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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF N-METHYLATED METABOLITES OF NICOTINE

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SUMMARY

An analytical method has been developed, using cation-exchange high-performance liquid chromatography, for the analysis of N-methylated metabolites of nicotine. This method has been used to detect and quantitate seven potential in vivo urinary metabolites of [2′-^{14}C]-nicotine, including four methylated nicotine derivatives, in the guinea pig.

INTRODUCTION

It has been known for many years that aza aromatic ring systems can undergo biological methylation reactions at the heteroatom [1—12]. However, little importance has been given to this route of biotransformation in drug metabolism studies, probably because of the difficulties involved in the isolation, characterization and quantitation of the resulting water-soluble, highly polar quaternary ammonium metabolites. Both nicotine (Fig. 1, 1a) and cotinine (Fig. 1, 2), two of the major pyridino alkaloids in tobacco leaf, have been reported by McKennis et al. [13] to form N-methylated quaternary pyridinium metabolites in the dog. However, no quantitative estimations of these metabolites have yet been carried out. In addition, this communication, which was published twenty years ago, appears to be the only study carried out on the in vivo methylation of nicotine. McKennis et al. [13] have also shown That cotinine (2) can be methylated to the N-methylcotinium (N-methyl-5′-oxonicotinium) ion (Fig. 1, 3) in man. These results indicate that biological methylation of nicotine and related compounds may be a significant route of metabolism in the organisms studied. Since quaternary ammonium compounds are often biologically active and because their formation is accompanied by a

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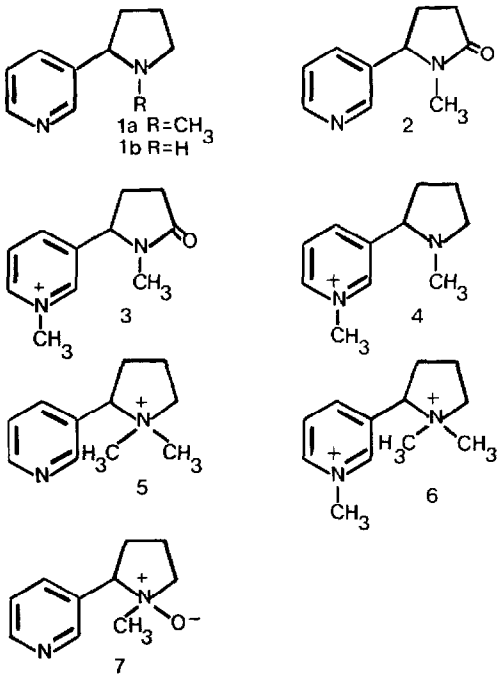

Fig. 1. Structural formulae of (1a) nicotine; (1b) nornicotine; (2) cotinine; (3) N-methyl-5′-oxonicotinium; (4) N-methylnicotinium; (5) N′-methylnicotinium; (6) N,N′-dimethylnicotinium; and (7) nicotine-1′-oxide.

marked change in physico-chemical properties such as charge, solubility and basicity, such metabolites could be of pharmacological and toxicological importance. In this respect it is surprising that relatively little attention has been focused on the in vivo methylation of nicotine and its relevance to the toxicology of tobacco products.

Biological methylation of nicotine could potentially give rise to a number of N-methylated products (Fig. 1) i.e. N-methylnicotinium (4), N-methyl-5′-oxonicotinium (3), N′-methylnicotinium (5) and N,N′-dimethylnicotinium (6) ions; of these, ions 3 and 4 have already been determined as nicotine metabolites in the dog [13]. However, no quaternary ammonium metabolites of nicotine have been isolated bearing a N′,N′-dimethylpyrrolidinium grouping, although a number of endogenous aliphatic tertiary amines are known to be substrates for methyltransferases that convert them into quaternary ammonium compounds [14, 15]. In order to determine the relative importance of N-methylation as a biotransformation route for nicotine, we have carried out in vivo metabolic studies with [2′-^{14}C] nicotine in the guinea pig. Urinary nicotine metabolites have been analyzed by an analytical procedure which allows the simultaneous determination of seven potential in vivo metabolites of nicotine, including four methylated nicotine derivatives, by cation-exchange high-performance liquid chromatography (HPLC). The details of this analytical procedure are described in this paper.

