Abstract

It was recently reported that toombak, a type of snuff used in the Sudan, contained unusually high levels of tobacco-specific nitrosamines. To estimate the internal dose of 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) received by individuals who use this type of tobacco, urine from a group of users was analyzed for 2 metabolites of NNK, 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNAL) and its O-glucuronide, NNAL-Gluc. NNK is a strong lung carcinogen believed to contribute to human lung cancer. NNK is also a lung nonoccupational carcinogen. Two diastereomers of NNAL-Gluc were present in all urine samples analyzed by gas chromatography with a nitrosamine selective detector. The average levels detected were 0.39 ± 0.14 (SD) nmol/ml urine (n = 7) and 0.88 ± 0.50 nmol/ml urine (n = 7), respectively. In a 24-h period, these individuals would excrete from 0.12 to 0.44 mg of these two metabolites (expressed per mg NNAL). Therefore, assuming chronic toombak use, the minimum daily dose of NNK to which these users were exposed was 0.12–0.44 mg. This is the highest documented uptake of a nonoccupational carcinogen. Two diastereomers of NNAL-Gluc were present in all urine samples analyzed. Previously, these two diastereomers were identified in the urine of an NNK-treated patas monkey but only one was detected in the urine of NNK-treated rats. The level of the 4-hydroxy-1-(3-pyridyl)-1-butanone releasing hemoglobin adduct was also quantified in these individuals. This adduct is believed to be a measure of NNK activation. The levels ranged from 68 to 323 fmol/g hemoglobin [mean, 148 ± 104 (SD)]. The wide range of adduct levels which were observed suggests that despite similar levels of NNK exposure, there are significant differences in the ability of individuals in this population to activate NNK, as well as potential differences in their cancer risk.

Introduction

Biochemical markers have been suggested as a means by which to more accurately assess the carcinogenic exposure of individuals and, therefore, their cancer risk. This approach has been used most successfully for human exposure to aflatoxin, a strong liver carcinogen (1). The accuracy of the marker which was used, the N’-guanine adduct of aflatoxin, was demonstrated in two populations with very high exposure to aflatoxin. A correlation was demonstrated between the level of aflatoxin in the food consumed and the level of the aflatoxin guanine adduct in the urine of the same individual (2). Exposure levels ranged from 0.1 to 10 μg aflatoxin/day.

It was recently reported that a type of smokeless tobacco used in the Sudan contains extraordinarily high levels of tobacco-specific nitrosamines. This tobacco ("toombak" in the local language) is used as an oral snuff. It is prepared by mixing sun-dried tobacco leaves, primarily Nicotiana rustica, with sodium carbonate. Approximately 1 g of this mixture is used at a time and left in the mouth for a period ranging from a few minutes to several hours, until it becomes bland, at which time it is replaced with fresh toombak. The level of NNK in this tobacco ranged from 0.62 to 7.87 mg/g and accounted for up to 50% of the tobacco-specific nitrosamines present (3). The highest level of NNK previously reported in other smokeless tobacco products was 0.014 mg/g and was less than 15% of the total tobacco-specific nitrosamines (4). Idris et al. (5) estimated, based on the average level of NNK in toombak (2.31 ± 1.55 mg/g), that a user of this tobacco would be exposed to 11.5 mg NNK per day or 0.16 mg/kg/day. This assumes the quantity of snuff used daily in the Sudan is half that in the United States, or 5 g/day. This is probably an underestimation. The level of tobacco-specific nitrosamines in the saliva of toombak users ranges from 0.7 to 30.6 μg/ml (5), or 10–100 times the levels found in saliva of users of other types of smokeless tobacco (6–8). These levels are consistent with an unusually high level of exposure to these carcinogenic nitrosamines.

