precision and accuracy were sufficient to determine concentrations as low as 1 ng/ml for all of the analytes except 2,3'-bipyridyl and metanicotine. With 2,3'-bipyridyl, precision and accuracy were unacceptable below 5 ng/ml. Quantitation of metanicotine was unacceptable, due to the low abundance of *m/z* 175 and lack of an abundant ion suitable for quantitation.

The low abundance of *m/z* 175 in the mass spectrum of the metanicotine propyl derivative is presumably due to the presence of the double bond, which encourages fragmentation by loss of a relatively stable allylic radical rather than by loss of an ethyl radical from the propyl group (Fig. 3). It was anticipated that replacing the propyl group with a group that would cleave to give a more stable radical would encourage formation of the *m/z* 175 ion. Consequently, we evaluated the phenethyl and 2-methoxyethyl derivatives, which would be expected to be more prone to fragment by formation of relatively stable benzyl or methoxymethyl radicals, resulting in greater abundance of *m/z* 175. Both the methoxyethyl and phenethyl derivatives were readily formed by reductive alkylation with the appropriate aldehydes (Fig. 3). The relative abundance of *m/z* 175 for the methoxyethyl derivative (2%) was a slight improvement over the relative abundance of *m/z* 175 for the propyl derivative (0.7%). However, for the phenethyl derivative, the relative abundance of *m/z* 175 was improved to 18%. The phenethyl and methoxyethyl derivatives of dihydrometanicotine were also evaluated. Both derivatives increased the relative abundances of *m/z* 177, to 64% and 66% for the methoxyethyl and phenethyl derivatives, respectively, as compared with 39% for the propyl derivatives (Table III).

Based on these data, the phenethyl derivative appeared most promising from the standpoint of increasing sensitivity, and it was evaluated further. This was done by analyzing urine samples containing the six alkaloids of interest as described above for the propyl derivatives, with the exception that phenylacetaldehyde was substituted for propionaldehyde. The data are presented in Table IV. Using the phenethyl derivatives, it was possible to quantitate metanicotine at concentrations as low as 1 ng/ml. For the other alkaloids, generally better precision and accuracy were obtained with the propyl derivatives, especially at low concentrations. Nornicotine was an exception; somewhat better results were obtained using the phenethyl derivative (Table IV).

The method has been applied to determination of nornicotine, anabasine, and anatabine in the urine of twenty-two cigarette smokers and eight smokeless tobacco users. In these subjects, concentrations of metanicotine, dihydrometanicotine, and 2,3'-bipyridyl were near or below the limit of quantitation of the assay. In cigarette smokers, nornicotine, anabasine, and anatabine concentrations averaged 78, 7.3, and 12 ng/ml, respectively. Much higher concentrations of all three alkaloids were found in urine of smokeless tobacco users, presumably due to absence of pyrolysis of the alkaloids in smokeless tobacco products. In eight subjects who used chewing tobacco, concentrations of nornicotine, anabasine, and anatabine averaged 293, 35, and 88 ng/ml, respectively, and in six subjects who used oral snuff, concentrations of the alkaloids averaged 145, 22, and 26 ng/ml, respectively. These data, along with concentrations and excretion of nicotine and its metabolite cotinine, are presented in Table V.

In summary, a sensitive analytical method for determination of minor alkaloids in urine has been developed, and concentrations of anabasine