MATERIALS AND METHODS

[2′-^{14}C]Nicotine free base (0.25 mCi, specific activity 60 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Initial metabolic studies were carried out on groups of five male Hartley guinea pigs (412 ± 51 g) which were deprived of food during experimentation, but given water ad lib. Animals were each injected intraperitoneally with approximately 12.5 μCi of [2′-^{14}C]nicotine free base in 1 ml of sterile water, and housed separately in custom-built glass metabolic cages supplied by the Crown Glass Company (Somerville, NJ, U.S.A.). Each unit incorporated an efficient urine and feces separator. Urine samples were collected over 24 h.

HPLC grade methanol was purchased from Fisher Scientific (Pittsburg, PA, U.S.A.), Gold Star triethylamine, analytical-grade sodium acetate and 1-nicotine were purchased from Aldrich (Milwaukee, WI, U.S.A.), cotinine was a gift from Dr. Kostenbauder, University of Kentucky (Lexington, KY, U.S.A.). Nornicotine (Fig. 1, 1b) was synthesized from ethyl nicotinate and N-vinylpyrrolidone via the method of Jacob [16]. Nicotine-1′-oxide (7) was prepared from 1-nicotine via the method of Phillipson and Handa [17], and consists of a mixture of the two possible diastereoisomers. N-methylnicotinium iodide, N′-methylnicotinium iodide and N,N′-dimethylnicotinium diiodide were prepared by the reaction of nicotine with methyl iodide using the conditions previously described by Seeman and Whidby [18]. N-methylcotininium iodide was prepared from cotinine and methyl iodide, using the method of McKennis et al. [13]. All chromatographic standards were prepared as 10 m*M* solutions in methanol.

Analyses were carried out on an Altex programmable HPLC system consisting of two Altex Model 110A pumps, an Altex Model 420 solvent programmer and an Altex Model 153 analytical UV detector operating at 254 nm, and UV output was recorded on an Omniscribe Model 5000 dual-channel recorder (Houston Instruments, Austin, TX, U.S.A.). Samples were introduced via a Rheodyne loop injector. All eluent buffers used were filtered and degassed in vacuo before use. Separations were carried out on a Partisil-10 SCX 10-μm particle cation-exchange column (Whatman), 25 cm × 4.6 mm I.D. to which was attached a CSK-I Whatman pellicular cation-exchange guard column (7 × 0.4 cm). Essential chromatographic operating parameters are to be found in the legends to the tables and figures.

Urine samples (20—50 μl) from animals dosed with [2′-^{14}C]nicotine were centrifuged at 2500 *g* and then injected onto the HPLC column. Radioactivity in column effluents was determined in one of two ways: (a) Directly, by the use of a Model HS Flo-1 radioactive flow-through detector (Radiomatic, Tampa, FL, U.S.A.), equipped with a Radiomatic Model ES stream splitter. In such cases the scintillation cocktail used was Flo-Scint III (Radiomatic), with a mixing ratio of 4:1, v/v, of a 50% split of effluent stream. The output of the detector was recorded simultaneously on the second channel of the recorder. (b) Indirectly, by collecting fractions into scintillation vials at 1-min intervals, using a Superac Fraction Collector (LKB, Baltimore, MD, U.S.A.) and adding 10 ml of 3a70B cocktail (RPI, Elk Grove, IL, U.S.A.) to each fraction. In this case ^{14}C-activity was measured using a Packard TriCarb Scintillation Counter,

and radiochromatograms were constructed by plotting the radioactivity in the fractions against their retention times. To determine the recovery of radioisotope from the analytical column, an identical volume of radioisotopic urine applied to the column was added directly to a scintillation vial containing an appropriate volume of column effluent and treated as described above.