NNK induces lung tumors in animals whether it is administered by i.p. or s.c. injection or given in the drinking water (9, 10). Rats treated with a total dose as low as 1.8 mg/kg develop lung tumors. Users of toombak would be exposed to this dose in less than 2 months. If the internal dose of the carcinogen were found to be even 1/100 of the reported amount, this would be the highest documented

1 The abbreviations used are: NNK, 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone, NNAL, 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone; NNAL-Gluc, 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone O-glucuronide; NNN, N’-nitrosonornicotine; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; isoNNAL, 4-(methyl nitrosamino)-4-(3-pyridyl)-1-butanone; NNK-N-oxide, 4-(methyl nitrosamino)-1-(3-pyridyl)-N-oxide; diol, 4-hydroxy-4-(3-pyridyl)-1-butanone; HPLC, high performance liquid chromatography; GC-TEA, gas chromatography-thermal energy analyzer; a nitrosamine selective detector; PAH, polynuclear aromatic hydrocarbons; TMS, trimethylsilyl.

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Materials and Methods

Chemicals. [5-3H]NNAL-Gluc (II) was collected from patas monkey urine (18). Unlabeled NNAL-Gluc (I) and NNAL-Gluc (II) were collected from NNK-treated rats and monkeys, respectively. [3,3'-D2]HPB and the tetrafluorobenzoate of HPB were synthesized (16, 19). NNAL, isoNNAL, and nitrosoguvacoline were synthesized and provided to us by Dr. Shantu Amin (20–22). Bis-trimethylsilyltrifluoroacetamide/1% trimethylchlorosilane was obtained from Regis Chemical Co. (Morton Valley, IL). µ-Glucuronidase, type Ixa was obtained from Sigma Chemical Co. (St. Louis, MO). 3-Acetylpyridine, pentafluorobenzyl chloride, and 2-pyridylcarbinol were obtained from Aldrich Chemical Co. (Milwaukee, WI). The last was converted to its acetate by treatment with acetic anhydride and trimethylamine in methylene chloride.

Collection of Samples. Urine and blood samples were obtained immediately following the use of toombak from 7 men who were chronic toombak-users. Collections were made at the Khartoum Cancer Hospital under the supervision of Dr. Idris. A 100-ml urine sample and a 10-ml blood sample were collected. Urine was frozen immediately at -20°C. The blood sample was centrifuged at 1000 X g and the RBC pellet was washed 3 times and then frozen at -20°C. The samples were packed in dry ice and hand carried by Dr. Idris from Khartoum, Sudan to the American Health Foundation in Valhalla, New York.

Analysis of Urine for NNAL and NNAL-Gluc. Urine was analyzed for these two NNK metabolites as described previously (14) with minor modifications. Briefly, a 20-ml aliquot of urine was extracted with ethyl acetate. Urine was then frozen at -20°C. The blood sample was centrifuged at 1000 x g and the RBC pellet was washed 3 times and then frozen at -20°C. The samples were packed in dry ice and hand carried by Dr. Idris from Khartoum, Sudan to the American Health Foundation in Valhalla, New York.

Analysis of Urine for NNAL and NNAL-Gluc. Urine was analyzed for these two NNK metabolites as described previously (14) with minor modifications. Briefly, a 20-ml aliquot of urine was extracted with ethyl acetate. Fifty ng of isoNNAL was added as an internal standard to the organic layer containing NNAL which was dried and further purified by HPLC. The aqueous layer was treated with µ-glucuronidase, spiked with 50 ng isoNNAL, extracted with methylene chloride, dried, and further purified by HPLC. After collection of the NNAL fraction from HPLC, which
easily collect the regions containing NNAL-Gluc (I) and other compounds in the urine sample. This allowed us to determine the retention time of these markers relative to the diol and NNK-N-oxide; the latter two compounds elute immediately prior to NNAL-Gluc (I) and just past NNAL-Gluc (II), respectively (Fig. 2). The diol and NNK-N-oxide peaks were clearly separated from the numerous UV peaks in the urine sample. Therefore, these compounds were used as retention time markers for the two glucuronides. A 20-μl aliquot of each sample was injected with a marker mix containing 50 ng/μl diol and 50 ng/μl NNK-N-oxide to determine the retention time of these markers relative to other compounds in the urine sample. This allowed us to easily collect the regions containing NNAL-Gluc (I) and NNAL-Gluc (II) using UV absorbing compounds present in an individual urine sample, as well as the added diol and NNK-N-oxide as markers. Then 350 μl of each sample was injected with 200 μl of the marker mix. Two fractions were collected, one from the top of the diol peak to 4 min past (44.8 min) and the second from 44.8 min to the end of the peak for the E-isomer of NNK-N-oxide (Fig. 2). To confirm the accuracy of our collections, one urine sample was spiked with [5-3H]NNAL-Gluc (II) and the retention time of the radioactive peak was shown to be within fraction 2, as determined by combined UV and radiolabel detection. The HPLC fractions containing each diastereomer were treated with β-glucuronidase (5000 units) overnight at 37°C, and the released NNAL was analyzed by GC-TEA as described previously (14).

