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ANALYSES OF NICOTINE AND COTININE IN TISSUES BY CAPILLARY GAS CHROMATOGRAPHY AND GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

Selective extraction and chromatographic techniques have been developed to measure low nanogram quantities of nicotine and cotinine in tissues. Analyses were performed by capillary column gas chromatography with a specific nitrogen—phosphorus detector and by gas chromatography—mass spectrometry. With close structural analogues for internal standards, high quantitative accuracy and precision were demonstrated for the range 5—1000 ng per g of tissue. The sensitivity limit was 2—3 ng/g for both compounds. The main advantage of these techniques compared to previously published methods is increased selectivity; the other methods were developed for analysis of biological fluids and are not readily adaptable to more complex biological matrices such as tissue homogenates. With the newly developed techniques, we were able to perform a pharmacokinetic study of nicotine and cotinine in mouse liver following a single intraperitoneal injection of nicotine.

INTRODUCTION

Owing to the widespread use of tobacco products, it is essential to acquire a thorough understanding of the pharmacological properties of nicotine, an important constituent of tobacco smoke [1]. To correlate specific responses with the presence of nicotine in various tissues of intact animals, the concentrations of this compound must be accurately determined. The analytical techniques presently available for the quantitation of nicotine in biological samples have been developed for analyses of blood and urine; the most sensitive and selective of these involve extraction followed by quantitation with gas chromatography utilizing a specific nitrogen—phosphorus detector (GC—NPD) [2—5] or with gas chromatography—mass spectrometry (GC—MS) [6, 7]. Similar techniques have

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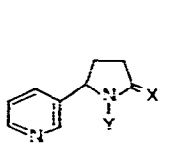
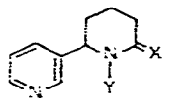


X = H,H
Y = H nornicotine
CH$_3$ nicotine
CD$_3$ NIC-d$_3$
X = O
Y = H norcotinine
CH$_3$ cotinine
CD$_3$ COT-d$_3$



X = H,H
Y = H anabasine
CH$_3$ MA
X = O
Y = CH$_3$ OMA

Fig. 1. Structures of compounds cited in the text.

been employed to quantitate cotinine (Fig. 1), a major metabolite of nicotine [5, 8]. Radioimmunoassay methods also have been developed to measure these compounds [9, 10], but because the specific antibodies are not readily available, chromatographic techniques are more generally useful for these analyses than immunochemical techniques. Our attempts to adapt published procedures to the quantitation of nicotine and cotinine at low nanogram levels in liver and brain tissues from mice were unsuccessful because of the simultaneous extraction of compounds which interfere with the analyses.

Tissue homogenates are more complex biological matrices than physiological fluids, requiring analytical methods which combine selective and efficient extractions with selective and sensitive chromatographic techniques. The present report describes the development and application of methods to quantitate low levels of nicotine and cotinine in tissue samples. Extraction schemes were developed to provide maximum recoveries of these compounds with minimum contamination of the extracts by interfering compounds. These methods, together with improved chromatographic and mass spectrometric techniques, allowed reliable quantitative analyses of nicotine and cotinine in tissue homogenates with a demonstrated sensitivity limit of 2—3 ng per g of tissue. The methods were applied to a pharmacokinetic investigation of nicotine and cotinine in mouse liver following a single intraperitoneal injection of nicotine.

EXPERIMENTAL

Reagents and standards

Nicotine (Sigma, St. Louis, MO, U.S.A.) was purified by fractional distillation under reduced pressure and stored at −20°C. A deuterated analogue, 1′-trideuteromethyl-nornicotine (NIC-d$_3$, Fig. 1), was prepared by the N-alkylation of 50 mg of nornicotine (Dr. N. Castagnoli, University of California, San Francisco, CA, U.S.A.) using sodium hydride and iodomethane-d$_3$ (99.5 atom % deuterium, Merck, St. Louis, MO, U.S.A.) in a manner which is analogous to a previously published procedure [11]. Methylanabasine (MA) was prepared similarly by the N-methylation of anabasine (Tridom Chemical, Hauppauge, NY, U.S.A.) with methyl iodide. Nicotine was oxidized to cotinine as described [12]. The internal standards 1′-trideuteromethyl-norcotinine (COT-d$_3$) and 1-methyl-6-(3-pyridyl)-2-piperidone (OMA, Fig. 1) also were prepared by oxida-