TABLE IV

INTRA-DAY PRECISION AND ACCURACY FOR DETERMINATION OF TOBACCO ALKALOIDS IN URINE

Alkaloid	Concentration (ng/ml)			Accuracy[b] (%)		C.V.[b,c] (%)	
	Actual[a]	Measured[b] propyl derivatives	Measured[b] phenethyl derivatives	Propyl derivatives	Phenethyl derivatives	Propyl derivatives	Phenethyl derivatives
Anabasine	1	0.81 (0.80)	0.69 (ND)	81 (80)	69	10.0 (9.7)	44.0
	2	1.56 (1.64)	2.75 (2.21)	78 (82)	138 (111)	10.0 (12.0)	29.0 (18.0)
	5	5.04 (5.05)	4.80 (4.96)	101 (101)	96 (99)	7.1 (4.0)	8.8 (7.8)
	10	11.4 (11.0)	9.62 (9.56)	114 (110)	96 (96)	11.0 (10.0)	9.9 (6.9)
	50	52.5 (52.3)	43.1 (41.7)	105 (105)	86 (83)	7.0 (8.6)	8.6 (9.7)
	100	97.1 (97.5)	104 (105)	97 (98)	104 (105)	10.0 (11.5)	11.0 (11.0)
Anatabine	1	0.84 (0.90)	0.53 (0.60)	84 (90)	53 (60)	19.0 (10.0)	76.0 (46.0)
	2	2.09 (1.88)	1.96 (1.88)	105 (94)	98 (94)	17.0 (6.8)	37.0 (26.0)
	5	4.92 (4.94)	5.12 (5.13)	98 (99)	103 (103)	6.3 (9.9)	28.0 (19.0)
	10	10.1 (10.8)	10.6 (11.9)	101 (108)	106 (119)	6.6 (6.9)	4.2 (4.9)
	50	52.9 (52.5)	46.9 (44.5)	106 (105)	94 (89)	4.7 (4.8)	4.5 (5.0)
	100	97.3 (97.7)	102 (106)	97 (98)	102 (106)	7.7 (8.8)	6.8 (10.0)
Nornicotine	1	1.19 (1.13)	0.93 (0.90)	119 (113)	93 (90)	18.1 (14.0)	20.0 (20.0)
	2	1.51 (1.50)	2.03 (2.12)	76 (75)	102 (106)	35.0 (33.0)	5.7 (7.6)
	5	5.02 (5.01)	4.97 (4.94)	100 (100)	99 (9)	10.0 (13.0)	11.0 (10.0)
	10	10.5 (10.2)	11.0 (11.1)	105 (102)	110 (111)	11.0 (11.0)	6.7 (6.9)
	50	54.3 (54.5)	50.7 (52.6)	109 (109)	101 (105)	5.7 (6.3)	5.5 (7.0)
	100	96.7 (96.9)	99.0 (98.9)	97 (97)	99 (99)	6.3 (7.4)	4.9 (3.8)

Metanicotine	1	–	1.09 (0.97)		109 (97)		28.0 (26.0)
	2	–	2.12 (2.20)		106 (110)		23.0 (16.0)
	5	–	5.02 (4.97)		100 (99)		30.0 (18.0)
	10	6.52 (6.12)	7.5 (8.15)	65 (61)	75 (81)	110.0 (77.0)	14.0 (11.0)
	50	48.5 (45.5)	51.6 (49.4)	97 (91)	103 (99)	63.0 (27.0)	3.7 (2.3)
	100	101.0 (98.8)	102 (105)	101 (99)	102 (105)	35.0 (34.0)	27.0 (24.0)
Dihydrometanicotine	1	1.01 (0.97)	1.55 (1.35)	101 (97)	155 (135)	12.0 (9.5)	41.0 (29.0)
	2	1.84 (1.81)	2.89 (2.59)	92 (91)	145 (130)	4.4 (8.1)	17.0 (15.0)
	5	4.98 (4.98)	6.07 (4.95)	100 (100)	121 (99)	5.4 (9.8)	9.7 (35.0)
	10	11.4 (10.9)	11.7 (12.4)	114 (109)	117 (124)	7.3 (8.2)	11.0 (11.9)
	50	53.8 (55.6)	44.0 (38.7)	108 (111)	88 (77)	4.0 (9.6)	17.0 (5.5)
	100	97.0 (96.1)	102 (105)	97 (96)	102 (105)	6.8 (7.4)	15.0 (9.2)
2,3′-Bipyridyl[d]	1	0.75 (0.68)	0.85 (0.73)	75 (68)	85 (73)	43.0 (28.0)	56.0 (53.0)
	2	1.52 (1.66)	2.02 (1.84)	76 (83)	101 (92)	45.0 (20.0)	11.0 (35.0)
	5	5.09 (5.10)	5.19 (5.14)	101 (102)	104 (103)	5.1 (4.2)	4.7 (21.0)
	10	11.3 (11.6)	9.47 (7.61)	113 (116)	95 (76)	8.8 (5.0)	8.6 (11.0)
	50	52.0 (54.4)	51.7 (60.2)	104 (109)	103 (120)	3.6 (4.9)	1.4 (4.5)
	100	98.0 (96.8)	102 (101)	98 (97)	102 (101)	5.9 (6.8)	14.0 (32.0)

