Metabolites of the Tobacco-specific Nitrosamine 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butaneone in Smokers’ Urine

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Abstract
Metabolites of the tobacco-specific nitrosamine, 4-(methylamino)1-(3-pyridyl)-1-butaneone, a potent pulmonary carcinogen, have been quantified in the urine of 11 smokers. They were not detected in nonsmokers’ urine. The metabolites, 4-(methylamino)1-(3-pyridyl)-1-butanol and its glucuronide, were detected in quantities of 0.23-1.0 and 0.57-6.5 μg/24 h, respectively. The results of this study provide the first evidence for metabolites of tobacco-specific nitrosamines in human urine.

Introduction
It has been estimated that up to 90% of lung cancer deaths in the United States are attributable to cigarette smoking (1). The tobacco-specific nitrosamine NNK (Fig. 1) is believed to play an important role in the induction of lung cancer in smokers because it is a potent pulmonary carcinogen in rats, mice, and hamsters inducing tumors at total doses similar to the estimated doses to which smokers are exposed (2, 3). NNK may also be involved in oral and pancreatic cancer associated with the use of tobacco products (3, 4). Although the metabolism of NNK has been extensively studied in laboratory animals, relatively little is known about its uptake and metabolism in humans. We believe that such information is critical to an understanding of mechanisms of cancer induction in humans.

Materials and Methods

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The abbreviations used are: NNK, 4-(methylamino)1-(3-pyridyl)-1-butaneone; NNAL, 4-(methylamino)1-(3-pyridyl)-1-butanol; NNAL-Gluc(I) and NNAL-Gluc(II), two diasteromers of [(4-methylamino)1-(3-pyridyl)but-1-yl]-β-D-glucopyranosyluronic acid; NNAL-TMS, trimethylsilyl ether of NNAL; iso-NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; HPLC, high performance liquid chromatography; GC-TEA, combined gas chromatography-thermal energy analyzer; GC-MS-SIM, combined gas chromatography-mass spectrometry-selected ion monitoring.

Volunteers. Eleven smokers and 7 nonsmokers ranging in age from 20 to 65 years were recruited. The protocol for collection of urine was approved by the American Health Foundation Institutional Review Board for protection of human subjects.

Analysis of Urine by GC-TEA. Twenty-four-h urine samples were collected in 3 liter amber specimen containers (Baxter Scientific Products Division, McGaw Park, IL) to which 10 ml of a solution of 20% ammonium sulfamate (Sigma) in 3.6 N H₂SO₄ had been added to inhibit artifactual nitrosation. Samples were stored at room temperature during collection. Aliquots of 100 ml were adjusted to pH 7 with 10 N NaOH. To this was added 0.5 ml of an aqueous solution of [5-3H]NNAL-Gluc(II) (2.1 Ci/mmol; 21,000 dpmm) as internal standard. The resulting solution was extracted 3 times with equal volumes of ethyl acetate. The aqueous portion (A-1) was saved. A solution of [5-3H]NNAL (2.1 Ci/mmol; 100,000 dpm) in 50 μl of methanol was added to the combined ethyl acetate extracts, as internal standard for unconfounded NNAL. The ethyl acetate layers were dried (Na₂SO₄) and concentrated to dryness by rotary evaporation. The residue (Fig. 2, Fraction 1) was dissolved in two 0.5-ml aliquots of H₂O, which were combined and set aside for subsequent HPLC purification and analysis for NNAL. Twenty μl of "antifuse B emulsion" (Sigma) were added to the aqueous portion, A-1, which was then concentrated by rotary evaporation (water bath temperature, 35°C) to approximately 70% of its initial volume. The purpose of this step was removal of most of the ethyl acetae, traces of which inhibited β-glucuronidase activity. β-Glu-
The collected material was concentrated to dryness on a Model SVC200H Speedvac concentrator (Savant Instruments, Farmingdale, NY) at approximately 35°C. The residue was transferred to a 100-μl glass-lined microcentrifuge tube. The collected material was concentrated to dryness, and silylated.

The residue (Fig. 2, Fraction 2) was taken up in CH2Cl2. This was concentrated, and the residue was silylated and analyzed as above for NNAL-TMS.

