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Determination of the nicotine metabolite *trans*-3′-hydroxycotinine in urine of smokers using gas chromatography with nitrogen-selective detection or selected ion monitoring

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ABSTRACT

A gas chromatographic method for the determination of the nicotine metabolite *trans*-3′-hydroxycotinine is described. The method involves conversion of the metabolite to the *tert.*-butyldimethylsilyl derivative, chromatography on a fused-silica capillary column, and determination using nitrogen–phosphorus detection or electron ionization mass spectrometry with selected ion monitoring. A structural analogue, *trans*-3-hydroxy-1-methyl-5-(2-pyridyl)pyrrolidin-2-one (*trans*-3′-hydroxy-*ortho*-cotinine), was used as an internal standard. Using selected ion monitoring, good precision and accuracy were obtained for determination of *trans*-3′-hydroxycotinine in urine over the concentration range 10–10 000 ng/ml. There was a good correlation between concentrations determined by selected ion monitoring and by nitrogen–phosphorus detection in urine of smokers, although low concentrations determined using nitrogen–phosphorus detection tended to be somewhat higher, suggesting some interference from urinary constituents. Concentrations and 24-h excretion of *trans*-3′-hydroxycotinine in the urine of 22 cigarette smokers are reported and compared to concentrations and excretion of nicotine, cotinine, nicotine 1′-N-oxide, nornicotine, and cotinine N-oxide.

INTRODUCTION

The tobacco alkaloid nicotine is one of the most widely consumed drugs. Yet, there is much to be discovered about the metabolic disposition of this simple compound. The major route of nicotine metabolism involves oxidation of the pyrrolidine ring to the γ-lactam derivative cotinine [1]. Cotinine is extensively metabolized [2], with only 10–20% excreted unchanged in urine. The major urinary metabolite of nicotine in humans appears to be *trans*-3′-hydroxycotinine (compound I, Fig. 1) [3–5]. To study the metabolic disposition of nicotine in human subjects using tobacco or receiving nicotine by various routes, we required a method for the determination of *trans*-3′-hydroxycotinine that would be convenient for a large number of samples, and yet be sufficiently sensitive and accurate for pharmacokinetic studies. In this paper we describe gas chromatographic (GC) methodology for the determination of *trans*-3′-hydroxycotinine in bio-

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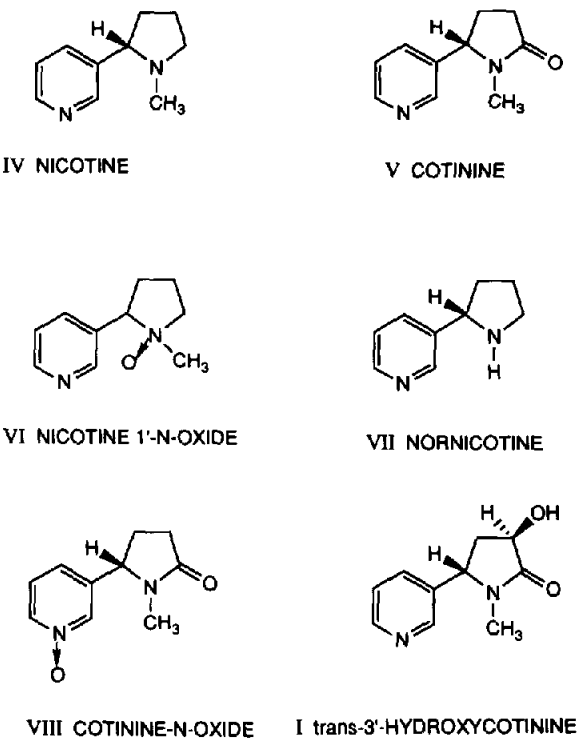

Fig. 1. Structures of nicotine and metabolites.

logical fluids and report data on the urinary excretion of the metabolite in 22 cigarette smokers.

EXPERIMENTAL

Materials

All organic chemicals, unless specifically noted, were from Aldrich (Milwaukee, WI, USA). Inorganic reagents were reagent grade, and solvents used for extractions were Fisher high-performance liquid chromatography (HPLC) grade. Microanalysis were performed by Galbraith Labs. (Knoxville, TN, USA). *trans*-3'-Hydroxycotinine was prepared as previously described [5].

Preparation of internal standards

Synthesis of (±)-*cis*-3'-hydroxy-*ortho*-cotinine [(±)-*cis*-3-hydroxy-5-(2-pyridyl)pyrrolidin-2-one, compound II, Fig. 2] was carried out by a method analogous to the synthesis of *cis*-3'-hydroxycotinine reported by Dagne and Castagnoli [6].

