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ISOLATION AND ANALYSIS OF N-OXIDE METABOLITES OF TERTIARY AMINES: QUANTITATION OF NICOTINE-1′-N-OXIDE FORMATION IN MICE

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SUMMARY

To investigate the formation and elimination of nicotine-1′-N-oxide (NNO) in mice treated with a single injection of nicotine, sensitive and selective methods were developed to quantitate this polar and heat-labile metabolite. The compound was isolated from tissue homogenates as a dodecyl sulfate ion pair with C_{18} extraction cartridges and analyzed on an amino bonded-phase high-performance liquid chromatographic column with a mobile phase consisting of isopropanol—water. Overall recoveries of NNO were 64—76% from biological media. Several methods of detection were evaluated; radiolabeling was necessary to achieve the sensitivity required for pharmacokinetic studies in mice. The *cis* and *trans* isomers of NNO were separated on a Partisil PAC column and enzymatic selectivity was evaluated for the formation of these isomers in mice.

INTRODUCTION

Many compounds containing tertiary aliphatic or aromatic amino groups and those with heteroaromatic nitrogens can be metabolized to N-oxides in the liver [1]. Examples of substances that undergo N-oxidation by the microsomal flavin-containing monooxygenase system are trimethylamine, N,N-dimethylaniline and chlorpromazine [2, 3]. Several compounds containing a pyridyl group have been reported to undergo N-oxidation by the cytochrome P-450 monooxygenases [4]. Nicotine, with both pyridyl and tertiary amino groups, is oxidized preferentially at the latter position to produce nicotine-1′-N-oxide (NNO) (Fig. 1). This oxidation occurs in liver microsomes from several animal species [5, 6] and the metabolite has been found in urine from cats [7] and humans [8] treated with nicotine or tobacco smoke. Another important path-

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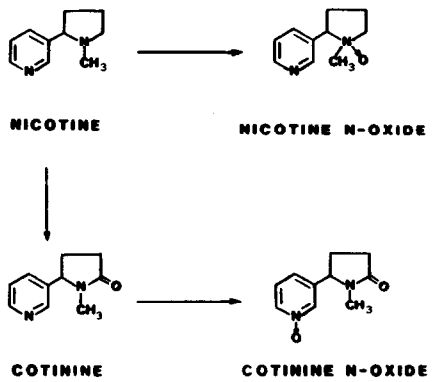


Fig. 1. Structures of nicotine and its metabolites discussed in the text.

way of nicotine metabolism is the conversion to cotinine (Fig. 1) [5, 6], and the subsequent N-oxidation of the pyridyl group to cotinine N-oxide has also been reported [9].

Our laboratory has been investigating the effects of genetic differences on the disposition of nicotine by determining the pharmacokinetic properties of this pharmacologically important constituent of tobacco and its primary metabolites in several inbred strains of mice [10, 11]. Because only small doses are tolerated, highly sensitive and selective analytical techniques were necessary to quantitate these compounds in blood and tissues of single animals. NNO and N-oxides in general are thermally labile, and the inherent polarity of the N-oxide group results in low extraction efficiencies by organic solvents. These characteristics, combined with low concentrations in complex biological matrices, complicate quantitative analyses. In studies not requiring such high sensitivity, analyses have been conducted by thin-layer chromatography (TLC) and paper chromatography of NNO produced from radiolabeled nicotine [7]. An alternative quantitative method has involved removal of unmetabolized nicotine from the sample followed by reduction of NNO with titanium trichloride; the nicotine produced is then analyzed by gas chromatography (GC) [8]. Polarographic methods have been developed for chloropromazine N-oxide [12] and indicine N-oxide [13], but these methods require extensive sample preparation and sensitivities extending to the low nanogram range have not been demonstrated. The N-oxides of nicotinamide and pyridine have been analyzed by high-performance liquid chromatography (HPLC) with a C_{18} column [14]; these polar compounds have low affinities for the C_{18} stationary phase, and elute relatively quickly along with other polar compounds in the samples.

