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RADIOMETRIC–HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR NICOTINE AND TWELVE OF ITS METABOLITES

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SUMMARY

A sensitive, reproducible radiometric–high-performance liquid chromatographic assay has been developed to measure concentrations of nicotine and twelve of its metabolites in biological fluids. Following administration of nicotine ([2-^{14}C]pyrrolidine) to rats, the assay was used in a pharmacokinetic investigation.

INTRODUCTION

Nicotine metabolism is complex [1], at least eighteen metabolites having been postulated (Fig. 1). Nevertheless, most studies on nicotine pharmacokinetics [2–5] focus almost exclusively on nicotine and/or its two principal metabolites, cotinine and nicotine-1′-N-oxide. Several reports on nicotine metabolism [6–10] suggest that certain pathways other than those of cotinine and nicotine-1′-N-oxide formation contribute significantly.

To investigate nicotine pharmacokinetics, a more rapid, sensitive and reliable assay would be desirable to measure simultaneously concentrations of nicotine and its principal metabolites, as well as its so-called minor metabolites. Since no such assay for nicotine and most of its metabolites is presently available, we developed, and herein report details of, a new radiometric–high-performance liquid chromatographic (HPLC) assay for determining simultaneously concentrations of nicotine and twelve of its metabolites in biological fluids.

EXPERIMENTAL

Chemicals and reagents

Racemic *R,S*-(±)-nicotine ([2-^{14}C]pyrrolidine) stored in ethanol (0.32 mg/ml) under argon was obtained from New England Nuclear (Boston, MA,

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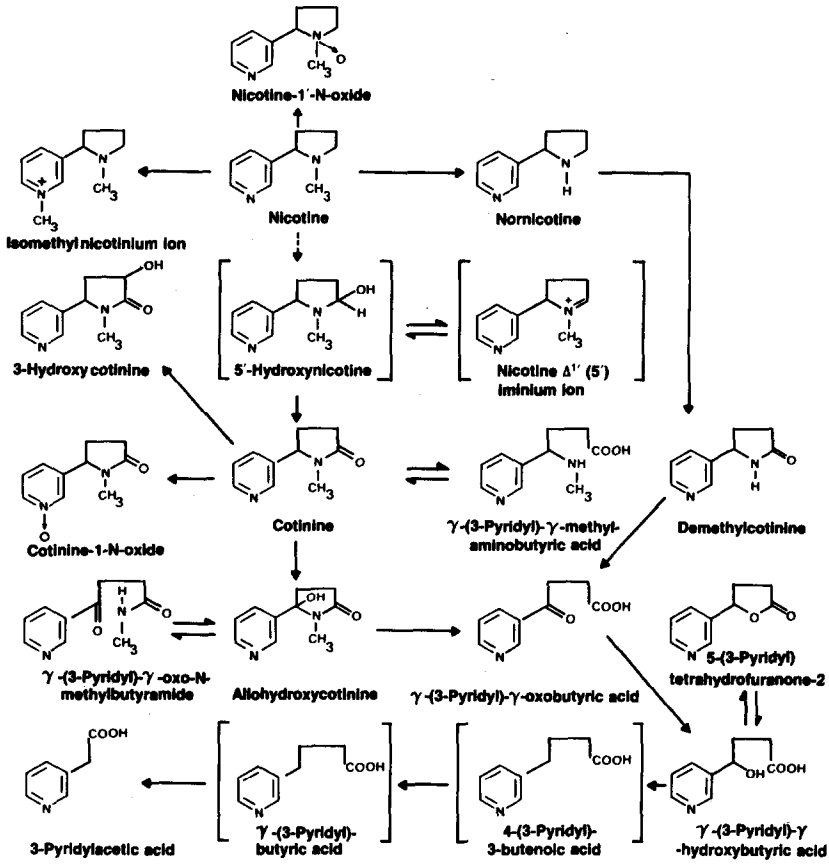


Fig. 1. Pathways of nicotine metabolism in mammals. Proposed intermediates in brackets; all others, except 5-(3-pyridyl) tetrahydrofuranone-2, have been detected as metabolites.

