Tobacco-Specific Nitrosamines and Their Pyridine-*N*-glucuronides in the Urine of Smokers and Smokeless Tobacco Users

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Abstract

Tobacco-specific nitrosamines are believed to play a significant role as causes of cancer in people who use tobacco products. Whereas the uptake of one tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, has been shown by analysis of its metabolites in urine, there are no published studies on urinary levels of *N*′-nitrosonornicotine (NNN), *N*′-nitrosoanatabine (NAT), and *N*′-nitrosoanabasine (NAB) or their metabolites in human urine. We developed a method for quantitation of NNN, NAT, NAB, and their pyridine-*N*-glucuronides NNN-*N*-Gluc, NAT-*N*-Gluc, and NAB-*N*-Gluc in human urine. Total NNN (NNN plus NNN-*N*-Gluc) was assayed using 5-methyl-*N*′-nitrosonornicotine as internal standard. Urine was treated with β-glucuronidase. Following solvent partitioning and solid-phase extraction, total NNN was determined using gas chromatography with nitrosamine-selective detection. Total NAT and total NAB were quantified in the same samples. Separate quantitation of NNN and NNN-*N*-Gluc was accomplished by extraction of the urine with ethyl acetate before β-glucuronidase hydrolysis; NNN was analyzed in the ethyl acetate extract, and after enzyme treatment, NNN released from NNN-*N*-Gluc was quantified in the extracted urine. Separate analyses of NAT, NAT-*N*-Gluc, NAB, and NAB-*N*-Gluc proceeded similarly. Analyte identities were confirmed by gas chromatography-tandem mass spectrometry. Mean levels of total NNN, NAT, and NAB in the urine of 14 smokers were (pmol/mg creatinine) 0.18 ± 0.22, 0.19 ± 0.20, and 0.040 ± 0.039, respectively, whereas the corresponding amounts in the urine of 11 smokeless tobacco users were 0.64 ± 0.44, 1.43 ± 1.10, and 0.23 ± 0.19, respectively. Pyridine-*N*-glucuronides accounted for 59% to 90% of total NNN, NAT, and NAB. The results of this study show the presence of NNN, NAT, NAB, and their pyridine-*N*-glucuronides in human urine and provide a quantitative method for application in mechanistic and epidemiologic studies of the role of tobacco-specific nitrosamines in human cancer. (Cancer Epidemiol Biomarkers Prev 2005;14(4):885–91)

Introduction

Tobacco products contain a wide range of chemical carcinogens (1-5). Among these, tobacco-specific nitrosamines, a group of carcinogens formed from tobacco alkaloids during the curing and processing of tobacco, are present in both unburned tobacco and tobacco smoke, and are likely to play a significant role in cancer induction by tobacco products (6-8). The most carcinogenic of the commonly occurring tobacco-specific nitrosamines are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN), whereas *N*′-nitrosoanabasine (NAB) is a weak carcinogen and *N*′-nitrosoanatabine (NAT) apparently is inactive (7). Based on their occurrence in tobacco products, on their carcinogenicity in laboratory animals, and on biochemical evidence, NNK is believed to be involved in causing lung and pancreatic cancer and perhaps other cancers in smokers, whereas NNN is thought to be a cause of esophageal cancer (9, 10). NNN and NNK are also believed to play an important role in oral cancer induction by smokeless tobacco products (6, 10, 11).

Development of specific biomarkers is crucial for obtaining quantitative information on levels of carcinogen uptake and metabolism. Thus, the results of numerous studies of NNK metabolism ultimately led to development of a specific urinary biomarker of NNK uptake by humans, the sum of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides (NNAL-Glucs). The NNAL biomarker has been extensively applied in studies of NNK uptake in smokers, smokeless tobacco users, and nonsmokers exposed to secondhand tobacco smoke (12). However, no biomarker was available to assess uptake of NNN in humans, because all NNN metabolites which occur in reasonable quantities in urine are also metabolites of nicotine or other tobacco alkaloids, which occur in far higher concentrations in tobacco products than does NNN (7, 13). A specific biomarker of NNN uptake would be crucial in studies investigating the causes of oral and esophageal cancer in people who use tobacco products. Similarly, biomarkers of NAB and NAT uptake could provide important information on tobacco-specific nitrosamine exposure in humans.

The major tobacco alkaloid nicotine and its principal metabolite cotinine are metabolized to pyridine-*N*-glucuronides. Nicotine-*N*-Gluc and cotinine-*N*-Gluc account for 3% to 5% and 12% to 17%, respectively, of total urinary nicotine metabolites in humans (14-16). Recently, NNAL-*N*-Gluc has been quantified in the urine of smokers and smokeless tobacco users (17). In view of the metabolic conversion of nicotine, cotinine, and NNAL to pyridine-*N*-glucuronides in humans, it seemed likely that NNN could be metabolized in the same way. Because a small amount of unchanged NNN was consistently observed in the urine of animals treated with NNN (7), the sum of free NNN and NNN-*N*-Gluc could serve as a specific urinary biomarker of human NNN uptake. The purpose of this study was to develop a sensitive method for quantitation of NNN and NNN-*N*-Gluc in the urine of smokers and smokeless tobacco users. We found