N-Methyl-2-pyridinecarboxaldehyde nitrone hydrochloride. This compound was obtained as a crystalline solid which was homogeneous by thin-layer chromatography (TLC) (silica gel, ethyl acetate–methanol–concentrated ammonium hydroxide, 85:10:1).

5-Carbethoxy-2-methyl-3-(2-pyridyl)-1,2-oxazoline. This compound was obtained as a clear oil, homogeneous by TLC.

(±)-cis-3'-Hydroxy-ortho-cotinine. The product (about 6 g) was a mixture of the *cis*- and *trans*-isomers which congealed to a waxy solid with a melting point (m.p.) of 75–91°C. GC and NMR analysis (see below) showed this to be about 80% *cis*-isomer. This crude product was recrystallized three times, each time from an equal weight of isopropanol to give 1.05 g of a fine white crystalline material with an m.p. of 107–112°C which was still a 90:10 mixture of isomers. Further purification was achieved by converting to the acetate, prepared by heating 1.0 g of the above material in a mixture of 5 ml of acetic anhydride and 1 ml of pyridine on the steam bath for 1.5 h. The product was poured into 200 ml of water, acidified with dilute sulfuric acid, and washed with 3 × 50 ml of methyl *tert*.-butyl ether. After being made basic with dilute ammonium hydroxide, the product was extracted with 3 × 50 ml of methylene chloride. The extracts were evaporated to an oil, which, on scratching with a glass rod under a drop of cyclohexane, crystallized. This was recrystallized twice from boiling 90:10 cyclohexane–toluene to give 0.75 g of (±)-*cis*-3'-hydroxy-*ortho*-cotinine acetate as fine white crystals with an m.p. of 111–112°C. GC analysis showed this product to be greater than 98% pure *cis*-isomer. Analysis: $C_{12}H_{14}N_2O_3$ requires C = 61.52, H = 6.02, N = 11.96; found: C = 61.55, H = 6.16, N = 11.88. A solution of 200 mg of the above acetate ester in 8 ml of butylamine was heated for 1.5 h at 100°C. The volatiles were removed under vacuum, and the residue was chromatographed on a short silica gel column and eluted with a mixture of ethyl acetate–methanol–ammonium hydroxide (80:20:2). The fractions containing the product were pooled, the solvents removed under vacuum, and the residue dissolved in 2 ml of warm

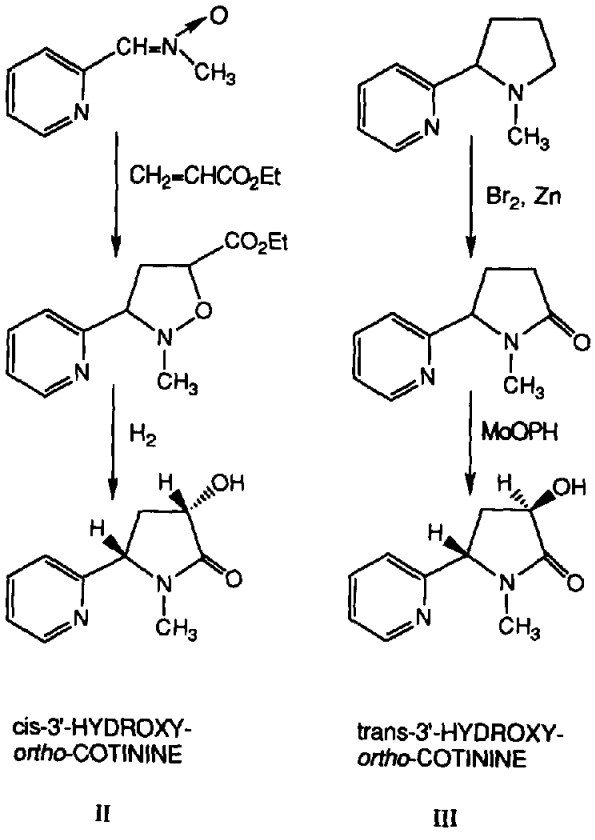

Fig. 2. Synthesis of internal standards.