U.S.A.). Its specific activity was 50.2 mCi/mmol. Radiochemical purity (>97%) was established by HPLC using a Waters Resolve C_8 Radial-Pak cartridge and water–methanol–0.1 *M* acetate buffer (pH 4.0)–acetonitrile (65:29:4:2, v/v) as mobile phase. Unlabelled nicotine purchased from K & K Labs., (Plainview, NY, U.S.A.), was purified by fractional distillation under reduced pressure. Cotinine and 3-pyridylacetic acid came from Aldrich (Milwaukee, WI, U.S.A.). Demethylcotinine, γ-(3-pyridyl)-γ-oxobutyric acid, γ-(3-pyridyl)-γ-oxo-N-methylbutyramide, γ-(3-pyridyl)-γ-methylaminobutyric acid, isomethylnicotinium ion and 3-hydroxycotinine were supplied by Dr. Georg Neurath (Hamburg, F.R.G.). Nicotine-1'-N-oxide, cotinine-N-oxide, γ-(3-pyridyl)-γ-hydroxybutyric acid and cotinine methonium ion were synthesized and purities checked according to established methods [8, 10–12]. Their identities were confirmed by mass spectral and NMR analysis. Nornicotine was a gift from Dr. P.A. Crooks (University of Kentucky, Lexington, KY, U.S.A.). Donated and purchased samples of nicotine and metabolites were certified to be of high chemical purity (>98%) by Drs. Crooks and Neurath. HPLC-grade methanol and acetonitrile came from J.T. Baker (Phillipsburg, NJ, U.S.A.). Triethylamine (Sigma, St. Louis, MO, U.S.A.)

and sodium acetate (Fisher Scientific, Pittsburgh, PA, U.S.A.) were of analytical-reagent grade. All HPLC eluent buffers and solvents were filtered through 0.2-μm filter disks (Millipore, Bedford, MA, U.S.A.) and degassed in vacuo before use.

Instrumentation

All analyses were performed on a Waters (Bedford, MA, U.S.A.) liquid chromatographic system comprised of a WISP 710B autosampler, two M45 solvent delivery systems controlled by an M680 automated gradient controller and a M440 absorbance detector. Absorbance at 254 nm was monitored on a Chromatopac C-R3A data processor (Shimadzu, Columbia, MD, U.S.A.) with sensitivity set at 0.005 a.u.f.s. For plasma samples, a 250×4.5 mm, 5-μm IBM cyano RP analytical steel column (IBM Instruments, Danbury, CT, U.S.A.) was used; for urine injections, a 150×4.5 mm, 5-μm IBM Optima cyano RP cartridge was connected in series with and preceded the cyano RP steel column. A 50×4.5 mm, 5-μm IBM Optima cyano RP cartridge served as guard column for both plasma and urine injections. We used a binary gradient mobile phase of two solvent systems designated A and B. Solvent A was water–methanol–0.1 *M* acetate buffer (pH 4.0)–acetonitrile (187.5:11:1:0.5, v/v). Solvent B was water–methanol–0.5 *M* acetate buffer (pH 4.0)–acetonitrile (187.5:11:1:0.5, v/v) adjusted to pH 5.0 with triethylamine (0.10%, v/v).

Radioactivity in the HPLC effluent was monitored with an LB 505 Berthold radioactivity flow monitor system (Berthold Analytical, Nashua, NH, U.S.A.) using a 400-μl PTFE flow-cell packed with G glass scintillator beads for heterogeneous counting. The radioactivity signal was stored and integrated by an LB 510 Berthold chromatography data station. For reliable quantitations, the limit of sensitivity was set at 50 dpm above a background of 20 dpm. Counting efficiencies for nicotine and metabolites were established by comparing their radiodetector counts with dpm values of respective fractions collected and measured using a Beckman LS100 scintillation counter.

Mass spectrometry was performed with a Kratos MS 950 mass spectrometer (Kratos Analytical, Ramsey, NJ, U.S.A.) operated in the electron ionization (EI) mode. Samples were introduced by the direct inlet system. Before mass spectral analysis, nicotine and the relatively non-polar basic metabolites cotinine, demethylcotinine, nornicotine, 3-hydroxycotinine and γ-(3-pyridyl)-γ-oxo-N-methylbutyramide were extracted from urine. This extraction was performed with methylene chloride, following urine alkalinization to pH 13. Nicotine-1′-N-oxide and isomethylnicotinium ion were purified from urine by a solid-phase extraction method [2] using silica gel extraction columns (J.T. Baker). The water wash from the silica gel extraction contained cotinine-N-oxide and the polar acidic metabolites 3-pyridylacetic acid, γ-(3-pyridyl)-γ-oxobutyric acid and γ-(3-pyridyl)-γ-methylaminobutyric acid. Further purification of the polar acidic metabolite fraction was achieved by sequential elution from quaternary amine (N^+) anion-exchange extraction columns (J.T. Baker) with methanol, following a distilled water wash. Metabolite A desorbed from quaternary amine extraction columns in preliminary methanol washes. We injected aliquots of such purified

fractions of nicotine metabolites under our HPLC conditions and isolated peaks corresponding to the individual metabolites. Fractions corresponding to nicotine, cotinine, demethylcotinine, nornicotine, 3-hydroxycotinine and γ-(3-pyridyl)-γ-oxo-N-methylbutyramide were desalted from the mobile phase by reextraction with methylene chloride following alkalinization to pH 13. Other metabolite fractions were desalted by chromatography on a Waters Resolve C_8 Radial-Pak cartridge using methanol–water mixture as mobile phase. The cartridge was eluted initially with distilled water and then the methanol content of the mobile phase gradually increased to elute the radioactive peak.

