DETERMINATION OF NICOTINE *N*-1-GLUCURONIDE, A QUATERNARY *N*-GLUCURONIDE CONJUGATE, IN HUMAN BIOLOGICAL SAMPLES

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SUMMARY

[Methyl-d_3]-*N*-1-β-D-glucopyranosyl-(±)-nicotinium inner salt {(±)-[methyl-d_3]nicotine *N*-1-glucuronide} was synthesized from (±)-[methyl-d_3]nicotine *via* reaction with methyl-2,3,4-tri-*O*-acetyl-1-bromodeoxy-α-D-glucopyranouronate, followed by deprotection with 1 M aqueous NaOH and purification by preparative TLC. Nicotine *N*-glucuronide was identified and determined directly in smokers' urine. A solid phase extraction method was used to partially isolate the material from urine. Subsequent determination was by thermospray-LC/MS using the synthetic d_3-labeled nicotine *N*-glucuronide as internal standard. The identified urinary component had the same retention time as a synthetic standard and gave the same mass spectrum. The thermospray mass spectrum was characterized from the protonated molecular ion (*m/z* 339) and the protonated aglycone ion (*m/z* 163). Quantitative results from this direct method were compared with those from an indirect method, which calculated the nicotine glucuronide in the biological sample from the amount of nicotine released following treatment of the sample with the deconjugating enzyme, β-glucuronidase. On average, the concentration of nicotine *N*-glucuronide determined by the direct method was 34% greater than that determined by the indirect method. Concentrations of nicotine *N*-

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glucuronide in urine ranged from 2.2 to 7.6 nmol/ml with a limit of detection of 1.3 nmol/ml.

KEY WORDS

deuterated nicotine *N*-glucuronide, nicotine metabolite, thermospray-LC/MS analysis, urinary biotransformation product

INTRODUCTION

Nicotine, widely consumed throughout the world as a major component of tobacco, has a highly complex metabolic profile in man /1/. Determining nicotine uptake is important in quantifying exposure to tobacco smoke by smokers, and also in determining exposure to second-hand tobacco smoke by nonsmokers /2/. The first step in quantifying exposure is understanding the fate of nicotine in the body. Wide inter-individual variability in the excretion of nicotine metabolites /3,4/ suggests that a single metabolite cannot be successfully used as a biomarker to assess nicotine exposure. Thus, methods that determine many nicotine metabolites have been developed /5-10/, with reported recoveries of 81-98% of nicotine in the form of urinary nicotine and its metabolites /11-12/.

An important part of nicotine metabolism in humans occurs through phase II reactions that produce glucuronide conjugates of nicotine and of two of its major metabolites, *trans*-3′-hydroxycotinine and cotinine, as demonstrated by their occurrence in the urine of smokers and snuff users /3,13-15/. Most of the evidence for the presence of these glucuronides has been by indirect methods, which involve the use of either enzyme (β-glucuronidase) or alkaline hydrolysis to release the corresponding aglycone, which is then detected by various analytical means /16/. While indirect methods for the determination of glucuronide conjugates can provide good quantitative estimates, they suffer from several deficiencies. Since they depend on quantification of the aglycone before and after treatment with β-glucuronidase and determination of glucuronide concentration by difference, such methods are rather time and labor intensive. In addition, incomplete hydrolysis, other sources of aglycone, lack of internal standards, and errors associated with

calculating concentrations by difference all contribute to uncertainties in the analytical outcome. A method of direct determination would reduce some of these uncertainties, and would afford a shorter analysis time.

