Clinical Chemistry 48:9
1460–1471 (2002)

Drug Monitoring and Toxicology

Simultaneous Analysis of Nicotine, Nicotine Metabolites, and Tobacco Alkaloids in Serum or Urine by Tandem Mass Spectrometry, with Clinically Relevant Metabolic Profiles

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Background: **Assessment of nicotine metabolism and disposition has become an integral part of nicotine dependency treatment programs. Serum nicotine concentrations or urine cotinine concentrations can be used to guide nicotine patch dose to achieve biological concentrations adequate to provide the patient with immediate relief from nicotine withdrawal symptoms, an important factor in nicotine withdrawal success. Absence of nicotine metabolites and anabasine can be used to document abstinence from tobacco products, an indicator of treatment success.**

Methods: **The procedure was designed to quantify nicotine, cotinine, *trans*-3′-hydroxycotinine, anabasine, and nornicotine in human serum or urine. The technique required simple extraction of the sample with quantification by HPLC–tandem mass spectrometry.**

Results: **The procedure for simultaneous analysis of nicotine, its metabolites, and tobacco alkaloids simultaneously quantified five different analytes. Test limit of quantification, linearity, imprecision, and accuracy were adequate for clinical evaluation of patients undergoing treatment for tobacco dependency. The test readily distinguished individuals who had no exposure to tobacco products from individuals who were either passively exposed or were abstinent past-tobacco users from those who were actively using a tobacco or nicotine product.**

Conclusions: **Nicotine, cotinine, *trans*-3′-hydroxycotinine, nornicotine, and anabasine can be simultaneously and accurately quantified in either serum or urine by HPLC–tandem mass spectrometry with imprecision <10% at physiologic concentrations and limits of quantification ranging from 0.5 to 5 μg/L. Knowledge of serum or urine concentrations of these analytes can be used to guide nicotine replacement therapy or to assess tobacco abstinence in nicotine dependency treatment. These measurements are now an integral part of the clinical treatment and management of patients who wish to overcome tobacco dependence.**

During the past three decades, there has been an increasing focus on cigarette smoking and the adverse health consequences associated with it. Most people are aware of the dangers of smoking, and many are aware that nicotine causes addiction. Because nicotine is the primary causative agent in addiction to tobacco products *(1)*, assessment of nicotine metabolism and disposition has become an integral part of nicotine dependence treatment.

One cigarette contains an average of 8.4 mg of nicotine. When tobacco is burned, nicotine is aerosolized into tar droplets that deliver ~1.6 mg of nicotine per cigarette. The hydrogen ion concentration (pH) of tobacco determines how much free base is delivered. The flue-cured tobaccos used in cigarettes are acidic (pH 5.5), whereas the air-dried tobaccos used in pipe and cigar tobaccos are alkaline (pH 8.5). Burned alkaline tobacco products yield higher free-base nicotine concentrations in smoke compared with acidic tobacco products *(2, 3)*.

Inhaled tobacco smoke reaches the small airways and alveoli of the lungs, where 82–92% of nicotine is absorbed in a pH-dependent fashion. Nicotine absorbed into the pulmonary circulation distributes rapidly to brain and heart tissues. Nicotine reaches the central nervous system within 20 s of tobacco smoke inhalation. Nicotine has

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Received February 22, 2002; accepted May 8, 2002.

short distribution (8 min) and elimination (2 h) half-lives. During the first 2 min of nicotine absorption, the arterial concentration exceeds the venous concentration by 6- to 10-fold. Rapid delivery of nicotine to the brain produces the intense, positive pharmacologic response that most cigarette smokers seek *(4, 5)* and is thought to be a key factor in nicotine dependence *(6)*. The fraction of nicotine that reaches the systemic circulation is <5% protein bound and exhibits a volume of distribution averaging 2.6 L/kg *(3, 7)*.

Numerous reports [summarized in Refs. *(2, 8)*] document that smokers adjust their smoking behavior to maintain a specific nicotine concentration throughout the day. Spit tobacco (ST) users exhibit similar behavior *(9, 10)*. Experiments to change the rate of elimination of nicotine through acidification demonstrate that study participants will self-administer tobacco products to compensate for decreases in serum nicotine concentrations by increasing the depth of inhalation and length of breath holding. This indicates that self-administration of nicotine is driven by the need to maintain serum nicotine concentrations *(11)*. Smokers compensate when smoking low-tar/low-nicotine delivery cigarettes to maintain serum nicotine concentrations, and there is a threshold dose of nicotine below which a new smoker will not become dependent *(4)*. The tobacco industry recognized that this threshold dose existed, knowing that smokers would switch to other brands if a cigarette did not deliver enough nicotine per cigarette to make smokers dependent *(3)*.

Smokers or ST users self-titrate their tobacco use to achieve steady-state venous nicotine blood concentrations in the range of 30–50 μg/L; the full range of concentrations seen in the majority of smokers or ST users is 5–100 μg/L. The peak venous nicotine concentration after the first morning cigarette is typically 10 μg/L. The arterial/venous concentration ratio is greater after the first cigarette, which increases the amount of nicotine delivered to the brain. As smokers consume cigarettes during the day, nicotine accumulates in the blood, reaching a plateau averaging 40 μg/L throughout the day. If a smoker consumes tobacco until bedtime, significant nicotine concentrations persist throughout the night *(12–14)*. Urine concentrations of nicotine and cotinine correlate with cigarette use in active smokers. Lawson et al. *(14)* described the typical urine output of cotinine, which was proportional to the number of cigarettes per day or nicotine patch dose. On a 44-mg patch, 5 mg of cotinine per day was a typical excretion rate. The urine cotinine concentration is useful to guide replacement therapy *(14–16)*.

Nicotine metabolism occurs predominantly in the liver by cytochrome P450 isoenzymes, aldehyde oxidase, flavin monooxygenase, and glycosylation. Cytochrome P450 2D6 was previously thought to be the primary isoenzyme responsible for nicotine metabolism. Benowitz et al. *(17)* studied cytochrome P450 2D6 metabolism in a small number of individuals and found that genotyping did not differentiate the relative degrees of nicotine dependence in those subjects. However, Berkman et al. *(18)* and Carlson et al. *(19)* reported that cytochrome P450 2A6 is important, and Tyndale et al. *(20)* reported a clear distinction between addicted smokers and nonsmokers based on the cytochrome P450 2A6 genotype. Multiple cytochrome P450 isoenzymes are involved in nicotine metabolism, and multiple metabolites exist.

The liver represents the primary site of metabolism of nicotine, although the lung and kidney also contribute to metabolism. A small fraction of nicotine is metabolized to nornicotine. Seventy percent of circulating nicotine is metabolized to cotinine by cytochrome P450 and aldehyde oxidase. Smokers and ST users maintain cotinine blood concentrations averaging 250–350 μg/L. Cotinine has an average half-life of 20 h and does not appear to have any direct pharmacologic action *(2, 21, 22)*. Cotinine is further metabolized to *trans*-3′-hydroxycotinine, and cotinine and *trans*-3′-hydroxycotinine are excreted as glucuronide esters *(7, 23)*. On average, 30% of the nicotine that enters the liver exits unchanged, and 70% leaves the liver in the form of metabolites. Events that change hepatic flow, such as eating, posture, and exercise, reduce hepatic blood flow and reduce the rate of nicotine metabolism *(5)*.

