

### Short Communication

## Analysis of Total 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanol (NNAL) in Human Urine

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### Abstract

A new method was developed for the analysis of metabolites of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in human urine. The metabolites are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides (NNAL-*O*-Gluc and NNAL-*N*-Gluc). The sum of these metabolites, total NNAL, was measured with this method. Urine was treated with  $\beta$ -glucuronidase, which converts NNAL-*O*-Gluc and NNAL-*N*-Gluc to NNAL. After solvent partitioning and further purification on a liquid-liquid extraction cartridge and by high-performance liquid chromatography, total NNAL was quantified by gas chromatography with nitrosamine selective detection. The new method is accurate and precise, and the results are in good agreement with those obtained using the traditional method, which quantifies NNAL and its glucuronides separately. Levels of total NNAL  $\pm$  SD (pmol/mg creatinine) were  $2.60 \pm 1.30$  ( $n = 41$ ) in smokers,  $3.25 \pm 1.77$  ( $n = 55$ ) in snuff-dippers, and  $0.042 \pm 0.020$  ( $n = 18$ ) in nonsmokers exposed to environmental tobacco smoke. The new method is faster and more sensitive than the traditional method and should greatly facilitate studies on human uptake of NNK.

### Introduction

The tobacco-specific NNK<sup>1</sup> (Fig. 1) is a highly effective lung carcinogen in rats and induces lung tumors in mice and hamsters as well (1). It also causes tumors of the pancreas, liver, and nasal mucosa in rats, and tumors of the oral cavity when administered together with *N'*-nitrososornicotine. The

presence of NNK in unburned tobacco, as well as mainstream and side-stream cigarette smoke, has been extensively documented in multiple international studies (2). NNK is arguably an important contributor to the etiology of lung and pancreatic cancer in smokers, oral cancer in smokeless tobacco users, and lung cancer in people exposed to ETS (1–10).

Urinary metabolites of NNAL and its glucuronides (NNAL-Gluc, the sum of NNAL-*O*-Gluc and NNAL-*N*-Gluc; Fig. 1) are useful biomarkers of NNK uptake in humans, and there is growing evidence on their quantitation (reviewed in Ref. 11). As new tobacco products with reportedly lower levels of NNK are being introduced, the demand for application of this assay to quantify human NNK uptake is increasing. Distinct advantages of the NNAL and NNAL-Gluc biomarker include absolute tobacco specificity, direct relevance to carcinogen uptake, and consistent detection in exposed individuals. In studies reported to date, NNAL and NNAL-Gluc have been quantified separately. This may be important when one is interested in determining the extent of detoxification of NNAL by glucuronidation. However, for estimating NNK uptake, the total of NNAL plus NNAL-Gluc is the critical parameter. Therefore, we have developed a new assay for total NNAL (NNAL plus NNAL-Gluc) in human urine.

### Materials and Methods

**Apparatus.** HPLC was carried out using a Waters (Waters Corp., Milford, MA) system equipped with an Agilent model 1100 autosampler (Agilent Technologies, Wilmington, DE), an ISCO Foxy, Jr. fraction collector (ISCO, Inc., Lincoln, NE), and a 150 mm  $\times$  4.6 mm Bondclone C18 (Phenomenex, Inc., Torrance, CA) column. The column was eluted with H<sub>2</sub>O and methanol at a flow rate of 1 ml/min. The solvent program was as follows: a linear gradient from 90% to 55% H<sub>2</sub>O over 20 min, then to the initial composition over 2 min, then re-equilibration for 15 min. 2-Pyridylcarbinol acetate (retention time, 11 min) and 3-acetylpyridine (retention time, 16 min) were used to indicate the eluant collection window for NNAL. Solvents were removed with a model SVT200H Speedvac (ThermoSavant, Farmingdale, NY).

The gas chromatograph-thermal energy analyzer consisted of a HP 6890 gas chromatograph (Agilent Technologies) interfaced to a model 543 Thermal Energy Analyzer (Orion Research, Beverly, MA). The pyrolyzer and interface temperatures of the thermal energy analyzer were 500°C and 275°C, respectively. The separation was performed on a 15 m  $\times$  0.25 mm i.d., 0.25  $\mu$ m film thickness, SPB-1701 (Aldrich) or a DB-1701 column (J & W Scientific, Folsom, CA) attached to a 2 m  $\times$  0.53 mm i.d. deactivated retention gap. The injection port temperature was 225°C. Pressure pulsed splitless injection was used (14 p.s.i. pressure pulse for 1 min). The injection liner was a 4 mm i.d. single goose-neck liner containing 1 cm of deactivated glass wool. A pressure program was used to keep

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<sup>1</sup> The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; ETS, environmental tobacco smoke; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; HPLC, high-performance liquid chromatography; i.d., inner diameter; GC, gas chromatography; MS/MS, tandem mass spectrometry; GC-TEA, gas chromatography-thermal energy analysis; *iso*-NNAL, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; BSTFA, bis-trimethylsilyltrifluoroacetamide containing 1% trimethylchlorosilane; NPPA, *N*-nitrosopentyl-3-picolylamine; TMS, trimethylsilyl; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone.