Previous work in our laboratory has reported on the synthesis of cotinine *N*-1-glucuronide inner salt /17/, *cis*-3′-hydroxycotinine *N*-1-glucuronide inner salt /17/, and *trans*-3′-hydroxycotinine *N*-1-glucuronide inner salt /18/. In addition, we have prepared [methyl-d_3]-*N*-1-β-D-glucopyranosyl-(±)-cotininium inner salt [(±)cotinine *N*-1-glucuronide] and obtained direct evidence for the occurrence of cotinine *N*-1-glucuronide in smokers' urine using thermospray-LC/MS /17,19,20/. In studies by Schepers *et al.* /21/, the glucuronide conjugate of *trans*-3-hydroxycotinine has also been detected in the urine of smokers. It was suspected that nicotine *N*-glucuronide conjugated in the same manner through the pyridine nitrogen was also present in smokers' urine; thus, a synthetic standard of *N*-1-β-D-glucopyranosyl-(*S*)-(-)-nicotinium inner salt (nicotine *N*-glucuronide) was prepared /22/. Other workers have also suggested this structure for the glucuronide conjugate of nicotine /23/. Chemical tests carried out previously on smokers' urine by Benowitz *et al.* have indicated the presence of quaternary pyridinium salts of nicotine /4/. Seaton *et al.* have demonstrated the coelution in rat bile of a ^{14}C-labeled peak with a synthetic nicotine *N*-glucuronide after the rat was treated with ^{14}C-nicotine /23/.

Tsai and Gorrod /24/ have recently reported evidence for the biosynthesis of the *N*-1-β-glucuronide conjugate of *S*-(-)-nicotine by marmoset hepatic microsomes. The analytical methodology utilized [2′-^{14}C]-nicotine as a substrate, and the enzymatically formed glucuronide was detected by high-pressure liquid-radiochromatography. *S*-(-)-Cotinine and (±)-*trans*-3′-hydroxycotinine could not be biotransformed into their respective *N*-1- and 3′-*O*-β-glucuronide conjugates using this microsomal preparation. No glucuronide conjugates of *S*-(-)-nicotine, *S*-(-)-cotinine or (±)-*trans*-3′-hydroxycotinine could be detected when activated microsomal preparations of rabbit, guinea-pig or rat were used as the enzyme source.

We report here *direct* evidence that nicotine glucuronide is produced in humans as the *N*-1-glucuronide with linkage through the pyridine nitrogen. We have prepared both unlabelled and d_3-labelled nicotine *N*-glucuronide inner salt. Using these synthetic standards, we

have developed a method for the determination of nicotine *N*-glucuronide in smokers' urine.

MATERIALS AND METHODS

Chemicals

In the synthetic work, (*S*)-(-)-nicotine free base, sodium hydroxide, nitromethane, and n-butyl lithium (2.5 M in hexane) were obtained from Aldrich Chemical Company (Milwaukee, WI); (*S*)-(-)-nicotine was distilled under vacuum before use. High purity methanol, methylene chloride, chloroform, acetic acid, and ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA). Dowex 50X1-100, 50-100 mesh cation-exchange resin was obtained from Sigma Chemical Company (St. Louis, MO). Deuterium oxide NMR solvent and [methyl-d_3]iodomethane (99.5 atom %D) were purchased from Aldrich Chemical Company. Tetrahydrofuran was obtained from Aldrich Chemical Company and distilled over sodium and benzophenone before use. Racemic nornicotine was synthesized from myosmine, which was prepared as previously described /25/ *via* the method of Jacob /26/. Methyl-2,3,4-tri-*O*-acetyl-1-bromo-deoxy-α-D-glucopyranuronate was prepared as reported by Caldwell *et al.* /17/, based on the procedure of Bollenback *et al.* /27/. Silica gel plates (250 μm layer), fluorescent at 254 nm, were purchased from Whatman Laboratory Sales (Hillsboro, OR). Preparative layer chromatography plates (2 mm layer, 20x20 cm), fluorescent at 254 nm, were obtained from EM Science (Gibbtown, NJ). Solvent systems utilized for TLC monitoring of reaction products were as follows: (a) chloroform/methanol (95:5 v/v); (b) chloroform/methanol/water (65:32.5:12.5 v/v). ^{1}H NMR spectra were obtained on a Varian Unity 300 MHz spectrometer (Palo Alto, CA). Spectra were acquired at 21°C in D_2O and all chemical shifts are reported in parts per million downfield of internal TSP.

