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Note

Identification and quantitative analysis of cotinine-N-oxide in human urine

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Through the broad use of tobacco, nicotine has become one of the most pervasive alkaloids in our environment. Every person is continuously exposed to nicotine. Active smokers have blood concentrations of 15–45 ng/ml [1] while non-smokers, due to passive exposure to tobacco smoke, have low but real (up to 5 ng/ml) concentrations [2].

Despite this ubiquitous, chronic exposure, the ultimate fate of nicotine in man has been poorly defined. Some 2–25% of absorbed nicotine (depending upon urinary pH) is excreted unchanged [3,4]. In humans, nicotine is metabolized primarily to cotinine which is, in turn, extensively metabolized [5,6]. Three metabolites of cotinine have been reported in human urine, but no quantitative analytical procedures have been published for their measurement. These are *trans*-3′-hydroxycotinine [5,7], cotinine methonium ion [8] and γ-(3-pyridyl)-γ-oxo-N-methylbutyramide [9]. The structures of these metabolites are shown in Fig. 1 with the numbering conventions being illustrated for the structures of nicotine and cotinine. A small amount of norcotinine is excreted in urine [5], but this latter base is probably derived from nornicotine in tobacco as it is not seen following administration of cotinine to man [9,10]. A few percent of ingested nicotine is excreted as nicotine-N′-oxide [11–13].

We present a method for the isolation and high-performance liquid chromatographic (HPLC) determination of an additional metabolite, cotinine-N-oxide, heretofore not described in man. The analytical procedure employs two structurally related synthetic analogues, the N-oxides of N-methyl-N-(3-pyridyl)acetamide (and propionamide) as internal standards. The structures of these are shown in Fig. 2.

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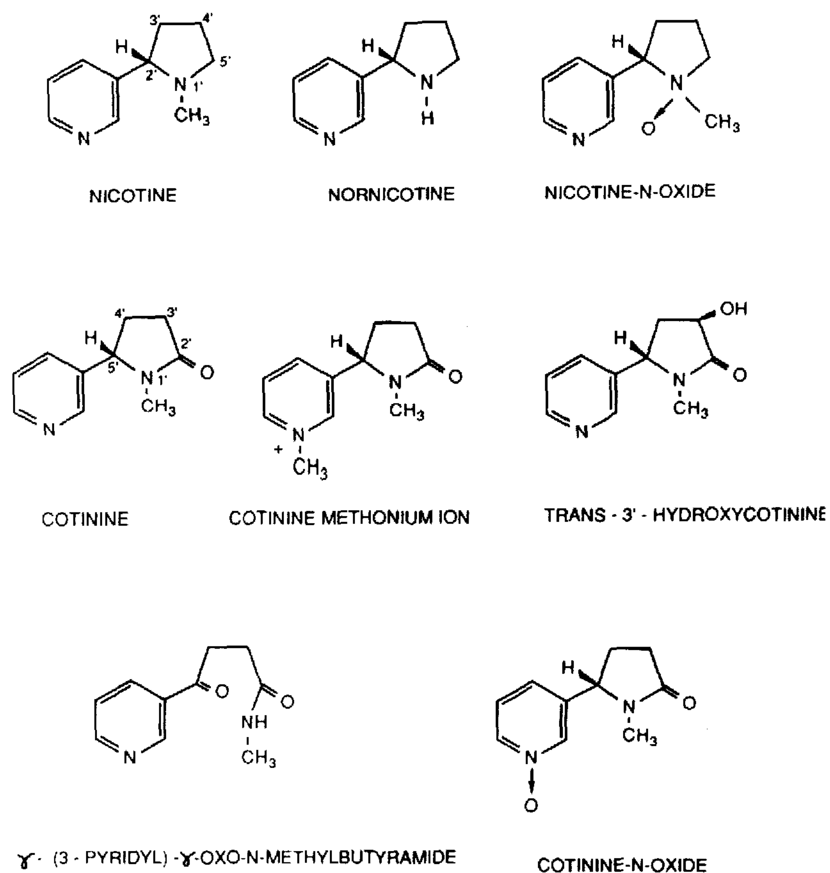

Fig. 1. Structures of nicotine and its major metabolites.