tion of NIC-d_3 or MA, respectively, under similar conditions. All synthetic products were purified by preparative thin-layer chromatographic plates (Analtech, Newark, DE, U.S.A.) and a solvent system ethyl acetate—methanol—ammonium hydroxide (85:10:5 for NIC-d_3 and MA; 70:25:5 for cotinine and OMA). The purified compounds were analyzed by GC—MS. Ethyl acetate (reagent grade, J.T. Baker, Phillipsburg, NJ, U.S.A.) was used without further purification, but toluene (reagent grade, J.T. Baker) was redistilled. Ammonium carbonate (HPLC grade, J.T. Baker) was pulverized with a mortar and pestle and washed twice by stirring with ethyl acetate. The solutions 4 *N* sodium hydroxide, 0.2 *N* sodium acetate (at pH 9.5) and 0.1 *N* hydrochloric acid were purified by extraction with ethyl acetate. Water was purified by ion exchange and charcoal filtration (Continental Water Systems, El Paso, TX, U.S.A.) followed by distillation.

Extraction of nicotine

The extraction scheme is shown in Fig. 2. Homogenates of mouse tissues were prepared by adding 4 ml of water to 1 g of liver or a whole brain (average weight about 0.5 g) in a motor-driven PTFE and glass homogenization apparatus. All samples were treated with 300 μl of 4 *N* sodium hydroxide immediately after adding the water to ensure that no further metabolism of nicotine occurred. After homogenization, 250 mg of MA dissolved in 100 μl of ethyl acetate were added as an internal standard for quantitation. The samples were extracted with 6 ml of ethyl acetate by vortexing for 20 sec and additional mechanical mixing for 20 min. The phases were separated by centrifugation at 1500 *g* for 5 min, the organic phase was transferred to a clean tube with a disposable pipet, and the homogenate was reextracted with 2 ml of 0.2 *N* acetate. The combined organic fractions were extracted with 2 ml of 0.2 *N* acetate buffer at pH 4.8 by vortexing for 2 min. The phases were separated by centrifugation and the organic phase was reextracted with 2 ml of the acetate buffer. The combined aqueous phases were saturated with powdered ammonium carbonate and extracted with 3 ml of toluene by vortexing for 3 min. The samples were centrifuged and the aqueous phase was reextracted with 3 ml of toluene. The combined toluene fractions were concentrated to approximately 100 μl by evaporation at 30—40°C under a gentle stream of prepurified nitrogen. The tubes were removed from the evaporation apparatus twice during the concentration procedure and vortexed to redissolve any nicotine which might have adhered to the glass surface. All glassware used in the extraction procedure was either disposable or thoroughly washed with soap and water, soaked in a cleaning solution (Contrad 70, Scientific Products, Houston, TX, U.S.A.) overnight, extensively rinsed with water and dried in an oven at 110°C.

Extraction of cotinine

The procedure developed for the extraction of cotinine from liver, brain and blood samples differed from the procedure for nicotine extractions in a few details. The internal standard added to each homogenate was 150 mg of COT-d_3 dissolved in 100 μl of ethyl acetate. Tissue homogenates were saturated with ammonium carbonate instead of adding sodium hydroxide (step A, Fig. 2). The back-extraction (step E) was performed with 0.1 *N* hydrochloric acid and the

