

## Short Communication

# Analysis of Total 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanol in Smokers' Blood

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### Abstract

The sum of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides (total NNAL) is an excellent biomarker for uptake of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Although numerous studies have examined levels of total NNAL in the urine of people who use tobacco products, few have quantified this biomarker in blood, and the available methods used relatively large amounts of blood. A method is urgently needed for the analysis of total NNAL in blood, the fluid most commonly stored in molecular epidemiologic studies. We developed a liquid chromatography-electrospray

ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for the analysis of total NNAL in 1-mL samples of plasma. LC-ESI-MS/MS provides both high-sensitivity and structural information supporting analyte identity. The method is practical and sensitive, with a detection limit of 8 fmol total NNAL/mL plasma. Levels of total NNAL averaged  $42 \pm 22$  (SD) and ranged 1.7 to 88 fmol/mL plasma in 16 smokers; NNAL was not detected in the plasma of five nonsmokers. These results show that total NNAL can readily be quantified in 1-mL plasma samples. (Cancer Epidemiol Biomarkers Prev 2005;14(11):2669–72)

### Introduction

Extensive analytic studies clearly show that substantial amounts of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK; Fig. 1) are present in unburned tobacco and tobacco smoke (1, 2). NNK is a potent lung carcinogen in rats, inducing tumors independent of the route of administration, and also produces tumors of the pancreas, liver, and nasal cavity (3). It causes lung tumors in susceptible and resistant strains of mice and in hamsters (3). A mixture of NNK and the related tobacco-specific nitrosamine *N'*-nitrosornicotine induced oral cavity tumors in rats (4). Biochemical studies show similarities in NNK metabolism in rodents and humans (3, 5). Collectively, the available evidence for the involvement of NNK in tobacco-induced cancers is strong, and the IARC classifies NNK and *N'*-nitrosornicotine in group 1 as carcinogenic to humans (2).

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL; Fig. 1) and its glucuronides (NNAL-Glucs) are metabolites of NNK in rodents and humans, and numerous studies have quantified levels of these compounds in the urine of smokers, smokeless tobacco users, and nonsmokers exposed to environmental tobacco smoke (6, 7). This work clearly shows that urinary total NNAL, the sum of NNAL and NNAL-Glucs, is a good biomarker for uptake of NNK. Advantages of this biomarker include its direct relationship to a potent carcinogen, relative ease of measurement, and tobacco specificity. However, only two studies have analyzed NNAL in blood. One showed the presence of NNAL in the blood of smokers,

whereas the second quantified NNAL and NNAL-Glucs in the blood of smokeless tobacco users (8, 9). Both studies used relatively large (5–10 mL) quantities of plasma for the analysis.

We are interested in the relationship between NNK dose and lung cancer in humans. This could be investigated in nested case-control studies by measuring total NNAL. Currently ongoing prospective molecular epidemiologic studies often store serum or plasma samples but less frequently collect urine. Therefore, there is an urgent need for the development of methodology to quantify total NNAL in blood, using relatively small samples. In this article, we describe a liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for quantitation of total NNAL in plasma.

### Materials and Methods

**Apparatus.** LC-ESI-MS/MS was carried out on Finnigan TSQ Quantum Discovery Max and Finnigan TSQ Quantum Ultra instruments (Thermo Electron Corp., Waltham, MA) interfaced with an Agilent Model 1100 capillary high-performance liquid chromatography system and a Model 1100 micro autosampler (Agilent, Palo Alto, CA). The high-performance liquid chromatograph was fitted with a  $150 \times 0.5$  mm (inner diameter) Luna C18(2) 3  $\mu$ m column (Phenomenex, Torrance CA) eluted with a gradient from 2% to 30% methanol in H<sub>2</sub>O over 5 minutes and then held at 30% methanol until 25 minutes at a flow rate of 10  $\mu$ L/min. A disposable precolumn filter (Phenomenex) was used to protect the high-performance liquid chromatography column. The column was maintained at 25°C. MS/MS variables were as follows: positive ion electrospray mode with selected reaction monitoring for  $m/z$  210  $\rightarrow$  180 for NNAL,  $m/z$  214  $\rightarrow$  184 for [pyridine-D<sub>4</sub>]NNAL, and  $m/z$  224  $\rightarrow$  194 for 5-(methylnitrosamino)-1-(3-pyridyl)-1-pentanone (C<sub>5</sub>-NNAL), at 0.5 amu scan width. The collision gas was Ar at a pressure of 1 mTorr, with collision energy of 12 eV. The quadrupoles were operated at a resolution of 0.7 amu. The ion transfer tube temperature was 200°C, the spray voltage was 2,700 V, the current was 0.1  $\mu$ A,