The present paper summarizes the results of our efforts to develop procedures for the efficient and selective isolation of NNO from blood and tissue homogenates. Extracts were analyzed by HPLC with an amino bonded-phase column, which produced good retention, peak shape and selectivity, and several methods for detecting NNO were evaluated. The techniques were applied to a pharmacokinetic study of NNO formation in mice, including a determination of enzyme selectivity in the formation of the *cis* and *trans* isomers of NNO. With slight modifications, these methods should be useful to study a wide range of N-oxides in biological samples.

EXPERIMENTAL

Materials

Nicotine (Sigma, St. Louis, MO, U.S.A.) was purified by fractional distillation under reduced pressure and stored at −20°C. Cotinine was prepared from nicotine as described [15]. NNO was synthesized by oxidation of nicotine with 10% hydrogen peroxide in methanol at 25°C for 48 h. The product was purified by preparative TLC on silica gel G with ethyl acetate—methanol—ammonium hydroxide (5:4:1). Nicotine dihydrochloride (methyl-^{14}C), specific activity 10.1 mCi/mmol, was obtained from ICN (Irvine, CA, U.S.A.). Radiolabeled NNO was prepared by oxidizing [^{14}C] nicotine and purifying the product as described above. Sodium dodecyl sulfate and sodium octyl sulfate were obtained from Eastman-Kodak (Rochester, NY, U.S.A.) and sodium heptane sulfonate was from Alltech (Deerfield, IL, U.S.A.). Solvents were HPLC grade from Fisher (St. Louis, MO, U.S.A.). Silica and C_{18} Sep Pak extraction cartridges (Waters Assoc., Milford, MA, U.S.A.) were attached to luer-tipped syringes and eluted by gravity flow. The C_{18} cartridges were prepared by washing successively with 2-ml volumes of methanol and water.

Instrumentation

Radioactivity was determined with a Beckman Model LS 8000 liquid scintillation counter and counting efficiency measured with standard [^{14}C] toluene (Amersham, Arlington Heights, IL, U.S.A.). HPLC was performed with a Beckman Model 110A pump and Hitachi Model 100-10 UV detector fitted with an 8-μl HPLC flow cell. Chromatograms were recorded on a Houston Instruments omniscribe strip chart recorder. The analytical columns, Alltech NH_2, Whatman (Clifton, NJ, U.S.A.) Partisil-10 PAC and Brownlee (Santa Clara, CA, U.S.A.) RP-2 were 250 × 4.6 mm with 10-μm packings. A 46 × 3.2 mm guard column packed with Whatman Co:Pell PAC was employed with the former two columns. Electrochemical detection was performed with a Bioanalytical Systems (West Lafayette, IN, U.S.A.) Model LC-9 detector employing a TL-6A gold—mercury thin-layer cell and an $Ag/AgCl_2$ reference electrode. Mass spectrometry (MS) was carried out with a Hewlett-Packard Model 5984A instrument operated in the chemical ionization (CI) mode with isobutane. Samples were introduced with a heated probe.

Animal experiments

Male DBA mice, 60—75 days of age, were injected intraperitoneally with a 1.0 mg/kg dose of [^{14}C] nicotine (specific activity 2.9 μCi/μmol) in 0.25 ml of normal saline. Animals were killed by cervical dislocation at specific times after injection, livers were removed immediately, weighed and homogenized with a Potter—Elvehiem apparatus in 4 ml of ice-cold distilled water containing 5.0 μg of unlabeled NNO as carrier. Liver weights ranged from 0.90—1.5 g. To develop the extraction techniques, livers, brains and blood were removed from untreated mice and spiked with various amounts of [^{14}C] NNO and 5.0 μg of unlabeled NNO. The N-oxide was then isolated and analyzed as described under Results and discussion.

RESULTS AND DISCUSSION

Isolation from biological samples

Solid phase methods were investigated for extracting NNO from aqueous media. Liver homogenates were spiked with [^{14}C] NNO and prepared for extraction by first preparing a tissue-free supernatant. This procedure was facilitated by the addition of acids or organic solvents; the latter produced higher and more consistent recoveries of NNO in the supernatants (Table I). Solvent was then evaporated under a stream of nitrogen at low temperatures (35—40°C) to avoid decomposition of this thermally labile compound. Lipophilic substances were removed by extraction with ethyl acetate under basic (pH 11—12) and acidic (pH 1—2) conditions, and the aqueous samples were applied to Sep-Pak extraction cartridges containing an octadecyl-bonded stationary phase. The polar N-oxide was not retained on this hydrophobic phase but was efficiently adsorbed on cartridges packed with underivatized silica. Removal of NNO from silica could not be accomplished with methanol, acetonitrile or isopropanol, but a mixture of methanol—isopropyl amine (50:50) was effective. Other polar compounds found to be present in the fraction of NNO isolated by this method interfered with HPLC analysis. In order to prepare purer extracts of the metabolite, C_{18} Sep-Pak cartridges were reinvestigated in conjuction with ion-pairing reagents so that polar compounds not forming extractable ion pairs could be separated from the metabolite.