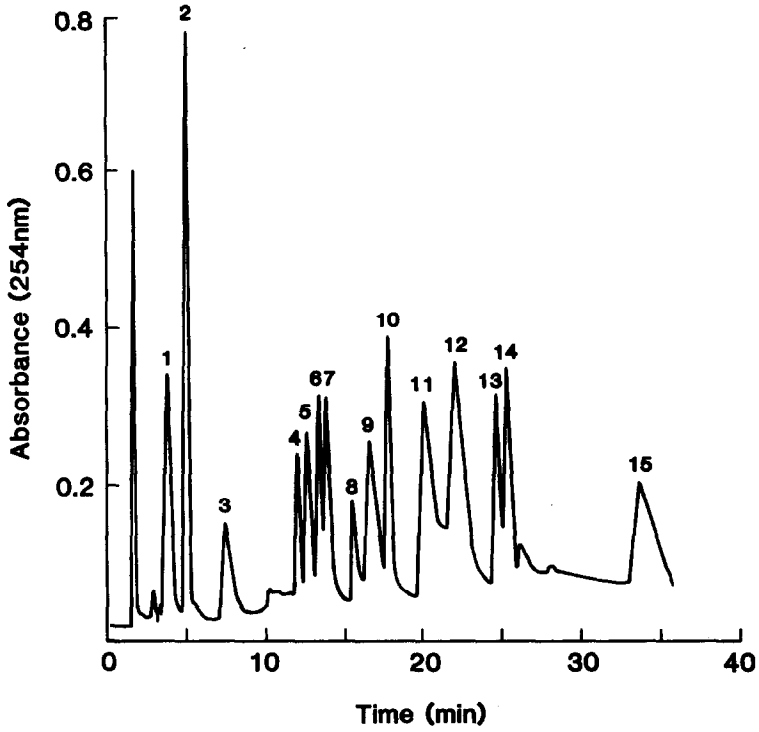
Animal experiments

Initial metabolic studies were performed on male Sprague–Dawley rats (Charles Rivers Labs., Newfield, NJ, U.S.A.) weighing 175–200 g. Animals were housed and maintained as previously described [13]. Under sodium pentobarbital (30 mg/kg intraperitoneally) anesthesia, the right common carotid and caudal arteries were cannulated [14]. Each rat was placed in an individual plastic metabolism cage (Maryland Plastics, NY, U.S.A.) and allowed to recover from surgery for 24 h. Each rat received via the caudal artery cannula a single 0.1 mg/kg dose of nicotine ([2-^{14}C]pyrrolidine) containing 5 μCi. Blood (200 μl) was drawn from the carotid artery cannula at 0.0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12 and 24 h following [^{14}C]nicotine administration. Blood samples were treated with sodium citrate and plasma fractions were stored at −20°C. Total voided urine specimens were colleted after nicotine administration at 0, 6, 12, 24, 36, 48, 72, 96 and 120 h and stored at −20°C until analysis.

Prior to injections in the liquid chromatographic system, plasma samples were treated with acetonitrile (1:1, v/v) to precipitate proteins, and urine samples were filtered through 0.22-μm Millex-GS filter units (Millipore).

RESULTS AND DISCUSSION

Fractionation of nicotine and its metabolites by the new HPLC procedure is depicted in Figs. 2 and 3. Fig. 2 shows separation by HPLC of unlabelled nicotine from authentic standards of its identified metabolites and an analogue 6-hydroxynicotine, on a 150×4.5 mm, 5-μm IBM cyano RP steel column. For this separation, the solvent program consisted of an initial 10-min linear gradient from 98% solvent A and 2% solvent B to 75% A and 25% B, run at a flow-rate of 1 ml/min. A stepwise modification to 50% A and 50% B was performed here and maintained for 12 min. At 22 min, the program was changed to 100% A and run at 2 ml/min for 3 min. From 25 to 30 min, 100% B was run at 2 ml/min. Initial conditions were then reestablished by equilibrating the column with 98% A and 2% B, initially at a flow-rate of 2 ml/min for 8 min and finally at 1 ml/min for 2 min. The rather unusual step at 22 min was necessary to afford separation of nornicotine from nicotine (Fig. 2). This solvent program for unextracted standards also served to determine nicotine and metabolite(s) in plasma. However, in urine, resolution of nicotine from its metabolites was inadequate with the above solvent program using the cyano RP steel column by itself. To optimize separa-