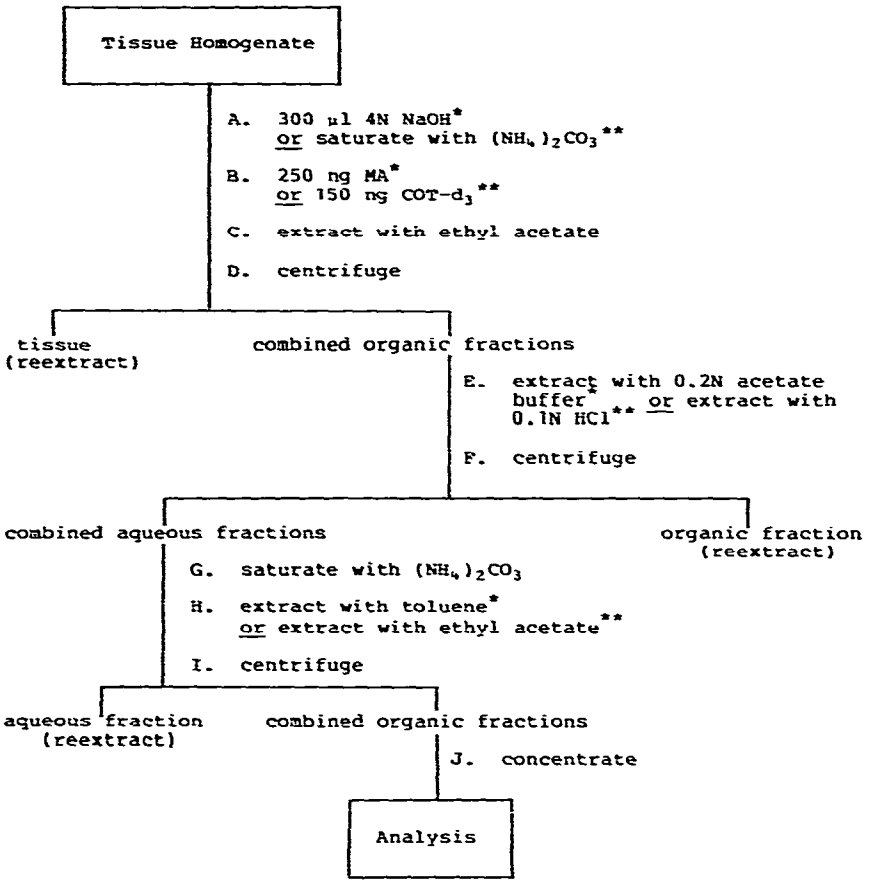


Fig. 2. Scheme for the extractions of nicotine and cotinine from tissue homogenates; (*) denotes procedure for nicotine extraction, (**) denotes procedure for cotinine extraction.

final extraction with ethyl acetate instead of toluene (step H). Finally, the extracts were evaporated to 20—40 µl prior to analysis.

Instrumentation

The gas chromatograph was a Hewlett-Packard Model 5710A equipped with an NPD and a Model 18740B capillary column controller. Analyses were performed on a 12 m × 0.2 mm I.D. fused silica capillary column which had been deactivated with Carbowax 20M and coated with a dimethyl silicone liquid phase (Hewlett-Packard). Peak areas were determined by automatic integration with a Spectra Physics Model 4100 computing integrator. Analyses also were performed with a Hewlett-Packard Model 5984A GC—MS—data system operated in both electron impact (EI) and chemical ionization (CI) modes. A 1.8 m × 2 mm I.D. glass column, packed with 3% OV-22 on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.) was interfaced to the mass spectrometer with a glass jet separator (for EI work) or with a direct line (for CI work). Helium carrier gas flow-rates were 30 or 12 ml/min, respectively.

Analyses of nicotine and cotinine

The capillary GC—NPD analyses of nicotine were performed using splitless injection techniques and a fused silica injection port liner. The injection port was maintained at 250°C, the detector at 300°C, and the column was programmed from 80—150°C at 4°C/min. Analyses of cotinine by GC—NPD were performed under identical conditions, except that the column temperature was programmed from 120—200°C at 4°C/min. Determinations of nicotine by GC—MS were carried out with the injection port at 250°C, the column 170°C, the transfer line at 200°C and the ion source at 150°C. The mass spectrometer was operated in the EI mode at 70 eV ionizing energy and with selected ions monitored at m/z 133 (nicotine) and 136 (NIC-d_3) with 100 msec dwell times. Cotinine was determined by GC—MS with the injection port at 250°C, the GC column programmed from 240—270°C at 8°C/min, the transfer line at 250°C and the ion source at 180°C. The mass spectrometer was operated in the CI mode by introducing isobutane as the reagent gas via the direct insertion probe to a source pressure of approximately 1 Torr. Ions were monitored at m/z 177 (cotinine) and 180 (COT-d_3). Peak areas were measured by the standard system software.