isopropanol. This was treated with a solution of isopropanol containing five drops of 60% perchloric acid, producing white crystals. The solvent was heated to a boil, and the solids were finely ground. After cooling, these were removed, washed first with 5 ml of isopropanol, then with 5 ml of methyl *tert.*-butyl ether. After drying under vacuum, the product weighed 227 mg, had an m.p. of 178–180°C, and was isomerically pure (>98%) by GC. Analysis: $C_{10}H_{13}ClN_2O_6$ requires C = 41.04, H = 4.48, N = 9.57; found C = 41.08, H = 4.40, N = 9.61. The *cis*-relationship of the 3'-hydroxyl group to the 5'-(2-pyridyl) group was established unequivocally by the ^1H NMR spectrum of the hydrogen atoms on the 4'-position of the pyrrolidone ring. The 4'-hydrogen atom of *cis*-3'-hydroxy-*ortho*-cotinine that lies *cis* to the 5'-(2-pyridyl) group is a multiplet of six lines centered at δ 1.92. The 4'-hydrogen atom lying *trans* to the 5'-(2-pyridyl) group is a multiplet of eight lines centered at δ 3.08. The corresponding spectra for these two hydrogen atoms in *cis*-3'-hydroxycotinine are a six-line multiplet at δ 1.83 and an eight-line multiplet at δ 3.06. In contrast, the two 4'-hydrogen atoms in *trans*-3'-hydroxycotinine are a multiplet of sixteen lines centered at δ 2.51 [5,6]. Attempts to isomerize *cis*-3'-hydroxy-*ortho*-cotinine by the method described in ref. 6 were unsuccessful.

Synthesis of (±)-trans-3'-hydroxy-ortho-cotinine [(±)-trans-3-hydroxy-5-(2-pyridyl)pyrrolidin-2-one, compound III, Fig. 2]

ortho-Myosmine [2-(2-pyridyl)-1-pyrroline]. A solution of 32.5 g of methyl picolinate and 30 g of N-vinylpyrrolidinone in 200 ml of tetrahydrofuran was added portionwise, down through a reflux condenser, into a flask containing a well stirred suspension of sodium hydride which had been generated from 16 g of a 50% mineral oil suspension by washing three times with dry toluene. External cooling was applied as needed to contain the exothermic reaction. After the addition was complete and the hydrogen gas evolution had almost stopped, the reaction mixture was brought to reflux for 15 min, cooled, and treated with 100 ml of 10% hydrochloric acid. The bulk of the organic solvent was removed under vacuum, treated with 100 ml of 10% hydrochloric acid, and held at reflux for 16 h. After cooling, the reaction was made basic with ammonium hydroxide and extracted with 2 × 100 ml of methylene chloride. The pooled extracts were stripped of solvent under vacuum and the resulting black oil distilled at 75–80°C at 0.1 mmHg to give 9.5 g of 2-(2-pyridyl)-1-pyrroline (*ortho*-myosmine) as an amber oil that solidified on standing.

(±)-ortho-Nicotine [N-methyl-2-(2-pyridyl)-pyrrolidine]. A solution of 9.0 g of *ortho*-myosmine in 200 ml of a 4:1 methanol–acetic acid mixture was cooled to −78°C, and 1 g of sodium borohydride was added in small portions over the course of 10 min. The reaction mixture was brought up to 0°C. This was treated with 10 ml of aqueous formaldehyde followed by 0.5 g of sodium borohydride. The formaldehyde addition and

hydride reduction cycle was repeated two additional times, to complete the N-methylation as determined by TLC. The reaction was quenched in water, made basic with ammonium hydroxide, and extracted with 2 × 100 ml of methylene chloride. These pooled extracts were, in turn, extracted with dilute sulfuric acid, the aqueous phase made basic with ammonium hydroxide, and again extracted with 2 × 100 ml of methylene chloride. The solvent was removed under vacuum, and the residue distilled at 120–130°C and 50 mmHg to give 7.5 g of (±)-N-methyl-2-(2-pyridyl)pyrrolidine [(±)-*ortho*-nicotine] as a white oil. A solution of 300 mg in 10 ml of isopropanol, acidified with 60% perchloric acid, gave a white crystalline salt which weighed 500 mg after filtering, ether-washing, and air-drying. An analytical sample, from isopropanol, was the diperchlorate monohydrate and had an m.p. of 167–169°C with decomposition. Analysis: $C_{10}H_{16}Cl_2N_2O_8 \cdot H_2O$ requires C = 31.51, H = 4.76; found: C = 31.79, H = 4.82.