Renal clearance of unmetabolized nicotine depends on urinary pH and can account for 2–35% of nicotine excretion. Nicotine is reabsorbed from the proximal tubules, where the pH is higher. In studies of human subjects with uncontrolled urine pH, renal clearance accounted for 10–15% of the direct elimination of nicotine. Renal clearance of cotinine is not pH dependent and represents a minor route of elimination: only 17% of nicotine is eliminated as cotinine in the urine. Cotinine undergoes further metabolism to *trans*-3′-hydroxycotinine; *trans*-3′-hydroxycotinine represents the major renal excretion product of nicotine *(6, 9, 24)*.

Cotinine and *trans*-3′-hydroxycotinine are present in biological fluids with apparent elimination half-lives of 20 and 15 h, respectively *(2, 12, 24, 25)*. Anabasine is a tobacco alkaloid present in tobacco products. Nornicotine is both a tobacco alkaloid and a nicotine metabolite *(26)*. Virtually all tobacco products contain these alkaloids, and direct exposure through active smoking or chewing tobacco exposes the user to these alkaloids. The presence of anabasine in biological fluids indicates active tobacco use *(27)*. Anabasine is not found in urine specimens from patients using nicotine replacement therapy, such as a patch, gum, inhaler, or nasal spray.

The serum nicotine concentration *(12)* or urine cotinine concentration *(14)* can be used to guide dosage in nicotine patch therapy to achieve the concentrations present when the patient was actively using a tobacco product. Absence of nicotine metabolites or anabasine can be used to document abstinence from tobacco products. Here we report a tandem mass spectrometry (MS/MS)-based lab-

oratory procedure developed to support clinical studies to evaluate treatment of nicotine dependence *(15, 16, 28–39)*. This report also summarizes the nicotine metabolic profiles observed in several of those studies, showing how analysis of nicotine, nicotine metabolites, and a tobacco alkaloid can be used in clinical evaluation of patients undergoing treatment for nicotine dependence.

Materials and Methods

REAGENTS

Nicotine, nicotine-d_3, cotinine, cotinine-d_3, anabasine, and nornicotine were purchased from Sigma Chemical Company. *trans*-3′-Hydroxycotinine was a gift from the American Health Foundation (Valhalla, NY). Formic acid ammonium salt was purchased from Sigma. Acetonitrile, ammonium hydroxide, and methanol were from Merck. Formic acid (88%) was from Fisher Scientific. Clean Screen extraction columns (part no. ZSDAU020) were from United Chemical Technologies, Inc. Bovine calf serum was from Pel Freez Biologicals.

CALIBRATORS AND CONTROLS

Stock reference solutions of nicotine, nicotine-d_3, cotinine, cotinine-d_3, anabasine, nornicotine, and *trans*-3′-hydroxycotinine were prepared in methanol. These reagents were prepared and stored at −20 °C in sealed plastic vials. Working internal standard was prepared in type I water to contain 2 mg/L each of nicotine-d_3 and cotinine-d_3. Working serum-based calibrators were prepared to contain four ascending concentrations of nicotine, cotinine, and *trans*-3′-hydroxycotinine. Serum control pools were prepared in bovine serum. Working urine-based calibrators were prepared to contain four ascending concentrations of nicotine, cotinine, *trans*-3′-hydroxycotinine, anabasine, and nornicotine. Urine control pools were prepared to contain typical physiologic concentrations of these compounds in urine from a volunteer not exposed to tobacco or nicotine products.

SAMPLE PREPARATION

Samples were prepared in a manner similar to that described previously by Baskin et al. *(40)*. One milliliter of calibrator, control, or patient sample, 0.1 mL of working internal standard, and 4 mL of 0.1 mol/L acetic acid were mixed in a 16 × 125 mm glass test tube for 2 s. Extraction columns were prepared by washing with 4 mL of methanol followed by 4 mL of 0.1 mol/L acetic acid, taking care not to dry the columns. Samples were poured into the columns and drawn slowly through the bed. The columns were washed with 4 mL of 0.1 mol/L acetic acid followed by 4 mL of methanol. Analytes were eluted into 16 × 100 glass test tubes with 4 mL of freshly prepared 20 mL/L ammonium hydroxide in methanol. Samples were evaporated to dryness at 30 °C with nitrogen. Samples were reconstituted in 0.2 mL of acetonitrile for chromatography.

COLUMN SEPARATION

Nicotine and anabasine are structurally similar and have similar molecular weights and daughter ions, so column separation was important. Adequate separation was achieved with a Polyhydroxyethyl A HPLC column [100 cm × 4.6 mm (i.d.); 5-μm bead size; 100-Å pore size; PolyLC Inc.). The mobile phase consisted of 200 mL of type 1 water, 1800 mL of acetonitrile, 1.3 g of ammonium formate, and 15 mL of formic acid (88%). This column functioned as a normal-phase separation medium, with additional water causing the analytes to elute faster.

LIQUID CHROMATOGRAPHY-MS/MS

The HPLC system consisted of a high-pressure liquid pump and autosampler (Series 200; Perkin-Elmer) and a tandem mass spectrometer (API 2000; PE Sciex). Eluant flow rate was 1.0 mL/min at ambient temperature. The injection volume was 0.05 mL. The HPLC column was connected directly to the heated nebulizer source of the liquid chromatography-MS/MS (LC-MS/MS) system. The source temperature was 500 °C, the nebulizer gas flow rate was 5 mL/min, nebulizer current was 2.0 V, gas 1 flow rate was 60 mL/min, and gas 2 flow rate was 60 mL/min. The curtain gas flow rate was 45 mL/min. Detection of ions at the electron multiplier occurred in two periods using the positive ion mode. Signal output, representing two separate ion pairs for cotinine (*m/z* 177.3/98.0 and *m/z* 177.3/80.1) and *trans*-3′-hydroxycotinine (*m/z* 193.3/134.0 and *m/z* 193.3/80.1) and a single ion pair for cotinine-d_3 (*m/z* 180.2/101.0), was simultaneously monitored during the first 3.5 min of sample elution. Signal output was simultaneously monitored for two separate ion pairs for nicotine (*m/z* 163.2/105.9 and *m/z* 163.2/80.1) and single ion pairs for anabasine (*m/z* 162.9/80.1), nornicotine (*m/z* 149.1/80.1), and nicotine-d_3 (*m/z* 166.3/87.2) during the last 4 min of sample elution. Total elution time monitored was 7.5 min. Nicotine-d_3 was used as the internal standard for nicotine, and cotinine-d_3 was used as the internal standard for all other analytes.

QUANTIFICATION

The concentration of each analyte was determined by calculating the ratio of each analyte ion-pair peak area relative to its respective internal standard peak area. Each analytical batch was calibrated by performing analysis of the calibrators outlined in the "Calibrators and Controls" section above; results from calibrator analysis were used to create a calibration curve using simple linear regression analysis. The slopes and intercepts from the resulting calibration equations were used to calculate control and specimen results.