For the analytical method, methanol (American Burdick & Jackson, Muskegon, MI) and high purity water (Milli Q Filtration System, Millipore Corporation, Bedford, MA) were used as solvents. Reagent grade ammonium acetate was purchased from Mallinckrodt (Paris, KY). β-Glucuronidase from *Helix pomatia* (100,000 Fishman

units/ml) was purchased commercially (Sigma Chemical Co., St. Louis, MO).

Synthesis of unlabeled and labeled nicotine *N*-1-glucuronides

Preparation of N-1-β-D-glucopyranosyl-(S)-(-)-nicotinium inner salt

A solution of (*S*)-(-)-nicotine (50 mg, 0.31 mmol) in methylene chloride (0.4 ml) was added to methyl-2,3,4-tri-*O*-acetyl-1-bromo-deoxy-α-D-glucopyranuronate (**1**) (367 mg, 0.93 mmol). The resulting solution was stirred at 40°C under nitrogen for 4 days. The reaction mixture was partitioned between water (1 ml) and chloroform (1 ml). The aqueous layer was washed with chloroform (2 x 0.5 ml) and evaporated to dryness to afford a crude sample of the desired conjugated product. Analytically pure *N*-1-(2,3,4-tri-*O*-acetyl-6-methyl-β-D-glucopyranuranosyl)-(*S*)-nicotinium bromide (45 mg, 26% yield) was obtained as an amorphous glass by preparative chromatography on silica gel plates (2.5 g) (system a; $R_f = 0.3$): ^{1}H NMR δ 9.20 (1H, s, pyridinium H-2), 9.01 (1H, d, pyridinium H-6), 8.61 (1H, d, pyridinium H-4), 8.10 (1H, t, pyridinium H-5), 6.22 (1H, d, H-1"), 5.30-5.75 (3H, m, H-2", H-3", H-4"), 4.65-4.75 (1H, m, H-5"), 3.65 (3H, s, $COOCH_3$), 3.50-3.65 (1H, m, H-2'), 3.10-3.20 (1H, m, H-5'a), 2.20-2.50 (2H, m, H-3'a, H-5'b), 1.70-2.20 (3H, m, H-3'b, H-4'), 2.12 (3H, s, NCH_3), 2.01, 1.95, 1.80 (9H, 3xs, $3xOCOCH_3$). Elemental analysis: Calc. for $C_{23}H_{31}N_2O_9Br$, C, 49.38; H, 5.59; N, 5.01. Found C, 49.69; H, 5.51; N, 4.89. The above intermediate (45 mg, 0.13 mmol) was dissolved in 1 M NaOH (0.2 ml) and the solution was stirred at room temperature for 18 h. The mixture was then neutralized with dilute aqueous acetic acid, and applied to a column containing a strong cation-exchange resin (Dowex 50X1-100). The column was eluted with 4 bed volumes of distilled water, followed by 2 M aqueous ammonia solution. Lyophilization of the combined basic eluates under high vacuum afforded 27 mg of *N*-β-1-D-glucopyranosyl-(*S*)-nicotinium inner salt [*S*-(-)-nicotine *N*-1-glucuronide] as an amorphous solid (95% yield). ^{1}H NMR δ 9.01 (1H, s, pyridinium H-2), 8.90 (1H, d, pyridinium H-6), 8.55 (1H, d, pyridinium H-4), 8.05 (1H, t, pyridinium H-5), 5.65 (1H, d, H-I"), 3.91-4.02 (1H, m, H-5"), 3.45-3.75 (4H, m, H-2", H-3", H-4", H-2'), 3.10-3.25 (1H, m, H-5'a), 2.25-2.60 (2H, m, H-3'a, H-5'b), 2.18 (3H, s, NCH_3), 1.82-2.01 (3H, m, H-

3'b, H-4'). Elemental analysis: Calc. for $C_{16}H_{22}N_2O_6$, C, 56.80; H, 6.55; N, 8.28. Found C, 57.11; H, 6.50; N, 8.01.

