

Blood–brain barrier penetration abolished by *N*-methyl quaternization of nicotine

(nicotine quaternization/brain uptake index/methylnicotines/HPLC)

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ABSTRACT The present study determined the effect of organically quaternizing either of the two tertiary nitrogen sites of nicotine to assess the *in vivo* effects of the permanently ionized states of the synthesized *N*-[¹⁴C]methylnicotines on brain uptake in rat after intracarotid injection. Male Sprague–Dawley rats were used to measure the brain uptake index (BUI) by single-pass clearance in brain after rapid injection at pH 7.4 into the left common carotid artery (expressed as a percentage) relative to simultaneously injected ³HOH. The BUI of [¹⁴C]mannitol, a control for the method background, was measured to be 2.6 ± 0.6 . At physiological pH, in striking contrast to the [*pyrrolidine*-2-¹⁴C]nicotine BUI of 120 ± 3 , the *N*-[¹⁴C]-methylnicotines had a BUI of 3.0 ± 0.6 , which was not significantly different from the method background and which indicated abolition of blood–brain barrier penetration of nicotine with the sensitivity of the BUI method.

Many alkaloids contain nitrogen(s) that exist in a positively charged form at physiological pH. Many of these substances are also known to have considerable brain uptake (1). In a study of [*pyrrolidine*-2-¹⁴C]nicotine at various pH values from 4.7 to 10.4, the brain uptake index (BUI) values varied from 49 ± 10 to 127 ± 5 , according to the fraction of neutral (uncharged) nicotine molecules available for blood–brain barrier (BBB) penetration (2). The present study was undertaken to determine if organic quaternization of either nitrogen or nicotine would reduce the fraction of neutral nicotine and its penetration of the BBB at physiological pH 7.4 by the BUI method.

Prior reports have reviewed theoretical or experimental factors that influence nicotine activity, quaternary nicotine ions, and uncharged molecules at physiological pH values (3–7). Pharmacological studies have found that the effects of *N*-methylnicotines were apparently confined to the peripheral nervous system (8–14). Many biological studies have suggested, based upon the occurrence of peripheral effects without central effects, that *N*-methylnicotines did not penetrate the BBB (15–19). Studies of nicotine metabolites in a number of species have shown the presence of *N*-methylnicotines (20–23).

In the present study nicotine was methylated with [¹⁴C]-methyl iodide to synthesize two quaternized and therefore permanently charged cationic derivatives. These quaternized derivatives were nicotine isomethiodide (NIM), in which the pyridine nitrogen was methylated, and nicotine monomethiodide (NMI) in which the pyrrolidine nitrogen was methylated. High-pressure liquid chromatographic (HPLC) procedures were developed to assay these derivatives; product structure was confirmed by nuclear magnetic resonance (NMR) spec-

troscopy. Partition coefficients (PC) of unlabeled nicotine and *N*-[¹⁴C]methylnicotines were measured.

MATERIALS AND METHODS

Animals. Studies were performed with 250- to 300-g adult male Sprague–Dawley rats (Harlan–Sprague–Dawley) that were housed in a temperature/humidity/light-controlled (6:00 a.m. to 6:00 p.m.) animal room with free access to food and water until the morning of the experiment.

Drugs and Chemicals. Nembutal (sodium pentobarbital solution, 50 mg/ml) was from Abbott. [¹⁴C]Methyl iodide (50 μ Ci/mmol; 1 μ Ci = 37 kBq) and [¹⁴C]mannitol (50 μ Ci/mmol) were from DuPont/NEN. Chloroform, CHCl₃, was from Fisher Scientific. Methanol (HPLC grade) was from Burdick and Jackson. Choline chloride, diethyl ether, *n*-hexane, methyl iodide, (*S*)-(-)-nicotine, 1-octanol, tetrahydrofuran HPLC grade, and C²HCl₃ were from Aldrich. 1-Hexanesulfonic acid, sodium salt, and Hepes were from Sigma. Hionic-fluor liquid scintillation cocktail and Soluene tissue solubilizer were from Packard Instrument. All water used in this experiment was from a Sybron/Barnstad (Newton, MA) water purifier. Unlabeled and ¹⁴C-labeled NIM and NMI were synthesized.