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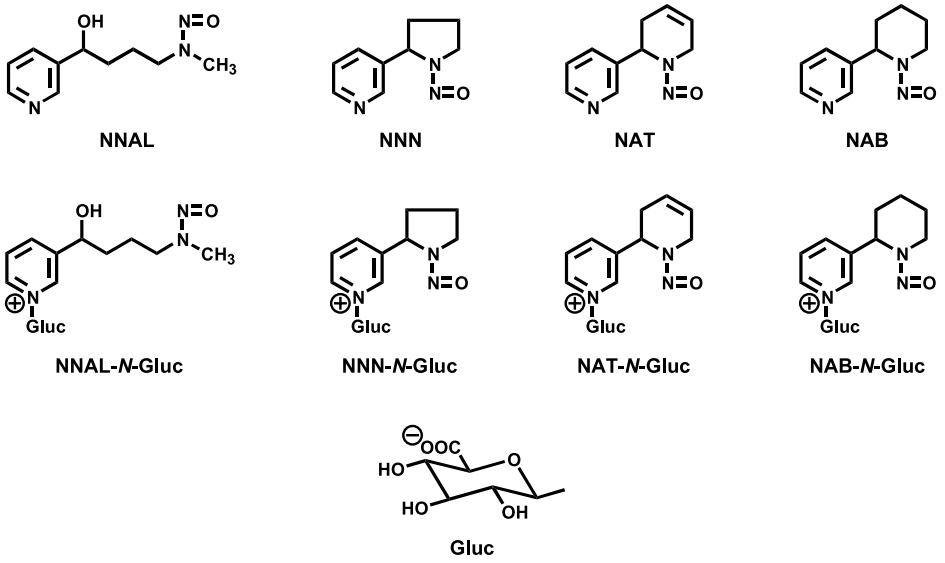

Figure 1. Structures of compounds discussed in the text.

that the same assay could also be used to quantify NAT, NAT-*N*-Gluc, NAB, and NAB-*N*-Gluc in human urine. Structures of the compounds analyzed here are illustrated in Fig. 1.

Materials and Methods

Caution. NNN and NAB are carcinogenic and mutagenic and should be handled with extreme care, using appropriate protective clothing and ventilation at all times.

Apparatus. NNN, NAT, and NAB were analyzed by gas chromatography with nitrosamine-selective detection (GC-TEA) using a HP 6890 gas chromatograph (Agilent Technologies, Wilmington, DE) interfaced with a model 543 Thermal Energy Analyzer (Orion Research, Beverly, MA). The gas chromatograph was equipped with a DB-1301 capillary column (30 m × 0.32 mm × 0.25 μm; J&W Scientific, Folsom, CA) and a 2 m × 0.53 mm deactivated fused silica precolumn. The flow rate was 2.6 mL/min He; splitless injection port temperature was 225°C. The following oven temperature program was used: 80°C for 2 min, then 12°C/min to 150°C, 7 min at 150°C, 12°C/min to 200°C, 10 min at 200°C.

Analysis of cotinine by gas chromatography-mass spectrometry (MS)–selected-ion monitoring was carried out with a model 6890 gas chromatograph equipped with an autosampler and interfaced with a model 5973 mass-selective detector (Agilent Technologies, Palo Alto, CA). The gas chromatograph was equipped with a DB-5 MS fused silica capillary column (15 m × 0.25 mm × 0.25 μm). The splitless injection port temperature was 250°C; the oven temperature was 70°C for 0.5 min, increased to 180°C at 10°C/min, held for 3 min, 50°C/min to 300°C, and returned to initial conditions. The flow rate was 1 mL/min He.

Identities of NNN, NAT, and NAB were confirmed by gas chromatography-tandem mass spectrometry, which was carried out with a Finnigan TSQ-7000 instrument operated in the positive ion chemical ionization mode. Daughter ion scans were done to monitor the transitions *m/z* 178 → 147 and 178 → 117 for NNN; *m/z* 190 → 160 and 190 → 144 for NAT; and *m/z* 192 → 162 and 192 → 146 for NAB, operating Q3 in the selected ion monitoring mode at a scan rate of 0.5 scan/s. The gas chromatograph (HP Model 5890 GC) was equipped with a 15 m × 0.25 mm i.d. DB 1301 column (0.25-μm film thickness) from J&W Scientific connected to a 2 m × 0.32 mm i.d. deactivated precolumn. The injection mode was splitless, the constant flow rate was 2.0 mL/min He, and the injection port temperature was 225°C. The temperature program was as follows: 80°C for 2 min, 20°C/min to 155°C, 2°C/min to 190°C, and 20°C/min to 250°C. The final temperature was held for 5 min.

Chemicals and Enzymes. NNN, NAB, and 5-methyl-*N*′-nitrosonornicotine (5-MeNNN) were synthesized as previously described (18, 19). NAT was obtained from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). [CD_3]Nicotine, [CD_3]cotinine, nornicotine, anatabine, anabasine, and β-glucuronidase (type IX-A from *Escherichia coli*) were purchased from Sigma Chemical Co. (St. Louis, MO).