RECOVERY, LIMIT OF QUANTIFICATION, AND LINEARITY STUDIES

Recovery was evaluated by adding each analyte into human serum or urine at four different concentrations (Table 1). Three replicates of each serum pool were

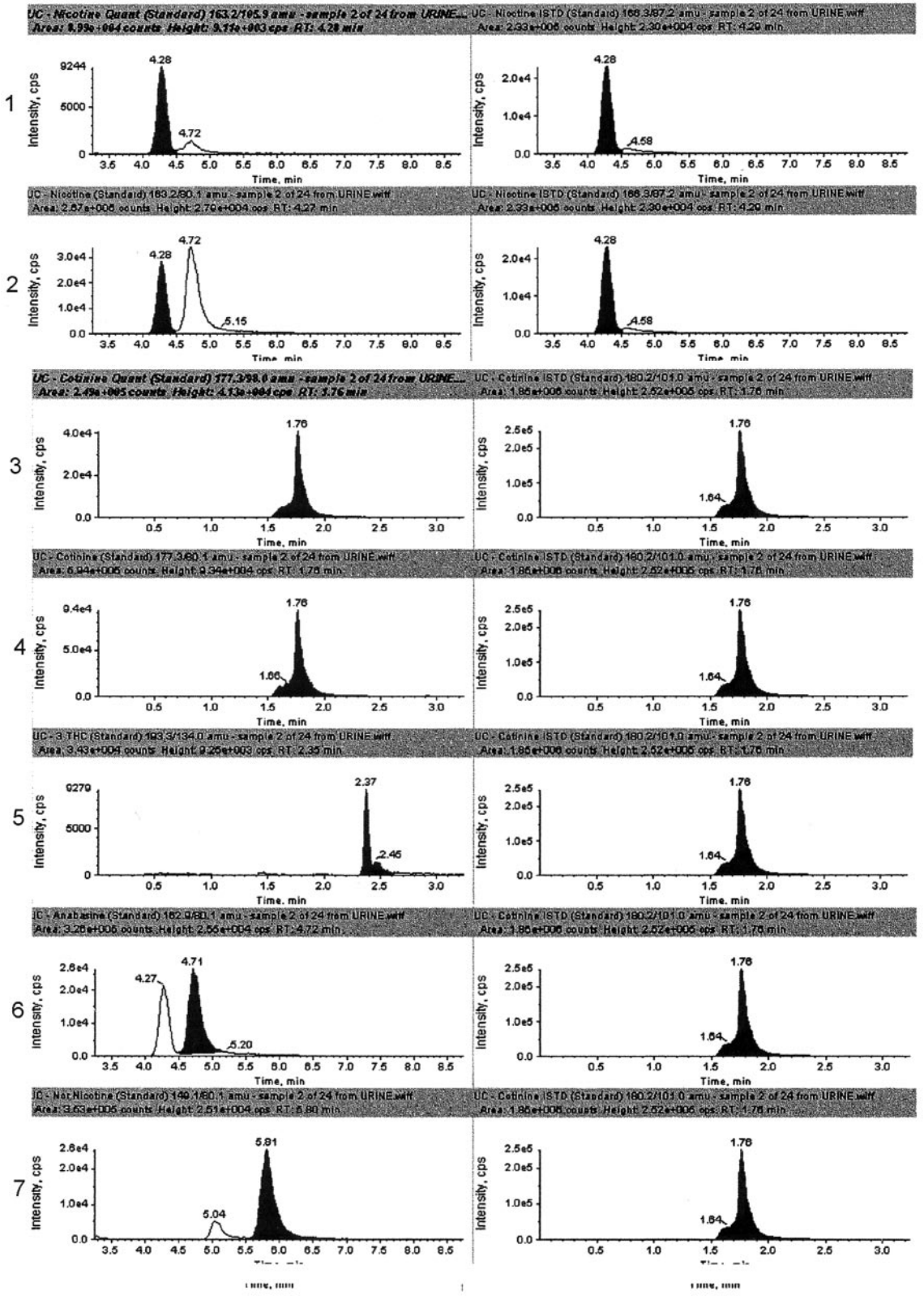

Fig. 1. Mass spectrograms of nicotine and nicotine-d_3 (*panels 1* and *2*), cotinine and cotinine-d_3 (*panels 3* and *4*), *trans*-3′-hydroxycotinine and cotinine-d_3 (*panel 5*), anabasine and cotinine-d_3 (*panel 6*), and nornicotine and cotinine-d_3 (*panel 7*).

Table 1. Recovery.[a]

Analyte concentration, μg/L	Recovery, %						
	Nicotine	Cotinine	*trans*-3-Hydroxycotinine	Anabasine	Nornicotine	Nicotine-d_3	Cotinine-d_3
5	45	82	30	100	102	49	77
50	51	68	22	73	88	51	66
100	42	65	17	74	97	39	63
1000	37	64	16	68	55	37	62
Mean	40	69	21	77	83	43	67

[a] Recovery from urine was derived by dividing the mean integrated LC-MS/MS peak area count observed for each analyte in the extracted samples by area counts observed for each unextracted sample. Results are expressed as percentages. Each recovery percentage represents the average of three replicates at each concentration. Similar recovery data were observed for serum (data not shown).

extracted as described in the "Sample Preparation" section outlined above. Triplicate unextracted analyte aliquots were also transferred directly into the LC-MS/MS mobile phase to serve as recovery references.

The limit of quantification was evaluated by adding the analytes of interest to human serum or urine collected from a non-tobacco user (Table 2). The serum or urine was tested before the addition, using the procedure outlined here to demonstrate that the ion-pair signal at the retention time unique to any of the analytes was no different from background, indicating the specimens were tobacco free.

Linearity was evaluated by analyzing tobacco-free human serum to which nicotine, cotinine, and *trans*-3′-hydroxycotinine had been added at five different concentrations on 3 different days (Table 3). A similar approach was used to evaluate linearity from tobacco-free human urine to which nicotine, cotinine, *trans*-3′-hydroxycotinine, anabasine, and nornicotine had been added (Table 4).

Results

MS/MS

A major advance in analytical technology has occurred over the past few years with the introduction of stable liquid chromatography systems coupled to tandem mass spectrometers, frequently referred to as LC-MS/MS. In this technology, the effluent of a liquid chromatograph is subjected to low-energy ionization to form charged molecular ions. These molecular ions are introduced into a mass spectrometer, where ions are separated by mass. The mass spectrometer is electronically tuned to focus the molecular ion of interest on an exit slit. When a molecular ion exits the first mass spectrometer, it enters a collision chamber where it reacts with an inert gas to fragment into charged daughter ions. The daughter ions are then directed into a second mass spectrometer, which separates the daughter ions and focuses them on a detector.