The limit of reliable quantitation for the radiochemical detector under the conditions of the analysis, was reached at a peak height of 80—100 dpm above a background of 20 dpm. The reproducibility of five consecutive injections of a sample affording a single peak of 300 dpm total area, gave an S.D. of 39 dpm and a coefficient of variation of 13.5%.

RESULTS AND DISCUSSION

The determination of in vivo metabolites of nicotine is usually carried out by gas—liquid chromatography [19—20]. Using this technique, various refinements in the methodologies employed have led to the development of sensitive and reliable procedures for the determination of nicotine and its metabolites in biological fluids. However, this method of analysis will only allow the direct determination of volatile components in biological samples. Polar metabolites, such as conjugates of nicotine and its phase I metabolites, and quaternary methyl metabolites, cannot be analyzed using this method unless appropriate derivatization to a more volatile compound is carried out. HPLC analysis has been used in recent years for the determination of nicotine and its metabolites [30—34]. The majority of these analytical systems have utilized a reversed-phase octadecyl silica packing and metabolites have been separated by virtue of their lipophilicity. Unfortunately, this type of analytical system is not suitable for the determination of the charged, highly water-soluble N-methylated quaternary metabolites of nicotine.

The analysis of quaternary ammonium ions by HPLC has received much attention during the last decade [35—37]. Most separations have been carried out using ion-pair reversed-phase chromatography. Our attempts to develop an analytical methodology for the quantitation of methylated metabolites of nicotine using ion-pair reversed-phase chromatography using a variety of sulfonic acid counter-ions were unsuccessful due to the extremely high affinity of the resulting ion-pairs of the methylated nicotine standards for the reversed-phase packing. Since we had previously been successful in developing a method for the analysis of N-methylated metabolites of pyridine by cation-exchange HPLC [38, 39] we decided to investigate the possible utility of this analytical system for the determination of methylated metabolites of nicotine.

Initial attempts to develop an efficient chromatographic system for the separation of nicotine, three oxidative metabolites and four potential methylated metabolites (Fig. 1, 3—6) of nicotine, were carried out on a Partisil-10 SCX 10-μm particle cation-exchange column using a 0.3 *M* ammonium acetate—methanol (70:30) buffer adjusted to pH 4.5 with glacial acetic acid. This system was used previously by us for the determination of methylpyridinium ion. However, poor chromatograms were obtained using this system; the methylated quaternary nicotine standards eluted with extremely long retention times and were observed as broad, tailing peaks. No significant

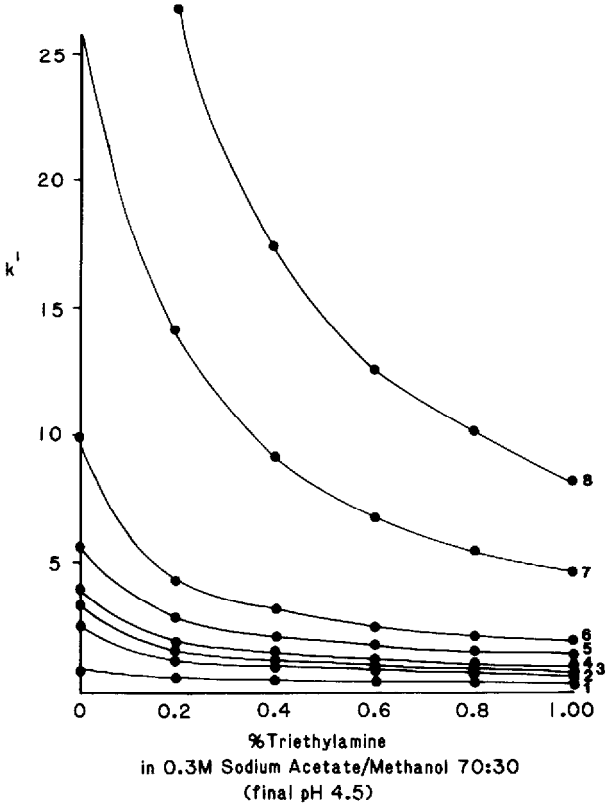

Fig. 2. Changes in k' values with increasing concentration of triethylamine from cation-exchange HPLC analysis of authentic standards of potential nicotine metabolites. Key: 1, cotinine; 2, nornicotine; 3, nicotine-N′-oxide; 4, nicotine; 5, N-methylcotininium iodide; 6, N′-methylnicotinium iodide; 7, N-methylnicotinium iodide; 8, N,N′-dimethylnicotinium diiodide.