BUI Method. The BUI procedure (1) was performed with minor modifications. In brief, the rat was rendered unconscious with intraperitoneal Nembutal (50 mg/kg of body weight) and placed in the supine position. The left common carotid artery (rather than the right common carotid artery as originally described) was exposed and cannulated by a 27-gauge needle, and a bolus injection was delivered. The experimental injectate consisted of $\approx 0.3 \mu$ Ci of a ¹⁴C-labeled substance and $\approx 0.3 \mu$ Ci of ³HOH dissolved in 0.1 ml of 310 milliosmolar saline solution containing 10 mM Hepes buffer at pH 7.4. Three experimental groups of rats were used to measure brain uptake of one of the following: (i) [¹⁴C]mannitol, (ii) *N*-[¹⁴C]methylnicotines (60:40 ratio of [¹⁴C]NIM to [¹⁴C]NMI), or (iii) *N*-[¹⁴C]methylnicotines (60:40 ratio of [¹⁴C]NIM to [¹⁴C]NMI) with 4.4 mM choline chloride added to the injectate mixture. Five seconds after injection, the rat was decapitated, and the brain was dissected from the skull. The left hemisphere was extruded through a 20-gauge needle into a liquid scintillation vial and digested for ≈ 2 hr at 60°C with 1.5 ml of Soluene. After digestion, 10 ml of Hionic-fluor liquid scintillation cocktail was added, and the specimen was analyzed for ¹⁴C and ³H activity. An aliquot of the injectate mixture was also assayed for ¹⁴C and ³H activity. Radioactivity was measured with a liquid scintillation counter (LSC)

Abbreviations: BBB, blood–brain barrier; BUI, brain uptake index; NIM, nicotine isomethiodide (pyridine nitrogen is methylated); NMI, nicotine monomethiodide (pyrrolidine nitrogen is methylated); PC, partition coefficient.

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(model Wallac 1410, Pharmacia LKB). The ratio of ^{14}C to ^3H ($^{14}\text{C}/^3\text{H}$) disintegrations per minute (dpm), found in brain tissue was divided by the ratio found in the injectate and was multiplied by 100 to calculate BUI (%). Statistical significance for these groups was determined by using the one-way analysis of variance (ANOVA) (24).

1-Octanol/Water PC. Five milliliters of 1-octanol and 5 ml of distilled water were placed in a septum-stoppered hypovial (Pierce). To this biphasic solution was added $\approx 1.0 \mu\text{Ci}$ of synthesized N - ^{14}C methylnicotines (NIM, $0.60 \mu\text{Ci}$; NMI, $0.40 \mu\text{Ci}$). The solution was mixed in a Vortex mixer, and the phases were separated. A $20\text{-}\mu\text{l}$ aliquot of each phase was measured for ^{14}C radioactivity by liquid scintillation counting, and the PC was calculated. To correct for any lipophilic impurities in the 1-octanol layer, the aqueous phase was reextracted with 5 ml of 1-octanol, and the procedure was repeated until a consistent PC was obtained with three sequential extractions.

The PC of unlabeled authentic nicotine was measured by HPLC. Since nicotine is soluble in both lipophilic and aqueous phases, reextractions as described above for the N - ^{14}C methylnicotines were not performed. One milligram of nicotine was dissolved in 10 ml of 1:1 (vol/vol) 1-octanol/distilled water. The biphasic solution was mixed in a Vortex, and the two phases were separated. A $100\text{-}\mu\text{l}$ aliquot of each phase was removed and evaporated to dryness. The residue from each phase was dissolved in $100 \mu\text{l}$ of HPLC mobile phase (100% *n*-hexane), the ratios of the nicotine HPLC peak areas were measured and compared, and the PC was calculated.

Synthesis of N - ^{14}C Methylnicotines. ^{14}C Methyl iodide ($250 \mu\text{Ci}$; 4.09 mg , 0.031 mmol), which was dissolved in 2 ml of methanol at -80°C ; was added to $\approx 6.64 \mu\text{l}$ of unlabeled nicotine (6.7 mg , 0.041 mmol) that had been dissolved in $50 \mu\text{l}$ of methanol. The methanol mixture was stirred overnight and allowed to react and warm to room temperature. An aliquot of the reaction mixture was taken, and then the solvent was evaporated with a stream of nitrogen. The solid that resulted was washed with diethyl ether 10 times to remove unreacted nicotine. The ether-washed residue was used for HPLC assays of the ^{14}C NIM and ^{14}C NMI mixture, BUI determinations, and PC measurements.