The retention of NNO on C_{18} cartridges in the presence of sodium octyl sulfate was dependent on the pH and the concentration of ion-pairing reagent (Table II). The optimal pH was 2.0—2.1, which can be rationalized based on the existence of two basic sites within NNO. At a sufficiently low pH, both the 1′-oxygen and the pyridyl nitrogen would be protonated and the resulting dication would associate with two octylsulfate anions. If the medium is less acidic only a single ion pair would form, and if the medium is too acidic the reagent would be protonated. The effects of alkyl chain length of the reagent on retention of NNO by C_{18} cartridges are shown in Fig. 2. Increasing the number of carbon atoms from 7 to 12 resulted in ion pairs with increasing affinity for the resin. Dodecylsulfate—NNO ion pairs were employed in subsequent extractions because this lipophilic species permitted washing the

TABLE I

RECOVERIES OF NICOTINE N-OXIDE IN THE SUPERNATANT AFTER REMOVING TISSUE FROM LIVER HOMOGENATES ($n = 4$)

Each sample contained 1 g of liver homogenate in 4 ml of water with 0.50 μg of [^{14}C]NNO. Radioactivity was measured in the supernatant after adding the agent and removing the tissue by centrifugation.

Addition	Percentage recovery (mean ± S.D.)
Perchloric acid	57 ± 11
Hydrochloric acid	71 ± 10
Methanol	82 ± 4
Acetonitrile	81 ± 5

TABLE II

RETENTION CHARACTERISTICS OF NICOTINE N-OXIDE ON C_{18} EXTRACTION CARTRIDGES WITH SODIUM OCTYLSULFATE

Samples (4 ml) containing [^{14}C]NNO and the ion-pairing reagent were applied to the cartridges and radioactivity measured in the initial effluent and after eluting with 2 ml of each solvent. The pH experiment contained 8 mg of reagent and the concentration experiment was conducted at pH 2.0.

Cartridge eluent	Radioactivity (dpm) measured in cartridge effluents				
	Effect of pH		Effect of octylsulfate concentration		
	pH 2.5	pH 2.0	20 μg	1.0 mg	8.0 mg
Aqueous solution of NNO	1984	74	—	—	—
Water	196	80	3289	640	79
Methanol—water (20:80)	100	55	5276	100	63
Methanol	2150	5750	812	8915	9565

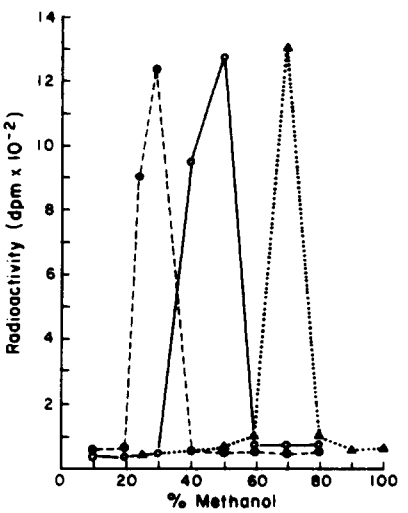


Fig. 2. The effects of alkyl chain length of ion-pairing reagents on the retention of NNO by C_{18} Sep-Pak cartridges. Aqueous solutions at pH 2.0 containing [^{14}C]NNO and an ion-pairing reagent were applied to the cartridges, eluted with 2-ml volumes of aqueous solvents. containing increasing percentages of methanol, and radioactivity measured in the effluents. The ion-pairing reagents were heptanesulfonate (— — —), octylsulfate (——) and dodecylsulfate (• • • •).

cartridges with methanol—water mixtures containing up to 50% methanol to remove polar substances before NNO was finally eluted with 100% methanol.