Analysis of NNAL Glucuronide Diastereomers. An HPLC system was developed to separate the 2 diastereomers of NNAL-Gluc. Samples were injected on a Whatman Partisphere ODS 5 μ column (4.6 mm x 25 cm) and eluted using the following series of linear gradients: at time 0 solvent was 100% 20 mM sodium phosphate, pH 7.0 and 0% methanol; in 16 min methanol was increased linearly to 6% and held for 30 min, then increased to 15% over the next 10 min, followed by a third linear gradient to 35% in 4 min, then held for 5 min. The flow rate was 1 ml/min. To confirm the usefulness of this separation for collecting NNAL-Gluc (I) and NNAL-Gluc (II) from urine, 20 μl of urine from a toombak user was spiked with both glucuronides as well as diol and NNK-N-oxide; the latter two compounds elute immediately prior to NNAL-Gluc (I) and just past NNAL-Gluc (II), respectively (Fig. 2). The diol and NNK-N-oxide peaks were clearly separated from the numerous UV peaks in the urine sample. Therefore, these compounds were used as retention time markers for the two glucuronides. A 20-μl aliquot of each sample was injected with a marker mix containing 50 ng/μl diol and 50 ng/μl NNK-N-oxide to determine the retention time of these markers relative to other compounds in the urine sample. This allowed us to easily collect the regions containing NNAL-Gluc (I) and NNAL-Gluc (II) using UV absorbing compounds present in an individual urine sample, as well as the added diol and NNK-N-oxide as markers. Then 350 μl of each sample was injected with 200 μl of the marker mix. Two fractions were collected, one from the top of the diol peak to 4 min past (44.8 min) and the second from 44.8 min to the end of the peak for the E-isomer of NNK-N-oxide (Fig. 2). To confirm the accuracy of our collections, one urine sample was spiked with [5-3H]NNAL-Gluc (II) and the retention time of the radioactive peak was shown to be within fraction 2, as determined by combined UV and radiolabel detection. The HPLC fractions containing each diastereomer were treated with β-glucuronidase (5000 units) overnight at 37°C, and the released NNAL was analyzed by GC-TEA as described previously (14).

Analysis of HPB-releasing Hemoglobin Adduct. A hemoglobin solution was prepared from the frozen RBC pellet (16). Briefly, the cells were lysed in an equal volume of HPLC grade H2O (Burdick and Jackson, Muskegon, MI). The resulting solution was stirred briskly and mixed on a vortex mixer; a 40% volume of 0.67 mM sodium phosphate buffer (pH 7) was added and the solution was centrifuged for 25 min at 20,000 x g. The supernatant was dialyzed against 4 1-L volumes of H2O over a 24-h period. The hemoglobin solution was frozen at 20°C in 5-ml aliquots until analysis.

The amount of HPB-releasing adduct in this hemoglobin was analyzed as described previously (14, 15) with modifications. HPB was released from hemoglobin by treatment with 0.1 N NaOH; the sample was neutralized and the protein was precipitated by the addition of 2 M NaH2PO4. In the original method of analysis, the HPB present in the supernatant was partially purified by multiple extractions. The extractions were replaced by the use of a 1000-mg C18 SepPrep column (Fisher Scientific, Springfield, NJ). After application of the supernatant to the column it was washed with an equal volume of H2O, followed by 1 ml of methanol to elute HPB. The methanol was added to 4 ml of H2O and extracted three times with an equal volume of methylene chloride. The methylene chloride layer was dried and the sample preparation was continued as described previously (16, 23). All samples were resuspended in toluene and analyzed by gas chromatography-mass spectrometry with negative ion chemical ionization detection and selected ion monitoring. A 30-m, 0.25-mm intradermal methylphenyl silicone (DB-17) bonded phase column (0.15 μm film thickness, J&W Scientific, Folsom, CA) was used, and injection was by an autosampler (Hewlett-Packard Model 7673B).