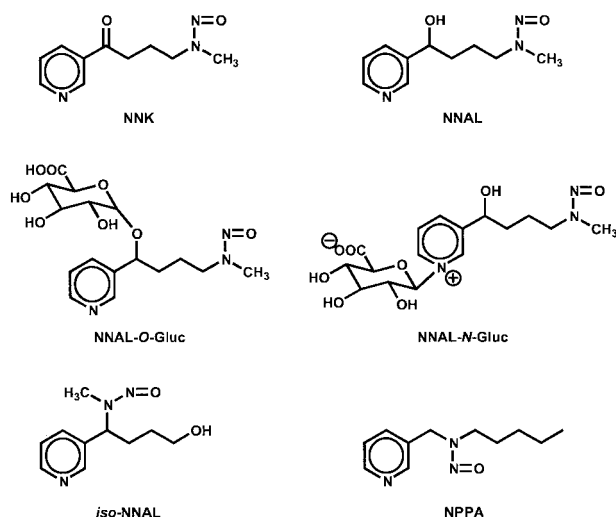


Fig. 1. Structures of compounds discussed in the "Introduction" and "Materials and Methods."

the flow rate of helium through the column at a constant rate of 0.8 ml/min. The oven temperature was initially held at 80°C for 2 min and was then ramped to 180°C at 20°C/min, then to 210°C at 2°C/min. From 210°C, it was increased to 250°C at 20°C/min and held for 5 min.

GC-MS/MS analysis was carried out on a TSQ-7000 instrument (ThermoElectron, San Jose, CA) using the same GC column and conditions as described for GC-TEA. The MS was operated in the positive-ion chemical ionization mode. The transition  $m/z$  282  $\rightarrow$   $m/z$  162 was monitored using conditions essentially identical to those described previously, except that the resolution was set at 0.5 a.m.u. (12).

**Chemicals and Enzymes.** NNAL, *iso*-NNAL, and NPPA were obtained from Toronto Research Chemicals (Toronto, ON, Canada). (*R*)-NNAL-*O*-Gluc was prepared by incubation of NNAL with rat liver microsomes and cofactors as described (13). BSTFA was purchased from Regis Technologies, Inc. (Morton Grove, IL). 2-Pyridylmethyl acetate, 3-acetylpyridine, and  $\beta$ -glucuronidase type IX-A from *Escherichia coli* were purchased from Sigma-Aldrich. Chem-Elute columns (5 ml) were obtained from Varian, Inc. (Walnut Creek, CA).

**Subjects.** Urine samples from smokers and snuff-dippers were obtained at baseline from subjects participating in studies that were designed to evaluate reduction of exposure to tobacco carcinogens. Urine samples from people exposed to ETS were obtained after a visit to a gambling casino.<sup>2</sup> All studies were approved by the University of Minnesota Research Subjects' Protection Programs Institutional Review Board Human Subjects Committee.

**Analysis of Total NNAL in Urine.** Urine from smokers or snuff-dippers (4.5 ml) was added to a 15 ml conical glass centrifuge tube (Kimble-Kontes, Vineland, NJ). The pH was determined with pH paper and adjusted to 6–8 if necessary. Two nanograms of *iso*-NNAL and a freshly prepared solution of  $\beta$ -glucuronidase (12,000 units) in 1 ml of H<sub>2</sub>O were added.