Concentrations of cotinine-N-oxide in urines from a population of smokers are also presented and compared with the concentrations of nicotine, cotinine and the nicotine metabolite, nicotine-N′-oxide.

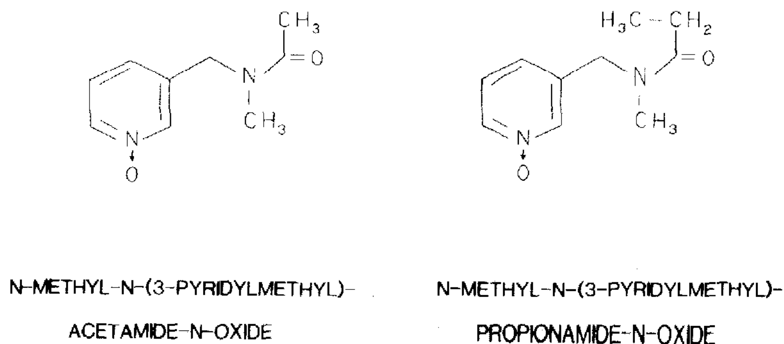

Fig. 2. Structures of the internal standards.

EXPERIMENTAL

Instrumentation

HPLC separations were carried out using Alltech 25 cm×4.6 mm I.D., 5-μm silica columns (Alltech Assoc., Deerfield, IL, U.S.A.) employing a Beckman Model 110A pump, a Model 160 absorbance detector (at 254 nm), and an Altex injector with a 20-μl loop (all from Beckman Instruments, Berkeley, CA, U.S.A.). The elution solvent was HPLC-grade acetonitrile (Burdick and Jackson, Muskegon, MI, U.S.A.) containing 1.5% β-methoxyethylamine (Aldrich, Milwaukee, WI, U.S.A.). Gas chromatographic (GC) analyses were performed on an HP 5880A Series gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Materials

All organic chemicals were from Aldrich, and inorganic reagents and laboratory solvents were analytical grade quality. Microanalyses were performed at Galbraith Labs. (Knoxville, TN, U.S.A.) and were run in duplicate. Average values are presented here. Concentrations of nicotine, cotinine and nicotine-N′-oxide in urine were measured by GC [13,14]. Cotinine-N-oxide was synthesized and purified by the method of Dagne and Castagnoli [15].

Experimental subjects

Twenty-five paid volunteers were living on a research ward. During an ad libitum smoking period, urine samples were collected over two successive 24-h periods. Urine collections were acidified and frozen until analyzed.

Preparation of internal standards

Synthesis of N-methyl-N-(3-pyridylmethyl)acetamide-N-oxide. A mixture of 2.44 g N-methyl-N-(3-pyridylmethyl)amine (20 mmol) and 2.30 g acetic anhydride (22.6 mmol) was heated for 0.5 h on a steam bath, added to 200 ml water and made basic with 8 *M* sodium hydroxide. The mixture was extracted with 3×75 ml methylene chloride, the extracts were pooled, and the solvent was removed in vacuo. The residue (2.0 g of a viscous yellow oil) was distilled yielding 1.60 g of a white, hygroscopic oil, b.p. 95–105°C at 0.035 mmHg. On standing open to the air, this material absorbed approximately 1 equiv. of water. Analysis: $C_9H_{12}N_2O + H_2O$ requires C=59.32, H=7.74; found: C=59.67, H=7.62.

To a solution of 1.55 g of the above amide in 8 ml methylene chloride (a turbid solution resulted due to the water that had been adsorbed), 2.30 g of 85% *m*-chloroperbenzoic acid (20% excess) was added, and the mixture refluxed for 1 h. An additional 20 ml of methylene chloride were added, and the organic phase extracted with 2×10 ml water. To these pooled aqueous extracts was added an equal volume of 50% aqueous sodium hydroxide, and this basic solution was extracted with 2×20 ml acetonitrile. These latter extracts were pooled, and the solvent was removed in vacuo, providing 0.8 g of a light amber-colored oil, which was pumped as free as possible of volatiles at ambient temperature, at 0.035 mmHg. About one half molecule of water remained in the sample. Analysis: $C_9H_{12}N_2O_2 + 1/2\ H_2O$ requires C=57.13, H=6.92; found: C=57.03, H=7.31.