Synthesis of Unlabeled N -Methylnicotines. N -Methylnicotines (NIM and NMI) were synthesized according to the original method (25). Briefly, freshly distilled (*S*)-(-)-nicotine (8.11 g , 0.05 mol) was dissolved in 50 ml of methanol and treated at room temperature over night with an equimolar amount of methyl iodide (7.09 g , 0.05 mol). The methanol was removed by rotary evaporation yielding a viscous amber mass, which was used for calibration of HPLC retention times of nicotine, NIM, and NMI and for continuous extraction.

A portion of the viscous amber mass was dissolved in water and extracted continuously with CHCl_3 for 4 days. The aqueous phase from the continuous extraction was frozen and lyophilized. This residue was dissolved in the HPLC mobile phase and assayed for methylation products, NIM and NMI, and unreacted nicotine.

A portion of the CHCl_3 that was removed was assayed by HPLC for unreacted nicotine, NIM, and NMI. The remainder of the CHCl_3 was dried over MgSO_4 , filtered, and removed by rotary evaporation, leaving a solid material.

The solid was triturated with diethyl ether, which removed the unreacted nicotine, and the residue was stored under N_2 at -80°C . This residue was used for HPLC assay for unreacted nicotine, NIM, and NMI; conformation of HPLC retention times of the synthesized ^{14}C NIM and ^{14}C NMI; and structural analysis of NIM by NMR. A 3-mg sample of the stored residue was dissolved in C^2HCl_3 and subjected to NMR spectroscopy with a Bruker (San Jose, CA) model AC-F 200 MHz spectrometer.

HPLC. HPLC procedures were developed to separate nicotine, NIM, and NMI based upon systematic procedures (26). For nicotine, NIM, and NMI HPLC assays, a $250 \times 4 \text{ mm}$ stainless-steel column and a $30 \times 4 \text{ mm}$ guard column containing alkylamine bonded to $60\text{-}\text{\AA}$ irregular silica (MicroPak NH2-10; Varian) were used together with an isocratic mobile phase of tetrahydrofuran/methanol, 186:77 (vol/vol), containing 0.019 M 1-hexanesulfonic acid, sodium salt. For nicotine PC determination, the mobile phase was replaced by 100% *n*-hexane.

For HPLC assay of ^{14}C mannitol, a $300 \times 7.8 \text{ mm}$ stainless-steel column containing sulfonated divinylbenzene, calcium form (Rezex Cal Monosaccharide; Phenomenex, Belmont, CA), was used with a mobile phase of water.

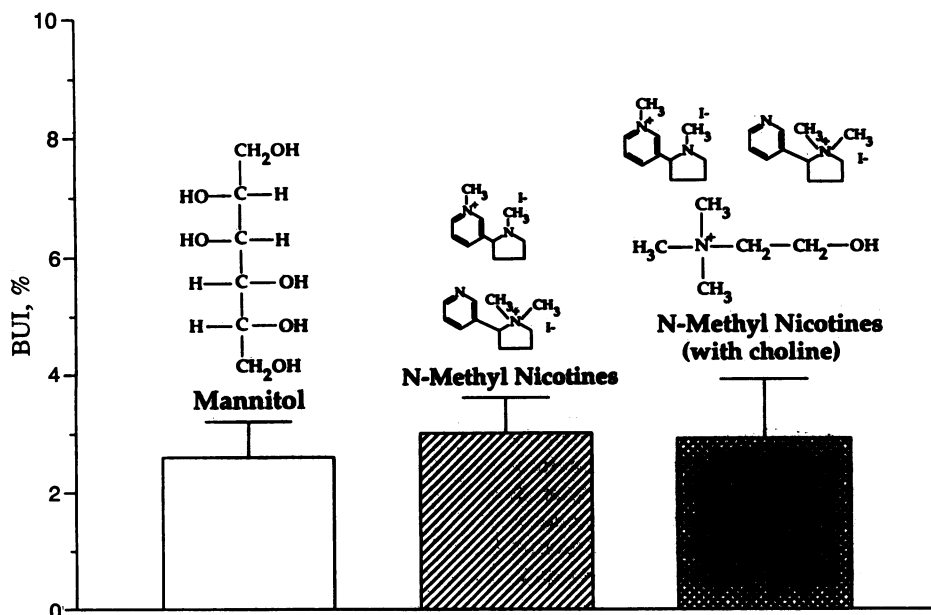


FIG. 1. Histogram of BUI of ^{14}C mannitol, N - ^{14}C methylnicotines, and N - ^{14}C methylnicotines with 4.4 mM unlabeled choline chloride added to the injectate. Chemical structures of all compounds are shown above the bar graphs.