[a] Spiked non-smokers' urine target concentration.

[b] Determined using peak-area ratio of analyte to internal standard; mean of six replicate analyses. Values in parentheses determined using peak-height ratios.

[c] Coefficient of variation based on six replicate analyses.

[d] This alkaloid is not converted to an N′-alkyl derivative. Determined simultaneously with the other analytes converted to the corresponding N′-alkyl derivatives.

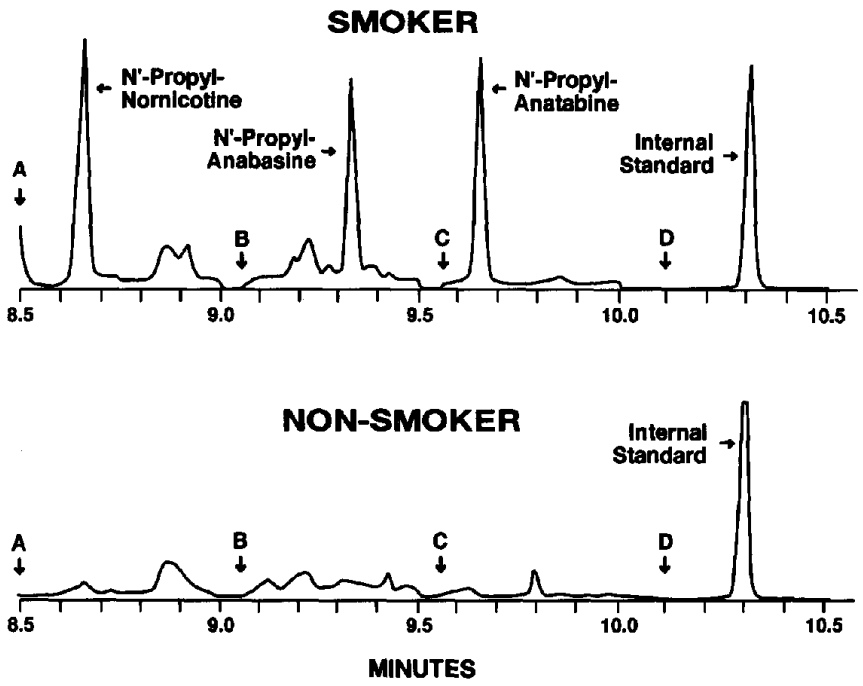

Fig. 4. Ion chromatograms of urine extracts treated with propionaldehyde and sodium borohydride. Upper panel, smoker's urine containing 86 ng/ml nornicotine, 13 ng/ml anabasine, 19 ng/ml anatabine, and 100 ng/ml internal standard, 2-(3-pyridyl)-hexahydroazepine. Lower panel, non-smoker's urine containing internal standard. Peaks: A = start of acquisition *m/z* 161; B = start of acquisition *m/z* 175; C = start of acquisition *m/z* 173; D = start of acquisition *m/z* 189.

TABLE V

URINARY CONCENTRATION AND EXCRETION OF TOBACCO ALKALOIDS IN SMOKERS AND SMOKELESS TOBACCO USERS

Data are based on 24-h urine collection and use of tobacco *ad libitum*.