The mixture was incubated with shaking at 37°C overnight. The pH of the urine was adjusted to  $2 \pm 0.5$  with 1 N HCl, and it was partitioned twice with equal volumes of CH<sub>2</sub>Cl<sub>2</sub>. The CH<sub>2</sub>Cl<sub>2</sub> extracts were discarded. The aqueous phase was adjusted to pH  $7 \pm 0.5$  with 1 N NaOH and applied to a Chem-Elute cartridge (10 ml). The cartridge was eluted with three 8-ml aliquots of CH<sub>2</sub>Cl<sub>2</sub>, and the pooled eluants were collected in a 50-ml disposable glass centrifuge tube (Kimble-Kontes). The CH<sub>2</sub>Cl<sub>2</sub> was removed with the Speedvac. The residue was transferred with 50  $\mu$ l of methanol, 200  $\mu$ l of 0.1 M potassium phosphate buffer (pH 7), and 250  $\mu$ l H<sub>2</sub>O into a filter unit autosampler vial (Whatman, Inc., Clifton, NJ). Five microliters of an aqueous solution of the collection markers consisting of 50  $\mu$ g of 2-pyridylmethyl acetate and 50  $\mu$ g of 3-acetylpyridine were added to each vial. The sample was injected on the HPLC system, monitored at 254 nm, and the fraction between the apices of the two marker compounds (11–16 min) was collected. This fraction containing NNAL and *iso*-NNAL was collected in a 15-ml glass centrifuge tube and was concentrated to dryness on the SpeedVac. The residue was transferred with two 75- $\mu$ l portions of methanol to a 300- $\mu$ l, 12  $\times$  32 mm polypropylene microvial (Waters), and the methanol was removed on the Speedvac. Five microliters of BSTFA and 2 ng of NPPA were added. The vial was capped, heated at 50°C for 60 min, and mixed intermittently. Two microliters were injected on the GC column from a cooled autosampler tray (Leap Technologies, Carboro, NC). GC data were collected on a PeakSimple data system (Alltech Associates, Inc., Deerfield, IL).

For analysis of urine of nonsmokers exposed to ETS, 20 ml of urine were analyzed, and 50,000 units of  $\beta$ -glucuronidase were added. The samples were purified using two 10-ml Chem Elute cartridges.

Two positive control samples of either urine from a non-smoker supplemented with NNAL and (*R*)-NNAL-*O*-Gluc or urine from a smoker were included in each set of samples to monitor assay variation. Negative control samples (water or urine from a nonsmoker) were included with the ETS samples.

**Analysis of NNAL and NNAL-Gluc in Urine.** The traditional assay was carried out as described (14), except that the normal-phase HPLC-collection step was omitted.

## Results

The analytical scheme is summarized in Fig. 2. *iso*-NNAL was added to urine as the internal standard. Treatment with  $\beta$ -glucuronidase converted NNAL-*O*-Gluc and NNAL-*N*-Gluc to NNAL (15, 16). Adjustment to pH 2–3 protonated NNAL on the pyridine ring. Therefore, it remained in the aqueous phase, whereas relatively nonpolar neutral and acidic organic compounds were extracted into CH<sub>2</sub>Cl<sub>2</sub> and discarded. Then the pH was readjusted to 7 and the fraction containing NNAL was further enriched by partitioning the aqueous solution with CH<sub>2</sub>Cl<sub>2</sub> on a Chem Elute diatomaceous earth-based liquid-liquid extraction cartridge. Final enrichment was accomplished by reverse-phase HPLC. The fraction containing NNAL was then silylated and analyzed by GC-TEA. Silylation converted NNAL and *iso*-NNAL to their corresponding TMS ethers, NNAL-TMS and *iso*-NNAL-TMS.

A typical GC-TEA chromatogram of total NNAL in urine from a smoker is illustrated in Fig. 3. The internal standard *iso*-NNAL-TMS and injection standard NPPA, as well as the analyte NNAL-TMS, were the major peaks that eluted in the region from 8–13 min. NPPA served to monitor changes in detector sensitivity and injection precision. The NNAL-TMS

<sup>2</sup> K. E. Anderson, J. Kloris, L. Murphy, S. G. Carmella, S. Han, C. Link, R. L. Bliss, S. E. Murphy, and S. S. Hecht. Metabolites of a tobacco-specific lung carcinogen in nonsmoking casino patrons, submitted for publication.

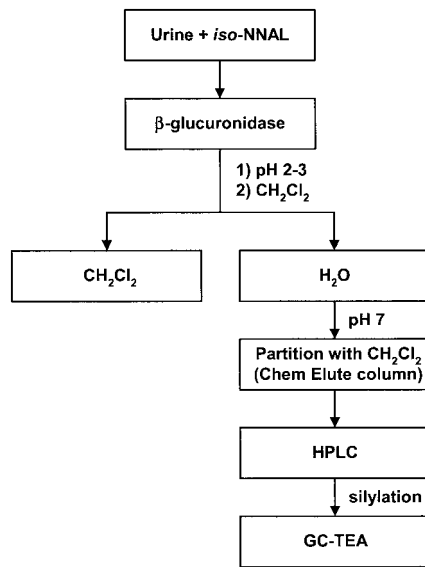


Fig. 2. Analytical scheme for total NNAL in human urine.