Introduction of all samples was by injection into a 100- μ l loop valve injector (model 7125; Rheodyne, Cotati, CA). Typical injections contained 1–2 μ g of material. The HPLC apparatus consisted of a model CDS-402 chromatography data station, an LC-5000 liquid chromatograph, and a model UV-50 variable wavelength detector (Varian). The UV-50 detector was set at 262 nm for nicotine and methylnicotines assays and at 190 nm for mannitol assays.

In tandem as a second detector for labeled material was a BD model Flo-One β -radioactivity flow detector (Radiomatic Instruments and Chemical, Tampa, FL). The output of the radioactivity flow detector was monitored by the CDS-402, and the output of the UV-50 was monitored with a potentiometric recorder (Brinkmann 2542) at a chart speed of 1 cm/min.

For all HPLC assays of nicotine and methylnicotines, the flow rate was 1 ml/min and the column was operated at 60°C. For [14 C]mannitol assays, the flow rate was 1 ml/min and the column was operated at 85°C.

RESULTS

Fig. 1 (histogram) shows the BUI results from the three experimental groups of animals receiving injectates at pH 7.4. The [14 C]mannitol, used to determine the background value

of the method, had a BUI of 2.6 ± 0.6 ($n = 4$). Assay of [14 C]mannitol by HPLC, retention time 11.1 min, indicated a purity of >99.9%. The mixture of *N*- 14 C-methylated nicotines (60:40 ratio of [14 C]NIM to [14 C]NMI) had a BUI of 3.0 ± 0.6 ($n = 5$). The BUI of *N*- 14 C-methylated nicotines (60:40 ratio of [14 C]NIM to [14 C]NMI), with 4.4 mM choline chloride added to the injectate, was 2.9 ± 1 ($n = 6$). This group of rats, which were injected with *N*- 14 C-methylated nicotines, with 4.4 mM choline chloride added, tested whether any saturable component of the uptake was measurable above background.

All three BUI groups (Fig. 1) of rats were tested for significance in the differences of their BUI data as described (24); the result of $P = 0.73$ represented no significant uptake of *N*- 14 C-methylated nicotines above the background of the BUI method determined with [14 C]mannitol.

The 1-octanol/water PC of *N*-[14 C]methylnicotines determined by liquid scintillation counting was 0.002. The 1-octanol/water PC of unlabeled nicotine determined by HPLC was 0.48. For PC measurement only, the HPLC retention time of nicotine was 6.2 min with a mobile phase of 100% *n*-hexane.

The synthesized *N*-[14 C]methylnicotines used for BUI measurement and assayed by HPLC indicated that two isomers were present in a 60:40 ratio favoring the [14 C]NIM product (Fig. 2a). There was essentially no unreacted nicotine.