Peak No.	Retention Time (min)	Peak Identity	Amount in injection (μg)
1	4.1	cotinine-N-oxide	0.012
2	5.7	3-pyridylacetic acid	0.020
3	7.7	γ-(3-pyridyl)-γ-oxobutyric acid	0.030
4	12.1	γ-(3-pyridyl)-γ-hydroxybutyric acid	0.180
5	12.7	3-hydroxycotinine	0.030
6	13.4	γ-(3-pyridyl)-γ-methylaminobutyric acid	0.100
7	13.9	γ-(3-pyridyl)-γ-oxo-N-methylbutyramide	0.030
8	15.5	demethylcotinine	0.020
9	16.6	cotinine	0.005
10	17.7	6-hydroxynicotine	0.1650
11	19.9	cotinine methonium ion	0.200
12	22.0	nicotine-1'-N-oxide	0.030
13	24.5	nornicotine	0.080
14	25.2	nicotine	0.080
15	33.4	isomethylnicotinium ion	0.240

Fig. 2. HPLC separation of unlabelled standards of nicotine and metabolites on 250×4.5 mm 5-μm IBM cyano RP steel column. See text for details of solvent program.

tions of nicotine from its metabolites in urine, it was necessary to connect an additional cyano RP cartridge in series with the cyano RP steel column and modify slightly the above gradient program. The solvent conditions up to 22 min were maintained as above. After 22 min, 100% B was directly run at 1 ml/min for 8 min and then slowed to 0.7 ml/min for 15 min. Column reequilibration (98% A and 2% B) was thus started at 45 min, maintained at 1.5 ml/min for 8 min and finally for 2 min at 2 ml/min. Fig. 3 is a typical radiochromatogram illustrating the metabolic disposition of [^{14}C]nicotine in a 6–12 h urine sample obtained from a rat that received a single intraarterial dose of nicotine ([2-^{14}C]pyrrolidine), 0.1 mg/kg.

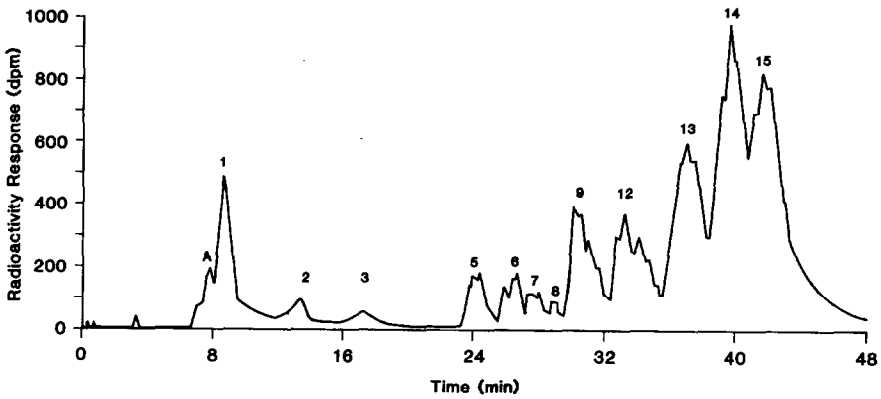


Fig. 3. HPLC separation of [^{14}C]nicotine and metabolites in a 50-μl injection of 6–12 h urine sample from a rat administered nicotine ([2-^{14}C]pyrrolidine), 0.1 mg/kg. See text and Fig. 2 for solvent program details and key to individual metabolites.

Previously we reported a novel HPLC analysis of nicotine and its two major metabolites, cotinine and nicotine-1′-N-oxide [2]. It was possible, using a C_{18} reversed-phase column to separate these three compounds under isocratic conditions with a water–methanol–acetate buffer–acetonitrile system as mobile phase [2]. Our rationale in this present attempt to separate additional nicotine metabolites from each other was to explore, using a chromatographic system similar or close to the previously reported conditions, with which we were familiar and had achieved success [2]. The shorter retention times of the more polar metabolites of nicotine on the C_{18} column rendered our previously used system(s) inappropriate for separating these metabolites from one another. Slight changes of the mobile phase, however, indicated that the retention of these metabolites could be prolonged sufficiently to improve their separation on reversed-stationary phases, such as the C_{18}, C_8 and cyano RP columns. Of these three reversed stationary phases, the cyano RP column yielded the best peak characteristics for the metabolites.

To optimize metabolite separation on this column, we measured how individual metabolite retentions were affected by the individual variables comprising the water–methanol–acetate buffer–acetonitrile system. These variables included pH, ionic strength and solvent composition of the mobile phase. Figs. 4–7 depict effects of these several physiocochemical variables on the retention capacity (k') of nicotine and its metabolites on the cyano reversed-phase column. Our mobile phase components exerted complex effects on individual retentions of nicotine and its metabolites. This complexity was expected because nicotine metabolites range widely in polarity and charge [4, 9, 15, 16]. These studies served to identify optimum conditions for our assay.