Synthesis of N-methyl-N-(3-pyridylmethyl)propionamide-N-oxide. N-Methyl-N-(3-pyridylmethyl)amine was reacted with propionic anhydride as described above for the analogous reaction with acetic anhydride, yielding (from 2.44 g amine and 3.2 g propionic anhydride after distillation at 95–105°C at 0.035 mmHg) 2.62 g of a white oil. Again, the sample was hygroscopic, absorbing just under a mole of water on standing. Analysis: $C_{10}H_{14}N_2O + 0.85\ H_2O$ requires C=62.05, H=8.17; found: C=62.18, H=8.45. This amide was converted to the N-oxide and isolated as described for the acetyl homologue above. The product, an amber oil, was freed from volatiles at ambient temperature at 0.035 mmHg (4 h). Water remained in the product, about 0.7 moles per mole. Analysis: $C_{10}H_{14}N_2O_2 + 0.7\ H_2O$ requires C=58.07, H=7.50; found: C=58.17, H=7.50.

Both internal standards were impossible to dry completely, could not be crystallized and could not be isolated as non-hygroscopic salts. They were used as internal standards with concentrations only approximately known. The aqueous stock solutions of these appeared to be stable (judged by chromatographic and absorptive properties) and they were used as isolated above, without further purification.

Isolation procedure

Urine samples had been acidified to a pH of 2–3 by addition of 6 *M* hydrochloric acid and frozen until analysis. Urine (2 ml) was added to a centrifuge tube together with the two internal standards (40 μl of a 10 μg/ml solution). Acetonitrile (2 ml) and 2 ml of 50% sodium hydroxide were added, and the mixture was vortexed for 5 min and then centrifuged.

Silica gel extraction columns (Baker 7086, Baker, Phillipsburg, NJ, U.S.A.) were conditioned by washing first with 2 ml of a mixture of methanol and concentrated ammonium hydroxide (9:1, v/v), followed by 2 ml of acetonitrile. The clear top phase of the centrifuged sample was transferred onto a prepared silica gel column, allowed to pass into the column by gravity, and the column was then washed with 1 ml of acetonitrile. The column was eluted with 4 ml of acetonitrile containing isopropylamine (9:1). This eluate was evaporated to dryness at a bath temperature 60°C under a stream of dry nitrogen. When the solvents had evaporated, the residual solids on the walls of the tube were washed down with an additional 0.5 ml of the acetonitrile–isopropylamine mixture. Again, the solvent was removed under a stream of dry nitrogen in the water bath.

Using a 50-μl Hamilton syringe (from Alltech Assoc), 40 μl of acetonitrile were used to wash down the residues of this last evaporation, and as much as possible (30–35 μl) was taken back up again into the syringe. This sample was loaded into a 20-μl loop and injected directly onto the HPLC column. Typical retention times were 17.3, 20.9 and 23.5 min for the propionamide internal standard, cotinine-N-oxide and the acetamide internal standard, respectively. The chromatogram of a typical urine extract is given in Fig. 3.

Standard curves were obtained by the addition of known amounts of reference cotinine-N-oxide (equivalent to final concentrations of 0, 50, 200, 500 and 2000 ng/ml) to non-smokers' urine, and these samples were carried through the analytical scheme. The lower limit of sensitivity was 10 ng/ml. Peak heights were

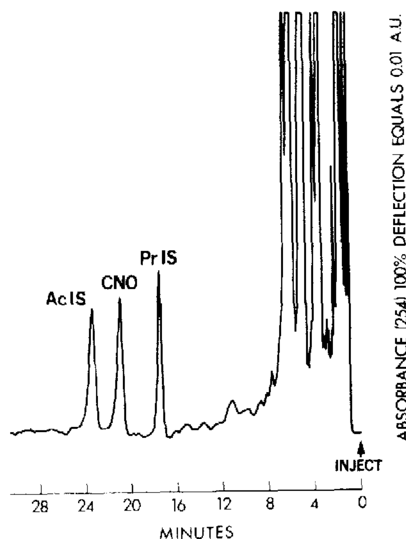

Fig. 3. Chromatogram of an extract of a smoker's urine: PrIS is the propionyl internal standard, AcIS is the acetyl internal standard and CNO is cotinine-N-oxide.