Analysis of Urinary Cotinine. Urinary cotinine analysis was by radioimmunoassay and was carried out in the Clinical Biochemistry Facility of the American Health Foundation (24, 25). The levels of cotinine reported include some trans-3'-hydroxycotinine due to cross-reactivity of the antibody used. Creatinine was analyzed on a Kodak Ektachem 500 clinical analyzer.

Results

Urine and blood samples were collected from 7 individuals who had used toombak within the last hour. Urine was analyzed for NNAL and NNAL-Gluc as a measure of NNK exposure, and hemoglobin was analyzed for the HPB-releasing adduct as a reflection of NNK and NNN α-hydroxylation, i.e., activation. Urinary cotinine levels were also analyzed for each as a measure of tobacco exposure, specifically nicotine exposure (25).

The level of free NNAL present in the urine of one of these individuals relative to that in a cigarette smoker is...
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The average level of the HPB-releasing adduct was 149 ± 104 fmol/g hemoglobin. These levels did not correlate with cotinine (r = 0.31) or NNAL plus NNAL-Gluc (r = 0.54). This is consistent with this adduct being a measure of individual activation of NNK and not exposure. The hemoglobin adduct may also form from NNN, a large amount of which is present in toombak (243 ± 90 µg/g) (27).

The relatively high level of NNAL-Gluc in the urine of toombak users made it possible to analyze this urine for the 2 diastereomers of NNAL-Gluc (Fig. 1). Previously it was shown that urine from NNK-treated patas monkeys contained both NNAL-Gluc (I) and NNAL-Gluc (II) in a 1:4 ratio, and that urine from NNK-treated rats contained only NNAL-Gluc (I) (18, 32). To determine the distribution of these two diastereomers in toombak-users, a reverse phase HPLC system which clearly separated NNAL-Gluc (I) and NNAL-Gluc (II) was developed. This separation is illustrated in Fig. 2. The NNAL-Gluc standards were co-injected with diol and NNK-N-oxide to determine if these two metabolites could be used as retention time markers. NNAL-Gluc (I) eluted just past diol and the second isomer of NNAL-Gluc (II) coeluted with the earlier eluting isomer (E) of NNK-N-oxide. Therefore, diol and NNK-N-oxide were added to each urine sample and two fractions were collected as indicated in Fig. 2. Fractions 1 and 2 from HPLC analyses were each treated with β-glucuronidase and analyzed for released NNAL by GC-TEA. The amount of each glucuronide was quantified in all 7 toombak users. The data are presented in Table 2. In all cases both diastereomers were present and the amount of NNAL-Gluc (II) was always greater. The NNAL-Gluc (II):NNAL-Gluc (I) ratio ranged from 2.7 to 1.3 (average, 1.9 ± 0.5).

![Fig. 3. GC-TEA traces of urinary NNAL from a Sudanese toombak user and a United States smoker. I.S.: injection standard nitrosoguvacoline and internal standard iso-NNAL.](image)

The levels of NNAL, NNAL-Gluc, and cotinine present in the urine of seven toombak users are presented in Table 1. The total NNAL plus NNAL-Gluc in a 20-ml urine sample for each individual was determined and expressed as nmol/liter. This was done to give a conservative estimate of 24-h excretion since the urine analyzed was obtained in a single collection from these chronic toombak users. Typically a normal healthy adult will produce 800–1800 ml urine in a 24-h period (26). Therefore, assuming chronic toombak use, these individuals excreted an average of 270 ± 130 µg NNAL plus NNAL-Gluc/day (expressed per µg NNK) and consequently were exposed to at least this amount of NNK. Toombak was analyzed from 6 samples provided to us by the users at the same time urine and blood samples were collected. The level of NNK in these samples ranged from 195 to 365 µg/g toombak (data not shown) (27). A small amount of NNAL (0 to 1% of the NNK) was also present in the toombak. This would not contribute significantly to the NNK excreted in the urine. A user of 10 g of this tobacco daily would be exposed to 1900–3600 µg NNK compared to a 1 pack/day United States cigarette smoker’s exposure of approximately 5 µg/day.