Nicotine and cotinine in mouse liver

Male C3H mice (60 days of age) were obtained from the Institute for Behavioral Genetics of the University of Colorado. Nicotine dissolved in normal saline was injected with an intraperitoneal dose of 1 mg/kg of body weight. Control mice were injected with saline only. The animals were killed by cervical dislocation at various times after injection. Livers were removed rapidly, homogenized, and nicotine and cotinine quantitated as described above. Nicotine and cotinine half-lives were calculated from the linear portions of semilogarithmic plots of tissue concentration vs. time as described [13].

RESULTS AND DISCUSSION

Isolation of nicotine from tissue

With ^{14}C-labeled nicotine, it was determined that the precipitation of tissue with a strong acid prior to extraction step C (Fig. 2) resulted in up to 30% losses of nicotine because of tissue adsorption. For this reason, all extractions were performed on whole homogenates. The formation of homogenate—solvent emulsions, a problem with many solvents, did not occur when ethyl acetate was used as the extraction solvent with homogenates maintained at a pH > 10. More than 90% of the ^{14}C-labeled nicotine could be recovered from liver homogenates after two extractions under these conditions. Back-extraction (step E) was necessary to clean up the sample. This was carried out with a weakly acidic buffer so that the extraction would be selective for those compounds which are sufficiently strong bases (such as nicotine, pK_a 7.9 [14]) to be converted to water-soluble conjugate acids. Nicotine was transferred back into an organic solvent (step H) by saturating the aqueous phase with ammonium carbonate and extracting with toluene. The ammonium carbonate served two purposes: to adjust the pH to about 9.2 and to increase the extraction efficiency due to a salting-out effect. Toluene was chosen for this extraction be-

cause the recoveries of nicotine were high, and the simultaneous extraction of more polar compounds which might interfere with the GC analyses was minimized. In addition, the relatively high boiling point of this solvent (111°C) facilitated capillary GC analysis by splitless injection techniques, for which the GC column must be maintained at a sufficiently low temperature during sample injection to produce a chromatographic solvent effect [15]. Unlike chlorinated solvents [16], toluene is compatible with the nitrogen—phosphorus detector, producing a very small response without degrading its performance.

The final concentration step (J) must be performed carefully so that the volume is not reduced to less than 50—100 μl, otherwise substantial losses of nicotine can occur probably due to volatilization. The overall recoveries of nicotine were determined by adding 250 mg to liver homogenates (equivalent to 1 g of liver), extracting nicotine by the methods described, and adding the standard MA to the concentrated samples immediately before GC analysis. The results were compared to the nicotine/MA peak area ratios obtained with standard solutions; recoveries were 81 ± 9% (mean ± S.D., n = 6).

Isolation of cotinine from tissues

Cotinine is more polar and water-soluble than nicotine, thereby requiring more rigorous extraction conditions for its efficient recovery from tissue homogenates. The first extraction step (Fig. 2) was performed after saturating the homogenate with ammonium carbonate to basify the aqueous phase and salt-out cotinine. Because cotinine is a weaker base than nicotine, the back-extraction (step E) was carried out with a strong acid. In the last extraction (step H), ethyl acetate was substituted for toluene, as cotinine was not efficiently extracted by the latter solvent. Due to the lower volatility of cotinine compared to nicotine, the volume of the final extract could be reduced to about 10 μl with no significant loss of the compound. Overall recoveries were determined, as for nicotine, by extracting cotinine from spiked liver homogenates and adding the standard (COT-d_3) to the concentrated sample immediately before analysis. Recoveries were 87 ± 7% (mean ± S.D., n = 5).