improvement could be achieved, even after a variety of modifications to the mobile phase, such as pH variation, change in molarity of ammonium acetate and change in the proportion and/or nature of the organic modifier, were made. The chromatography was improved somewhat when sodium acetate buffer was used in place of ammonium acetate buffer. However, very long retention times were still observed for the methylated nicotine standards (see Fig. 2). To overcome this problem we hypothesized that by inclusion of a water-soluble, non-UV-absorbing protonated base into the buffer system, it might be possible to displace the tightly bound cationic nicotine standards from the sulfonic acid binding sites on the Partisil-10 SCX column, thus decreasing the retention time of these ions, on the column. Fig. 2 shows the effect of gradually increasing the percentage of the tertiary base triethylamine in a 0.3 *M* sodium acetate—methanol (70:30) buffer, pH 4.5, on the retention times of authentic nicotine derivatives. The triethylamine concentration was varied from 0 to 1%, v/v. As can be seen, a drastic decrease in the retention times of the more tenaciously bound quaternary methylated nicotine derivatives is obtained when the percentage of triethylamine in the buffer

increases. The retention times of the other standards are less markedly effected. The triethylamine also had the effect of sharpening up broad, poorly resolved peaks to give a much superior chromatogram. We also investigated the effect of the ionic strength of the sodium acetate buffer on the above chromatographic system (see Fig. 3). Generally, shorter retention times were obtained as the ionic strength of the buffer increased, but this effect was less pronounced than the triethylamine effect. Variation of the proportion of methanol in the buffer afforded some surprising results (see Fig. 4). At low percentages of methanol (0—20%) little effect was seen on the retention times of the standards. However, at methanol percentages above 30%, a marked increase in the retention times of N-methylnicotinium iodide and N,N'-dimethylnicotinium diiodide was seen, while little effect was observed on the retention times of the other standards. We attribute this observation to a solubility phenomenon, the above two compounds probably having poorer solubility in methanol than in water. The effect of buffer pH over the range 4.0—7.0 was also examined. The results are shown in Fig. 5. Generally, longer retention times were observed for all standards as the pH of the buffer increased. N-Methylnicotinium iodide and N,N'-dimethylnicotinium diiodide were again the compounds most affected by pH changes. Taking these data into consideration an optimum system was chosen using an isocratic buffer system consisting of 30% methanol in 0.3 *M* sodium acetate buffer, pH 4.5, to initially elute the less strongly bound standards from the cation-exchange column. This was followed by a gradient of triethylamine rising rapidly from 0 to 1.0% over

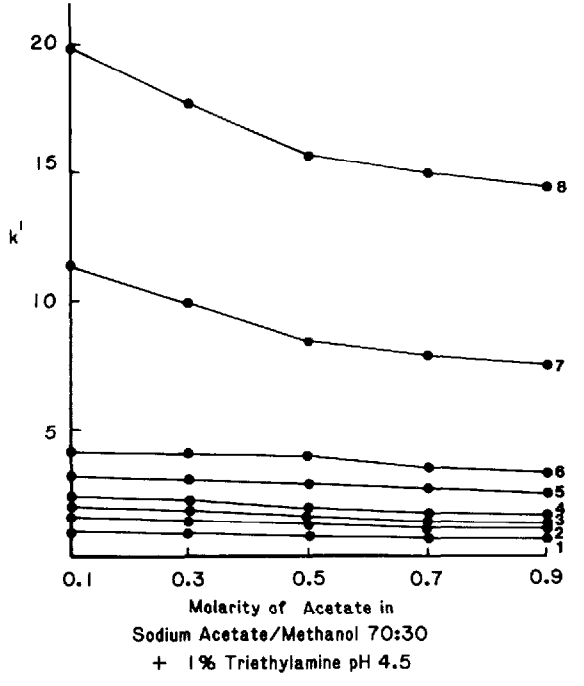

Fig. 3. Changes in k' values with increasing molarity of sodium acetate from cation-exchange HPLC analysis of authentic standards of potential nicotine metabolites. For key see Fig. 2.