![Fig. 4.](image)

The amount of cotinine/ml urine in toombak users correlates with the amount of total NNAL plus NNAL-Gluc/ml urine (r = 0.94; P < 0.03). In the previous study in smokers the correlation was not as strong (r = 0.62). The level of urinary cotinine in toombak users is not significantly greater than the levels which have been reported in smokers (28), despite the fact that toombak contains an average of 30 mg nicotine/g and mainstream smoke from United States filter cigarettes contain approximately 1 mg nicotine/cigarette (29). This observation is consistent with the comparable levels of urinary cotinine in United States snuff dippers and smokers. United States snuff contains between 10 and 30 ng nicotine/g tobacco (30, 31).

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In the patas monkey, the average excretion of NNAL-Gluc in a 24-h urine sample accounted for 22% of the urinary metabolites or about 6% of the dose. NNAL was not detected in the same urine sample. Both diastereomers of NNAL-Gluc were quantified in monkey urine; 4 times more NNAL-Gluc (II) was excreted than NNAL-Gluc (I). As mentioned previously, NNK-treated rats excreted detectable levels of NNAL-Gluc (I) only. The urine of toombak users contained NNAL-Gluc (II) and NNAL-Gluc (I) in a 1.9:1 ratio. This suggests that the patas monkey is a better model in which to study NNK metabolism.

Detectable levels of the HPB-releasing hemoglobin adduct were measured in all 7 toombak users. Previously this adduct was measured in the blood from 22 United States snuff dippers immediately following chewing. It was detected in 20 of 22 individuals and therefore may vary significantly among individuals as well as among populations. The two small groups we are comparing are from two very different populations. The United States group consisted of young white men while the toombak users are black North Africans.

The minimum mean NNK exposure of the toombak users in the present study was 270 µg NNK/day as estimated from urinary NNAL and NNAL-Gluc. The total level of these two metabolites expressed per mg creatinine is 207 ± 158 ng. In a recent study of urinary aflatoxin levels in The Gambia the total level of aflatoxin metabolites ranged from 0.2 to 20 ng/mg creatinine (2). It has been estimated that this population has one of the highest exposures to aflatoxin (32). The exposure to NNK in the Sudan is more than 10 times the level of aflatoxin exposure in the Gambia. Aflatoxin is a strong liver carcinogen and an association between human liver cancer and aflatoxin has been established (reviewed in Ref. 32). Lung cancer is estimated to cause 149,000 deaths in the United States alone and a much greater number worldwide (10). The majority of the latter deaths are due to the use of tobacco products. A major lung carcinogen in tobacco products is NNK. Therefore, tobacco exposure, and potentially NNK exposure, are major causes of cancer worldwide. While the easiest means of significantly reducing the incidence of these cancers is to eliminate the use of tobacco, this is not going to happen in the near future. Therefore, it is important to understand the etiology of tobacco-related cancers. This requires the accurate assessment of carcinogen exposure.

The Sudanese population which uses toombak provides a unique opportunity in which to study whether NNK exposure leads to the induction of lung cancer in humans. It is estimated that 42% of adult males in the Sudan use toombak and only a small percentage of these individuals smoke. This group is exposed to high levels of NNK, the internal dose of which is documented in this publication, but has little exposure to other lung carcinogens, specifically PAH, present in tobacco smoke. Therefore, in a future molecular epidemiology study of toombak-users, one may be able to establish NNK as a human lung carcinogen in much the same way aflatoxin has been identified as a human liver carcinogen.

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