Analyses

Total NNN (NNN plus NNN-N-Gluc). Urine (36 mL) was adjusted to pH 7.0 ± 0.5 and 36,000 units of β-glucuronidase type IX-A from *E. coli* (Sigma Chemical) were added to each sample. The solution was incubated overnight with gentle shaking at 37°C. On the next day, 2 ng 5-MeNNN internal standard, dissolved in acetonitrile, were added, and the urine was extracted thrice with equal volumes of methylene chloride. The tubes were shaken gently on a bench top shaker for 10 min, and the resulting emulsions were reduced by low-speed centrifugation. The methylene chloride extracts were combined into a fresh 50-mL glass centrifuge tube and ~5 g sodium sulfate were added. The mixture was briefly shaken, and after ~0.5 hour, the methylene chloride extract was transferred to a fresh 50-mL glass centrifuge tube and concentrated to dryness in portions on a model SVT200 H Speedvac concentrator (Thermo Savant, Farmingdale, NY). The dry residue was redissolved in 8 mL high-performance liquid chromatography–grade water, and the pH was adjusted to 2 to 3 with ~300 μL of 1 N HCl. Then, the sample was partitioned thrice with methylene chloride; organic layer going to waste. The aqueous portion was adjusted to pH 7 with 1 mL of potassium phosphate buffer (prepared from 0.1 mol/L KH_2PO_4 adjusted to pH 7 with H_3PO_4), applied to a 10-mL ChemElut cartridge (Varian, Harbor City, CA), and eluted with 4 × 10 mL methylene chloride into a clean 50-mL glass centrifuge tube. The combined eluants were concentrated to dryness (Speedvac concentrator). Residues were dissolved in 0.5 mL of methylene chloride and further purified by solid-phase extraction using Sep-Pak Plus Silica cartridges (Waters Co., Milford, MA), preequilibrated with methylene chloride. The cartridges were washed with 5 mL methylene chloride/ethyl acetate (50:50), and NNN was eluted with 10 mL of ethyl

acetate. The ethyl acetate eluants were concentrated to dryness (Speedvac). The dry residues were transferred into gas chromatograph microvials with 3 × 50 μL methanol, dried, and redissolved in 5 μL of acetonitrile. Four μL of the prepared sample were injected into GC-TEA.

Separate NNN and NNN-N-Gluc. The method is similar to that described above, except that free NNN is extracted from the urine before β-glucuronidase treatment. Urine (36 mL) was adjusted to pH 7.0 ± 0.5 and then partitioned thrice with equal volumes of ethyl acetate. Two ng of 5-MeNNN internal standard were added to the combined extracts, which were dried with sodium sulfate, and concentrated to dryness. The dry residue was stored at −20°C for further processing. The extracted urine containing NNN-*N*-Gluc was reduced to approximately two thirds of its original volume (Speedvac) to remove residual ethyl acetate, which may inhibit β-glucuronidase activity. The urine was further treated overnight with β-glucuronidase as above. After 5-MeNNN internal standard was added, the urine was extracted thrice with equal volumes of methylene chloride. The methylene chloride extracts containing NNN released from NNN-*N*-Gluc were dried with sodium sulfate and concentrated to dryness. Starting with the next step, the residues of both ethyl acetate and methylene chloride extracts were treated in the same manner. The dry residue was redissolved in 8 mL water and the solution was further cleaned up and analyzed as in the total NNN assay.

Total and Free Cotinine. Total and free cotinine were analyzed by a method similar to those described previously (20-22). For the total cotinine assay, 0.1 mL of 1 N NaOH was added to 0.4 mL urine, and the mixture was incubated at 80°C for 30 min. Fifty ng of [CD_3]cotinine (Sigma Chemical) were added to a 5-mL glass centrifuge tube (Kimble, Vineland, NJ) containing 0.3 mL H_2O and 0.4 mL of 25% aqueous K_2CO_3. The base-treated urine (0.2 mL) was added to the tube, and the mixture was extracted once with 1 mL methylene chloride. The methylene chloride layer was separated and mixed with 200 μL methanol. This solution was concentrated under a gentle stream of nitrogen to a total volume of 100 to 200 μL. The samples were transferred to gas chromatograph microinsert vials and analyzed by GC-MS–selected ion monitoring. Free cotinine was analyzed by a similar method, excluding base treatment. Urine (0.1 mL) was added to a 5-mL glass centrifuge tube containing 0.4 mL H_2O, 0.4 mL 25% K_2CO_3, and [CD_3]cotinine. The mixture was further treated in the same manner as in the total cotinine assay.

Other Analyses. Total NNAL was quantified as described (23). Creatinine was assayed by Fairview-University Medical Center Diagnostic Laboratories (Minneapolis, MN) using Vitros CREA slides.

Statistical Analyses. Pearson product moment correlation coefficients and statistical significance of the correlations of combined data from smokers and smokeless tobacco users were determined using SigmaPlot 2001, v. 7.101 (SPSS, Inc., Chicago, IL). Data in the text and tables are expressed as mean ± SD.