Preparation of Urine for Analysis by GC-MS-SIM. Morning void (approximately 800 ml) from smokers 2 and 3 (Table 1) was combined. Fraction 2 was obtained as described above, with allowances for the larger scale being made. The aqueous portion of the urine was treated with β-glucuronidase to release NNAL from NNAL-Gluc(I) and NNAL-Gluc(II). The released NNAL was further purified by normal phase HPLC with a 4.6- x 250-mm Microsorb-MV 5-um silica column (Rainin Instrument Co., Woburn, MA) with elution by solvents A (50/50:CHCl3/hexane) and B (20% isopropyl alcohol in solvent A). The solvent program was 0-35% B in 20 min and was then held for 5 min. The flow rate was 1 ml/min. Eluent was collected from 24 to 45 min, concentrated to dryness, and silylated.

Results

The analytical method is summarized in Fig. 2. Fraction 1, obtained by extraction with ethyl acetate, contained unconjugated NNAL. The aqueous portion of the urine was treated with β-glucuronidase to release NNAL from NNAL-Gluc(I) and NNAL-Gluc(II). The released NNAL was enriched by partitioning to give Fraction 2. Each fraction was further purified by HPLC prior to silylation and analysis by GC-TEA. Typical GC-TEA traces from analyses of Fraction 2 are illustrated in Fig. 3.

The identity of NNAL in Fraction 2 of smokers’ urine was confirmed by GC-MS-SIM. Major peaks in the chemical ionization spectrum of standard NNAL-TMS are m/z 282 (M + 1; relative intensity, 72)

Table 1 NNAL and NNAL-Gluc in smokers’ urine

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Cigarettes/day</th>
<th>NNAL (nmol/μg)</th>
<th>NNAL-Gluc (nmol/μg)</th>
<th>Cotinine (mg/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>36</td>
<td>2.2 (0.46)</td>
<td>11 (4.4)</td>
<td>15.3</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>29</td>
<td>1.8 (0.37)</td>
<td>14 (5.5)</td>
<td>20.0</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>20</td>
<td>1.7 (0.35)</td>
<td>17 (6.5)</td>
<td>6.82</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>25</td>
<td>2.1 (0.43)</td>
<td>4.4 (1.7)</td>
<td>3.39</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>20</td>
<td>3.3 (0.69)</td>
<td>10 (3.9)</td>
<td>7.31</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>29</td>
<td>4.8 (1.0)</td>
<td>10 (3.9)</td>
<td>8.80</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>25</td>
<td>1.1 (0.23)</td>
<td>11 (4.2)</td>
<td>2.22</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>25</td>
<td>3.3 (0.70)</td>
<td>1.5 (0.57)</td>
<td>2.28</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>12</td>
<td>4.2 (0.87)</td>
<td>3.6 (1.4)</td>
<td>5.92</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>20</td>
<td>1.4 (0.29)</td>
<td>8.6 (3.3)</td>
<td>2.71</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>15</td>
<td>1.3 (0.28)</td>
<td>7.2 (2.8)</td>
<td>4.22</td>
</tr>
</tbody>
</table>

a Values include 3'-hydroxycotinine.

b Numbers in parentheses, μg.

c Levels of NNK in mainstream smoke of the cigarettes used by these volunteers were available. These were multiplied by cigarettes/day to obtain estimated daily dose of NNK, as follows (nmol): 4, 16.4; 6, 22.7; 9, 7.7; 10, 13.0.
Retained using the electron impact mode (data not shown).

Some experiments were carried out without β-glucuronidase, or with sulfatase instead of β-glucuronidase, or with β-glucuronidase in the presence of saccharic acid 1,4-lactone, an inhibitor of β-glucuronidase activity. No NNAL was detected in Fraction 2 of these samples. In another series of experiments, the aqueous portion of one smoker’s urine was injected on HPLC and the eluents corresponding in retention time to NNAL-Gluc(I), NNAL-Gluc(II), and NNAL were collected. The NNAL-Gluc(I) and (II) fractions were treated with β-glucuronidase and the resulting mixtures as well as the NNAL fraction were analyzed by GC-TEA after silylation. NNAL-TMS was detected in all three fractions, which is consistent with the presence of NNAL-Gluc(I) and (II) as well as unconjugated NNAL in smokers’ urine.