Representative ion-pair mass spectrograms from extracts of human urine containing nicotine, cotinine, *trans*-3′-hydroxycotinine, anabasine, and nornicotine are shown in Fig. 1. Two distinct ion pairs were analyzed for nicotine and cotinine and one ion pair for *trans*-3′-hydroxycotinine, anabasine, nornicotine, nicotine-d_3, and cotinine-d_3. The ratio of ion-pair area to internal standard area was determined as outlined in the "Quantification" section above. The results from the two ion pairs analyzed for nicotine and cotinine were expected to be similar; these two ions were monitored for quality assessment. If the quantified results were different between the two ions, the cause was either poor integration or poor column separation. Similar mass spectrograms were derived from extracts of human serum containing nicotine, cotinine, and *trans*-3′-hydroxycotinine (not shown).

Recovery, Limit of Quantification, Imprecision, and Linearity

Ratios of the results of LC-MS/MS analysis comparing unextracted and extracted samples were used to estimate recovery (Table 1). Mean recoveries from urine were 40% for nicotine, 69% for cotinine, 21% for *trans*-3′-hydroxycotinine, 77% for anabasine, 83% for nornicotine, 43% for nicotine-d_3, and 67% for cotinine-d_3. Analyte recovery from serum was similar (data not shown).

The imprecision for analysis of 10 replicates of five different human serum pools to which low concentration of all analytes had been added is outlined in Table 2. The limit of quantification, defined as the concentration where imprecision is <20%, was 2 μg/L for nicotine, cotinine, and nornicotine, 0.5 μg/L for anabasine, and 5 μg/L for *trans*-3′-hydroxycotinine. Similar imprecision was observed for urine (data not shown; imprecision for urine at higher analyte concentrations is described later in Table 5).

Table 2. Imprecision at low concentrations.[a]

	Target value, μg/L				
	0.5	1.0	2.0	3.0	5.0
CV, %					
Nicotine	24	21	18	15	12
Cotinine	19	16	15	12	9
t-3-OH-Cotinine[b]	52	39	31	27	20
Anabasine	19	17	13	10	9
Nornicotine	27	21	19	16	14

[a] Imprecision was calculated from analysis of 10 replicates of serum to which all analytes had been added at the concentrations noted. Similar data were observed for urine samples (data not shown).

[b] *t*-3-OH-Cotinine, *trans*-3′-hydroxycotinine.

Table 3. Linearity for serum.[a]

	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Least-squares equation	Regression constant
Nicotine, μg/L	2	5	20	50	100	$y = 1.09x + 0.6$	0.999
Cotinine, μg/L	10	100	500	1000	5000	$y = 0.86x + 60$	0.997
trans-3′-Hydroxycotinine, μg/L	50	200	500	1000	5000	$y = 0.96x - 17$	0.983

[a] Simple linear regression analysis was performed to define the least-squares equation and regression constants. Results represent the means for three replicates of serum with analytes added at the concentration noted.

Simple linear regression analysis was performed to calculate least-squares equations and regression constants to document linearity (Tables 3 and 4). Regression constants describing the relationship between expected and analyzed results were consistently >0.99 for nicotine, cotinine, anabasine, and nornicotine in both serum and urine. *trans*-3′-Hydroxycotinine showed greater variance, with regression constants consistently >0.98. Simultaneous analysis by MS/MS for nicotine, cotinine, *trans*-3′-hydroxycotinine, anabasine, and nornicotine by the procedure outlined here was linear from physiologically undetectable concentrations to the highest concentrations expected in specimens from tobacco users. Similar results were observed when human and bovine serum were used as the matrix, verifying that bovine serum could be used for preparation of calibrators.

Within-run imprecision was assessed by analyzing pools of nicotine-free urine with nicotine, cotinine, *trans*-3′-hydroxycotinine, anabasine, and nornicotine added at three different concentrations (Table 5). Thirty replicates were extracted within a single batch and analyzed in sequence by MS/MS. Within-run imprecision (CVs) ranged from 4.9–11% for nicotine, 7.2–9.6% for cotinine, 18–22% for *trans*-3′-hydroxycotinine, 14–17% for anabasine, and 17–20% for nornicotine. Between-run imprecision was assessed over many consecutive days for serum and urine, using a single pool of serum or urine to which the analytes had been added (Table 6). The mean between-run imprecision for the procedure described here in serum specimens was 7.5% for nicotine, 4.9% for cotinine, and 18% for *trans*-3′-hydroxycotinine. The mean between-run imprecision in urine specimens was 10% for nicotine, 4.4% for cotinine, 20% for *trans*-3′-hydroxycotinine, 12% for anabasine, and 13% for nornicotine.

INTERFERENCE

The drugs tested for potential interference are listed in footnote 1. Interference testing was performed by direct LC-MS/MS analysis without extraction of a methanolic solution containing each drug at a concentration of 1 g/L. Any drug found to give a detectable signal greater than that equivalent to the lowest nicotine or metabolite calibrator (see the section on linearity) was added into urine at 10 mg/L and carried through the complete extraction and MS/MS analysis. Only bupivacaine showed any indication of interference. Bupivicaine significantly interfered with the baseline of the internal standard cotinine-d_3. All analytes except nicotine would be incorrectly quantified in the presence of bupivacaine.

ACCURACY

Accuracy was assessed by comparing results obtained by the proposed method for serum specimens collected from human study participants who used tobacco products with results obtained by comparison methods. Fig. 2 shows the correlation of results for serum nicotine de-