Compound	Cigarette smoking (n = 22)			Chewing tobacco (n = 8)			Oral snuff (n = 6)		
	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.	Range
Nornicotine, ng/ml	78	57	4–215	293	239	123–841	145	66	81–223
Nornicotine, μg/24 h	146	99	7–378	388	238	138–574	331	175	214–644
Anabasine, ng/ml	7.3	6.5	<1–31	35	41	11–133	22	11	10–40
Anabasine, μg/24 h	12	11	0–40	44	40	15–138	50	32	27–115
Anatabine, ng/ml	12	9	<1–47	88	72	19–243	26	12	12–45
Anatabine, μg/24 h	18	16	<0–60	109	66	41–252	56	20	32–85
Nicotine, ng/ml	1041	878	73–3700	1047	754	209–2220	732	521	169–1580
Nicotine, μg/24 h	2038	1594	107–4718	1321	698	448–2302	1562	901	394–3150
Cotinine, ng/ml	1241	758	141–3010	1730	1598	487–5410	1269	632	478–2110
Cotinine, μg/24 h	2441	1481	234–5719	2311	1572	394–5610	2933	1694	1114–6089

and anatabine in urine of tobacco users have been reported for the first time. Determination of these alkaloids in urine is being used as an outcome measure to assess the efficacy of tobacco cessation programs that employ nicotine gum. The utility of this measure will be the subject of future publications.

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REFERENCES

1 F. Saitoh, M. Noma and N. Kawashima, *Phytochemistry*, 24 (1985) 477–480.
2 I. Schmeltz and D. Hoffmann, *Chem. Rev.*, 77 (1977) 295–311.
3 N. L. Benowitz and P. Jacob III, *Clin. Pharmacol. Ther.*, 35 (1984) 499–504.
4 N. L. Benowitz, H. Porchet, L. Sheiner and P. Jacob III, *Clin. Pharmacol. Ther.*, 44 (1988) 23–28.
5 N. L. Benowitz, P. Jacob III, C. Denaro and R. Jenkins, *Clin. Pharmacol. Ther.*, 49 (1991) 270–277.
6 Y. Zhang, P. Jacob III and N. L. Benowitz, *J. Chromatogr.*, 525 (1990) 349–357.
7 G. B. Neurath, M. Dunger, D. Orth and F. G. Pein, *Int. Arch. Occup. Environ. Health*, 59 (1987) 199–201.
8 P. Jacob III and N. L. Benowitz, in F. Adlkofer and K. Thurau (Editors), *Effects of Nicotine on Biological Systems*, Birkhauser Verlag, Basel, 1991, pp. 35–44.
9 A. H. Beckett, J. W. Gorrod and P. Jenner, *J. Pharm. Pharmacol.*, 24 (1972) 115–120.
10 P. Jacob III, *J. Org. Chem.*, 47 (1982) 4165–4167.
11 E. Späth and L. Mamoli, *Chem. Ber.*, 69 (1936) 1082–1085.
12 P. M. Quan, T. K. B. Karns and L. D. Quin, *J. Org. Chem.*, 30 (1965) 2769–2772.
13 E. Späth and F. Kesztler, *Chem. Ber.*, 70B (1937) 704–709.
14 E. Späth and G. Bobenberger, *Chem. Ber.*, 77 (1944) 362–369.
15 O. Hromatka, *Chem. Ber.*, 75 (1942) 522–530.
16 M. Ishikura, M. Kamada and M. Terashima, *Synthesis*, (1984) 936–938.
17 P. Jacob III, N. L. Benowitz, L. Yu and A. T. Shulgin, *Anal. Chem.*, 58 (1984) 2218–2221.
18 P. Jacob III, L. Yu, M. Wilson and N. L. Benowitz, *Biol. Mass Spectrom.*, 20 (1991) 247–252.