***Preparation of [methyl-d_3]-N-1-β-D-glucopyranosyl-(±)-nicotinium inner salt* (3)**

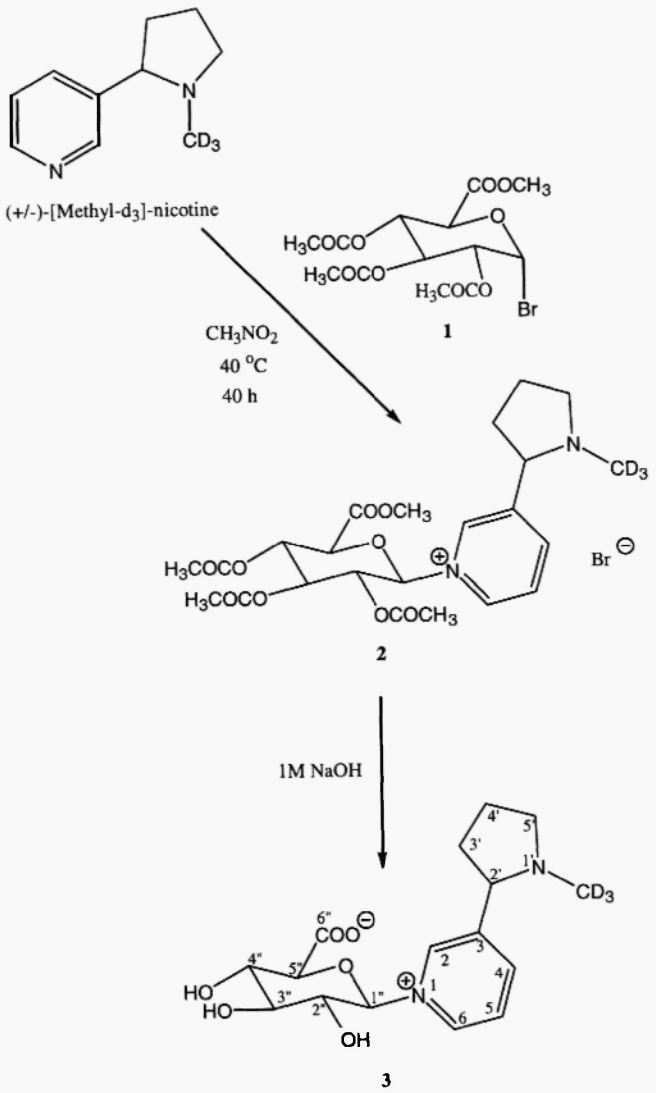
[Methyl-d_3]nicotine *N*-1-glucuronide was prepared by the synthetic route shown in Scheme 1. A solution of (±)-[methyl-d_3]-nicotine, prepared by condensation of [methyl-d_3]-iodomethane with (±) nornicotine at -70°C in dry tetrahydrofuran under nitrogen in the presence of 1 equivalent of n-butyl lithium (215.6 mg, 1.3 mmol) in nitromethane (2 ml) /28/, was added to **1** (1.56 g, 3.9 mmol). The reaction mixture was stirred at 40°C under nitrogen for 40 h, cooled to room temperature, and extracted with water (4 x 2 ml). The aqueous layer was evaporated to dryness to afford a mixture of **2** and the corresponding elimination product (ratio 1.5:1, respectively). This mixture was dissolved in 1 M NaOH (6 ml) and stirred at room temperature for 18 h. The basic solution was neutralized with dilute aqueous acetic acid; TLC analysis (system b, $R_f = 0.1$) showed mainly one area corresponding to **3**. Pure **3** (22 mg) was isolated from 0.5 ml of the basic reaction mixture by preparative silica gel TLC (system b). ^{1}H NMR δ 8.81-9.94 (2H, m, pyridinium H-2 and H-6), 8.45-8.54 (1H, d, pyridinium H-4), 7.96-8.06 (1H, t, pyridinium H-5), 5.60-5.68 (1H, d, H-1"), 3.90-4.02 (1H, m, H-5"), 3.41-3.61 (3H, m, H-2", H-3", H-2'), 3.02-3.10 (1H, m, H-5'a), 2.10-2.20 (2H, m, H-3'a, H-5'b), 1.60-1.85 (3H, m, H-3'b, H-4'); mass spectral analysis: *m/z* 166 ($M+H^+$-$C_6H_8O_6$, 100%), 342 ($M+H^+$, 6%).