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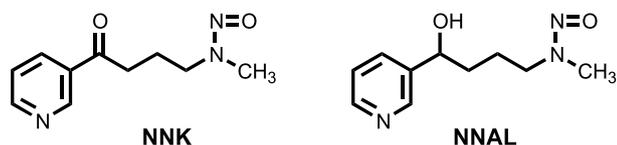


Figure 1. Structures of NNK and NNAL.

and the sheath gas was  $N_2$  at 15 units. The instrument was tuned using [pyridine- $D_4$ ]NNAL infused in 30% methanol in  $H_2O$ . The autosampler needle was washed with 80% acetonitrile/19% methanol/1% formic acid between injections. Polypropylene autosampler vials (250  $\mu$ L), were purchased from Waters Associates (Milford, MA).

**Chemicals and Enzymes.** NNAL and  $C_5$ -NNAL were obtained from Toronto Research Chemicals (Toronto, Ontario, Canada). [Pyridine- $D_4$ ]NNAL was synthesized from [pyridine- $D_4$ ]ethyl nicotinate (Cambridge Isotope Laboratories, Cambridge, MA) as described previously (10, 11).  $\beta$ -Glucuronidase type IX-A from *Escherichia coli* was purchased from Sigma-Aldrich (St. Louis, MO). Mixed mode cation exchange solid-phase extraction cartridges (60 mg) were purchased from Waters Associates.

**Subjects.** Plasma samples were obtained from smokers participating in a study examining the relationship of genotype to urinary metabolites, from smokers participating in a smoking reduction study, and from nonsmokers. The studies were approved by the University of Minnesota Research Subjects' Protection Programs Institutional Review Board Human Subjects Committee. Pooled smokers' plasma was purchased from Biochemed Pharmacologicals (Winchester, VA).

**Analysis of Total NNAL in Plasma.** Plasma (1 mL) was placed in a 10-mL glass conical tube with a Teflon-lined cap, and the pH was adjusted to 6 to 7 if necessary. [Pyridine- $D_4$ ]NNAL [50  $\mu$ L of 1 pg (4.7 fmol)/ $\mu$ L acetonitrile] was added to the sample.  $\beta$ -Glucuronidase (12,000 units, in 0.5 mL  $H_2O$ ) was added, and the mixture was incubated with shaking at 37°C overnight. The sample was adjusted to pH 2 to 3 with 1 N HCl. The mixed mode cation exchange cartridges were placed in a 16-port vacuum manifold and conditioned with 5 mL of methanol and 10 mL  $H_2O$ . The samples were slowly applied to the cartridges and eluted with 5 mL of 1 N HCl, 5 mL of methanol, and 5 mL 90:5:5  $H_2O$ /methanol/ $NH_4OH$ . These fractions were discarded. The NNAL-containing fraction was obtained by eluting with 5 mL of 30:65:5  $H_2O$ /methanol/ $NH_4OH$ . This fraction, in a 15-mL conical tube, was concentrated to dryness on a SpeedVac (Thermo-Savant, Waltham, MA). The residue was transferred in two 80- $\mu$ L aliquots of acetonitrile to a 0.45- $\mu$ m nylon Spin-X LC filtration vial (Corning, Corning, NY). The filtrate was transferred to the autosampler vial and concentrated to dryness (SpeedVac). It was prepared for LC-ESI-MS/MS by adding 6  $\mu$ L of 2% aqueous methanol containing 2.5 pg (11.2 fmol)/ $\mu$ L  $C_5$ -NNAL as injection standard, and 4  $\mu$ L were injected. The response of the MS system to NNAL and [pyridine- $D_4$ ]NNAL was linear in the range of 2 to 38 fmol per injection ( $R^2 = 0.999$ ), as determined by the ratio of peak areas of these analytes to that of  $C_5$ -NNAL, which was kept constant.

## Results

The method is outlined in Fig. 2. The internal standard, [pyridine- $D_4$ ]NNAL, was added to 1 mL of plasma, and the mixture was incubated with  $\beta$ -glucuronidase to catalyze hydrolysis of conjugated NNAL. A mixed mode cation

exchange solid-phase extraction cartridge was used to enrich NNAL from other plasma components. The eluants containing NNAL were then directly analyzed by LC-ESI-MS/MS, monitoring the transition  $m/z$  210  $[M + H]^+ \rightarrow 180 [(M + H) - NO]^+$ . A typical selected reaction monitoring chromatogram is shown in Fig. 3. The *E* (major) and *Z* (minor) rotamers of NNAL are clearly visible. The internal standard eluted 0.15 minute before NNAL.