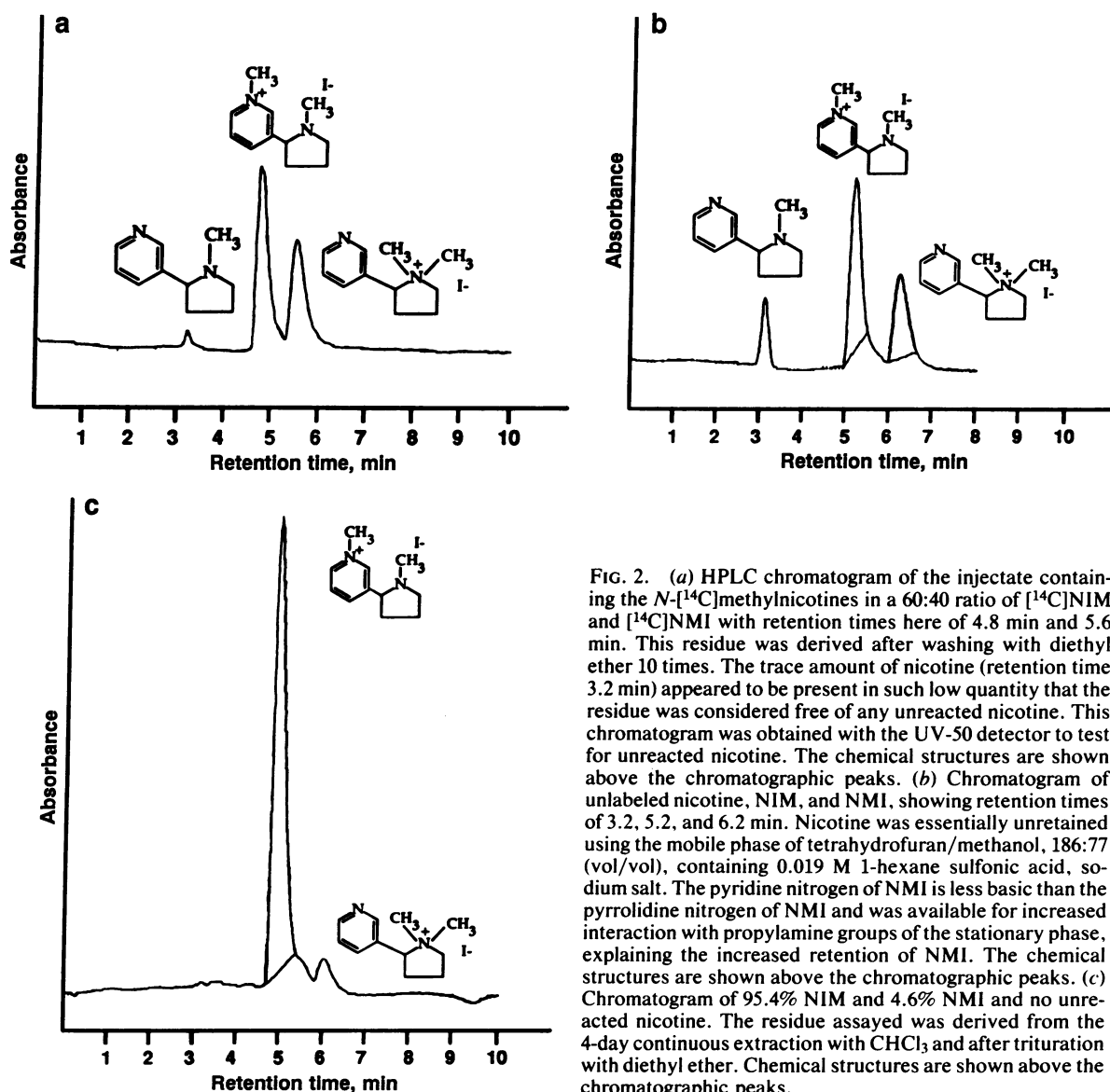


FIG. 2. (a) HPLC chromatogram of the injectate containing the *N*-[14 C]methylnicotines in a 60:40 ratio of [14 C]NIM and [14 C]NMI with retention times here of 4.8 min and 5.6 min. This residue was derived after washing with diethyl ether 10 times. The trace amount of nicotine (retention time 3.2 min) appeared to be present in such low quantity that the residue was considered free of any unreacted nicotine. This chromatogram was obtained with the UV-50 detector to test for unreacted nicotine. The chemical structures are shown above the chromatographic peaks. (b) Chromatogram of unlabeled nicotine, NIM, and NMI, showing retention times of 3.2, 5.2, and 6.2 min. Nicotine was essentially unretained using the mobile phase of tetrahydrofuran/methanol, 186:77 (vol/vol), containing 0.019 M 1-hexane sulfonic acid, sodium salt. The pyridine nitrogen of NMI is less basic than the pyrrolidine nitrogen of NMI and was available for increased interaction with propylamine groups of the stationary phase, explaining the increased retention of NMI. The chemical structures are shown above the chromatographic peaks. (c) Chromatogram of 95.4% NIM and 4.6% NMI and no unreacted nicotine. The residue assayed was derived from the 4-day continuous extraction with CHCl_3 and after trituration with diethyl ether. Chemical structures are shown above the chromatographic peaks.

The synthesis of unlabeled methylnicotines permitted standardization of reaction conditions, confirmation of labeled-product retention times, and structural determination of NIM by NMR (Fig. 2 *b* and *c* and Fig. 3).

From the crude product formed by the addition of CH₃ to nicotine in methanol, an amber mass was obtained from which HPLC retention times (Fig. 2*b*) were determined for three unlabeled compounds: nicotine (3.2 min), NIM (5.2 min), and NMI (6.2 min).