GC—NPD analyses of nicotine and cotinine

Previous methods for the GC analyses of nicotine and cotinine were developed with standard packed columns [2—5]. Capillary columns, however, provide higher analytical sensitivity and selectivity because of narrower peak shapes, higher resolution and reproducible retention times. Mouse liver extracts were analyzed by GC—NPD techniques with a dimethyl silicone-coated fused silica capillary column and splitless injection; the chromatograms are shown in Fig. 3. The excellent peak shapes for nicotine and MA are apparent, as well as the highly consistent retention times of these compounds. The inherent GC—NPD sensitivity limit was determined with standard solutions of nicotine to be approximately 50 pg injected on column. Fig. 3, however, shows that extracts of liver homogenates from animals not treated with nicotine contained 1—2 ng of the compound. The identity of this peak was verified by GC—MS methods. The materials used for extraction were highly purified; extractions of water in place of tissue samples demonstrated that most of the background nicotine was from tissue and not from contaminated reagents or solvents. It was, of course,

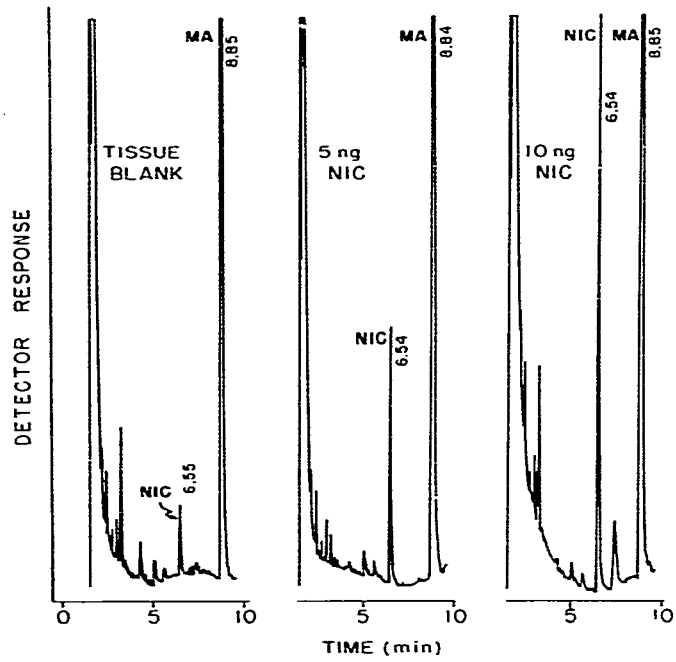


Fig. 3. Analyses of tissue extracts by capillary GC-NPD. Mouse liver homogenates were unspiked or spiked with 5 or 10 ng of nicotine (NIC) along with 250 ng of methylanabasine (MA) and extracted as described in the text.

also necessary to exclude all tobacco smoking from the laboratory and from the animal quarters. When mice were placed in wire cages without bedding, and food and water withdrawn 12 h before sacrificing them, the background levels of nicotine dropped substantially to less than 1 ng per g of liver. Because of this background nicotine in animals housed and fed normally, the effective sensitivity limit for determinations of injected nicotine was 2—3 ng per g of tissue.

The internal standard MA was chosen for GC quantitation because of its similarity to nicotine with regard to pK_a and solubility. A standard calibration curve was constructed with data obtained by analyzing mouse liver homoge-

TABLE I

STANDARD CALIBRATION CURVES FOR THE QUANTITATION OF NICOTINE AND COTININE IN LIVER HOMOGENATES

n = 5 at each concentration.

	Method	No. of concentrations*	Equation of the line	Correlation coefficient
Nicotine	GC—NPD	7	$Y = 0.0054X + 0.0430$	0.999
	GC—MS	7	$Y = 0.0042X + 0.0367$	0.998
Cotinine	GC—MS	8	$Y = 0.0082X + 0.0153$	0.999

*Each homogenate contained 1 g of mouse liver. Concentrations were within the range 5—1000 ng.

TABLE II

ACCURACY AND PRECISION OF THE ASSAYS FOR NICOTINE AND COTININE IN LIVER HOMOGENATES

Mean ± S.D., $n = 4$ at each concentration.

Amount of nicotine or cotinine added* (ng)	Nicotine determined (ng)	Cotinine determined (ng)
10	10.1 ± 2.1	9.8 ± 2.5
25	24.7 ± 1.5	—
50	50.7 ± 3.7	51.9 ± 3.5
250	252 ± 6	248 ± 8

*Each homogenate contained 1 g of mouse liver.

nates spiked with a quantity of nicotine in the range 5—1000 ng along with 250 ng of MA. The standard curve data are included in Table I. Verification of the extraction and quantitation technique was accomplished by spiking liver homogenates with known quantities of nicotine and analyzing the samples as described. The results (Table II) demonstrate the accuracy and precision of this method.