(±)-ortho-Cotinine [N-methyl-5-(2-pyridyl)-pyrrolidin-2-one]. To a solution of 4.4 ml of (±)-*ortho*-nicotine in 20 ml of 80% acetic acid were added 10 ml of elemental bromine and the mixture was heated on the steam bath for 4 h. An additional 5 ml of bromine were added and the heating continued for an additional 2 h. The reaction mixture was cooled in an ice bath and, with vigorous stirring, 30 g of zinc dust were added in very small portions and at a rate commensurate with the exothermic reaction that took place. The reaction mixture was allowed to come to room temperature, stirred an additional 0.5 h, and decanted from the unreacted zinc. The residual metal was washed with water, and the washings were combined with the decanted phase. This was diluted with 300 ml of water, made basic with aqueous sodium hydroxide, and extracted with 3 × 150 ml of methylene chloride. The extracts were pooled, the solvent removed under vacuum, and the residue distilled at 0.2 mmHg to give 1.0 g of *ortho*-nicotine (boiling at 60–95°C) and 1.6 g of N-methyl-5-(2-pyridyl)pyrrolidin-2-one [(±)-*ortho*-cotinine] as a yellow oil (boiling at 120–135°C). A solution of 0.4 g of this product in 20 ml of isopropanol was made acidic with concentrated perchloric acid and the formed oil scratched until crystallization set in. This was diluted with an equal volume of diethyl ether, filtered, and the isolated solids recrystallized from boiling methanol. The white perchlorate salt had an m.p. of 192–193°C. Analysis: $C_{10}H_{13}ClN_2O_5$ requires C = 43.41, H = 4.73; found: C = 43.33, H = 4.84.

(±)-trans-3′-Hydroxy-ortho-cotinine [(±)-trans-3-hydroxy-5-(2-pyridyl)pyrrolidin-2-one]. A solution of 3.3 ml of 1.5 *M* lithium diisopropylamide in 60:40 tetrahydrofuran–heptane was added to 10 ml of anhydrous tetrahydrofuran under argon, cooled to 0°C with stirring, and then treated with 0.45 g of (±)-*ortho*-cotinine. After 5 min stirring, the solution was cooled to −78°C and 1.5 g of oxodiperoxymolybdenum(pyridine)-(hexamethylphosphoric triamide), MoOPH, were added [7]. This was warmed to 0°C, during which the color changed from yellow to light green. Then 10 ml of saturated sodium sulfite were added followed by solid sodium chloride until no more would dissolve. The organic layer was separated, and the aqueous phase extracted with 2 × 20 ml of isopropanol. The combined organics were stripped of solvent under vacuum, yielding a residue that was a mixture of starting *ortho*-cotinine and product 3′-hydroxy-*ortho*-cotinine. This was treated with 5 ml of acetic anhydride and heated on the steam bath for 3 h. After cooling 15 ml of dilute sulfuric acid were added to bring the pH to < 2 and the suspension was washed with 2 × 15 ml of methylene chloride. The acid was neutralized with potassium carbonate and the basic solution extracted with 2 × 15 ml of methylene chloride. After removal of the solvent from the combined extracts, the residue was purified by column chromatography on silica gel, with an eluting solvent of ethyl acetate–methanol–ammonium hydroxide (85:10:1). On TLC, this solvent mixture provided a complete separation of all five compounds involved in this synthesis, *cis*-3′-hydroxy-*ortho*-cotinine, *trans*-3′-hydroxy-*ortho*-cotinine, *ortho*-cotinine, *cis*-3′-hydroxy-*ortho*-cotinine acetate and *trans*-3′-hydroxy-*ortho*-cotinine acetate, with R_F of 0.22,

0.30, 0.48, 0.60 and 0.72, respectively. Fractions from the column (5 ml) were collected, and those that contained the *trans*-acetate were pooled. Removal of the solvent under vacuum gave a residue that crystallized. This proved to be substantially pure *trans*-3′-hydroxy-*ortho*-cotinine acetate by TLC. This material, 23 mg of tan crystal, was heated in 5 ml of *n*-butylamine on the steam bath for 1 h which, after removal of the base and passage through a short clean-up silica gel column employing the above-described 85:10:1 solvent mixture, gave a white oil showing a single spot of *trans*-3′-hydroxy-*ortho*-cotinine by TLC. Dissolving in 1.5 ml of isopropanol and treatment with 1 ml of isopropanol containing 0.2 ml of 60% perchloric acid gave slow deposition of fine white needles. This was diluted with three volumes of diethyl ether, filtered, and air-dried giving 17.3 mg of (±)-*trans*-3′-hydroxy-*ortho*-cotinine perchlorate as white crystals. The melting point of the perchlorate salt was 233–235°C.