measured manually, determined by the generation of an artificial baseline beneath each peak by "tangent skimming" the machine-drawn baseline on either side. The height was measured from the top of the peak to this drawn baseline. Ultraviolet spectra were determined for methanol solutions employing the flow cell of a diode-array detector. The three compounds involved (the two internal standards and cotinine-N-oxide) had absorption maxima at 267 nm and, in serial dilutions from 2000 ng/ml (in factors of 4) down to 31.25 ng/ml, gave satisfactory agreement with Beer's law. The standard curves (peak-height ratio versus concentration, in spiked urine) showed acceptable linearity (correlation coefficient $r > 0.99$ for both internal standards and cotinine-N-oxide). The accuracy of the assay was established by analysis of non-smokers' urine samples spiked with known amounts of cotinine-N-oxide. The precision was determined by the variation of this value from run to run. At a concentration of 100 ng/ml, the mean value was 100.8 ng/ml with a between-run coefficient of variation of 10.0% ($n=4$) and 18.4% ($n=6$) for the acetyl and the propionyl internal standards, respectively. At a concentration of 500 ng/ml, the mean value was 496 ng/ml with the corresponding coefficients of variation being 3.0% ($n=4$) and 2.9% ($n=6$), respectively.

Reduction of metabolic cotinine-N-oxide to cotinine

Identification of the material being cotinine-N-oxide depended upon the recovery characteristics and the relative retention time on the HPLC column. To further establish the identity of this metabolite, cotinine-N-oxide was reduced back to cotinine. The HPLC fractions containing cotinine-N-oxide, from four smokers' urines, were pooled. The solvent was removed in vacuo and replaced with 2 ml water. This sample was split, and one portion was reduced with sodium bisulfite. The cotinine levels were determined by GC [14]. This method was

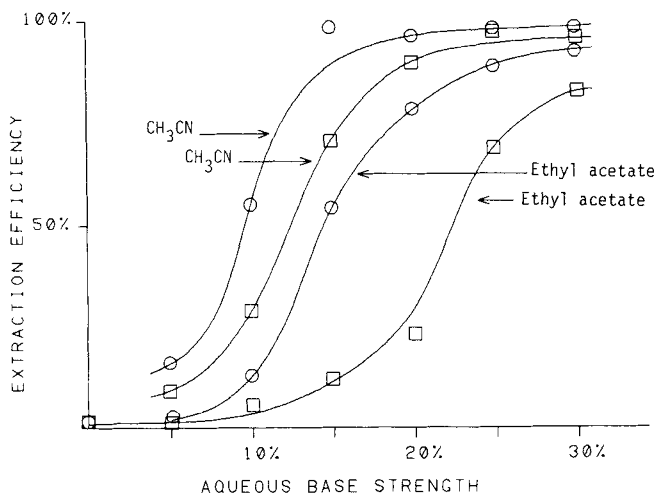

Fig. 4. Extraction efficiency of solvents for cotinine-N-oxide from aqueous base. (○) Propionamide internal standard; (□) cotinine-N-oxide.

modified to employ a fused-silica capillary column (25 m×0.32 mm I.D.; 5% phenylmethyl silicone) run with temperature programming from 90 to 275°C at a rate of 25°C/min, following a 0.5-min initial hold. The retention time for reference cotinine was 7.20 min. There was a large peak present at this retention time in the reduced sample which was not present before reduction.

RESULTS AND DISCUSSION

Quantitative analytical methods have been published for the determination of nicotine, cotinine and nicotine-N-oxide. The extraordinary polarity of cotinine-N-oxide has hindered the development of an extraction-based analysis for this metabolite. From aqueous systems (such as urine), extraction is extremely inefficient with even the best of the solvents evaluated. The efficiency is not appreciably improved by salting out. Several potential extraction solvents were compared by the determination of partition coefficients of cotinine-N-oxide between solvent and water. Most solvents that are immiscible with water (ethers or esters) had partition coefficients (organic phase to aqueous phase) of less than 0.1. Of the halocarbons explored, methylene chloride was the best, but the partition still strongly favored the aqueous phase. Numerous efforts were made to "salt out" the cotinine-N-oxide by increasing the ionic content of the aqueous phase. Only aqueous sodium hydroxide was satisfactory in that it permitted some solvents (such as ethyl acetate) to give reasonable extraction efficiencies and permitted the investigation of water-miscible solvents such as acetonitrile. The extraction efficiencies of these latter two solvents for cotinine-N-oxide and the propionamide internal standard are given in Fig. 4. It is evident that at 25% sodium hydroxide, either solvent is acceptable for the internal standard, but acetonitrile was better for the extraction of cotinine-N-oxide. Further, the instability of ethyl acetate in contact with 25% base argues against its use.