[1] Drugs tested for potential interference: acetaminophen, acetazolamide, acetohexamide, *N*-acetylprocainamide, acetylsalicylate, allobarbital, alprazolam, amiodarone, amitriptyline, amobarbital, amphetamine, antipyrine, aprobarbitol, baclofen, barbital, barbituric acid, bendroflumethazide, benzamide, benzocaine, benzoyleconine, benzthiazide, benztropine, bisacodyl, bromazepam, brompheniramine, bupivicaine, bupropion, butabarbital, butalbital, caffeine, carbamazepin-10,11-epoxide, carbamazepine, carisoprodol, celontin, chlordiazepoxide, chlorimipramine, chlorophenothiazine, chloropromazine, chloropropamide, chlorothiazide, chlorpheniramine, chlorzoxazone, cinoxacine, cisapride, citalopram, citrizine, clonazepam, clozapine, cocaine, coumarin, cyclobenzaprine, cyclosporin A, cyclothiazide, cyheptamide, demoxepam, desipramine, dextromethorphan, diacetylmorphine, diazepam, diclofenac, dicumarol, dicyclomine, diflunisal, diltiazem, diphenhydramine, disopyramide, doxepin, doxylamine, encainide, ephedrine, ethacrynic acid, ethosuximide, felbamate, fenoprofen, fentanyl, flecainide, fluconazole, flunitrazepam, fluoxetine, fluphenazine, flurazepam, flurbiprofen, fluvoxamine, furosemide, gabapentin, gemfibrozil, glipizide, glutethimide, glybenclamide, griseofulvin, guaiphenesin, guanosine, halazepam, haloperidol, hexobarbital, hydralazine, hydrochlorothiazide, hydrocodone, hydroflumethazide, hydromorphone, hydroxyzine, ibuprofen, imipramine, indapamide, indomethacin, isoniazide, itraconazole, ketoconazole, ketoprofen, lamotrigine, levofloxacin, levorphanol, lidocaine, lorazepam, maprotiline, meclofenamic acid, medazepam, mefenamic acid, meperidine, mephentermine, mephobarbital, meprobamate, meropenem, methadone, methamphetamine, methapyriline, methaqualone, metharbital, methoxyphenamide, methoxypsoralen, methyl nitrazepam, methyl salicylate, methylclothiazide, methyprylon, metoclopramine, metoprolol, metronidazole, mexilitine, midazolam, morphine, mycophenolic acid, naltrexone, naproxen, nifedipine, nitrazepam, nitroglycerine, norchlorimipramine, nordoxepin, norfluoxetine, normethsuximide, nortriptyline, norverapamil, noscapine, olanzapine, orphenadrine, oxazepam, oxycodone, oxymorphone, palcidyl, paroxetine, pentazoczine, pentobarbital, pentoxifylline, perphenazine, phenazopyridine, phencyclidine, phenobarbital, phensuximimide, phentermine, phenylbutazone, phenylephrine, phenylethylamine, phenylpropanolamine, phenylsuccinide, phenyltoloxamine, phenytoin, piperazine, polythiazide, prednisone, primidone, probucol, procainamide, procaine, prochlorperazine, promazine, promethazine, propofenone, propoxyphene, propranolol, protriptyline, pseudoephedrine, quetiapine, quinidine, quinine, ranitadine, resorcinol, resperidone, ritalin, salicylate, secobarbital, sertraline, sildenafil, strychnine, succinimide, sulfadiazine, sulfamethoxazole, sulfanilamide, sulfapyridine, sulfisoxazole, sulindac, temazepam, thalidomide, theophylline, thiopental, thioridazine, thiothixene, tiagabine, ticlopidine, tocainide, tolazamide, tolbutamide, tolmetin, topiramate, tramadol, trazodone, triazolam, trichloromethiazide, trifluoperazine, triflupromazine, trimethoprim, trimipramine, trovafloxacine, valproic acid, venlafaxin, verapamil, vigabatrine, warfarin, yohimbine, Zantac, zolmetriptan, zolpidem, zomepirac.

Table 4. Linearity for urine.[a]

	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Least-squares equation	Regression constant
Nicotine, μg/L	5	20	100	500	1000	$y = 1.02x - 0.8$	0.999
Cotinine, μg/L	10	50	200	1000	3000	$y = 0.99x - 2.2$	0.999
trans-3′-Hydroxycotinine, μg/L	50	200	500	2000	5000	$y = 0.96x + 17$	0.987
Anabasine, μg/L	5	20	100	500	1000	$y = 0.89x - 0.2$	0.998
Nornicotine, μg/L	5	20	100	500	1000	$y = 1.13x + 0.6$	0.996

[a] Simple linear regression analysis was performed to define the least-squares equation and regression constants for calibration curves. Results represent the means for three replicates of urine with analytes added at the concentration noted.

rived by the method presented here compared with the method of Lawson et al. *(12)*. Fig. 2 also shows the correlation of results for serum cotinine derived by the method presented here compared with the method of Machecek and Jiang *(41)*. Excellent correlations ($r > 0.98$) were observed between the proposed method and the comparison methods.

SAMPLE STABILITY

Combining fresh human serum specimens containing nicotine, cotinine, and *trans*-3′-hydroxycotinine from multiple tobacco users facilitated the creation of 10 different pools of natural human serum with adequate volumes to assess analyte stability in serum. Ten individual specimens of human urine containing nicotine, cotinine, *trans*-3′-hydroxycotinine, anabasine, and nornicotine from tobacco users were used to assess analyte stability in urine. Each of these pools was aliquoted into a separate capped, plastic ampoule for storage under various conditions. The mean of results derived from analysis of three replicates of each pool on the day the pools were created defined the baseline concentrations for each pool. Individual aliquots were stored at ambient temperature, refrigerated (4–6 °C), or frozen (−20 °C). One ampoule representing each storage duration and temperature was opened and analyzed on days 1, 3, 7, and 14 after aliquoting. The mean results for all 10 pools for each time duration, temperature condition, and specimen type were compared with the baseline values and expressed as percentages of baseline values (Table 7).

Table 5. Within-run imprecision.[a]

	Target value, μg/L	Mean, μg/L	SD, μg/L	CV, %
Nicotine	25	24.8	1.61	6.5
	100	98.7	4.83	4.9
	500	477	51.95	11
Cotinine	50	54.9	3.94	7.2
	100	103	7.59	7.4
	500	492	47.09	9.6
trans-3′-Hydroxycotinine	100	112	22.56	20
	200	185	32.97	18
	1000	886	198.67	22
Anabasine	20	22.5	3.61	16
	80	79.7	11.29	14
	400	431	74.95	17
Nornicotine	20	17.5	3.19	18
	80	71.1	14.11	20
	400	434	73.88	17

[a] Imprecision was calculated from analysis of 30 replicates of urine containing added analytes at the concentrations noted.

CONCENTRATIONS IN NON-TOBACCO USERS

Thirty healthy volunteers were recruited to provide serum and single-void urine specimens collected at the same time to serve as reference specimens to define "normal" concentrations for nicotine, nicotine metabolites, or tobacco alkaloids in humans not exposed to tobacco products. These volunteers were recruited from a pool of healthy volunteers who routinely provide biological specimens for the purpose of evaluating normal ranges at the Mayo Clinic. The individuals recruited were approximately equally divided between gender and ranged in age from 20 to 60 years. All recruited participants claimed that they did not use tobacco products, had no others living with them who used tobacco products, and did not frequent areas where tobacco smoke was prevalent for at least 1 week before specimen collection.

In this "normal" population, all serum results for nicotine, cotinine (with one exception), *trans*-3′-hydroxycotinine, anabasine, and nornicotine were <2 μg/L. One

Table 6. Between-day imprecision.[a]

	Target value, μg/L	Mean, μg/L	SD, μg/L	CV, %	n[b]
Serum					
Nicotine, μg/L	17	18.7	1.40	7.5	76
Cotinine, μg/L	50	48.4	2.37	4.9	76
trans-3′-Hydroxycotinine, μg/L	50	49.7	8.70	18	76
Urine					
Nicotine, μg/L	17	18.9	1.89	10	65
Cotinine, μg/L	50	50.0	2.19	4.4	65
trans-3′-Hydroxycotinine, μg/L	80	74.9	15.03	20	65
Anabasine, μg/L	15	17.6	2.03	12	65
Nornicotine, μg/L	28	27.5	3.57	13	65

[a] Imprecision was calculated from analysis of single specimens of serum or urine, with all analytes added at concentrations noted, on multiple days.
[b] n, number of days.