The complete procedure for isolating NNO from tissue homogenates is summarized in Fig. 3. Blood samples (1 ml) were first diluted with 2 ml of water and the protein was removed by addition of acetonitrile (2 ml), cooling to 5°C and centrifugation. Biological samples containing known quantities of NNO were extracted and overall recoveries determined after chromatographic analysis as discussed below.

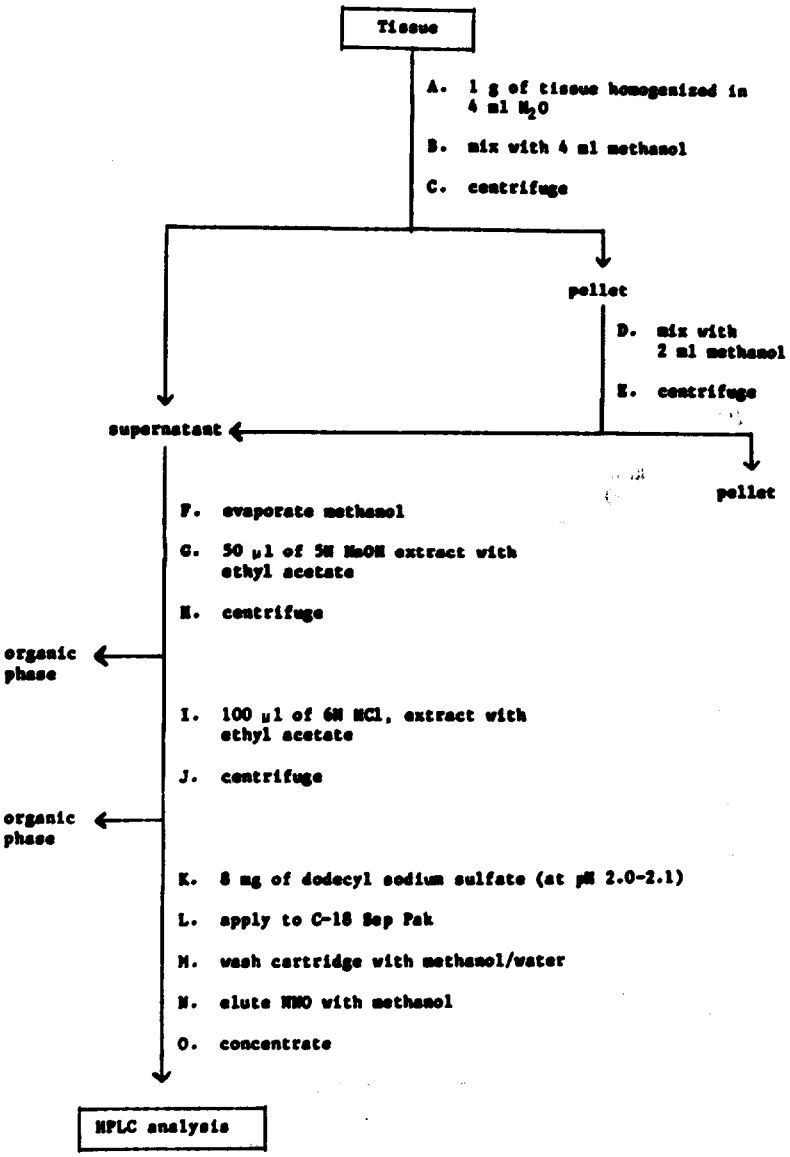
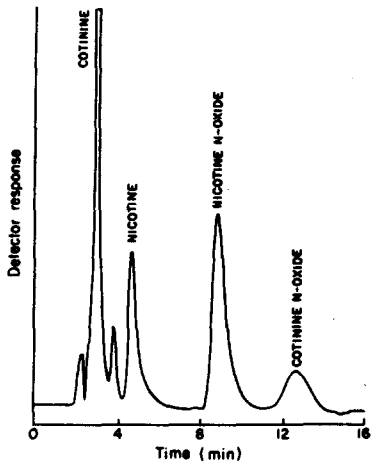


Fig. 3. Complete extraction scheme for isolating NNO from mouse tissues.