Subjects

The six subjects in this study included male and female full flavor or full flavor "low tar" cigarette smokers ranging in age from 38 to 52 years. All subjects smoked *ad libitum* their regular brand of cigarettes, which had yields of 0.6 to 1.3 mg FTC nicotine per cigarette. The subjects collected 24-hour urine samples and recorded the number of cigarettes smoked daily. Consumption ranged from 16 to 48 cigarettes per day and summation of urinary nicotine and its metabolites by a reported method /3/ gave daily yields of 13 to 60 mg nicotine equivalents excreted. A previously characterized, standard smokers'

SCHEME 1

Synthesis of (±)-[methyl-d_3]-nicotine glucuronide



urine, prepared as a pooled sample from nine smokers /29/, was also included in this study.

Sample preparation

For direct determination of nicotine *N*-glucuronide, the internal standard, [methyl-d_3]-nicotine *N*-glucuronide, was added to *5* ml aliquots of urine at a concentration of 3.4 nmol/ml. The sample was acidified with 1 ml of buffer (0.5 M ammonium acetate, pH 5). A Florisil cartridge (Waters, Milford, MA) was prepared by wetting with methanol followed by 3 ml of buffer. The sample was loaded onto the cartridge, which was then washed with 3 ml buffer, 3 ml of 0.5% ammonium hydroxide, and 3 ml methanol. The analyte was eluted with 3 ml of 20% ammonium hydroxide. Approximately 0.75 ml of the final eluate was transferred to a 0.2 μm Microfilterfuge tube (Rainin Instrument Company, Woburn, MA). The sample was force-filtered by centrifugation at 16,500 *g* for 3 min (Eppendorf Model 5415, Eppendorf GmbH, Hamburg, Germany). An injection of 10 μl of this filtrate was made in duplicate onto the LC/MS system. A series of standards was prepared by adding to a nonsmoker's urine [methyl-d_3]nicotine *N*-glucuronide at 3.4 nmol/ml and nicotine *N*-glucuronide from 0 to 10.1 nmol/ml. The standards were prepared as described above. Nicotine *N*-glucuronide was also determined indirectly by a previously described method utilizing β-glucuronidase hydrolysis to release nicotine from its conjugated form /3/. In this method, the amount of nicotine *N*-glucuronide was determined by the difference in nicotine concentration before and after enzymatic hydrolysis.

A larger aliquot of smoker's urine (50 ml) was prepared in the same manner to obtain a full mass spectrum for confirmation. The final eluate was lyophilized (model 75035, Labconco Corporation, Kansas City, MO) and reconstituted in 0.5 ml of 0.5% ammonium hydroxide. Fifty ml aliquot parts of a blank (nonsmoker) urine and a blank urine fortified with the nicotine *N*-glucuronide standard at 14 nmol/ml were prepared likewise. Recovery of nicotine *N*-glucuronide from the spiked urine sample was 32%.

Thermospray LC/MS

Mass spectrometric analyses of standards and samples were performed on a Hewlett-Packard 5988A quadrupole system with a

1090 liquid chromatograph and thermospray option (Palo Alto, CA) by a modification of a previously reported method /3,10/. For the determination of nicotine *N*-glucuronide, the water/methanol gradient began with 3% methanol and increased to 30% methanol at 3 min, to 45% methanol at *5* min, and to 90% methanol at 5.5 min. Post-column addition of 0.1 M ammonium acetate buffer was accomplished using a water/methanol "reversed-gradient" to match that of the analytical column. A Waters model 510 pump (Milford, MA) was used for post column addition of 0.4 ml/min of 0.1 M ammonium acetate. A Rheodyne model 7161 injector (Cotati, CA) was used to introduce the sample to the column. The mass spectrometer was operated in the filament-off mode at a source temperature of 250°C. Ions monitored were *m/z* 163 and 166 [$(M+H)^+$ for nicotine and [methyl-d_3]nicotine, respectively] and *m/z* 339 and 342 {$(M+H)^+$ for nicotine *N*-glucuronide and [methyl-d_3]-nicotine *N*-glucuronide, respectively} with a dwell time of 0.24 seconds for each ion. For extracts from the larger samples, the mass spectrometer was scanned repetitively from 150 to 400 mass units at 1.2 s per scan.