Instrumentation

Gas chromatographic-mass spectrometric (GC–MS) analyses were performed using electron ionization on a Hewlett Packard (HP) 5890 gas chromatograph with an HP 5970 quadrupole mass-selective detector (Hewlett-Packard, Palo Alto, CA, USA). A 12 m × 0.2 mm I.D. HP fused-silica capillary column was used, with a stationary phase of 5% phenyl methyl silicone crosslinked (0.33 μm thickness). The column oven temperature was programmed from 70°C (after a 2-min hold) to 270°C at 20°C/min. The injection port temperature was 250°C. The sample (1 μl) was injected in the splitless mode using an HP 7673A automatic sampler. The septum purge on time was 0.8 min. A microcomputer-based workstation, HP 59970, was used for controlling the instrument and data reduction. Gas chromatographic–nitrogen–phosphorus detection (GC–NPD) analyses were performed on an HP 5880 gas chromatograph, with a nitrogen–phosphorus detector, using a 25 m × 0.32 mm I.D. HP fused-silica capillary column with a crosslinked 5% phenyl methyl silicone stationary phase of 0.52 μm film thickness. The column oven temperature was programmed from 90°C (after a 0.5-min hold) to 180°C at 25°C/min, then to 220°C at 10°C/min, and finally to 275°C at 25°C/min. ^{1}H NMR spectra were obtained on a General Electric 500-MHz spectrometer.

Experimental subjects

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

Urine analysis procedure

To a 1-ml aliquot of urine in a 13 × 100 mm culture tube 1 μg of the internal standard, *trans*-3′-hydroxy-*ortho*-cotinine (100 μl of a 10 μg/ml solution in 0.05 *M* hydrochloric acid), and 1 ml of 50% aqueous potassium carbonate were added. Then 2.5 ml of methylene chloride–isopropanol (50:50) were added and the mixture was vortex-mixed for 5 min, centrifuged at 2500 *g* for 5 min, and cooled in a dry-ice–acetone bath to freeze the aqueous layer. The organic phase was poured into a new tube and then evaporated to dryness in a vacuum evaporator at 60°C. A 1-ml portion of methylene chloride was added and evaporated, to assure that the residue was free of water and isopropanol. To this were added 200 μl of a solution containing 5% (w/v) *tert*.-butyldimethylsilyl chloride and 5% imidazole in anhydrous N,N-dimethylacetamide. The culture tube was capped, and the mixture was vortex-mixed briefly, then placed in a heating block at 80°C for 1 h. After cooling, 0.4 ml of toluene–butanol (90:10) and 1 ml of water were added, the mixture vortex-mixed for 1 min, centrifuged at 2500 *g* for 5 min, and the tube placed in a dry-ice–acetone bath to freeze the aqueous layer. The organic layer was poured into an autosampler vial for analysis either by GC–MS or by GC–NPD. With GC–MS, *m/z* 249 was monitored, the major fragment representing the loss of the *tert*.-butyl group from the *tert*.-butyldimethylsilyl derivative of *trans*-3′-hydroxycotinine and of the internal standard.

Calibration procedure

Quantification was achieved by integration of the ion chromatograms or detector responses, and constructing standard curves of peak-area ratio of analyte/internal standard *versus* concentration by linear regression. Standard curves were obtained by the addition of known amounts of *trans*-3′-hydroxycotinine to 0.05 *M* hydrochloric acid, to achieve final concentrations of 10, 30, 100, 300, 1000, 3000, and 10 000 ng/ml.

RESULTS AND DISCUSSION

Previous methods for the determination of *trans*-3′-hydroxycotinine in biological fluids have utilized GC–NPD (underivatized [8], as the acetate [3], or as the trimethylsilyl ether [9]), GC with electron-capture detection (as the heptafluorobutyrate [10]), or GC–MS (underivatized [8] or as the trimethylsilyl ether [9]). Three methods have employed HPLC [11–13], and one has employed HPLC–MS [14]. As most of the studies in our laboratory involve the use of stable isotopes, and this technique will be used in human studies with deuterium-labelled *trans*-3′-hydroxycotinine, we wished to develop an assay that could use either GC–MS or GC with nitrogen-selective detection.

trans-3′-Hydroxycotinine is a rather polar compound that is difficult to extract from urine. We found that a 50:50 mixture of methylene chloride–isopropanol effectively extracts *trans*-3′-hydroxycotinine from basic solutions and is a convenient solvent because it has a density less than that of water thus forming the upper layer and allowing easy phase separation.