Results

Animal studies consistently show that low amounts, generally <1% of the dose, of unchanged NNN are excreted in urine and there was no indication of the presence of NNN-*N*-Gluc (13). Based on this work, we expected that NNN and NNN-*N*-Gluc, if present in human urine, would be found in relatively low concentrations. Therefore, we began our work by developing a method for total NNN, using 36 mL of urine for analysis.

Analysis of Total NNN in a Nonsmoker's Urine to which NNN was Added. First, we applied the method to a nonsmoker's urine to which a known amount of NNN was added. A typical GC-TEA trace of the spiked urine sample is illustrated in Fig. 2A. The method produced clean chromatograms, with clear NNN and 5-MeNNN peaks and good recovery of both internal standard (63%) and spiked NNN (79%).

Analysis of Tobacco Users' Urine for Total NNN. The method was applied for analysis of total NNN in the urine of six smokers and six smokeless tobacco users. A typical chromatogram is presented in Fig. 2B. Peak 1 had the same retention time as NNN, established by coinjection with a standard solution. Generally, the GC-TEA traces obtained were clean and the NNN and 5-MeNNN peaks were well resolved. NNN was found in 8 of the 12 urine samples.

Other Compounds Found in Tobacco Users' Urine. Peaks 2 and 3 of Fig. 2B were consistently observed in urine samples

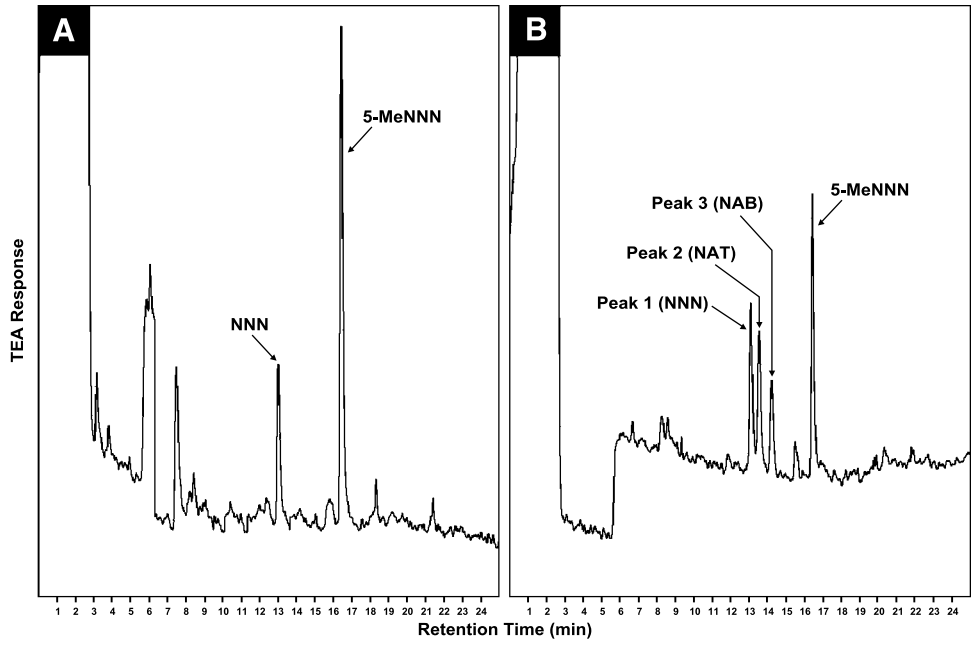

Figure 2. Chromatograms obtained upon GC-TEA analysis, using the total NNN assay, of (**A**) a nonsmoker's urine to which NNN (0.1 pmol/mL urine) was added; and (**B**) a smoker's urine.

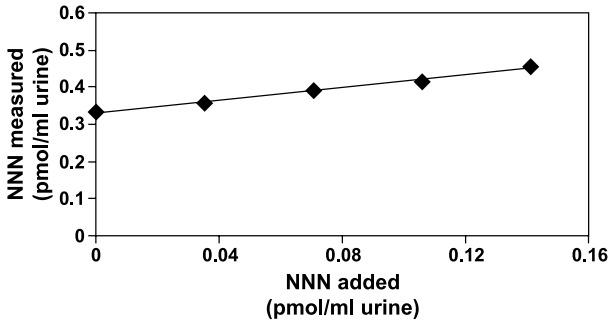

Figure 3. Relationship between levels of NNN added to a smoker's urine sample and levels measured (r = 0.99).

from smokers and smokeless tobacco users. These peaks corresponded in retention time to NAT and NAB. Because the urine work-up did not include any steps that could potentially affect the presence of NAT or NAB in the final sample, we suspected that these two peaks were NAT and NAB. This suggestion was supported by results obtained upon coinjection with standard solutions of NAT and NAB.

Investigating the Possibility of Artifactual Formation of NNN, NAT, and NAB. Nornicotine, anatabine, and anabasine were added to urine samples from a nonsmoker and they were analyzed for total NNN. Small, barely detectable peaks corresponding in retention time to NNN were observed in three of four urine samples to which nornicotine had been added. No peaks corresponding in retention time to NAT and NAB were observed in urine samples to which anatabine and anabasine had been added. This shows that artifactual NNN, NAT, and NAB formation is not responsible for the detection of these compounds found in smokers' urine nor can it affect quantitation.