RESULTS

The thermospray mass spectrum of nicotine *N*-glucuronide, similar to that of cotinine-*N*-glucuronide /19/, shows the protonated aglycone as the base peak at *m/z* 163 with a weak protonated molecular ion at *m/z* 339 (Fig. 1A). By monitoring these two ions, it was possible to detect small amounts of nicotine *N*-glucuronide. However, the urine matrix is quite complex and coeluting interferences obscured direct determination of nicotine *N*-glucuronide in most urine samples even by selected ion monitoring. A method for separating nicotine *N*-glucuronide from early eluting interferences was developed using Florisil solid phase extraction. By loading the urine at pH 5, the nicotine *N*-glucuronide was protonated and thus retained on the packing. Strong base was required to remove the compound from the cartridge, presumably as the zwitterion.

Figure 2 compares total ion chromatograms (150 to 400 mass units) for extracts prepared from 50 ml urine samples from a nonsmoker (blank), the same urine spiked with the nicotine *N*-glucuronide synthetic standard, and urine from a smoker. The standard coeluted with a well-resolved peak from the smoker's urine, which

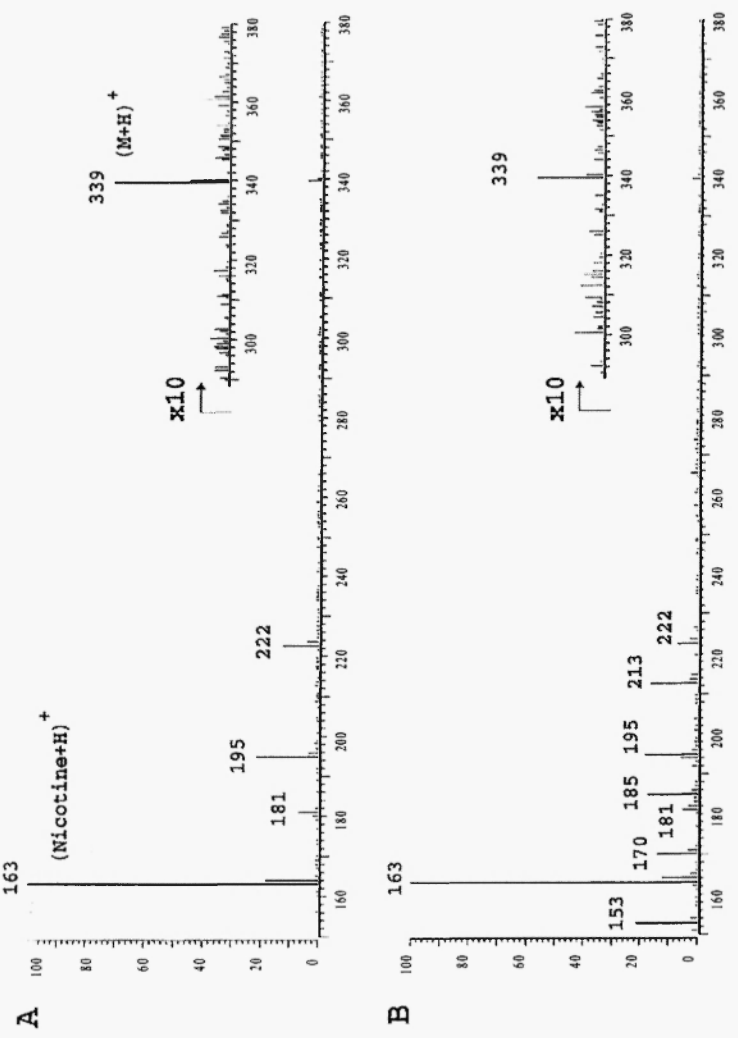


Fig. 1: Thermospray mass spectra of nicotine *N*-glucuronide from **A**) a synthetic standard and **B**) a preparation from a smoker's urine.