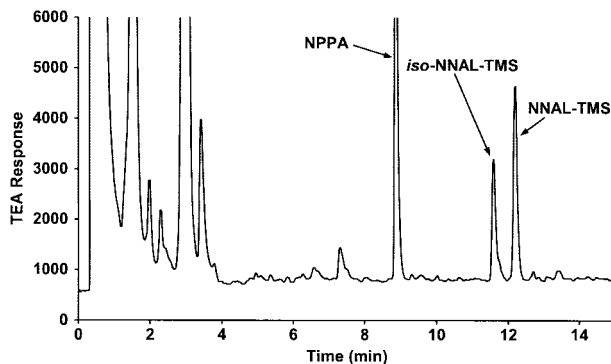


Fig. 3. GC-TEA chromatogram of the NNAL-containing fraction of human urine. *iso*-NNAL is the internal standard, and NPPA is the injection standard. NNAL and *iso*-NNAL appear as their TMS ether derivatives, NNAL-TMS and *iso*-NNAL-TMS.

peak illustrated in Fig. 3 corresponds to 3.5 pmol (730 pg). The detection limit of the assay for NNAL in urine from smokers was 0.1 pmol/ml, starting with 5 ml.

Precision of the new assay was determined by dividing a pooled urine sample from seven smokers into six aliquots and analyzing each for total NNAL. The results were  $3.27 \pm 0.21$  pmol/ml (RSD = 6.4%). Levels of NNAL and NNAL-Gluc were also determined in this sample using the original separate assay. The results were  $0.78 \pm 0.03$  pmol/ml NNAL (RSD = 4.4%),  $2.26 \pm 0.10$  pmol/ml NNAL-Gluc (RSD = 4.3%), and  $3.04 \pm 0.09$  pmol/ml total NNAL (RSD = 3.0%). The accuracy of the new assay was determined by assaying six replicates of urine from a nonsmoker, which had 1.05 pmol/ml NNAL and 1.11 pmol/ml (*R*)-NNAL-*O*-Gluc (2.16 pmol/ml total NNAL) added to it. Analysis gave  $2.06 \pm 0.093$  pmol/ml (RSD = 4.6%). Recoveries of internal standard averaged 50%.

The assay was applied to urine samples from smokers, snuff-dippers, and nonsmokers exposed to ETS in a public venue. The results are illustrated in Fig. 4, A and B.

These silylated urine samples were also analyzed by GC-MS/MS, with selected reaction monitoring of  $m/z$  282  $\rightarrow$   $m/z$  162. A typical chromatogram is illustrated in Fig. 5. The detection limit for NNAL-TMS by GC-MS/MS was 30 fmol on the column. The corresponding detection limit by GC-TEA was 120 fmol.

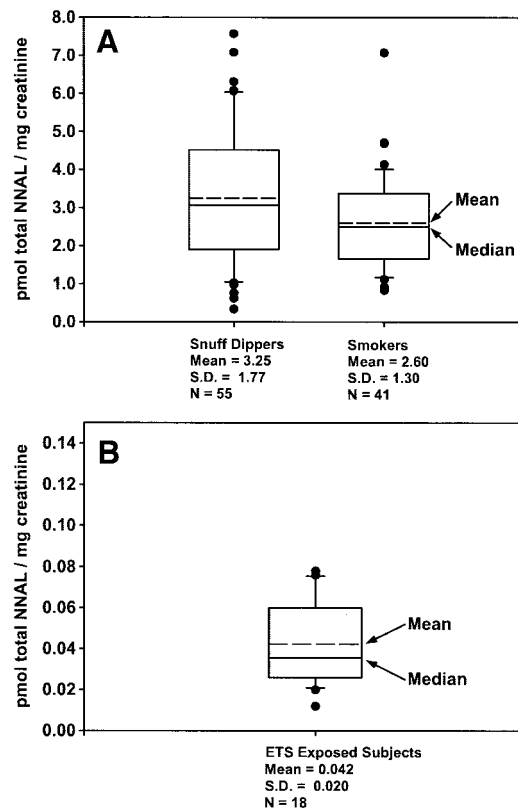


Fig. 4. Levels of total NNAL in the urine of snuff-dippers and smokers (A) and nonsmokers (B) exposed to ETS. Boxes, 25th and 75th percentiles; lines outside boxes, 10th and 90th percentiles; dots, outliers.