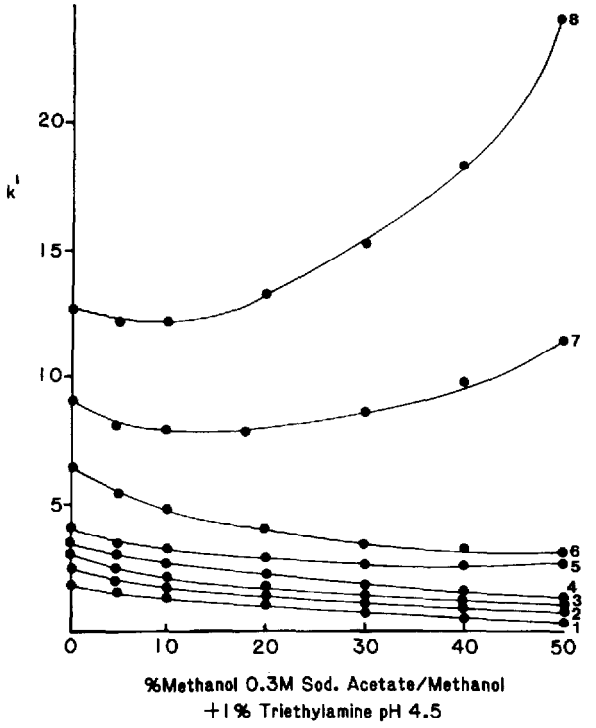

Fig. 4. Changes in k' values with increasing concentration of methanol from cation-exchange HPLC of authentic standards of potential nicotine metabolites. For key see Fig. 2.

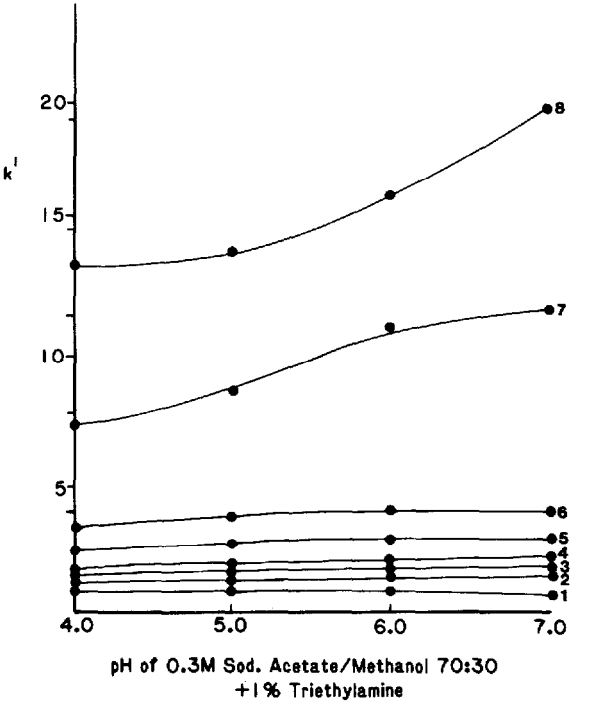

Fig. 5. Changes in k' values with increasing pH from cation-exchange HPLC of authentic standards of potential nicotine metabolites. For key see Fig. 2.