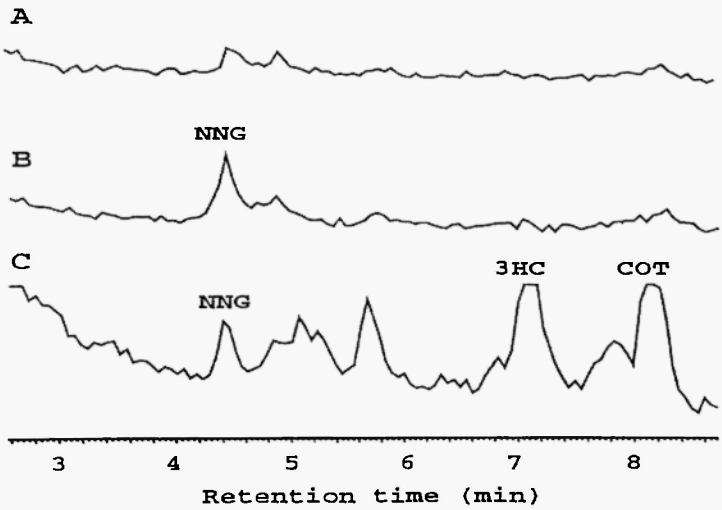


Fig. 2: Total ion chromatograms of urine samples prepared from **A)** a nonsmoker's urine, **B)** a nonsmoker's urine fortified with nicotine *N*-glucuronide at 2.8 nmol/ml, and **C)** a smoker's urine.

gave a very similar spectrum, as shown in Figure 1B, confirming its identity as nicotine *N*-glucuronide. In addition, treatment of both the standard and smoker's urine with β-glucuronidase resulted in the disappearance of this peak and, in the case of the standard, the production of nicotine, indicating a glucuronide linkage. The smaller peaks in the mass spectrum from the smoker's urine (Fig. 1B) suggest minor endogenous material coeluting with nicotine *N*-glucuronide. The eluent from the smoker's urine also contained *trans*-3′-hydroxycotinine and cotinine, as shown in Figure 2C. These are both significant urinary nicotine metabolites. The small coeluting peak in the blank urine (Fig. 2A) did not contain any of the ions in the mass spectrum of nicotine *N*-glucuronide.

The synthesis of [methyl-d_3]nicotine *N*-glucuronide permitted an accurate determination of nicotine *N*-glucuronide directly in urine. A standard curve was established using the ratio of *m/z* 339 to *m/z* 342, since these ion chromatograms had less chemical noise than the *m/z* 163 and 166 traces. The plot was linear with the regression equation: $y = (0.9457 \pm 0.0393)x + (0.0453 \pm 0.0564)$, $R^2 = 0.9931$. The limit of

detection was determined as 1.3 nmol/ml using the intercept plus three standard deviation units /30/. Nicotine *N*-glucuronide was also determined indirectly using a method that measures nicotine in the samples before and after treatment with β-glucuronidase /3/. Table 1 shows the results of both direct and indirect determinations. Agreement between the two values ranged from -34 to 75% with the direct method determining on average 34% more nicotine *N*-glucuronide in these samples than the indirect method. From the pre-hydrolysis concentration of nicotine and the concentration of nicotine *N*-glucuronide determined directly, the ratio of conjugated to non-conjugated nicotine was calculated. Ratios ranged from 0.21 to 0.79 for the samples in this study. Table 1 also lists the total amount of nicotine equivalents excreted by each smoker and the relative percent of nicotine *N*-glucuronide based on that amount. The average amount of total nicotine excreted as nicotine *N*-glucuronide was 6.26 ± 4.2% with a range of 0.9 to 14.3%.

DISCUSSION

The study presented here provides a method for the determination of nicotine *N*-glucuronide in smokers' urine as the *N*-1-β-D-glucopyranosylnicotinium inner salt, the same structural type of conjugate as cotinine *N*-1-glucuronide /17,19/, and adds to other reports of human urinary excretion of quaternary *N*-glucuronide conjugates of xenobiotics /31-37/.