It is more difficult to achieve narrow, symmetrical chromatographic peaks with cotinine than with nicotine due to the higher polarity and resulting column adsorption of this metabolite. Good peak shape was obtained, however, with a Carbowax-deactivated fused silica column (Fig. 4). A close structural analogue, OMA, was chosen as the internal standard for the GC work with cotinine. Extracts of mouse liver homogenates obtained by the methods dis-

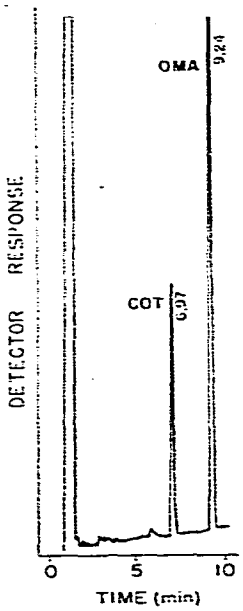


Fig. 4. Analysis of a standard sample of cotinine (COT) and the internal standard, OMA, by capillary GC.

cussed above were found to contain compounds which interfered with the GC—NPD analysis of cotinine at low nanogram levels. Since attempts to produce cleaner extracts resulted in substantially lower recoveries, no further work was carried out to quantitate cotinine by GC—NPD methods. Such techniques could be employed, however, if high sensitivity is not required.

GC—MS analyses of nicotine and cotinine

A method was developed for the GC—MS quantitation of nicotine in tissue extracts. Under EI conditions, the following mass spectral ions are potentially useful for measurement by selected ion monitoring: *m/z* 84 (base peak), *m/z* 133 (relative abundance 40%) and *m/z* 162 (M^+, relative abundance 20%). By analyzing tissue extracts, it was determined that monitoring *m/z* 84 did not provide adequate selectivity and *m/z* 162 did not provide adequate sensitivity for the analyses of low levels of nicotine. Therefore, all GC—MS work was performed by monitoring *m/z* 133 for nicotine and *m/z* 136 for NIC-d_3, the internal standard. The sensitivity limit was equivalent to that obtained by the GC—NPD method, 2—3 ng per g of tissue. The standard calibration data are included in Table I. This method was validated by spiking liver homogenates with known amounts of nicotine and analyzing them as described; the results are included in Table II. The GC—MS method is approximately equivalent to the GC—NPD method with regard to sensitivity, accuracy and precision. Therefore, the GC—MS method provides an alternative quantitative technique to the GC—NPD method for the analysis of nicotine in tissue extracts.

GC—MS analysis of cotinine under EI conditions was not sufficiently selective to quantitate low levels of this metabolite in tissue extracts. By monitoring the M+1 ion for cotinine (at *m/z* 177) under CI conditions with isobutane, however, good sensitivity and selectivity were achieved. The ion current profiles obtained by analyses of liver extracts are shown in Fig. 5. The sensitivity limit is at least 2—3 ng of cotinine per g of tissue, which is equivalent to the sensitivity obtained for nicotine determinations. The GC—MS work was performed with a packed OV-22 column; the use of a capillary column should lead to further improvement in GC—MS sensitivity. A standard calibration curve was

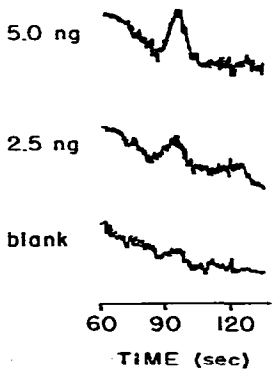


Fig. 5. Ion current chromatograms obtained by monitoring the M+1 ion of cotinine (*m/z* 177) in tissue extracts with chemical ionization GC—MS. Mouse liver homogenates were unspiked or spiked with 2.5 or 5 ng of cotinine and extracted as described in the text.

constructed with data obtained from the analyses of liver homogenates spiked with amounts of cotinine in the range 5—1000 ng per g of tissue. Homogenates also contained 150 mg of the internal standard COT-d_3. The data are summarized in Table I. As with the methods for nicotine quantitation, the extraction and GC—MS techniques for cotinine were validated by spiking tissue homogenates with known amounts of cotinine and performing the analyses. The results (Table II) also demonstrate the reliability of this method.