Identification of NNN, NAT, and NAB by Gas Chromatography-Tandem Mass Spectrometry. Some samples were analyzed by gas chromatography-tandem mass spectrometry to confirm the identity of NNN, NAT, and NAB. In this analysis, the $[MH]^+$ ions of NNN (m/z 178), NAT (m/z 190), and NAB (m/z 192) were allowed to enter the second quadrupole. Collision-induced dissociation gave m/z 147 and 117 for NNN, m/z 160 and 144 for NAT, and m/z 162 and 146 for NAB. The retention times of the peaks corresponding to m/z 178 → 147, 178 → 117, 190 → 160, 190 → 144, 192 → 162, and 192 → 146 were the same as those observed upon analyses of standard solutions of NNN, NAT, and NAB, as were the ratios of the peak areas. These results confirm the identity of NNN, NAT, and NAB quantified by GC-TEA.

Characteristics of the Method. The accuracy of the method was tested by spiking a smoker's urine (which contained 0.344 pmol NNN/mL urine) with different amounts of NNN. The correlation between spiked and measured NNN (r = 0.99) is shown in Fig. 3. Analysis of five nonspiked aliquots of the same urine produced coefficients of variation of 9% for NNN, 10% for NAT, and 12% for NAB. Mean recovery of 5-MeNNN internal standard was 52 ± 6.7%. The detection limits of the method were 0.032 pmol/mL urine for NNN, 0.014 pmol/mL urine for NAT, and 0.018 pmol/mL urine for NAB.

Separate NNN and NNN-*N*-Gluc Assay. The separate NNN and NNN-*N*-Gluc assay was first evaluated using four aliquots of a nonsmoker's urine, each spiked in duplicate with different known amounts of NNN. Recovery of the spiked NNN was 96 ± 1.5%. No NNN was found in the NNN-*N*-Gluc fraction, when NNN was added to the urine. The method was applied to a smoker's urine. Four aliquots of the same urine collection were taken for the separate NNN and NNN-*N*-Gluc assays. The coefficients of variation were 9.7% and 5.5% for NNN and NNN-*N*-Gluc, respectively. Peaks corresponding to NNN, NAT, and NAB were found in both the ''free'' and ''Gluc'' fractions (Fig. 4A and B).

Analysis of Urine from Smokers and Smokeless Tobacco Users. Twenty-five urine samples (14 from smokers and 11 from smokeless tobacco users) were analyzed for NNN and NNN-*N*-Gluc. The results are summarized in Table 1. Levels of cotinine, cotinine-*N*-Gluc, total NNAL, and creatinine in the

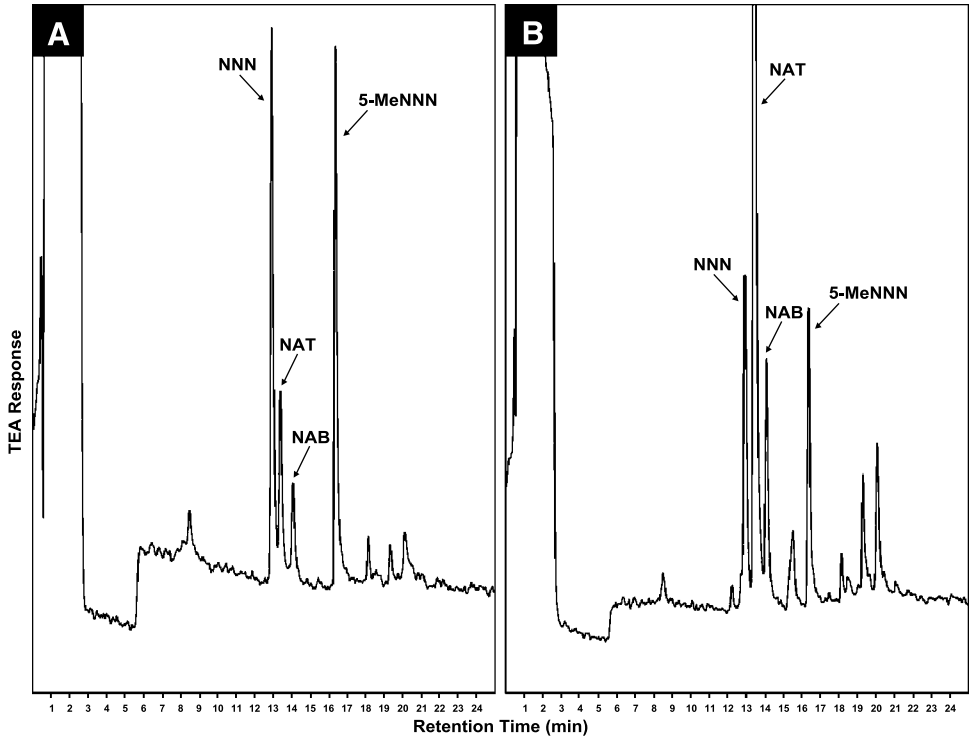

Figure 4. Chromatograms obtained upon GC-TEA analysis, using the separate NNN and NNN-*N*-Gluc assay, of (**A**) free NNN fraction of a smoker's urine and (**B**) NNN-*N*-Gluc fraction of a smoker's urine.