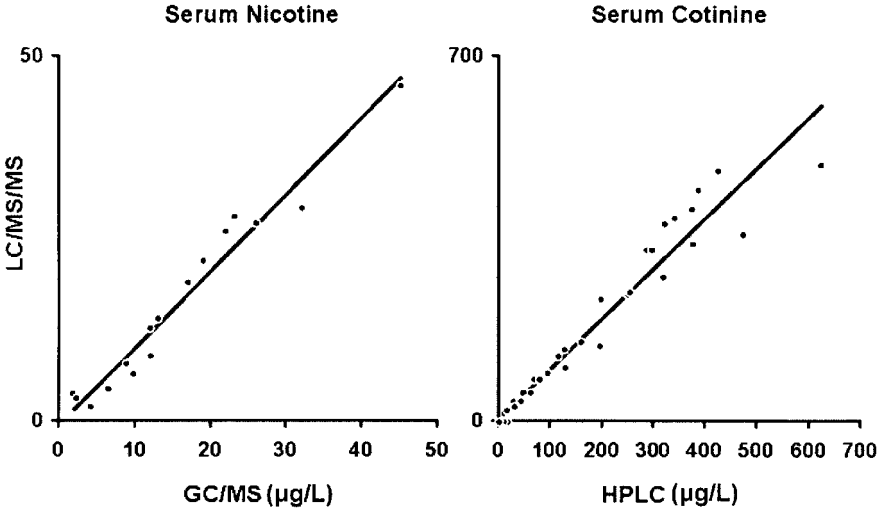

Fig. 2. Simple linear regression plots comparing nicotine analysis by gas chromatography-MS and LC-MS/MS (*left*) and cotinine analysis by HPLC and LC-MS/MS (*right*).

The equations for the lines are: (*left*), $y = 1.05x - 0.52$ ($r = 0.980$); (*right*), $y = 0.9651x + 25\,009$ ($r = 0.983$).

exception was a male 50 years of age who had a serum cotinine result of 2.1 μg/L. All other analyte values in his serum specimen were <2 μg/L. All urine results for nicotine, cotinine, anabasine, and nornicotine in this group were <3 μg/L, and *trans*-3′-hydroxycotinine was <30 μg/L with one exception. The exception (the same individual who caused the exception for serum) provided a random urine sample with a nicotine concentration of 10.2 μg/L, cotinine of 12.1 μg/L, and *trans*-3′-hydroxycotinine of 62.3 μg/L (Fig. 3).

Discussion

We report here for the first time the utility of using MS/MS to simultaneously quantify nicotine, cotinine, *trans*-3′-hydroxycotinine, nornicotine, and anabasine in human serum or urine. MS/MS offers the advantage of sensitivity and specificity because detection is based on molecular mass and chemical structure, properties that are unique to each molecule. The procedure for simultaneous analysis of nicotine and related compounds presented here takes advantage of these properties to allow for a single extraction and simultaneous quantification of five different analytes present in biological samples at low concentrations (~2 μg/L).

RECOVERY AND IMPRECISION

Analyte recovery ranged from 16% to 102% (Table 1). Incorporation of two deuterated internal standards exhibiting recoveries similar to the analytes normalized for recovery variance. The opportunity to normalize recoveries yielded imprecision ranging from 4% to 20% (Tables 2, 5, and 6). The imprecision associated with results >2 μg/L was well within the limits defined for acceptable analytical performance *(42)* and significantly exceeded

Table 7. Sample stability.[a]

	Change from baseline under specified storage condition and days of storage, %											
	Ambient				Refrigerated				Frozen			
	Day 1	Day 3	Day 7	Day 14	Day 1	Day 3	Day 7	Day 14	Day 1	Day 3	Day 7	Day 14
Serum												
Nicotine	+5.0	−13.0	−4.5	+9.4	+3.1	−2.0	−7.7	+2.3	−13.1	−9.7	+11.2	+5.4
Cotinine	−4.1	+6.4	+1.3	−6.7	−5.9	+5.4	−0.7	−2.3	−7.7	+4.8	−5.2	−3.9
t-3-OH-Cotinine[b]	+6.1	−29.9	−14.4	−21.7	+17.4	−45.2	−4.9	−37.1	+12.1	−19.1	−26.1	−48.2
Urine												
Nicotine	−2.1	−1.0	−5.7	+4.5	−3.7	+0.7	−8.6	+1.0	−12.0	−6.1	−4.3	−0.2
Cotinine	−11.9	−2.2	+6.9	+6.2	−13.7	−5.4	−4.1	+4.9	−8.5	−17.1	+11.3	−6.7
t-3-OH-Cotinine	−7.1	−14.2	+0.2	−21.9	−1.2	−11.5	−19.9	−31.8	−16.6	−53.0	−29.2	−78.6
Anabasine	−18.2	−7.7	−16.0	+9.1	−19.3	+8.4	−19.5	+15.1	−21.2	+14.8	−9.4	−12.1
Nornicotine	−18.4	−17.6	+13.8	−11.6	−17.1	+4.6	−13.3	+17.4	−15.2	−21.0	+11.1	+3.2

[a] Sample stability was evaluated in a serum pool and a urine pool obtained from tobacco users containing analyte concentrations in the ranges expected for typical tobacco users. Samples were analyzed in triplicate immediately after pooling; the mean of the triplicate results defined a baseline value for each sample. Aliquots were stored for a maximum of 14 days in separate containers for each storage condition [ambient, refrigerated (~4°C), and frozen (−20°C)], and then a single aliquot was analyzed to represent each storage condition. Results represent the average percentage of deviation from baseline after 1 day (Day 1), 3 days (Day 3), 7 days (Day 7), or 14 days (Day 14) of storage under each respective condition.

[b] *t*-3-OH-Cotinine, *trans*-3′-hydroxycotinine.