Retention times of nicotine and its metabolites, with the exception of cotinine-N-oxide, were shortened as the mobile phase pH was increased from 4.1 (pH of solvent A) to 5.0 (pH of solvent B) (Fig. 4). For the acidic metabolites, enhanced ionization probably accounts for decreasing retention on the reversed-phase col-

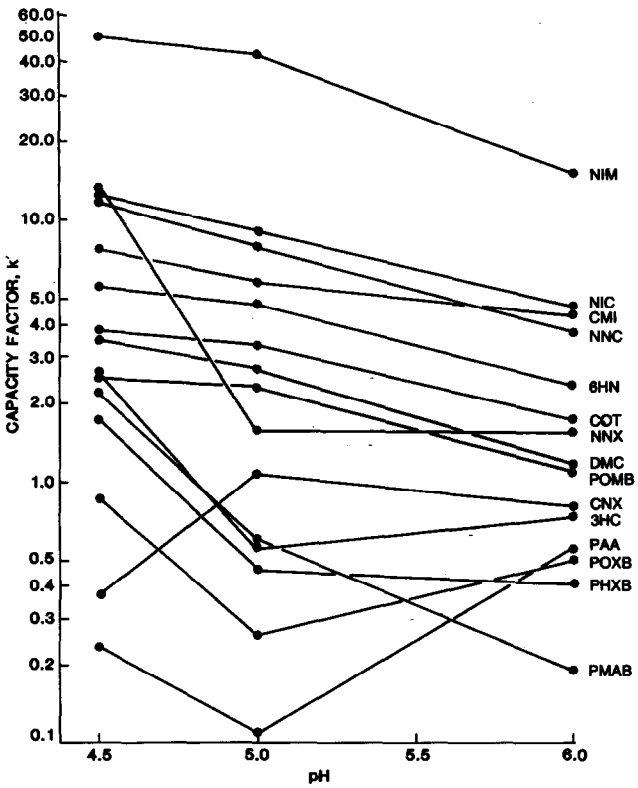


Fig. 4. Effect of changes in mobile phase pH on retention (k' values) of nicotine and metabolites on 250×4.5 mm, 5-μm IBM cyano RP steel column. Mobile phase was water–methanol–0.1 *M* acetate buffer (pH 4.0)–acetonitrile (187.5:11:1:0.5, v/v), adjusted to different pH values with triethylamine. Triethylamine compositions (%, v/v) of mobile phase were 0.03, 0.07 and 0.12 at pH values of 4.5, 5.0 and 6.0, respectively. The mobile phase was run under isocratic conditions at a flow-rate of 2 ml/min. Key: NIM, isomethylnicotinium ion; NIC, nicotine; CMI, cotinine methonium ion; NNC, nornicotine; 6HN, 6-hydroxynicotine; COT, cotinine; NNX, nicotine-1′-N-oxide; DMC, demethylcotinine; POMB, γ-(3-pyridyl)-γ-oxo-N-methylbutyramide; CNX, cotinine-N-oxide; 3HC, 3-hydroxycotinine; PAA, 3-pyridylacetic acid; POXB, γ-(3-pyridyl)-γ-oxobutyric acid; PHXB, γ-(3-pyridyl)-γ-hydroxybutyric acid; PMAB, γ-(3-pyridyl)-γ-methylaminobutyric acid.

umn at higher pH. In contrast, for the basic metabolites, shortened retention occurring with increasing mobile phase pH probably results from displacement by triethylamine of the more weakly basic nicotine metabolites from the polar cyano column. This property [15] led us to use triethylamine to modify the pH of the mobile phase.

Retention times of most nicotine metabolites on the column were shortened as ionic strength of the acetate buffer in the mobile phase was increased from 0.1 *M* (as in solvent A) to 0.5 *M* (as in solvent B) (Fig. 5). Methanol and acetonitrile concentrations apparently had less consistent effects on retention of nicotine and its metabolites (Figs. 6 and 7). Optimal separation of the metabolites was achieved with methanol and acetonitrile concentrations of 5.5 and 0.25%, respectively, selected for both solvent A and solvent B. Thus, to obtain the separations illus-