Table 1. NNN, NAT, NAB and their pyridine-*N*-glucuronides in the urine of smokers and smokeless tobacco users

Subjects	NNN (pmol per mg creatinine)	NNN-*N*-Gluc (pmol per mg creatinine)	NAT (pmol per mg creatinine)	NAT-*N*-Gluc (pmol per mg creatinine)	NAB (pmol per mg creatinine)	NAB-*N*-Gluc (pmol per mg creatinine)
Smokers						
1	0.059	ND	ND	0.15	ND	ND
2	ND	0.066	ND	0.11	ND	0.047
3	ND	ND	ND	ND	ND	ND
4	ND	0.042	ND	0.029	ND	ND
5	0.038	0.074	0.031	0.11	ND	0.032
6	0.066	0.075	0.059	0.11	0.01	0.02
7	ND	ND	ND	ND	ND	ND
8	0.43	0.36	ND	0.17	ND	0.085
9	0.024	0.048	ND	0.10	ND	0.042
10	0.018	0.025	0.01	0.032	0.01	0.02
11	0.078	0.10	0.12	0.068	ND	0.04
12	0.16	0.26	0.25	0.43	ND	0.14
13	0.26	0.20	0.31	0.23	ND	0.055
14	0.075	0.10	0.15	0.15	0.02	0.046
Mean ± SD	0.086 ± 0.12	0.096 ± 0.11	0.067 ± 0.104	0.12 ± 0.11	0.003 ± 0.006	0.038 ± 0.039
Smokeless tobacco users						
1	0.50	0.91	0.14	2.78	0.056	0.26
2	0.11	0.17	0.02	0.33	0.01	0.047
3	0.03	0.09	0.07	0.08	ND	0.02
4	0.31	0.48	0.078	1.82	0.052	0.35
5	0.12	0.24	0.039	1.56	0.02	0.19
6	0.47	0.65	0.11	2.20	0.086	0.44
7	0.14	0.14	0.037	0.64	0.02	0.06
8	0.58	0.65	0.15	2.77	0.11	0.38
9	0.19	0.54	0.059	2.23	0.026	0.22
10	0.16	0.25	0.023	0.09	0.02	0.03
11	0.15	0.19	0.029	0.49	0.02	0.058
Mean ± SD	0.25 ± 0.19	0.39 ± 0.27	0.069 ± 0.046	1.36 ± 1.06	0.037 ± 0.034	0.19 ± 0.16

Abbreviation: ND, not detected.

same urine samples are summarized in Table 2. Mean levels of NNN per mg creatinine in smokeless tobacco users (0.25 ± 0.19 pmol) were higher than in smokers (0.086 ± 0.12 pmol). Similarly, mean levels of NNN-*N*-Gluc per mg creatinine (0.39 ± 0.27 pmol) in smokeless tobacco users were higher than in smokers (0.096 ± 0.11 pmol). NNN-*N*-Gluc accounted for an average of 59.1 ± 26.0% of total NNN in smokers and 61.8 ± 7.7% of total NNN in smokeless tobacco users (Table 3). The

Table 2. Cotinine, cotinine-*N*-Gluc, total NNAL, and creatinine in the urine of smokers and smokeless tobacco users

Subject	Cotinine (nmol per mg creatinine)	Cotinine-*N*-Gluc (nmol per mg creatinine)	Total NNAL* (pmol per mg creatinine)	Creatinine (mg/mL urine)
Smokers				
1	3.86	6.81	0.80	1.38
2	3.49	3.31	0.73	1.72
3	6.04	2.17	2.09	1.5
4	2.80	0.54	1.13	3.06
5	5.16	8.85	1.12	0.84
6	7.76	8.00	2.79	1.48
7	12.2	8.55	1.10	0.52
8	6.18	16.6	1.43	0.35
9	2.99	6.16	1.10	1.74
10	4.39	6.34	1.66	2.35
11	3.63	4.02	2.09	2.18
12	7.71	12.8	2.22	0.75
13	3.93	14.3	2.53	1.33
14	3.80	4.95	0.65	1.48
Mean ± SD	5.28 ± 2.56	7.39 ± 4.62	1.53 ± 0.7	1.48 ± 0.7
Smokeless tobacco users				
1	13.6	17.9	4.45	0.75
2	1.21	2.61	1.68	2.57
3	1.79	4.54	0.98	1.91
4	6.09	6.57	3.08	1.13
5	2.82	7.22	4.71	3.20
6	5.49	6.79	2.21	0.78
7	4.39	0.95	3.08	2.45
8	9.57	14.8	6.06	0.90
9	3.75	9.02	2.13	1.75
10	0.82	3.05	0.34	1.87
11	1.81	4.29	1.27	2.56
Mean ± SD	4.67 ± 3.93	7.06 ± 5.18	2.73 ± 1.76	1.81 ± 0.8

*Total NNAL=NNAL plus NNAL-Gluc.