The possibility that NNAL or NNAL-Gluc could have formed artifically during analysis of the urine samples was considered although the amine precursors to NNAL and NNAL-Gluc are not known to be constituents of smokers’ urine. Urine was collected from a smoker and divided into three 50-ml aliquots, each of which was mixed with ammonium sulfamate/H2SO4 as described in “Materials and Methods.” The first aliquot was treated with 0.5 mg of 4-(methy lamino)-4-(3-pyridyl)-1-butanol, the amine precursor to iso-NNAL, and was allowed to stand for 24 h at room temperature. It was then analyzed for iso-NNAL. The purpose of this experiment was to determine the extent of nitrosation, under our collection and analysis conditions, of a secondary amine that is readily nitrosated and is structurally related to the secondary amine precursor to NNAL, which was not available. The yield of iso-NNAL in this experiment was 0.01%. The second 50-ml aliquot was treated with 4.5 mg NaNO2, which is equivalent to the amount of NaNO2 typically present in 50 ml of urine (15). [Urine samples from noninfected individuals do not normally contain nitrite (15)]. The third aliquot was not treated. These two aliquots were allowed to stand for 24 h at room temperature and were then analyzed. Levels of NNAL in these two aliquots were the same, whereas levels of NNAL-Gluc were about 20% higher in the NaNO2 treated than in the untreated aliquot. Collectively, these data indicate that artificial formation of NNAL or NNAL-Gluc under our conditions is minimal.

Recoveries of [5-3H]NNAL and [5-3H]NNAL-Gluc(II) using the method outlined in Fig. 2 were 36 ± 7.7 and 30 ± 5.2%, respectively. Repetitive analyses of one smoker’s urine gave values of 0.66 ± 0.064 μg/liter NNAL (n = 6) and 2.4 ± 0.41 μg/liter NNAL-Gluc (n = 7). The results of analyses of smokers’ urine are summarized in Table 1. One of seven nonsmokers appeared to excrete NNAL-Gluc (~0.7 μg/24 h); all other nonsmokers’ urines were negative.

Six urine samples from smokers were also analyzed for NNK. None was detected.

Discussion

The data presented here clearly demonstrate that NNAL and NNAL-Gluc are present in smokers’ urine. The amounts detected are in the expected range based on the levels of NNK in cigarette mainstream smoke. These data confirm the uptake of NNK by smokers and allow one to estimate its dose. This will be important in further studies evaluating the relationships of NNK to tobacco-induced cancer. The present results do indicate that total uptake of NNK in a lifetime of smoking is comparable to the lowest doses of this carcinogen which induce lung tumors in rodents (4).

It is possible that some of the NNAL and NNAL-Gluc in urine may come from NNAL in cigarette smoke. The presence of NNAL in cigarette smoke has not been reported, although it has been tentatively identified in snuff products, at levels which appear to be considerably lower than those of NNK (16). NNAL has also been detected in the saliva of Sudanese snuff-dippers (17). NNAL, like NNK, is a potent pulmonary carcinogen in the rat and mouse (18-20). It also induces ductal tumors of the pancreas in the rat (20).
The failure to detect NNK was not unexpected based on metabolism studies which have shown that little if any of this nitrosamine is excreted unchanged in laboratory animals (5, 6, 12). The detection of NNAL-Gluc in smokers’ urine is consistent with our recent studies of NNK metabolism in the patas monkey, which showed that NNAL-Gluc(I) and (II) were prominent urinary metabolites (6).

Previous studies have shown that hemoglobin adducts, known to result from metabolic activation of NNK, NNAL, and NNN, are elevated in approximately 20% of smokers (9). This presumably relates to higher levels of α-hydroxylation of these nitrosamines in some smokers. The data in Table 1 indicate that the ratio of NNAL to NNAL-Gluc varies from 0.1 to 2.3. It will be important to determine whether the ratio of free to conjugated NNAL influences levels of hemoglobin and DNA adducts in smokers.

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References
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