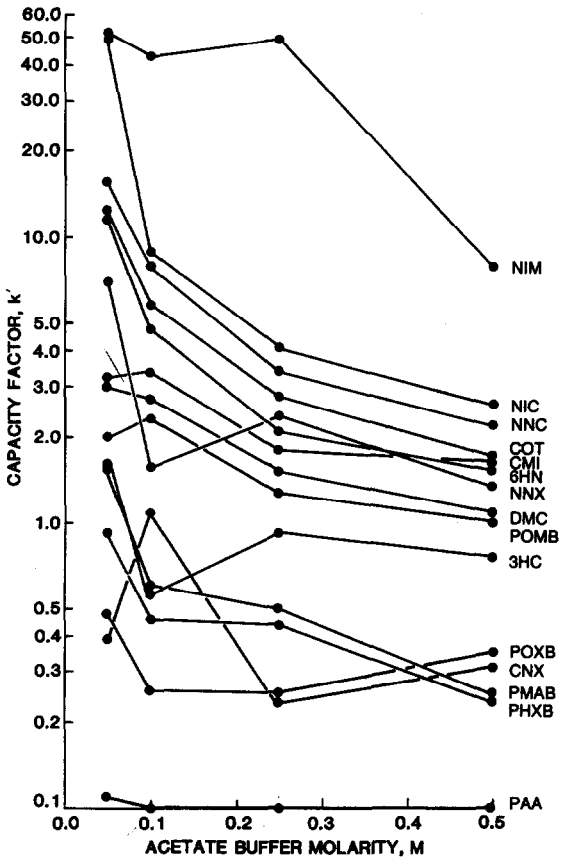


Fig. 5. Effect of changes in acetate buffer molarity on retention (k' values) of nicotine and metabolites on 250×4.5 mm, 5-μm IBM cyano RP steel column. Mobile phase was water–methanol–acetate buffer (variable ionic strengths, pH 4.0)-acetonitrile (187.5:11:1:0.5, v/v), adjusted to pH 5.0 with triethylamine. Acetate buffer molarities applied were 0.05, 0.1, 0.25 and 0.5 M and all were adjusted to pH 4.0 before inclusion in mobile phase. Mobile phase was run under isocratic conditions at a flow-rate of 2 ml/min. See Fig. 4 for key.

trated in Figs. 1 and 2, we altered pH and ionic strength of the mobile phase during the gradient run. Figs. 4–7 also serve as valuable guides to optimize conditions for measuring nicotine and metabolites on older or different-brand cyano columns.

Correlation of results obtained by this new HPLC method with radiometric thin-layer chromatographic (TLC) methods for nicotine, cotinine and nicotine-1′-N-oxide [6,7,11] was high (r=0.93, 0.90 and 0.91 for nicotine, cotinine and nicotine-1′-N-oxide, respectively).

Recovery of total ^{14}C-label derived from nicotine and metabolites from the HPLC cyano steel column and cartridge was 98±2% (n=5). This high value for sample recovery was estimated by comparing the amount of total radioactivity eluted with the known amount of radioactivity injected from a urine sample obtained from a rat administered nicotine ([2-^{14}C]pyrrolidine). Recovery of total

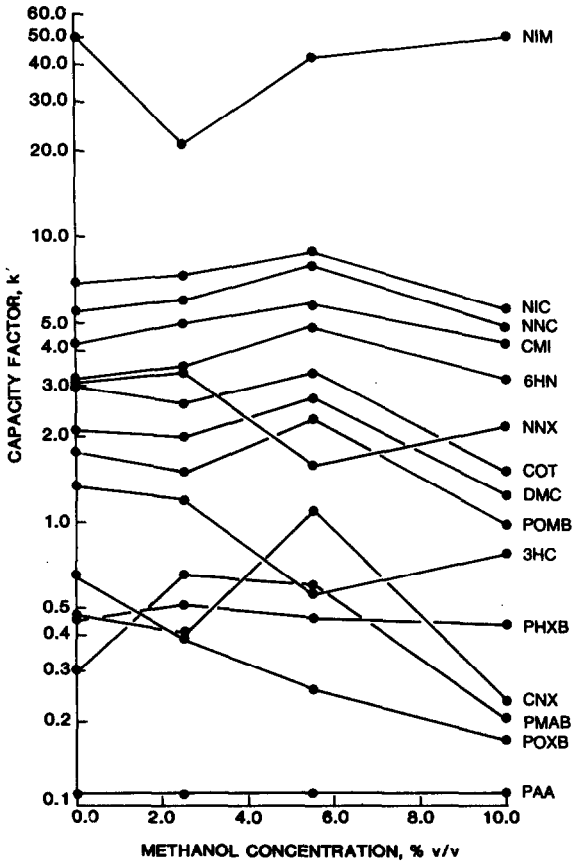


Fig. 6. Effect of changes in mobile phase methanol concentrations (0.0, 2.5, 5.5 and 10.0%, v/v) on retention (k' values) of nicotine and metabolites on 250×4.5 mm, 5-μm cyano RP steel column. Mobile phase was water–methanol–0.1 *M* acetate buffer (pH 4.0)–acetonitrile. Compositions (v/v, %) of acetate buffer and acetonitrile were fixed at 0.5 and 0.25, respectively. Water content in mobile phase was inversely adjusted to compensate for increasing methanol concentrations. Mobile phase pH, at each different methanol concentration, was adjusted to 5.0 with triethylamine before running isocratically at a flow-rate of 2 ml/min. See Fig. 4 for key.

urinary ^{14}C from the preliminary filtration procedure was 99±1% (n=5). Recoveries of [^{14}C]nicotine, cotinine and total ^{14}C from plasma by the acetonitrile precipitation method were 89±5, 92±3 and 83±6% (n=4), respectively.