The detection limit for standard NNAL injected into the LC-ESI-MS/MS system was 0.2 fmol (signal/noise = 3). The limit of quantitation of the assay was  $\sim 8$  fmol total NNAL per mL plasma. The intraday precision of the assay was determined by analyzing five aliquots of a smoker's plasma. The results were  $58 \pm 6.3$  fmol total NNAL/mL (relative SD = 10.9%). The interday precision based on analyses of the pooled smokers plasma (2-4 per set as positive controls in four sets of assays) was relative SD = 13.7%.

Assay accuracy was determined by spiking pooled smokers' plasma, which contained 27 fmol/mL total NNAL, with 25, 50, and 100 fmol/mL NNAL. Analysis produced the results illustrated in Fig. 4 ( $r = 0.99$ ,  $y$  intercept = 19 fmol/mL). Assay recoveries averaged  $28 \pm 21\%$  ( $n = 53$ ).

The assay was applied to plasma samples from 16 smokers and five nonsmokers. Data for total NNAL in smokers' plasma are summarized in Table 1. The mean was  $42 \pm 22$  (SD) fmol/mL. All nonsmoker samples were negative ( $< 8$  fmol/mL).

## Discussion

We have developed a sensitive and practical method for quantitation of total NNAL in blood. The high sensitivity of LC-ESI-MS/MS, which has a sub-femtomole detection limit for NNAL, makes this method feasible with 1-mL quantities of blood. LC-ESI-MS/MS also provides structural information in the form of the  $m/z$  210  $\rightarrow$  180 transition, supporting analyte identity. This is further buttressed by comparison of the *E/Z* ratio of NNAL rotamers and their retention times to those of the internal standard [pyridine- $D_4$ ]NNAL. Previous analyses of NNAL in blood have been done using gas chromatography with nitrosamine selective detection, a method that has been almost exclusively used for analysis of urine, but which lacks adequate sensitivity for small blood samples (6).

The use of a mixed mode cation exchange solid-phase extraction cartridge, which has both ion exchange and reverse-phase properties, greatly simplified our analysis, obviating the need for liquid-liquid partitioning or high-performance liquid chromatography purification steps. This technique was first introduced for NNAL analysis in urine by

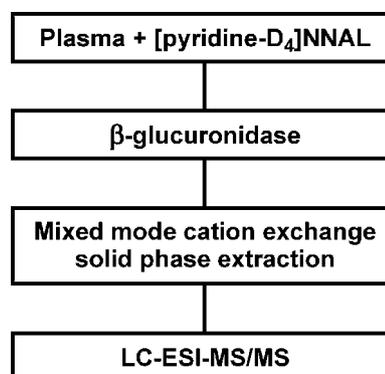
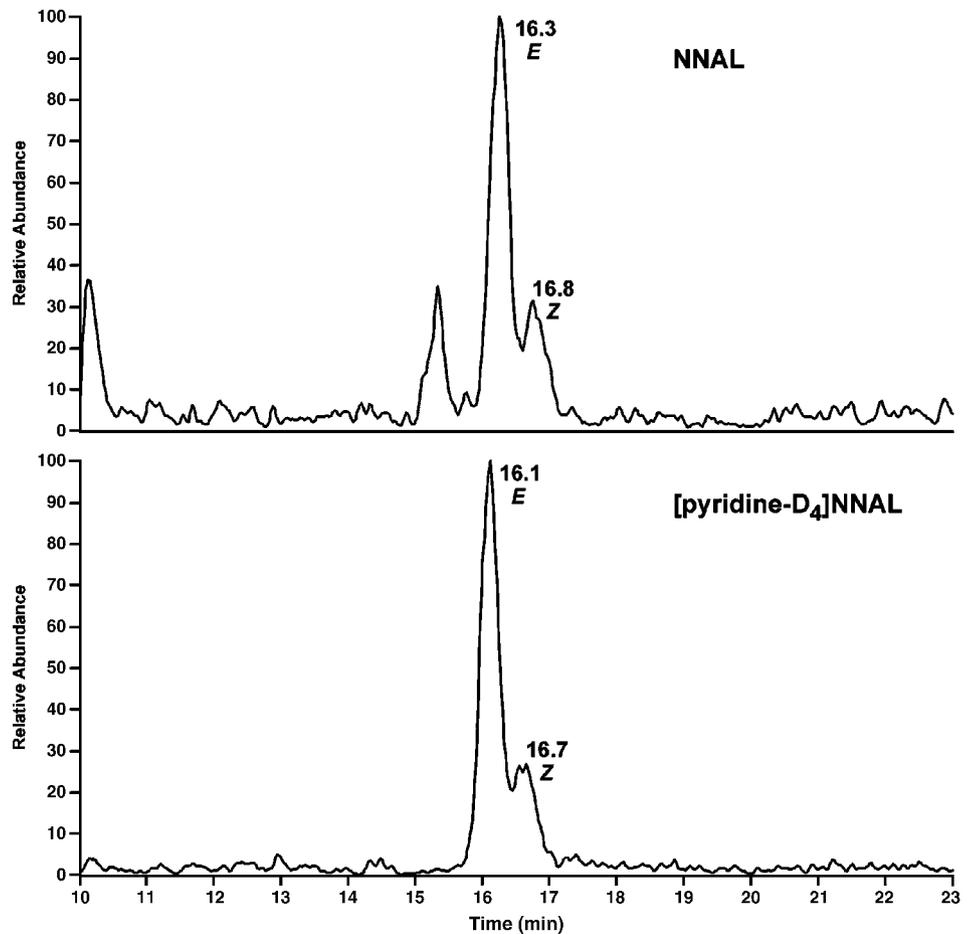