Since the presence of an hydroxy group may cause strong adsorption on surfaces of injection port liners or chromatographic columns, most of the published GC methods use a derivative to improve chromatographic properties. Reported methods have used either esters or silyl ethers. We first evaluated the acetate and propionate ester derivatives, since their hydrolytic stability allowed partitioning into aqueous acid for both cleaning up and concentrating the extracts. Unfortunately, these derivatives decomposed to a certain extent in the heated injection port of the gas chromatograph, giving rise to varying amounts of the unsaturated derivative 1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one, which results from the elimination of acetic acid or propionic acid. Consequently, we explored triakylsilyl derivatives, anticipating that silyl ethers would be less prone to elimination reactions. This was indeed found to be the case. The *tert.*-butyldimethylsilyl derivative proved to be especially useful, since the major ion produced on electron ionization resulted from the loss of the *tert.*-butyl group. Thus, all of the original 3′-hydroxycotinine molecule is maintained and isotopic labels, if present, would not be lost in the analysis. This relatively high-mass fragment (*m/z* 249) proved to be excellent for selected ion monitoring, resulting in clean chromatograms (Fig. 3) and high sensitivity.

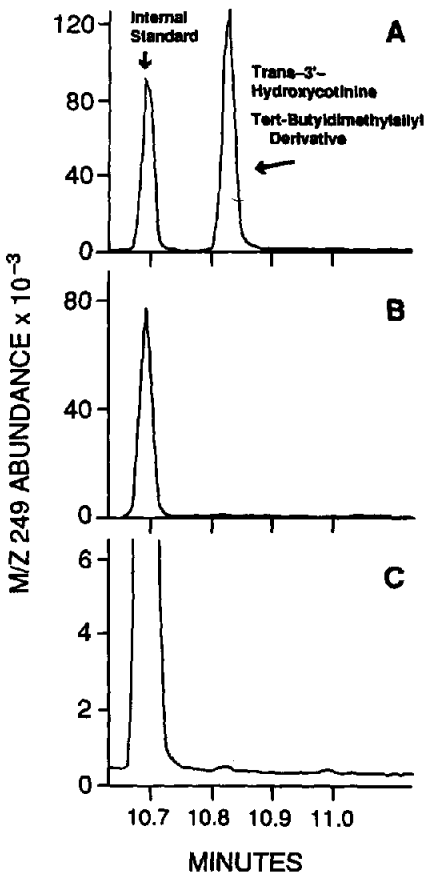

Fig. 3. Ion chromatogram (*m/z* 249) of derivatized extracts of (A) smoker's urine specimen containing *trans*-3′-hydroxycotinine, (B) non-smoker's urine specimen, and (C) the non-smoker's urine specimen in which the *y*-range has been scaled by 1:14.

Because of the difficulty of extraction and the need for derivatization, we felt that the use of a close structural analogue of *trans*-3′-hydroxycotinine as an internal standard would be highly desirable. The use of *cis*-3′-hydroxycotinine was considered, but it has been reported to be a minor metabolite of nicotine [15]. Previously, we had found that 1-methyl-5-(2-pyridyl)pyrrolidin-2-one ("*ortho*-cotinine") served as an excellent internal standard for the GC determination of cotinine, having solvent partitioning properties very similar to those of cotinine, yet being cleanly separated from it on capillary GC columns. Consequently, we anticipated that *trans*-3-hydroxy-1-methyl-5-(2-pyridyl)pyrrolidin-2-one (*trans*-3′-hydroxy-*ortho*-cotinine) would be a good choice as an internal standard for *trans*-3′-hydroxycotinine.

The synthetic route that led to *trans*-3′-hydroxy-*ortho*-cotinine is shown in Fig. 2. The procedure of Dagne and Castagnoli [6] for the synthesis of *cis*-3′-hydroxycotinine, applied to the N-methylnitrone of picolinaldehyde, yielded a mixture of *cis*- and *trans*-3′-hydroxy-*ortho*-cotinine in a ratio of 4:1. Attempts to isolate the *trans*-isomer, or to isomerize the *cis*- to *trans*-form, were not successful. Consequently, we oxidized *ortho*-cotinine directly to the hydroxy derivative using MoOPH, as previously described for the synthesis of *trans*-3′-hydroxycotinine [3]. This approach was successful. The desired *trans*-isomer was isolated as the crystalline acetate ester which was purified by recrystallization, and converted to the hydroxy compound by heating with butyl amine. This proved to be an excellent internal standard for both the GC–MS and the GC–NPD procedures, allowing the determination of 3′-hydroxycotinine in concentrations over the range 10–10 000 ng/ml.

Typical retention times were 10.69 and 10.82 min for the *tert.*-butyldimethylsilyl derivatives of the internal standard and *trans*-3′-hydroxycotinine, respectively, for GC–MS (Fig. 3) analysis and 13.02 and 13.33 with GC–NPD (Fig. 4).