A pharmacokinetic study of nicotine and cotinine in mouse liver

Mice were injected with a single intraperitoneal dose of 1 mg/kg of nicotine, killed at various intervals after administration and the liver nicotine and cotinine extracted by the methods described. Nicotine was quantitated by GC—NPD and cotinine by GC—MS techniques. The data were used to construct the semilogarithmic plots of concentration vs. time shown in Fig. 6. The disappearance of nicotine was biphasic with the half-life of the elimination phase calculated to be 9.2 min. At 45 min after injection, the levels of nicotine were approaching the lower limit of detection. The maximum tissue levels of cotinine were attained in 5 min and its disappearance was monophasic. Readily detectable quantities were still present in liver at 2 h after injection. The elimination half-life of this metabolite was 27 min.

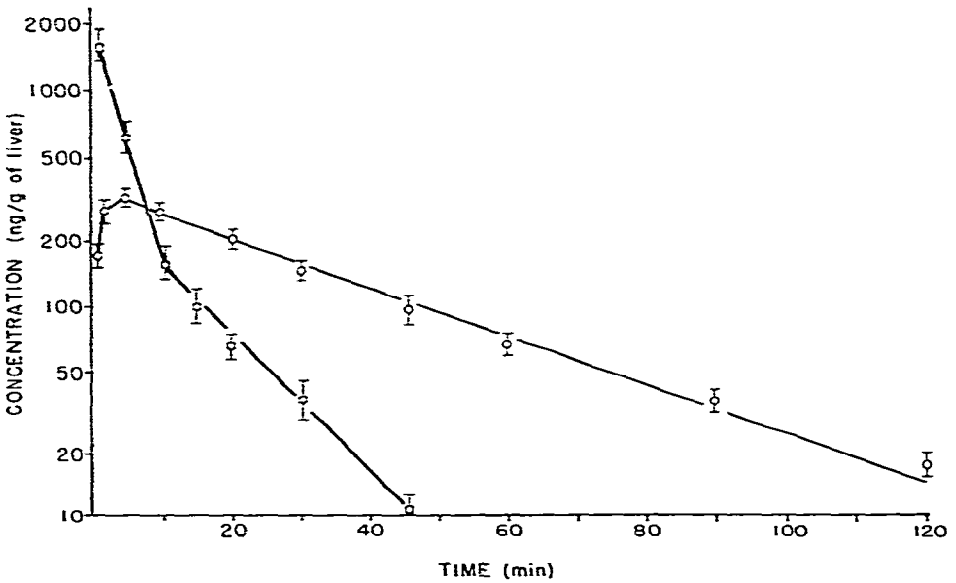


Fig. 6. Concentrations of nicotine (□) and cotinine (○) in livers of mice injected with nicotine vs. time after injection (mean ± S.E., $n = 6$).

CONCLUSION

Sensitive and selective analytical techniques have been developed for the quantitation of nicotine and cotinine in biological samples. The emphasis of this work was on the accurate measurement of low nanogram amounts of these compounds in tissues rather than in fluids, such as blood and urine [2—8]. Data

were presented to demonstrate that sensitivity for nicotine is limited by background levels of the compound. The internal standards for quantitation are much more similar structurally to nicotine and cotinine than most of those used previously. Nicotine extracts were accurately analyzed by both capillary GC—NPD and GC—MS techniques. Cotinine extracts, however, could only be quantitated by chemical ionization GC—MS methods to achieve the necessary analytical selectivity at low tissue levels of the metabolite. A pharmacokinetic investigation carried out to demonstrate the usefulness of these techniques revealed a very short half-life for nicotine in mouse liver after a single intraperitoneal injection of the compound; the half-life of the metabolite cotinine was three-fold longer. Further studies of the pharmacokinetics of nicotine and cotinine in other tissues and the physiological and environmental factors that affect the disposition of nicotine are in progress.

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