Within-day precision of the assay was assessed by analyzing in a single day five aliquots of a single pooled 48-h urine sample. Calculated within-assay coefficients of variation for nicotine and metabolites appear in Table I. Inter-day variations between replicate assays for nicotine and metabolites from the same 48-h urine sample measured daily for five days are also shown in Table I. Within-assay coefficients of variation for plasma nicotine and cotinine determinations were 6.2 and 7.5%, respectively. Inter-day precisions were estimated as 8.9 and 9.8% for plasma nicotine and cotinine, respectively. Both within-assay and inter-day precision

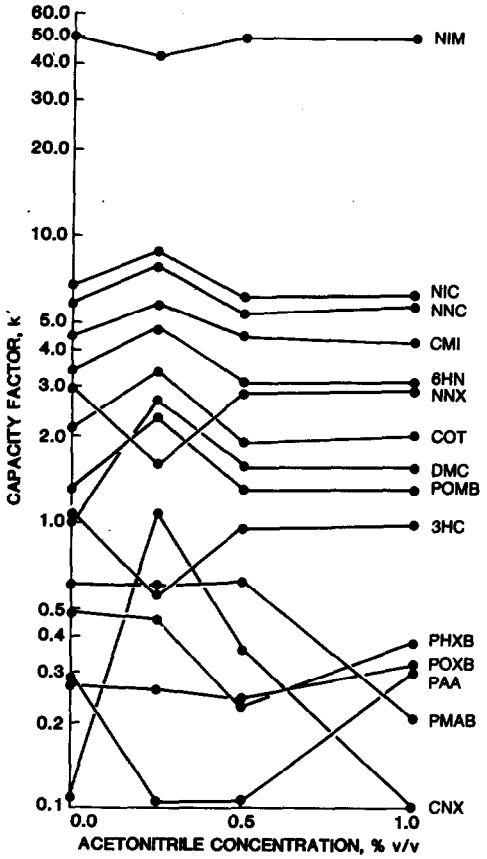


Fig. 7. Effect of changes in mobile phase acetonitrile concentrations (0.0, 0.25, 0.5 and 1.0%, v/v) on retention (k' values) of nicotine and metabolites on 250×4.5 mm, 5-μm IBM cyano RP steel column. Mobile phase and conditions were as for Fig. 6 except acetate buffer and methanol compositions were fixed at 0.5 and 5.5% (v/v), respectively while water content was adjusted inversely to compensate for increasing acetonitrile concentrations. See Fig. 4 for key.

estimates for nicotine and metabolites in urine and plasma are low and within accepted limits.

Specificity of the assay for nicotine and metabolites was intially verified by peak enrichment with authentic external standards on both IBM cyano RP columns and a Waters Resolve C_8 Radial-Pak cartridge. Identities of radiolabelled metabolites have been subsequently confirmed by mass spectral analysis. Metabolite A from its mass spectrum appears to be allohydroxydemethylcotinine.

These assay characteristics of our radiometric HPLC procedure demonstrate its sensitivity, rapidity, reliability and suitability for pharmacokinetic studies. Thus, in a preliminary application, this new assay was used to investigate pharmacokinetic profiles of nicotine and its metabolites in the rat (Fig. 8, Table II).

Radioactivity due to nicotine and cotinine was detected in substantial amounts in plasma samples from rats administered nicotine ([2-^{14}C]pyrrolidine), 0.1 mg/kg. Nicotine disappearance was biexponential (Fig. 8), with an elimination

TABLE I

COEFFICIENTS OF VARIATION FOR RADIOMETRIC-HPLC DETERMINATIONS OF NICOTINE AND METABOLITE LEVELS IN RAT URINE

Compound	Coefficient of variation (%)	
	Within-assay ($n=5$)	Inter-day ($n=5$)
Nicotine	5.1	9.4
Cotinine	7.8	9.1
Nicotine-1'-N-oxide	4.4	4.6
Cotinine-N-oxide	6.3	8.6
3-Pyridylacetic acid	5.4	7.0
γ-(3-Pyridyl)-γ-oxobutyric acid	3.9	4.9
3-Hydroxycotinine	7.2	9.9
γ-(3-Pyridyl)methylaminobutyric acid	5.2	9.0
Nornicotine	2.7	10.7
Demethylcotinine	8.2	12.5
γ-(3-Pyridyl)-γ-oxo-N-methylbutyramide	4.1	6.2
Isomethylnicotinium ion	5.8	17.3
Metabolite A	3.6	5.2

half-life (mean ± S.E.) of 1.0 ± 0.1 h. Cotinine appeared as the major metabolite in plasma and had a longer elimination half-life, 5.2 ± 0.4 h (Fig. 8). These results agree with earlier reports [5,17], further validating our assay. Nicotine-1'-N-