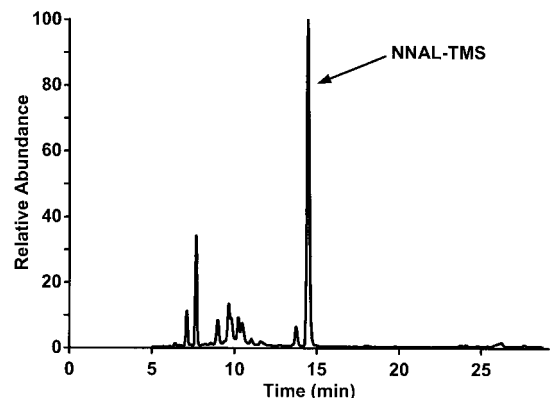


Fig. 5. GC-MS/MS chromatogram of the NNAL-containing fraction of urine from a smoker. The transition  $m/z$  282  $\rightarrow$   $m/z$  162 was monitored.

## Discussion

The results of this study clearly show that the new assay for total NNAL in human urine is accurate and precise. Results obtained using the new and traditional methods were comparable. The new assay has several advantages. The most important of these is the speed of analysis, which is approximately 2-fold that of the traditional method. The new method is more sensitive, because all NNAL is now measured in a single GC-TEA chromatogram. The acid partitioning step produces cleaner samples, resulting in less maintenance of the gas chromatograph-thermal energy analyzer and cleaner chromatograms. Finally, the addition of the internal standard in the first step should improve overall accuracy. The only disadvantage of the new method is that NNAL and NNAL-Gluc are not distinguished. Therefore, no information on the extent of glucuronidation is obtained. For many studies, however, total NNAL is sufficient, because the key question often relates to NNK uptake.

The results of the assays presented here can be compared with those obtained using the traditional method in previous studies of smokers, snuff-dippers, and nonsmokers exposed to ETS; these studies were carried out with different cohorts. The mean level of total NNAL in the urine of 27 smokers was  $2.69 \pm 1.31$  pmol/mg creatinine (17) compared to  $2.60 \pm 1.30$  pmol/mg creatinine obtained here. The mean amount of total NNAL in the urine of 11 snuff-dippers was  $3.78 \pm 3.19$  pmol/mg creatinine (18) compared to  $3.25 \pm 1.77$  pmol/mg creatinine urine measured here. Previous field studies of total NNAL in the urine of nonsmokers exposed to ETS have typically shown levels of  $\sim 0.05$  pmol/ml urine (14), a finding that is also consistent with the amounts found here.

In our hands, GC-TEA is a robust and reproducible method for the measurement of NNAL in urine. An alternate method of detection is MS/MS. MS/MS equipment is widely available, although it is more expensive than thermal energy analyzer instruments. Although we did not pursue the development of a GC-MS/MS method for total NNAL, the chromatogram illustrated in Fig. 5 indicates that this is feasible using the same sample preparation as outlined in Fig. 2. Moreover, the detection limit of GC-MS/MS is 4-fold better than that of GC-TEA. We have previously used GC-MS/MS for confirmation of the identity of NNAL in urine and have used liquid chromatography-MS/MS to evaluate the stereochemistry of NNAL in urine (12, 14, 19). Recently, a liquid chromatography-MS/MS method for the quantitation of NNAL and NNAL-Gluc in urine has also been described (20).

In addition to measurement of urinary total NNAL as described here, or NNAL and NNAL-Gluc separately, there are three other approaches for assessing NNK metabolism in humans. Methods are available for quantitation of NNAL-*N*-oxide in urine (21), HPB-releasing hemoglobin adducts (22), and HPB-releasing DNA adducts (23). NNAL-*N*-oxide is only a minor urinary metabolite and may have limited significance as a detoxification product of NNK in humans (22). Levels of HPB-releasing adducts indicate the extent of NNK metabolic activation by  $\alpha$ -methyl hydroxylation, which leads to pyridyl-oxobutylation of hemoglobin and DNA (1). The adduct methods are not completely specific to NNK, because the same products are formed by 2'-hydroxylation of *N*'-nitrosonornicotine. These methods are also not as practical as measurements of urinary NNK metabolites, because adduct levels are frequently quite low or undetectable. Urinary metabolites of NNK resulting from  $\alpha$ -hydroxylation are also formed from nicotine

and are, therefore, not useful for investigating NNK metabolism in people exposed to tobacco products (24). Quantitation of total NNAL, or NNAL and NNAL-Gluc, in urine is presently the most practical means to investigate human NNK metabolism.

In summary, we have developed a new assay for total NNAL in human urine. This assay should markedly facilitate studies on the uptake of the tobacco-specific carcinogen NNK in humans.

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