HPLC analysis

NNO was incompatible with columns containing hydrophobic stationary phases (C_8 and C_{18}) because the compound was not adequately retained to permit separation from other polar substances in the extracts. Moderately polar amino (NH_2) and aminocyano (PAC) bonded phases, however, exhibited good retention and selectivity characteristics for NNO. The former was employed for routine analyses with isopropanol—water mobile phases. A chromatogram illustrating the separation of NNO from nicotine, cotinine and cotinine N-oxide is shown in Fig. 4. The elution order is consistent with a normal-phase separation



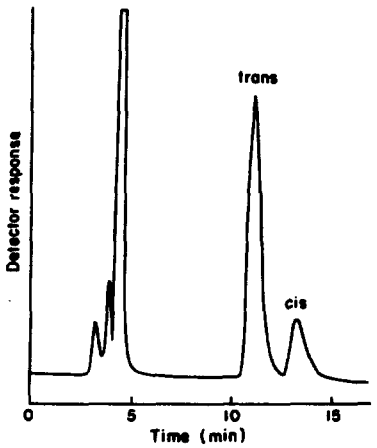


Fig. 4. Chromatogram from the HPLC analysis of standard compounds on an amino bonded phase column with isopropanol—water (75:25) as the mobile phase at 1.0 ml/min and detection at 254 nm.

Fig. 5. HPLC separation of *cis*- and *trans*-NNO on a partisil PAC column with methanol—water (95:5) as the mobile phase.

mechanism except for the short retention time of cotinine, which is more polar than nicotine on a reversed-phase column [16]. It was advantageous to eliminate the residual ion-pairing reagent from NNO extracts to avoid rapid degradation of the HPLC columns. This removal was accomplished by passing the extract [in 2 ml of isopropanol—water (12:88)] through another C_{18} Sep-Pak cartridge. The reagent was retained and the NNO analyzed in the effluent after concentration.

The oxygen atom of NNO can be introduced so that the N-methyl group is in a *cis* or *trans* relationship to the pyridyl group about the pyrrolidine ring. These isomers could be separated readily on a PAC column with methanol—water (Fig. 5). The peak assignments were based on data showing that the hydrogen peroxide oxidation of nicotine produces a larger quantity of *trans*- compared to *cis*-NNO [17]. It has been reported that the *trans* form also predominates when other oxidizing agents are employed, and that the isomers of NNO can be separated on a silica HPLC column, although no chromatograms were presented to demonstrate the separation and peak shape obtained [18].

The limit of detection for NNO with a UV monitor at 260 nm was in the range 20—25 ng, which was not sufficiently low for our pharmacokinetic experiments. Because N-oxides can be reduced electrochemically [12, 13], an amperometric HPLC detector with a gold—mercury thin-layer cell was investigated. Several chromatographic conditions were employed in searching for the optimal detector response. The most successful procedure involved a C_2 column with a thoroughly deoxygenated mobile phase containing a high percentage of water [isopropanol—0.1 *M* chloroacetic acid (10:90)] and 1 m*M* EDTA. The signal obtained for NNO increased with increasingly negative electrode potentials to a practical limit (because of high background signals) of —0.90 V. The sensitivity limit of approximately

25—30 ng did not represent an improvement over UV detection due to the relatively high potentials necessary to reduce NNO under HPLC conditions.

MS detection was also investigated. The column effluent corresponding to the elution time of NNO was collected, the solvent evaporated and the sample analyzed by direct probe CI-MS. Due to the thermal instability of NNO, the most successful method involved loading a few microliters of a methanol solution of NNO into a shallow (4 mm) quartz capillary tube, inserting the sample with the ion source at a relatively cool temperature (100 °C) and then heating the probe tip rapidly with isobutane as the reagent gas. Under these conditions, the most prominent ions were m/z 179 (MH^+, 30%), 163 ($MH^+ - O$, 100%) and 161 ($MH^+ - H_2O$, 82%). Analysis of methanol solutions containing various amounts of NNO indicated that 1.0 ng could be detected by selected-ion monitoring, but the response was non-linear over the range 1—100 ng. When tissues extracts were analyzed by this technique, there were signals which interfered with the low-level detection of NNO.