Figure 2. Analytic scheme for total NNAL in human plasma.



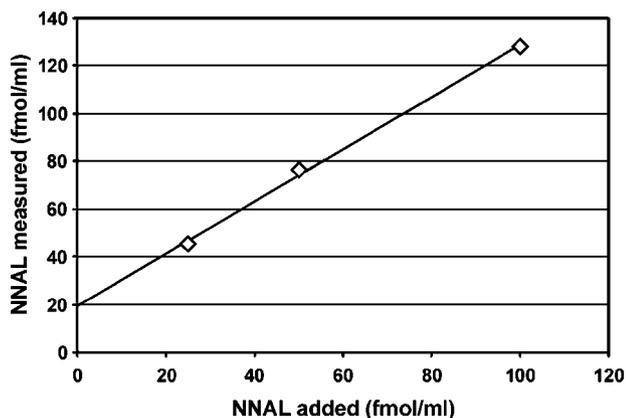
**Figure 3.** Chromatograms obtained by LC-ESI-MS/MS analysis of total NNAL in a smoker's plasma. Selected reaction monitoring of (top)  $m/z$  210  $\rightarrow$  180 for NNAL and (bottom)  $m/z$  214  $\rightarrow$  184 for [pyridine- $D_4$ ]NNAL, internal standard. Peaks with numbers indicating retention times are the *E* (major) and *Z* (minor) rotamers of NNAL and [pyridine- $D_4$ ]NNAL.

Byrd and Ogden (12). A further potentially time saving approach would be use of an LC mobile phase in which NNAL eluted earlier than the 16.3-minute retention time observed here. However, in our hands, this was not possible without encountering coeluting materials, which interfered with quantitation.

Total NNAL (i.e., free NNAL plus NNAL-Glucs) is the variable of interest for studies investigating the relationship between NNK dose and cancer, as we propose. A previous study of plasma from smokeless tobacco users found that total NNAL averaged 340 fmol/mL, eight times higher than observed here (9). This most likely reflects the higher amounts of NNK per unit of product in smokeless tobacco than in

cigarette smoke (1, 2). Levels of total NNAL in the urine of smokeless tobacco users are only slightly higher than in smokers, probably due to extensive further metabolism of NNAL in both groups (9). In the only previous report in smokers, levels of free NNAL in the plasma of four subjects ranged from not detected to 114 fmol/mL (8).

In summary, we have developed a method for the analysis of total NNAL in blood. Total NNAL is an excellent biomarker of uptake of the tobacco-specific carcinogen NNK; therefore, this method should be useful in epidemiologic studies investigating the relationship between dose of this carcinogen and cancer incidence in people who use tobacco products.



**Figure 4.** Relationship between levels of NNAL added to a smoker's plasma and measured amounts ( $r = 0.99$ ). Points, single determinations.

**Table 1. Total NNAL in smokers' plasma**

Subject no.	Gender	Cigarettes/d	NNAL (fmol/mL)
1	Female	30	88
2	Female	20	82
3	Female	20	61
4	Female	20	60
5	Female	16	52
6	Female	15	46
7	Female	25	39
8	Female	20	39
9	Male	10	36
10	Female	12	32
11	Male	25	30
12	Female	14	24
13	Male	25	24
14	Female	3	20
15	Female	12	17
16	Female	16	17

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