Standard curves were constructed by linear regression using the instrument data system. Two standard curves, one from 10 to 1000 ng/ml and the other from 1000 to 10 000 ng/ml, were used. This was necessary due to the wide range of concentrations and the fact that linear regression places greater weight on high values than low ones. For GC–MS analysis, the equations for the standard curves from 10 to 1000 ng/ml and from 1000 to 10 000 ng/ml were $y = 0.604x + 0.000350$ ($r^2 = 1.000$) and $y = 0.641x + 0.0226$ ($r^2 = 0.999$), respectively. The concentrations found in non-smokers' blank urines (about 10 ng/ml, presumably resulting from passive exposure to nicotine) define the lower practical limit of quantitation, although the intrinsic limit of detectability is less than 1 ng/ml. The limit of detection in plasma appears to be about 1 ng/ml. Precision and accuracy were determined by analyzing the urine of non-smokers made up to known concentrations of *trans*-3′-hydroxycotinine with synthetic standard [5]. The values are given in Table I.

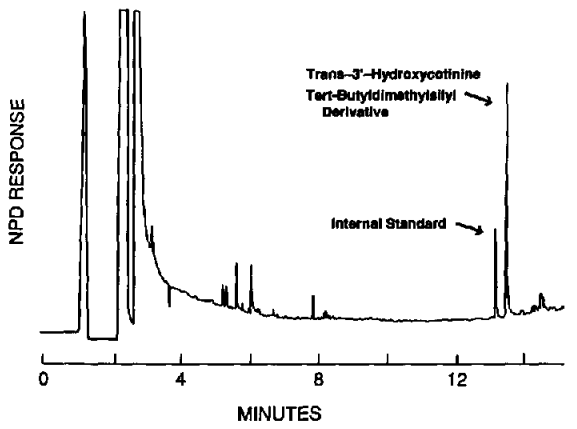

Fig. 4. GC–NPD chromatogram of derivatized extract of a smoker's urine specimen.

This method has been applied to the determination of *trans*-3′-hydroxycotinine concentrations in the urine of 22 cigarette smokers. Concentrations ranged from 193 to 11 200 ng/ml with a mean of 2910 ng/ml; the 24-h excretion total ranged from 437 to 14 770 μg per 24 h with a mean of 5642 μg per 24 h. These values for *trans*-3′-hydroxycotinine, with the 24-h excretion totals of nicotine [16], cotinine [16], nicotine 1′-N-oxide [16], nornicotine [17] and cotinine N-oxide [18] that had been determined for the same

TABLE I

INTRA-DAY PRECISION AND ACCURACY FOR GC–MS DETERMINATION OF *trans*-3′-HYDROXYCOTININE IN URINE

Concentration (ng/ml)			Accuracy (%)	C.V.[b] (%)
Actual[a]	Measured (mean)	Blank corrected		
Day 1				
0	16.1	–	–	15
10	27.4	11.3	113	7.0
20	36.0	19.9	99.5	8.7
50	71.9	55.8	112	7.0
250	250	234	93.5	3.3
1000	1010	991	99.1	2.2
5000	4590	4580	91.5	4.2
Day 2				
0	10.3	–	–	12
50	63.8	53.5	107	5.3
250	291	281	112	1.4
1000	910	900	90.0	5.6
5000	4930	4920	98.4	1.7

[a] Spiked non-smokers' urine; target concentration. There may be some *trans*-3′-hydroxycotinine present in blank urine due to exposure to environmental tobacco smoke.

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

TABLE II

URINARY EXCRETION AND CONCENTRATIONS OF NICOTINE AND METABOLITES IN 22 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.
Nicotine (IV)[a]	2618	212–5903	1882	1270	134–3132	919
Nicotine 1′-N-oxide (VI)[a]	1080	135–3192	687	574	63–1319	362
Cotinine (V)[a]	2842	440–6196	1512	1368	200–2873	740
Cotinine N-oxide (VIII)[b]	703	45–1718	392	370	25–820	210
Nornicotine (VII)[c]	157	12–361	100	85	6–207	62
trans-3′-Hydroxycotinine (I)[d]	5488	437–14770	3874	2910	193–11200	2414

[a] Ref. 16.

[b] Ref. 18.

[c] Ref. 17. As nornicotine is also a tobacco alkaloid, this value represents the sum of both metabolic formation and direct absorption from tobacco.

[d] By GC–MS.