Table 3. Percentage contribution of pyridine-*N*-glucuronides to total NNN, NAT, NAB, and cotinine

Subject	NNN	NAT	NAB	Cotinine
Smokers				
1	ND*	100	NA	63.9
2	100	100	100	48.7
3	NA†	NA	NA	26.4
4	100	100	NA	16.2
5	66.1	77.6	100	63.2
6	53	63.9	63	50.8
7	NA†	NA	NA	41.2
8	45.3	100	100	72.8
9	66.5	100	100	67.3
10	58.1	84.7	70.4	59.1
11	56.8	36.5	100	52.6
12	62.4	62.6	100	62.5
13	43	42.5	100	78.5
14	57.6	48.9	70.6	56.6
Mean ± SD	59.1 ± 26.0	76.4 ± 24.7	90.4 ± 15.6	54.3 ± 17.1
Smokeless tobacco users				
1	64.7	95.1	82.1	56.8
2	61.0	94.1	80.1	68.4
3	74.9	54.4	100	71.7
4	60.4	95.9	87.1	51.9
5	66.8	97.5	92.6	71.9
6	58.0	95.2	83.7	55.3
7	50.7	94.5	76.4	17.8
8	52.9	95.0	78	60.7
9	73.5	97.4	89.6	70.6
10	60.3	79.3	62.1	78.8
11	56.5	94.5	75.6	70.3
Mean ± SD	61.8 ± 7.7	90.3 ± 12.9	82.5 ± 10.0	61.3 ± 16.7

*Pyridine-*N*-glucuronide not detected.
†Not applicable, free nitrosamine and its pyridine-*N*-glucuronide were not detected in this sample.

amount of total NNN was 12.3% of total NNAL in smokers and 35.6 % of total NNAL in smokeless tobacco users (Table 4).

Free NNN correlated with free cotinine ($r = 0.44$, $P = 0.028$) and NNN-*N*-Gluc correlated with cotinine-*N*-Gluc ($r = 0.597$, $P = 0.0016$). Total NNN correlated with total cotinine ($r = 0.625$, $P = 0.00084$) and total NNAL ($r = 0.641$, $P = 0.00056$).

The results for NAT, NAT-*N*-Gluc, NAB, and NAB-*N*-Gluc are also summarized in Table 1. Mean levels of NAT per mg creatinine were similar in smokers (0.067 ± 0.104) and smokeless tobacco users (0.069 ± 0.046). However, mean levels of NAT-*N*-Gluc per mg creatinine were substantially higher in smokeless tobacco users (1.36 ± 1.06) than in smokers (0.12 ± 0.11). NAT-*N*-Gluc accounted for 76.4 ± 24.7% of total NAT in smokers and 90.3 ± 12.9% of total NAT in smokeless tobacco users (Table 3). The amount of total NAT was 13.3% of total NNAL in smokers and 50.3% of total NNAL in smokeless tobacco users (Table 4).

Mean levels of NAB per mg creatinine were higher in smokeless tobacco users (0.037 ± 0.034 pmol) than in smokers (0.003 ± 0.006; Table 1). Mean levels of NAB-*N*-Gluc per mg creatinine were also higher in smokeless tobacco users (0.19 ± 0.16) than in smokers (0.038 ± 0.039). NAB-*N*-Gluc accounted for 90.4 ± 15.6% of total NAB in smokers and 82.5 ± 10.0% of total NAB in smokeless tobacco users (Table 3). The amount of total NAB was 3% of total NNAL in smokers and 8.9% of total NNAL in smokeless tobacco users (Table 4).

There was no correlation between free NAT and free cotinine ($r = 0.037$, $P = 0.86$) or between free NAB and free cotinine ($r = 0.184$, $P = 0.38$). A significant positive correlation was observed between NAT-*N*-Gluc and cotinine-*N*-Gluc ($r = 0.565$, $P = 0.003$) and between NAB-*N*-Gluc and cotinine-*N*-Gluc ($r = 0.522$, $P = 0.007$). Total NAT strongly correlated with total cotinine ($r = 0.596$, $P = 0.002$) and total NNAL ($r = 0.732$; $P < 0.0001$). Total NAB also positively correlated with total cotinine ($r = 0.483$, $P = 0.014$) and total NNAL ($r = 0.659$, $P = 0.0004$). Strong correlations were also observed between total NNN and NAT ($r = 0.86$, $P < 0.0001$), between total NNN and NAB ($r = 0.86$, $P < 0.0001$), and between total NAT and NAB ($r = 0.94$, $P < 0.0001$).

Discussion

The results of this study show for the first time that NNN and its pyridine-*N*-glucuronide are present in the urine of smokers and smokeless tobacco users. These results provide a specific biomarker of human NNN exposure, which could be important for the investigation of esophageal and oral cancer risk in people who use tobacco products. Previous studies aimed at finding urinary biomarkers of NNN uptake were unsuccessful, because the metabolites were also formed from nicotine and related tobacco alkaloids (13, 24). This study also resulted in the discovery of NAT, NAB, and their pyridine-*N*-glucuronides in the urine of smokers and smokeless tobacco users, which presents the first opportunity to measure the uptake of these tobacco-specific nitrosamines in humans.