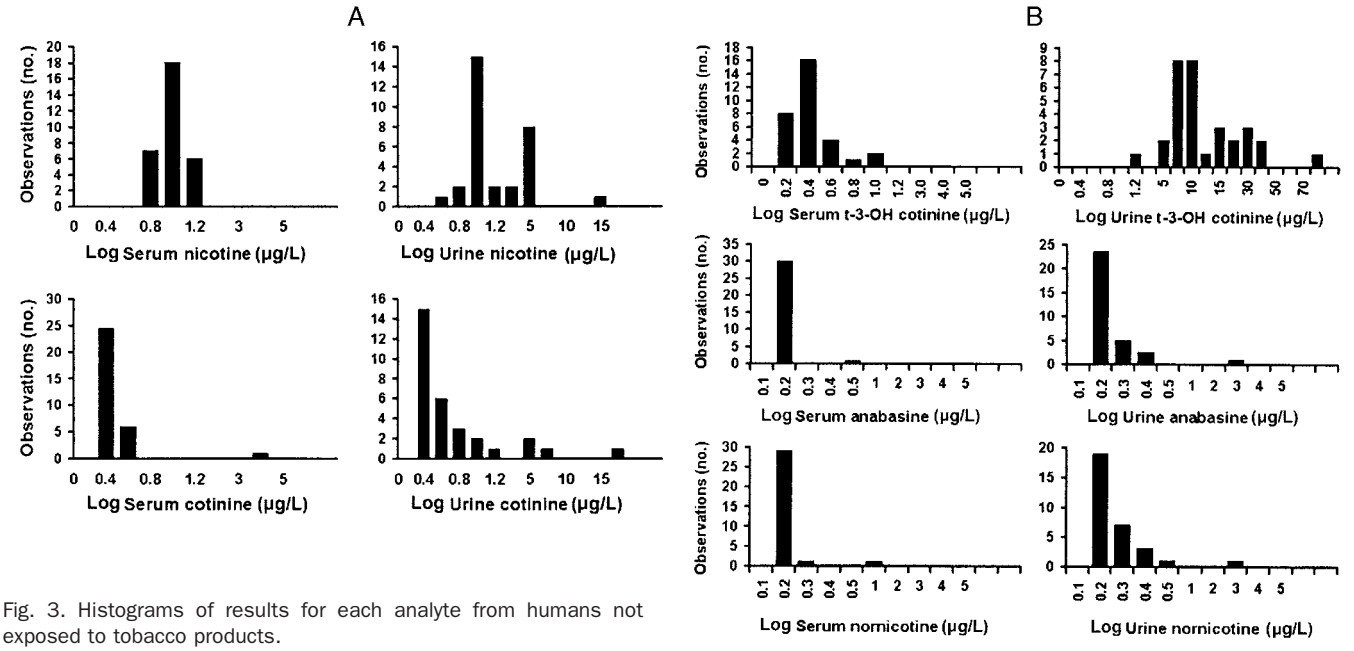

Fig. 3. Histograms of results for each analyte from humans not exposed to tobacco products.

the needs of clinical practice. Imprecision for the key clinically important analytes nicotine, cotinine, and anabasine was <12% throughout the concentration ranges of clinical interest. The largest imprecision was observed for *trans*-3′-hydroxycotinine; this was attributable to considerable difference in recovery of the internal standard and this analyte. Improvement could be anticipated if a deuterated internal standard for *trans*-3′-hydroxycotinine were available. Unfortunately, there is no commercial source for such an internal standard. Custom synthesis was estimated to cost more than $75 000, which exceeded the perceived value of more accurate analysis for this analyte at this time.

LIMIT OF QUANTIFICATION

For this report, we elected to use the definition of limit of quantification proposed by Shah et al. *(42)*: the limit of quantification is the concentration at which the analyte can be measured with an imprecision <20%. Our data (Table 2) indicate that 2 μg/L is the quantification limit for nicotine and nornicotine in urine, whereas 1 μg/L is the quantification limit for anabasine and cotinine, and 5 μg/L is the quantification limit for *trans*-3′-hydroxycotinine.

ACCURACY

Accuracy was demonstrated by correlation slopes of 1.0 ± 0.05 (r >0.98) for nicotine and cotinine extracted from biological specimens compared with results obtained by comparison methods (Fig. 2). No comparison methods for *trans*-3′-hydroxycotinine, anabasine, and nornicotine were available. On the basis of similarities among the chemical structures of nicotine, cotinine, anabasine, and nornicotine, we anticipate that the accuracies for anabasine and nornicotine would be similar to those observed for nicotine and cotinine.

LINEARITY

For serum, the linear ranges for this method were 2–100 μg/L for nicotine, 10–5000 μg/L for cotinine, and 50–5000 μg/L for *trans*-3′-hydroxycotinine (Table 3). For the reasons outlined below, we did not perform linearity studies for anabasine and nornicotine in serum. For urine, the linear ranges were 5–1000 μg/L for nicotine, 10–3000 μg/L for cotinine, 50–5000 μg/L for *trans*-3′-hydroxycotinine, 5–1000 μg/L for anabasine, and 5–1000 μg/L for nornicotine (Table 4).

CONTAMINATION

One would expect tobacco abstainers to have no nicotine or nicotine metabolites in their biological fluids. However, the ubiquitous availability of nicotine products in society provides numerous opportunities for abstainers to be passively exposed. Exposure to nicotine occurs wherever tobacco smoke exists *(43)*. As a further indication of the ubiquitous distribution of nicotine, we observed during test development that many of the reagent-grade chemicals in our laboratory stocks contained measurable nicotine. This required careful culling of contaminated reagents from our stocks before implementation of this procedure. It is difficult to completely avoid nicotine in our environment.

RESULTS IN UNEXPOSED HUMANS

We observed trace amounts of nicotine and nicotine metabolites in biological fluids of tobacco-free individuals recruited to provide biological specimens anticipated to be void of nicotine and metabolites (Fig. 3). The median

serum and urine nicotine concentrations observed in the non-tobacco users were 0.9 and 1.2 μg/L, respectively. Median serum and urine cotinine concentrations were 0.5 and 0.9 μg/L, respectively, and median serum and urine *trans*-3′-hydroxycotinine concentrations were 0.5 and 0.9 μg/L, respectively. Nornicotine and anabasine were undetectable (<0.5 μg/L) in the serum and urine of these recruits.

Passive exposure to tobacco smoke causes nonsmokers to accumulate and excrete measurable nicotine and metabolites. Toumi et al. *(43)* and Pokorski et al. *(44)* reported that urine cotinine in passively exposed nonsmokers was <30 μg/L and that *trans*-3′-hydroxycotinine was 300 μg/L. One member of our "normal range" cohort exhibited urine cotinine of 12.1 μg/L and *trans*-3′-hydroxycotinine of 63 μg/L. We believe these concentrations were attributable to passive exposure.

CLINICAL STUDIES

In an evaluation of treatment for nicotine addiction, Dale et al. *(45)* reported that monitoring tobacco-product use rates did not predict treatment success. As a result of this observation, we concluded that a better indicator of treatment success was needed. Riboli et al. *(46)* and Haufroid and Lison *(47)* found that urine cotinine >50 μg/L consistently differentiated abstinence from continued tobacco use.

Dale et al. *(9)* reported observations of nicotine, metabolites, and alkaloid concentrations in 65 ST users who were electively beginning treatment for tobacco dependence. Each ST user contributed serum and urine during ad libitum use for testing and anther set of samples after 2 weeks of tobacco abstinence. Abstinence was verified by carbon monoxide monitoring and psychological evaluation. Results from the Dale study *(9)* are depicted in histogram format in Fig. 4 and are offered in tabular format in Table 8 as reference for future studies.

REFERENCE RANGES

Results outlined in Figs. 3 and 4 and Table 8 provide useful reference ranges for future studies.