To circumvent the problems discussed above for detection of low nanogram amounts of NNO in biological media, radiolabeling was necessary. Water samples and several biological samples were mixed with various amounts of [^{14}C]NNO together with 5 μg of unlabeled NNO as carrier. The samples were processed as described (Fig. 3) and the column effluent corresponding to NNO was collected from the HPLC columns and radioactivity measured. The results are summarized in Fig. 6. Overall recoveries were not dependent on the concentration of [^{14}C]NNO and the mean values were 64—76% for the biological

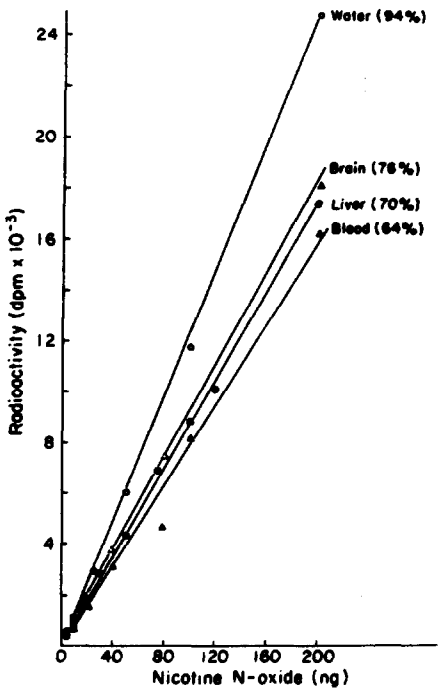


Fig. 6. Calibration curves and recoveries of [^{14}C]NNO obtained from water and biological media by application of the extraction and chromatographic methods. The mean recovery for NNO from each type of sample is shown in parentheses.

samples and 94% for the water samples. These plots were used subsequently to quantitate metabolically generated NNO in mouse tissues.

Applications of the method

Mice were injected with [^{14}C]nicotine at a dose of 1.0 mg/kg, killed at various times, and livers removed, homogenized and spiked with unlabeled NNO. These samples were extracted and analyzed for [^{14}C]NNO. The reconstructed chromatogram from a representative liver sample is compared to the chromatogram obtained with a UV detector in Fig. 7. The radioactive peak corresponds to the elution of NNO. No significant amounts of other metabolites were present in the extract. The concentrations of NNO in liver were plotted against time after injection with nicotine (Fig. 8). The maximum concentration, 80 mg/g of liver, was attained in 15 min and an elimination half-life of 15.9 min was calculated from the data. Several tissue concentrations on the elimination phase of the pharmacokinetic profile were below 20 ng/g, which emphasizes the need for a highly sensitive method of quantitation. As determined in our previous work, levels in blood and brain tissue of mice never exceeded 20 ng/g during the entire time course of the study [11]. Urine and feces were collected from six mice during a period of 20 h after nicotine administration; approximately 2.0—2.5% of the dose was excreted as NNO in urine and 0.03% in feces.

The relative amounts of *cis*- and *trans*-NNO formed from [^{14}C]nicotine in mice were determined by separation of the isomers on a PAC column. The results presented in Table III demonstrate that the amount of the *trans* isomer formed at three time points after nicotine administration was approximately two-fold greated than the *cis* isomer. The selectivity for