The aqueous layer from the 4-day continuous extraction was determined by HPLC to contain an unlabeled ≈1:1 mixture of both NIM and NMI and no unreacted nicotine.

Part of the chloroform used in the 4-day continuous extraction of unlabeled methylnicotines was assayed by HPLC and found to contain substantial unreacted nicotine, NIM as a major component, and NMI as a minor component.

The purified residue, which was obtained from the remaining CHCl₃ (after evaporation and trituration with diethyl ether), was determined by HPLC assay to contain 95.4% NIM, 4.6% NMI, and no unreacted nicotine (Fig. 2*c*).

The relatively pure (95.4%) unlabeled NIM residue was used for NMR structural analysis, and its spectrum (Fig. 3) was found to correspond with published data (27). The unlabeled pure NIM residue analyzed by NMR (Fig. 3) was also used to confirm the retention time of the [¹⁴C]NIM, which was 5.2 min.

The minimal detectable quantity for the *N*-methylnicotines by HPLC was ≈0.1 nmol when using ultraviolet detection.

DISCUSSION

This study represents a direct *in vivo* quantitative method for the determination of BBB penetration of *N*-[¹⁴C]methylnicotines. Table 1 summarizes aspects of this and other relevant BUI studies.

[¹⁴C]Choline, a quaternary amine, has been studied by the BUI method in this laboratory (28–31). [¹⁴C]Choline has been found to penetrate the adult rat BBB (BUI 6.27 ± 0.3) by a very-low-capacity carrier system, $K_m = 442 \pm 60 \mu\text{M}$ (30). In

the experiment reported here, additions of unlabeled choline to the injectate resulted in complete disappearance of uptake. The BUI of [¹⁴C]choline with 10 mM unlabeled choline added was 0.7 ± 0.05 and indicated no diffusion-mediated penetration (28).

To determine if quaternary amines other than choline could penetrate the BBB *in vivo*, *N*-[¹⁴C]methylnicotines were synthesized. Although the BUI of *N*-[¹⁴C]methylnicotines was not significantly greater than the BUI of [¹⁴C]mannitol, unlabeled choline (corresponding to 10 × K_m) was added to test whether the small increase in the BUI of *N*-[¹⁴C]methylnicotines could be attributed to the methylated nicotines being transported into brain by the choline carrier system (Table 1). Because the presence of 4.4 mM choline did not reduce the BUI of *N*-[¹⁴C]methylnicotines, it can be assumed that the uptake of *N*-[¹⁴C]methylnicotines had no saturable component.

Nicotine contains two tertiary nitrogens. The pK_a of the pyridine nitrogen is 3.22 at 20°C, and the pK_a of the more basic pyrrolidine nitrogen is 8.11 at 20°C (3). These values were not corrected for ionic activity. At pH 7.55 the pyrrolidine site was 74% charged; however, since nicotine was in rapid equilibrium with a neutral fraction of 26%, the BUI of [*pyrrolidine-2*-¹⁴C]nicotine (Table 1) was 120 ± 3 (2).

When the injectate was pH 4.7, the nicotine BUI was 49 ± 10 (2) and its neutral fraction was 0.05%. This decreased BUI of nicotine at pH 4.7 was probably due to the decreased neutral fraction and increased cationic fraction of nicotine, which probably did not cross the BBB.

By organically quaternizing either nitrogen, there was no residual neutral fraction, and the BUI of *N*-[¹⁴C]methylnicotines (3.0 ± 0.6) was statistically reduced to the background of the BUI method (2.6 ± 0.6 for [¹⁴C]mannitol; Table 1). The effects of changing the injectate pH over a range were not pursued with the *N*-[¹⁴C]methylnicotines because the BUI was minimal at physiological pH.

The 1-octanol/water PC for *N*-[¹⁴C]methylnicotines was decreased by a factor of 240, compared with the PC for