On average, the concentration of nicotine *N*-glucuronide determined by the direct method was 34% greater than that determined by the indirect method. The latter method depends on a small difference in two measurements and is thus inherently less precise. In addition, it depends on freeing nicotine from its conjugate, which, if not completely successful, will affect accuracy. The direct determination method is expected to be more accurate and precise since it is does not require a hydrolysis step, and is based on only one measurement. In particular, use of isotopically labeled internal standards (isotope dilution) has been shown to afford an excellent method of quantification /38/. The production of a linear response and a near unity slope over the calibration range constitutes a high degree of confidence in these data.

TABLE 1

Nicotine and nicotine *N*-glucuronide (NNG) determinations for six smokers and a standard smoker's urine (SSU) [a]

Subj.	Nicotine (nmol/ml)		NNG (nmol/ml)		% Diff	Ratio Conj/Unconj	NNG per 24 h (mg)	Total Nic. per 24 h (mg)	% of total Nic. as NNG
	Pre-enzyme	Post-enzyme	Indirect	Direct					
1	10.7	13.7	3.1	2.2	-34%	0.21	0.20	21.5	0.9%
2	10.1	12.7	2.5	4.6	58%	0.45	0.71	13.5	5.3%
3	14.0	18.6	4.6	7.3	47%	0.52	2.89	40.8	7.1%
4	8.1	12.3	4.2	5.6	29%	0.69	1.86	59.6	3.1%
5	5.8	9.6	3.7	3.5	-5%	0.61	2.28	35.6	6.4%
6	6.3	8.5	2.3	5.0	75%	0.79	1.81	12.7	14.3%
SSU	12.6	17.6	5.1	7.6	41%	0.61	-	-	6.8%
Avg	9.7	13.3	3.6	5.1	34%	0.55			6.3%
SD	3.1	3.8	1.1	2.0		0.19			4.2%

[a] Data are given in nmol/ml. Indirect determination of nicotine *N*-glucuronide is by difference in pre- and post-enzyme treatment of the urine. "Ratio Conj/Unconj" is the ratio of the conjugated (direct) to unconjugated nicotine. "NNG per 24 h" is based on the concentration of NNG and total volume of urine for each subject. "Total Nic. per 24 h" was determined by a reported method /3/.

The percent of nicotine excreted as nicotine *N*-glucuronide was quite variable among the smokers studied. Subject 1 had less than 1% of this particular metabolite and subject 6 had more than 14%. However, the average percentage of nicotine *N*-glucuronide, 6.3 ± 4.2% of the total amount of recovered nicotine and metabolites, was similar to other reported amounts of 4.6 ± 2.9% /4/, 3% /14/, and 3 ± 2% /3/. The ratio of conjugated to unconjugated nicotine was also variable, ranging from 0.21 to 0.79, again with subjects 1 and 6 in this study exhibiting the two extreme values. The average of 0.55 ± 0.19 is comparable with reported ratios of conjugated to unconjugated nicotine of 0.5 /4/, 0.25 /3/, and 1.0 /14/. Although the number of subjects analyzed was small, these data indicate significant inter-individual variability in phase II nicotine metabolism among smokers.

From our data, nicotine *N*-glucuronide can account for up to 14% of total nicotine excreted by most smokers; thus, it is important to have a reliable analytical method for nicotine *N*-glucuronide for quantification and to validate its occurrence. Quaternary *N*-glucuronides are zwitterions and have the characteristics of being polar, thermally labile, and nonvolatile. These properties make them difficult to analyze directly by conventional mass spectrometric techniques such as GC/MS. As demonstrated here, thermospray-LC/MS is a viable means of analysis for these compounds. The availability of authentic standards (d_3-labeled and unlabeled) is useful for the identification of the glucuronide conjugates of nicotine and phase I nicotine metabolites, and also for the development of methods for their quantification in biological matrices. The third major phase II nicotine metabolite is *trans*-3′-hydroxycotinine glucuronide, which has been reported to be conjugated at the 3′-oxygen position /21/. The determination of all these conjugates will add to our complete understanding of nicotine metabolism in humans.

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