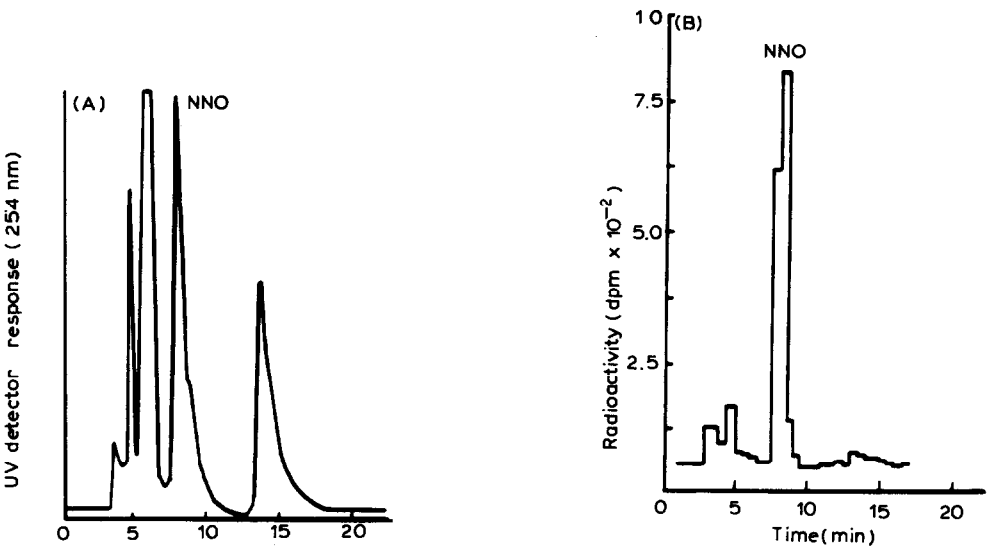


Fig. 7. (A) Chromatogram obtained by HPLC analysis with UV detection of the liver extract from a mouse injected with [^{14}C]nicotine. (B) Reconstructed chromatogram obtained by HPLC separation of the same sample with radioactivity measured in samples collected every 30 sec.

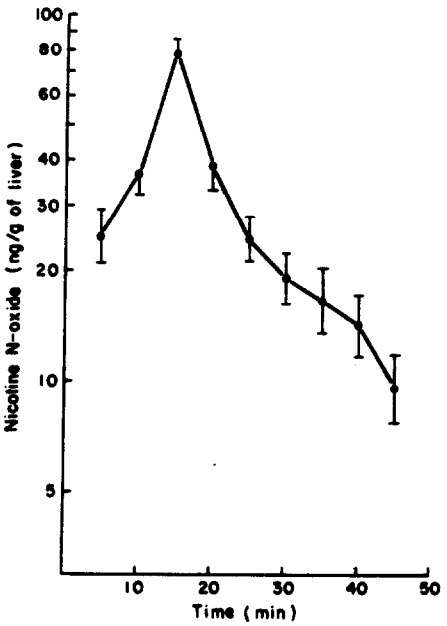


Fig. 8. Concentration of NNO in livers of DBA mice injected with nicotine versus time. Each point is the mean ± S.E. of determinations of three animals.

TABLE III

ISOMERS OF NICOTINE N-OXIDE FORMED IN MICE ($n = 3$)

Mice were injected intraperitoneally with [^{14}C] nicotine, livers removed at the times shown, and the isomers of NNO quantitated.

Time after injection (min)	Ratio of *trans/cis*-N-oxides (mean ± S.D.)
15	2.01 ± 0.08
25	1.94 ± 0.10
35	1.87 ± 0.07

formation of these products has been determined in vitro with 10 000 *g* supernatant fractions of liver from several species by paper chromatography [6]. The results show substantial species variation in the *cis/trans* ratio, but there are several factors that complicate the comparison of in vivo and in vitro data. These include the finding that the isomers of NNO are partially reduced back to nicotine at different rates by a liver supernatant fraction in the absence of oxygen [19]. The ratio determined in vivo, therefore, probably represents the net result of both the stereoselective oxidation of nicotine and the selective reduction of *cis*- and *trans*-NNO.

CONCLUSION

A method has been developed for the efficient and selective isolation of NNO from biological media. Recoveries averaged about 70% and the extracts were sufficiently pure to be analyzed by HPLC with minimal interference from

contaminants. Columns containing amino- or aminocyano-bonded stationary phases produced good chromatographic results with NNO. The method is an alternative to those used previously, which include paper chromatography [7] and chemical reduction to nicotine followed by GC [8]. With radiolabeled nicotine, the NNO produced could be quantitated at very low levels, which were limited only by the specific activity of nicotine. NNO was quantitated in mouse tissues at concentrations ranging from 5.0 to 80 ng/g of tissue [11]. For levels above approximately 30 ng/g, it should be possible to obtain accurate data by UV or electrochemical detection due to the high purity of the extracts. The data reported here and in the previous paper [11] represent the only comprehensive description of NNO pharmacokinetics in any mammalian species. Urinary excretion following nicotine administration to human subjects has been reported, but plasma levels of NNO were too low to be measured accurately [16]. The method we have developed, with minor modifications, should be readily adaptable to the analyses of other hydrophilic amine N-oxides in biological media.

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