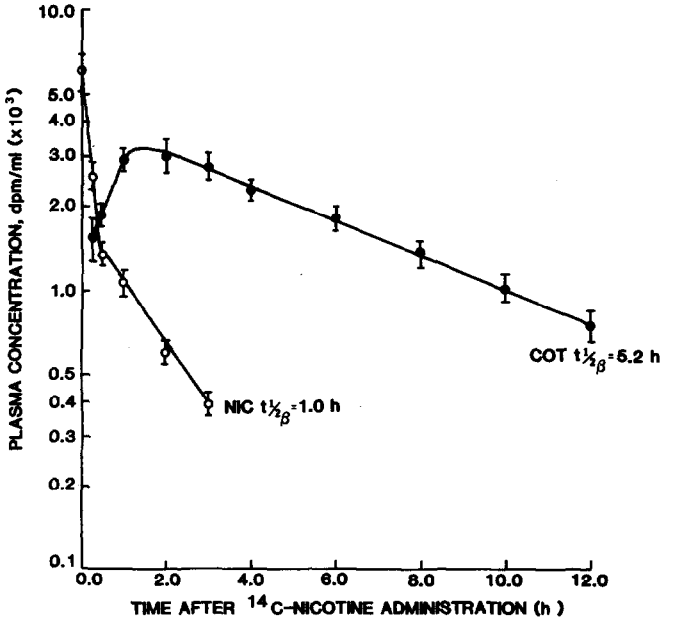


Fig. 8. Semilogarithmic plots of nicotine (○) and cotinine (●) plasma concentrations (mean ± S.E.) as a function of time following administration of nicotine ([2-^{14}C]pyrrolidine), 0.1 mg/kg.

TABLE II

96-h URINARY EXCRETION OF NICOTINE AND METABOLITES IN RATS FOLLOWING SINGLE INTRAARTERIAL DOSE OF NICOTINE ([2-^{14}C]PYRROLIDINE), 0.1 mg/kg

Compound	Recovery of administered radioactivity (mean ± S.E., $n=5$) (%)
Nicotine	11.3 ± 0.8
Cotinine	7.2 ± 0.9
Nicotine-1'-N-oxide	11.6 ± 0.9
Cotinine-N-oxide	9.3 ± 0.9
3-Pyridylacetic acid	2.2 ± 0.3
γ-(3-Pyridyl)-γ-oxobutyric acid	1.8 ± 0.1
3-Hydroxycotinine	4.5 ± 0.6
γ-(3-Pyridyl)-γ-methylaminobutyric acid	4.4 ± 0.5
Nornicotine	8.9 ± 0.9
Demethylcotinine	1.0 ± 0.1
γ-(3-Pyridyl)-γ-oxo-N-methylbutyramide	2.0 ± 0.1
Isomethylnicotinium ion	2.7 ± 0.3
Metabolite A	3.1 ± 0.4

oxide, cotinine-N-oxide and γ-(3-pyridyl)-γ-methylaminobutyric acid were detectable only in trace amounts in plasma.

HPLC profiles of metabolic disposition of nicotine in rat urine following [^{14}C]nicotine administration (Fig. 3) demonstrate extensive metabolism of nicotine to at least twelve different metabolites. γ-(3-Pyridyl)-γ-hydroxybutyric acid and cotinine methonium ion, which would have appeared at 22 and 32 min in the chromatographic run, respectively (Fig. 3), were not detected in any urine sample collected. Data in Table II show recoveries (%) of each metabolite from the total radioactivity administered. In rats cotinine is apparently not the major urinary metabolite of nicotine.

HPLC facilitated separation of numerous nicotine metabolites (Figs. 2 and 3), including the pool of previously unidentified polar metabolites first described as origin activity by Adir et al. [5]. Their radiometric TLC method failed to separate these polar metabolites from one another. Generally, HPLC methodology appears to have several advantages, such as improved resolution and increased sensitivity, over older conventional TLC methods for separating metabolites from parent drugs. Radiometric–HPLC methods have been applied extensively to investigate the kinetics and metabolism of many drugs, including oxazepam [18], quazepam [19], epichlorhydrin [20], caffeine [21] and oxyprenolol [22]. Recently, a high-performance cation-exchange chromatographic method was developed for determining largely N-methylated metabolites, as well as three oxidative metabolites, of nicotine [15,23]. Although selective for the N-methylated quaternary metabolites, the Partisil-10 SCX cation-exchange column used seemed not well suited for extended separation of additional nicotine metabolites as nearly 70% of the radioactivity in urine samples injected on the column coeluted with

cotinine in the void volume [15]. The cyano reversed-phase column we used was more versatile in separating most nicotine metabolites.

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