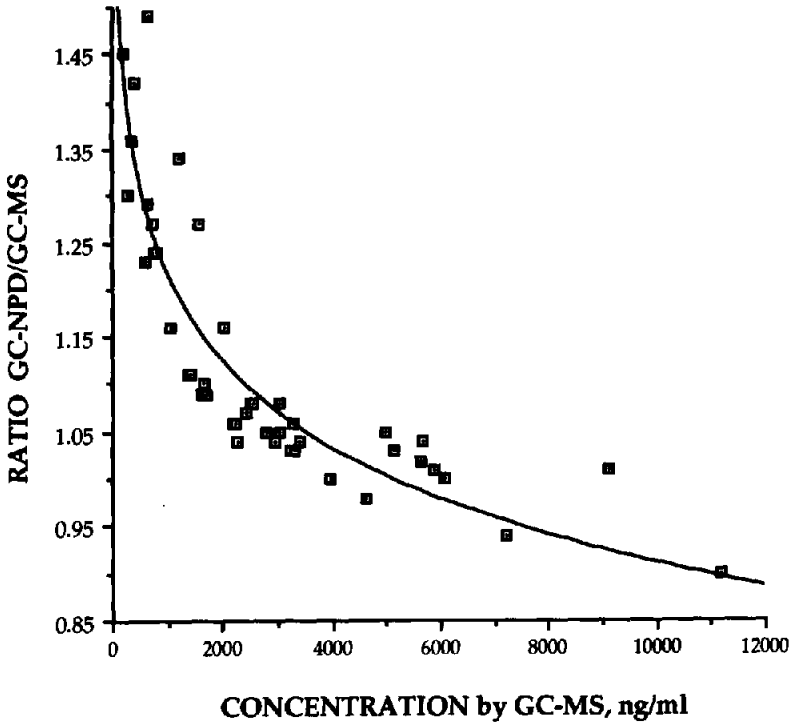

Fig. 5. Comparison of concentrations of *trans*-3′-hydroxycotinine in smokers' urine determined by GC–NPD and GC–MS.

subjects, are shown in Table II. In several smokers' urine samples, identity of *trans*-3′-hydroxycotinine was confirmed by obtaining a full-scan mass spectrum of underivatized hydroxycotinine, or by selected ion monitoring of the 3′-chloro derivative prepared by treating the extract with thionyl chloride [5].

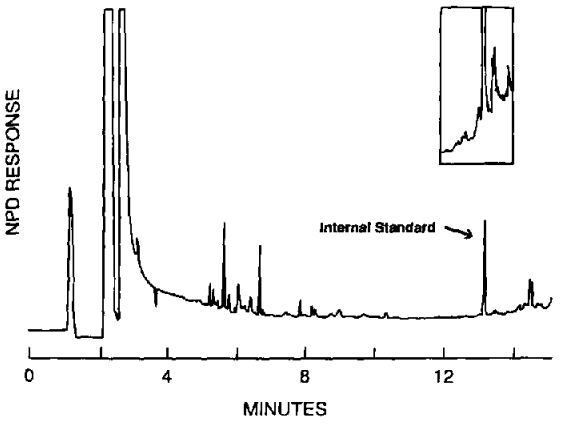

Fig. 6. GC–NPD chromatogram of derivatized extract of the same non-smoker's urine specimen shown in Fig. 3. Inset is a segment of the chromatogram including the retention time of *trans*-3′-hydroxycotinine, in which the *y*-range has been scaled by 1:14.

In general, there was an excellent correlation between concentrations of *trans*-3′-hydroxycotinine determined by GC–MS and GC–NPD. Concentrations of *trans*-3′-hydroxycotinine in urine of 22 cigarette smokers determined by GC and GC–MS averaged 3028 and 2910 ng/ml, respectively. However, there was a tendency for the GC–NPD values to be larger at the low end of the concentration range. A plot of the ratios of concentrations determined by GC–NPD and GC–MS against the GC–MS value is given in Fig. 5. Presumably, the discrepancy between GC–MS and GC is due to small amounts of urinary constituents that have the same retention time as derivatized *trans*-3′-hydroxycotinine. This possibility is supported by the observation that extracts of non-smokers' urine, analyzed by GC–NPD (Fig. 6), were not as clean as when analyzed by GC–MS with selected ion monitoring (Fig. 3B and 3C).

In summary, methods for the determination of

trans-3′-hydroxycotinine in urine using GC–NPD and GC–MS have been developed. They are straightforward and readily amenable to automated analysis of large numbers of samples. Studies of the metabolic generation and pharmacokinetics of *trans*-3′-hydroxycotinine in humans are in progress.

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