Amounts of total NNN, NAT, and NAB in the urine of smokeless tobacco users were considerably higher than in the urine of smokers (Table 4). This is consistent with the relatively high levels of tobacco-specific nitrosamines in smokeless tobacco products (6, 19, 25). However, the differences between levels of total NNN (3.5-fold), NAT (7.6-fold), and NAB (5.7-fold) in smokeless tobacco users and smokers were greater than the corresponding difference in total NNAL (1.8-fold). This could be due to endogenous formation of NNN, NAT, and NAB in smokeless tobacco users. We have previously shown that NNN, NAT, and NAB form endogenously in rats treated by gavage with the tobacco alkaloids nicotine, nornicotine, anatabine, and anabasine together with sodium nitrite, but that NNK did not form under these conditions (26). Typical levels of nornicotine, anatabine, and anabasine in tobacco are 0.6, 0.5, and 0.1 mg/g tobacco (27). In smokeless tobacco users, NNN, NAT, and NAB could form in the acidic stomach when the tobacco alkaloids nornicotine, anatabine, and anabasine and salivary nitrite are swallowed, but the formation of NNK from nicotine would be less likely under these conditions.

Compared with levels of total NNN, amounts of total NAT and NAB in the urine of smokeless tobacco users were surprisingly high. The ratio NAT/NNN in moist snuff was 0.56 to 0.91, whereas the ratio total NAT/total NNN in the urine of smokeless tobacco users was 2.3, 2.5 to 4.1 times

Table 4. Total NNN, NAT, NAB, NNAL, and cotinine in urine

Group	Mean ± SD				
	Total NNN (pmol/mg creatinine)	Total NAT (pmol/mg creatinine)	Total NAB (pmol/mg creatinine)	Total NNAL (pmol/mg creatinine)	Total cotinine (nmol/mg creatinine)
All	0.39 ± 0.40	0.73 ± 0.96	0.12 ± 0.16	2.06 ± 1.39	12.3 ± 7.2
Smokers	0.18 ± 0.22	0.19 ± 0.20	0.040 ± 0.039	1.53 ± 0.70	12.7 ± 6.0
Smokeless tobacco users	0.64 ± 0.44	1.43 ± 1.10	0.23 ± 0.19	2.73 ± 3.76	11.7 ± 8.8

higher than in tobacco (19). Similarly, the ratio NAB/NNN was 0.052 to 0.082 in smokeless tobacco and 0.35 in urine, a 4.2- to 6.6-fold difference (19). This may be due in part to differences in metabolism of NNN, NAT, and NAB. The percent contribution of pyridine-*N*-glucuronides to the total amount of NAT and NAB was higher than that of NNN, particularly in smokeless tobacco users (Table 3), suggesting that pyridine-*N*-glucuronidation is more facile for NAT and NAB than NNN. Further studies are required to investigate the comparative enzymology of pyridine-*N*-glucuronidation of NNN, NAT, and NAB. In the case of NNAL, the hepatic enzymes UGT2B7 and UGT1A9 seem to be important catalysts for conversion of NNAL to NNAL-*O*-Glucs, whereas UGT1A4 plays a significant role in the formation of NNAL-*N*-Glucs (28, 29). Significant correlations were observed between amounts of NNN-*N*-Gluc, NAT-*N*-Gluc, and NAB-*N*-Gluc and cotinine-*N*-Gluc, suggesting the involvement of a common enzyme. UGT1A4 has been identified as one catalyst of cotinine pyridine-*N*-glucuronidation (30).

In a study of NNAL-*N*-Gluc formation in humans, the contribution of NNAL-*N*-Gluc to total NNAL was significantly higher in smokers than in snuff dippers, indicating that smoking may induce pyridine-*N*-glucuronidation of NNAL (17). Smoking is known to induce pyridine-*N*-glucuronidation of 3′-hydroxycotinine and some drugs (31-33). At the same time, smoking has no effect on the pyridine-*N*-glucuronidation of nicotine or cotinine (31). In our study, pyridine-*N*-glucuronidation of NNN, NAT, and NAB was somewhat lower in smokers than in smokeless tobacco users.

Levels of total NNN, NAT, and NAB are compared with levels of total NNAL in Table 4. Considering all the urine samples analyzed, the amounts of total NNN, NAT, and NAB were 21.2%, 29.6%, and 5.6%, respectively, of total NNAL, these values being generally higher for smokeless tobacco users compared with smokers. Whereas these comparisons provide a general index of levels of the new biomarkers compared with the more extensively studied NNAL, it is difficult to directly relate these data to dose because of differences in metabolism. For example, in patas monkeys given similar doses of NNN and NNK, levels of unchanged NNN in urine were only about 2% to 3% of NNAL-Gluc (13, 34).

In summary, this study shows the presence of the tobacco-specific nitrosamines NNN, NAT, and NAB as well as their pyridine-*N*-glucuronides in the urine of smokers and smokeless tobacco users. A method is described for quantitation of these important compounds. This method should be useful in mechanistic and epidemiologic studies of tobacco-related cancer.

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