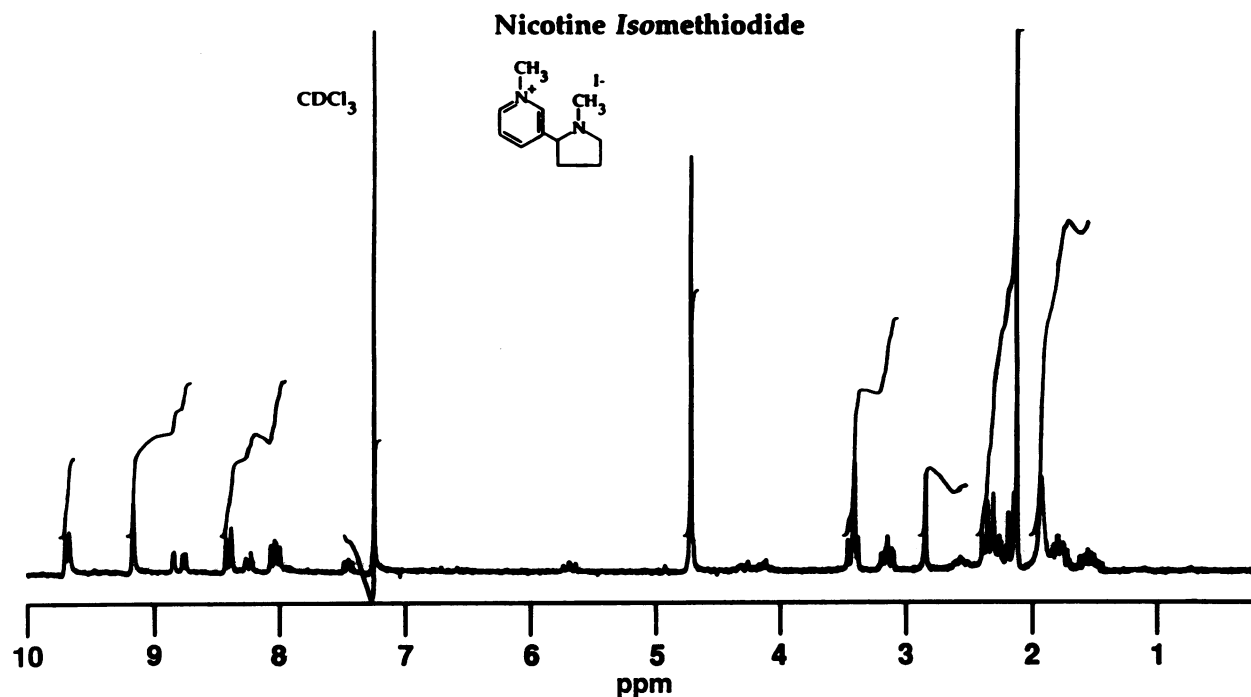


FIG. 3. NMR spectra of the isolated NIM in C²HCl₃ (CDCl₃) at 200 MHz. The peak at ≈7.3 ppm is that of the solvent C²HCl₃. Two methylation products or isomers (NIM and NMI; Fig. 2*b*) were formed by using molar equivalents of CH₃ and nicotine and the specified reaction conditions (25). The methyl peaks associated with NMI (24) were not detected by NMR.

Table 1. BUI values of mannitol, methylnicotines, choline, and nicotine at various pH values

¹⁴ C-labeled tracer*			Buffer		
Name	Conc., mM	pH	Name	Conc., mM	BUI, %
Mannitol	0.024	7.4	Hepes	10	2.6 ± 0.7
Methylnicotines	0.004	7.4	Hepes + Cho	10 4.4	3.0 ± 0.6 2.9 ± 1.0
Cho	0.004	5.5	Succinate	20	6.3 ± 0.5
		7.5	Hepes + Cho	20 10	6.3 ± 0.2 0.7 ± 0.05
		10.4	Caps [†]	10	127 ± 5
Nicotine	0.035	7.5	Hepes	10	120 ± 3
		4.7	Mes [‡]	10	49 ± 10

Cho, choline; Conc., concentration.

*Values are expressed as means ± SD, where $n = 4-8$ rats. Methylnicotines consisted of a 60:40 ratio of [¹⁴C]NIM to [¹⁴C]NMI. Values for the first two choline listings were obtained from Oldendorf *et al.* (27), while the values for the bottom choline listing were obtained from Oldendorf *et al.* (25); and values for nicotine were obtained from Oldendorf *et al.* (2).

[†]Caps, [3-(cyclohexylamino)propanesulfonic acid].

[‡]Mes, [2-(*N*-morpholino)ethanesulfonic acid].

unlabeled nicotine; this correlated with the low BUI of *N*-[¹⁴C]methylnicotines compared with that of [¹⁴C]nicotine.

This study was also undertaken to further the understanding of BBB penetration by tertiary amines and their quaternized analogs. The abolition of BBB penetration would make *N*-methylnicotines a potentially undesirable addictive product.

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