TABLE I

URINARY EXCRETION OF COTININE-N-OXIDE AND OTHER NICOTINE METABOLITES IN 25 CIGARETTE SMOKERS

Compound	Excretion (μg per 24 h)		
	Mean	S.D.	Range
Nicotine	2522	1827	212–5786
Nicotine-N′-oxide	1078	686	135–3192
Cotinine	2883	1539	441–6196
Cotinine-N-oxide	716	370	68–1536

The two-phase system of acetonitrile versus aqueous base resulted in effective extraction of cotinine-N-oxide into the organic phase, allowing its quantitative determination at levels that occur in the urine of smokers.

To select an internal standard, a number of amides of N-methyl-N-(3-pyridylmethyl)amine were prepared and converted to their N-oxides for comparison of their chromatographic behavior to that of cotinine-N-oxide. Only the N-oxide of the acetamide had a longer retention time than cotinine-N-oxide; all others (the N-oxides of the amides from formic acid, propionic acid, methoxyacetic acid and pivalic acid) preceded the cotinine-N-oxide peak. The homologous acids, acetic and propionic, most closely paralleled the partition characteristics of cotinine-N-oxide between base and acetonitrile. They were chosen as internal standards for quantitation.

Using this procedure, we have analyzed the urines of 25 subjects for concentrations of cotinine-N-oxide. These values, along with the observed values for the nicotine and other metabolites, are given in Table I. Concentrations of cotinine-N-oxide ranged from 20 to 1170 ng/ml, with a mean value of about 400 ng/ml. No cotinine-N-oxide was found in any non-smokers' urine (limit of sensitivity, 10 ng/ml).

Our findings indicate that cotinine-N-oxide is a metabolite of nicotine in humans, but quantitatively a minor one. The major end-product or end-products of nicotine metabolism in man have yet to be discovered.

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REFERENCES

1 M.A.H. Russell, C. Wilson, U.A. Patel, C. Feyerabend and P.V. Cole, Br. Med. J., 2 (1975) 414.
2 D. Foliart, N.L. Benowitz and C.E. Becker, N. Engl. J. Med., 308 (1983) 1105.
3 A.H. Beckett, M. Rowland and E.B. Triggs, Nature, 207 (1965) 200.
4 N.L. Benowitz and P. Jacob III, J. Pharmacol. Exp. Ther., 234 (1985) 153.
5 E.R. Bowman, I.B. Turnbull and H. McKinnis, J. Pharmacol. Exp. Ther., 127 (1959) 92.
6 N.L. Benowitz, F. Kuyt, P. Jacob III, R.T. Jones and A.-L. Osman, Clin. Pharmacol. Ther., 34 (1983) 604.
7 P. Jacob III, N.L. Benowitz and A.T. Shulgin, Pharm. Biochem. Behav., (1988) in press.
8 H. McKennis, L.B. Turnbull and E.R. Bowman, J. Biol. Chem., 238 (1963) 719.
9 E.R. Bowman and H. McKinnis, J. Pharmacol. Exp. Ther., 135 (1962) 306.
10 A.H. Beckett, J.W. Gorrod and P. Jenner, J. Pharm. Pharmacol., 24 (1972) 115.
11 A.R. Hibberd, A.E. Klein and J.W. Gorrod, Br. J. Cancer, 40 (1979) 816.
12 A.H. Beckett, J.W. Gorrod and P. Jenner, J. Pharm. Pharmacol., 23 (1971) 55S.
13 P. Jacob III, N.L. Benowitz, L. Yu and A.T. Shulgin, Anal. Chem., 58 (1986) 2218.
14 P. Jacob III, M. Wilson and N.L. Benowitz, J. Chromatogr., 222 (1981) 61.
15 E. Dagne and N. Castagnoli, J. Med. Chem., 15 (1972) 840.