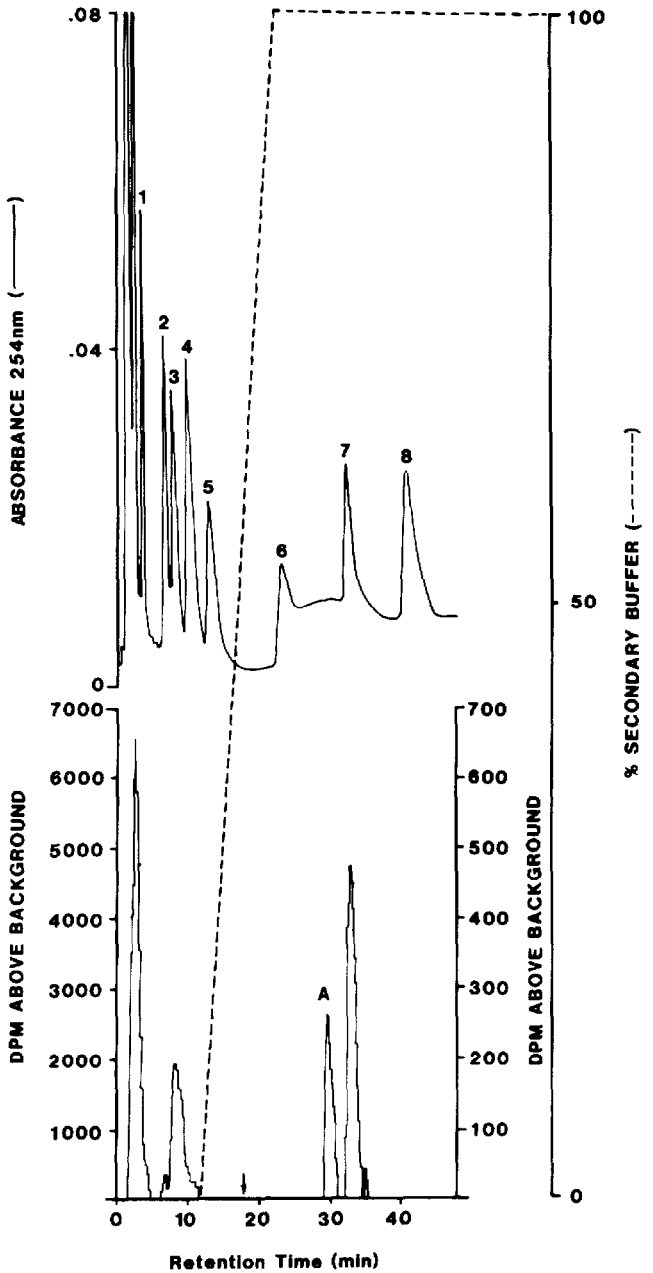

Fig. 6. Radiochromatogram of 6-h guinea pig urine, after intraperitoneal administration of [2′-^{14}C]nicotine, using cation-exchange HPLC. Column, Partisil-10 SCX (25 cm × 4.6 mm I.D.); eluent, primary buffer, methanol—0.3 *M* sodium acetate, pH 4.5 (30:70, v/v), secondary buffer, methanol—0.3 *M* sodium acetate, pH 4.5 (30/70, v/v) containing 1.0% v/v triethylamine (adjusted to pH 4.5); flow-rate 1 ml/min at 141.34 bars; the broken line shows the gradient profile; UV detection at 254 nm; ^{14}C-detection by direct analysis of column effluent, using a Flo-1, Model HS radioactive flow-through detector (see text for details). Peaks: 1 = cotinine; 2 = nornicotine; 3 = nicotine-N′-oxide; 4 = nicotine; 5 = N-methylcotininium ion; 6 = N′-methylnicotinium ion; 7 = N-methylnicotinium ion; 8 = N,N′-dimethylnicotinium ion; A = unidentified metabolite. The left-hand dpm scale refers to the radiochromatogram to the left of the arrow; the right-hand dpm scale refers to the radiochromatogram to the right of the arrow.

a 10-min period, to elute the more strongly bound methylated nicotine derivatives from the column (see Fig. 6). The above analytical system allows the analysis of seven potential metabolites of [^{14}C]nicotine.