Serum. (*a*) A nicotine concentration in the range 6–52 μg/L with cotinine in the range 106-1230 μg/L and *trans*-3′-hydroxycotinine in the range 28–581 μg/L was representative of individuals using either tobacco or nicotine replacement therapy. Nornicotine and anabasine were not detected (<1 μg/L) in the serum of active tobacco users. (*b*) Nicotine concentrations <2 μg/L, cotinine <3 μg/L, and *trans*-3′-hydroxycotinine <2 μg/L were representative of tobacco users who were abstinent longer than 2 weeks. Nornicotine and anabasine were not detected in the serum of abstinent tobacco users. (*c*) Nicotine concentrations <2 μg/L, cotinine <2 μg/L, and *trans*-3′-hydroxycotinine <0.5 μg/L were representative of unexposed non-tobacco users. Nornicotine and

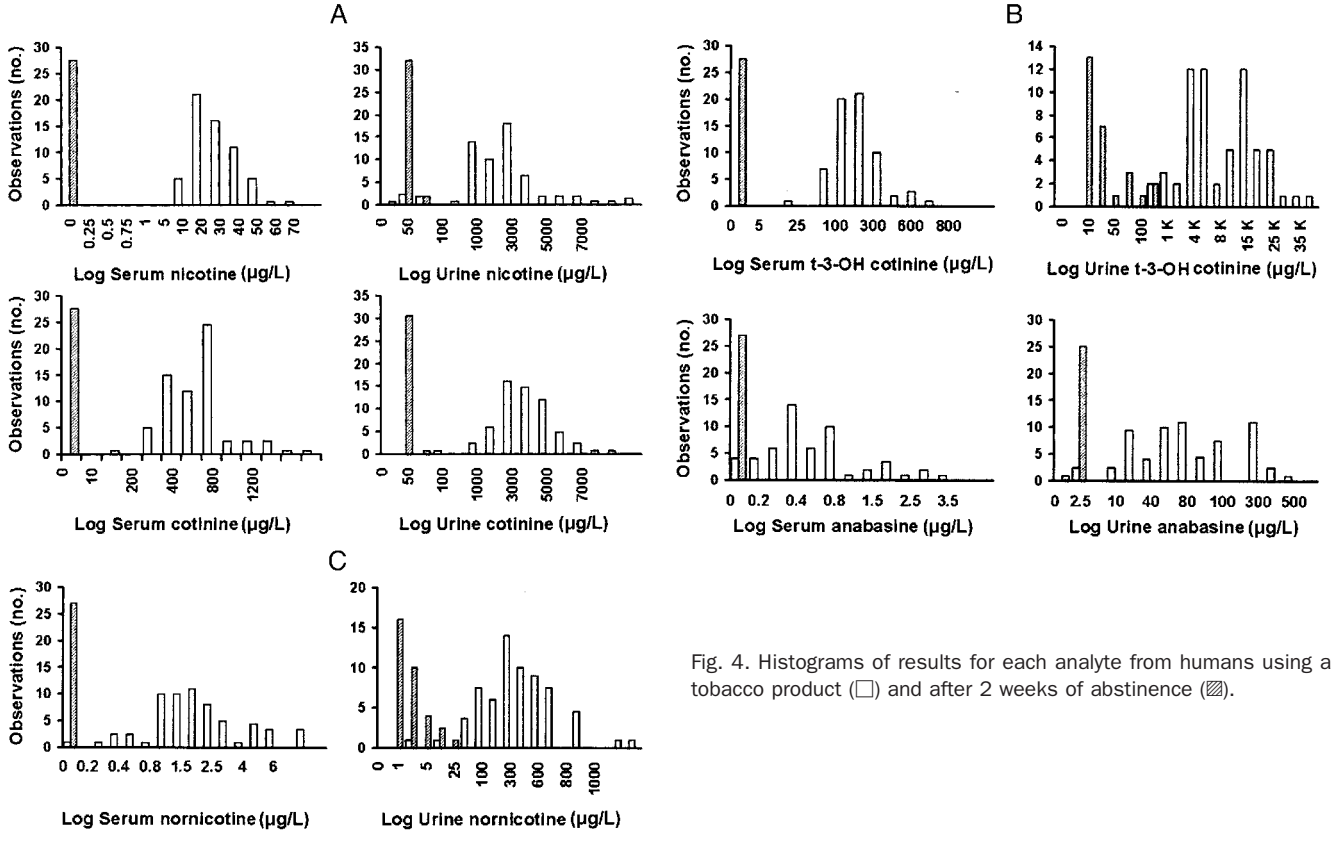

Fig. 4. Histograms of results for each analyte from humans using a tobacco product (□) and after 2 weeks of abstinence (▨).

Table 8. Reference ranges.[a]

	Reference range, μg/L			
	Non-Tobacco user	Passive exposure *(43, 44)*	Abstinent tobacco user	Active tobacco use
Serum				
Nicotine	<2	<2	<2	30–50
Cotinine	<2	<8	<2	200–800
t-3-OH-Cotinine[b]	<2	<2	<2	100–500
Urine				
Nicotine	<2	<20	<30	1000–5000
Cotinine	<5	<20	<50	1000–8000
t-3-OH-Cotinine	<50	<50	<120	3000–25 000
Anabasine	<2	<2	<2	10–500
Nornicotine	<2	<2	<2	30–900

[a] Suggested reference ranges for future studies based on results are outlined in Figs. 3 and 4 and Refs. *(43, 44)*.

[b] *t*-3-OH-Cotinine, *trans*-3′-hydroxycotinine.

anabasine were not detected (<0.5 μg/L) in the serum of unexposed non-tobacco users.

Urine. (*a*) Nicotine concentrations were 100-6780 μg/L, cotinine was 73–6680 μg/L, *trans*-3′-hydroxycotinine was 400–31 900 μg/L, nornicotine was 19–1010 μg/L, and anabasine was 2.8–257 μg/L in tobacco users. Similar results were observed in patients prescribed nicotine replacement therapy for nicotine, cotinine, and *trans*-3′-hydroxycotinine, but anabasine was not detected in the urine of individuals who were on nicotine replacement therapy. (*b*) Nicotine concentrations <30 μg/L, cotinine <23 μg/L, *trans*-3′-hydroxycotinine <120 μg/L, nornicotine <3 μg/L, and anabasine <2 μg/L were representative of tobacco users who abstained from using tobacco products for longer than 2 weeks. (*c*) Nicotine concentrations <2 μg/L, cotinine <5 μg/L, *trans*-3′-hydroxycotinine <50 μg/L, nornicotine <2 μg/L, and anabasine <2 μg/L were representative of unexposed non-tobacco users.

Assessment of nicotine metabolism and disposition has become an integral part of nicotine dependency treatment programs. Serum nicotine concentration or urine cotinine concentration can be used to guide dosage for nicotine patch therapy to achieve biological concentrations adequate to provide the patient with immediate relief from withdrawal symptoms, allowing a greater opportunity for withdrawal success. The absence of nicotine metabolites and anabasine can document abstinence from tobacco products, an indicator of treatment success. Nicotine, cotinine, *trans*-3′-hydroxycotinine, nornicotine, and anabasine can be simultaneously and accurately quantified in either serum or urine by MS/MS with imprecision <10% and limits of quantification of 0.5–5 μg/L. Knowledge of the serum or urine concentrations of these analytes can be used to guide nicotine replacement therapy or to assess tobacco abstinence in patients in nicotine treatment programs. These measurements are now an integral part of the clinical treatment and management of patients who wish to overcome tobacco dependence.

The work described here was approved by the Mayo Clinic Institutional Review Board (680-99, 1120-01, and 1684-01) and supported by grants from the NIH (Grants R01CA90884-01, R01AA95005, and NCTT6N99C4). We wish to acknowledge Joyce Freiheit, Rebecca Verthein, and Debra Judy for gathering information and preparing this manuscript for publication.

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