Because of the very low dose of carrier-free [^{14}C]nicotine utilized in the metabolic studies (ca. 0.078 mg per kg body weight, ca. 12 μCi), it was not possible to analyze the low levels of metabolites in the urine samples collected, by UV detection in the HPLC effluent. Therefore, the effluent generated after co-injection of urine and authentic standards onto the Partisil 10 SCX column, was monitored for ^{14}C by liquid scintillation counting for the detection and quantitation of the small amounts of metabolites present. A radiochromatogram obtained from the analysis of a 6-h urine sample from a male Hartley guinea pig dosed with [^{14}C]nicotine is illustrated in Fig. 6, and is typical of results obtained from the analyses of each of five animals within the experimental group. Results from the HPLC cation-exchange radiochromatograms of total 24-h urine samples showed that the N-methylnicotinium iodide standard peak generally had about 4% of the ^{14}C-label in the total 24-h urine associated with it and in one animal experiment as much as 8% (see Table I). No radioactivity co-eluted with any of the other methylated nicotine standards. However, an unknown metabolite (metabolite A) is present in the urine of guinea pigs treated with [2'-^{14}C]nicotine which is retained to a greater extent on the cation-exchange column than the N'-methylnicotinium ion. This metabolite accounted for about 2% of the ^{14}C-label excreted in 24-h urine, and was as much as 4% in some cases. This component is most probably a quaternary metabolite in view of its high affinity for the Partisil-10 SCX packing. The identification of this nicotine metabolite is presently being carried out in our laboratories and will be the subject of a further communication. Significant amounts of radioactivity also eluted with the cotinine (69.4%) and nicotine-N'-oxide (21.5%) standards. In the former case, it was not always possible to

TABLE I

ANALYSIS OF URINARY METABOLITES OF [2'-^{14}C]NICOTINE IN THE GUINEA PIG

Compound	Percent in 24-h total urine*	Reproducibility***
Cotinine	69.4 (±3.9)**	61.2 (±0.53)**
Nornicotine	1.6 (±1.10)	1.9 (±0.25)
Nicotine-N'-oxide	21.5 (±3.2)	24.9 (±0.41)
Nicotine	1.3 (±0.65)	0.75 (±0.10)
N-Methylcotininium ion	0.0	0.0
N'-Methylnicotinium ion	0.0	0.0
N-Methylnicotinium ion	4.2 (±0.96)	8.4 (±0.80)
Metabolite A	2.4 (±0.56)	2.5 (±0.13)
N,N'-Dimethylnicotinium ion	0.0	0.0

*Recovery of ^{14}C-label in 24-h total guinea pig urine was 76.9 ± 2.3% of the amount administered, where n = 5.

**S.E.M., n = 5.

***Repeat analyses of a single guinea pig urine sample after intraperitoneal injection of [2'-^{14}C]nicotine.

resolve the radioactivity eluting in the void volume from that associated with the cotinine standard; therefore, quantitation of ^{14}C-label under the cotinine peak may also include radioactivity attributable to very polar, non-basic water-soluble metabolites such as conjugates, which elute from the cation-exchange column with little or no retention. Small amounts of nornicotine (1.6%) and unmetabolized nicotine (1.3%) were also detected in the total 24-h urine (see Table I for details). These results indicate that nicotine is extensively metabolized in vivo in the guinea pig and that in addition to cotinine, the N′-oxide of nicotine is a major urinary metabolite. Average recovery of ^{14}C-label in the urine was about 77% of the total ^{14}C-label administered to animals as [2′-^{14}C] nicotine, and the recovery of radioactivity from analytical HPLC columns was always better than 95% of the ^{14}C-label in the urine sample applied to the column.

In conclusion, an HPLC cation-exchange system has been developed for the identification and quantitation of methylated metabolites of [^{14}C] nicotine in the guinea pig, an animal species known to be a good N-methylater of azaheterocycles [39]. This animal species produces only one identifiable methylation product, N-methylnicotinium ion, which constitutes 4% of the total ^{14}C-label found in total 24-h urine, after intraperitoneal injection of [2′-^{14}C] nicotine.

We are currently utilizing the above analytical procedure to investigate the in vivo methylation of nicotine in other animal species, and studies are also being carried out to determine the role of methylation in nicotine toxicity.

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