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Subpicogram per Milliliter Determination of the Tobacco-Specific Carcinogen Metabolite 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol in Human Urine Using Liquid Chromatography−Tandem Mass Spectrometry

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Exposure to secondhand tobacco smoke (SHS) has been linked to increased risk for a number of diseases, including lung cancer. The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is of particular interest due to its potency and its specificity in producing lung tumors in animals. The NNK metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in urine is frequently used as a biomarker for exposure. Due to its long half-life (40−45 days), NNAL may provide a long-term, time-averaged measure of exposure. We developed a highly sensitive liquid chromatography−tandem mass spectrometry method for determination of NNAL in human urine. The method involves liquid−liquid extraction followed by conversion to the hexanoate ester derivative. This derivative facilitates separation from interfering urinary constituents by extraction and chromatography and enhances detection with electrospray ionization mass spectrometry. The lower limit of quantitation is 0.25 pg/mL for 5-mL urine specimens. Applications to studies of people with a range of different SHS exposure levels is described.

Tobacco and tobacco smoke contain numerous toxic substances, including ~70 carcinogens.[1] More than 400 000 people in the United States and about 5 000 000 people worldwide die from tobacco-related diseases each year.[2,3] Most of these deaths occur in cigarette smokers, but other forms of tobacco use and exposure to secondhand tobacco smoke (SHS) are also significant causes of morbidity and mortality.[4] The association between cigarette smoking and lung cancer has been known for many years, well documented and publicized initially in the 1964 Surgeon General's Report.[5] A number of epidemiological studies have demonstrated an increased risk of lung cancer in nonsmokers having long-term exposure to SHS.[4,6]

Based on toxicology studies in animals, it has been proposed that polycyclic aromatic hydrocarbons and tobacco-specific nitrosamines (TSNAs are the major lung carcinogens in tobacco smoke.[7] The TSNA 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has received particular interest due to its potency as a carcinogen and its specificity in producing lung tumors in laboratory animals.[7,8] NNK is specific to tobacco and tobacco smoke, formed from nicotine by oxidation and nitrosation during the curing process, as well as being formed pyrosynthetically from nicotine during smoking (Figure 1).[8−10]

In humans and in laboratory animals, a major route of NNK metabolism is reduction of the keto group to give the secondary alcohol 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL (Figure 1). NNAL is further metabolized to N- and O-glucuronide conjugates, which are excreted in urine along with free NNAL.[7,8] A number of studies have documented the presence of NNAL and its glucuronides in urine of smokers and in urine of nonsmokers exposed to SHS. Since these are quantitatively

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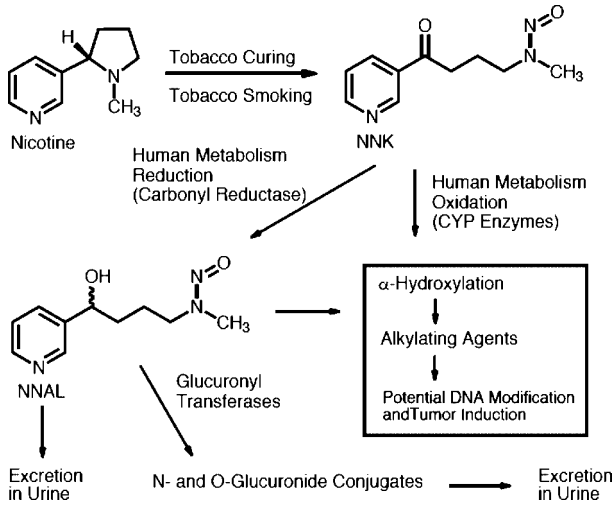

Figure 1. Formation and metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

important metabolites of NNK, their determination in urine is of considerable utility in studies of human exposure to this tobacco-specific carcinogen.[11,12] In addition, the very long biologic half-live of NNK, 40−45 days,[13] makes its metabolites useful as biomarkers for studies of long-term exposure to this carcinogen and to tobacco smoke in general. This is especially useful in studies of SHS exposure, in which exposure patterns are often irregular and the impact of exposure on disease outcomes occurs over a relatively long time period.

Depending upon the brand and the smoking conditions, a cigarette delivers 50−220 ng of NNK in the mainstream smoke, which is inhaled by the smoker[8,14−16] and 50−1440 ng in the sidestream smoke.[6,8,12] This results in urine concentrations (total NNAL, free + glucuronide) ranging from ∼50 to ∼3000 pg/mL, with means of ∼400−600 pg/mL in habitual smokers.[17] In nonsmokers, concentrations are much lower, from undetectable to ∼100 pg/mL, with means ranging from 2 to 20 pg/mL depending on the exposure levels.[18] Measuring low parts per trillion levels of NNAL in a complex biological matrix such as urine presents a considerable analytical challenge. In the pioneering work by Hecht and colleagues, GC with a nitrosamine-selective detector (thermal energy analyzer, TEA) was used. This method required extraction of large volumes of urine (50−100 mL), purification of the extract using two preparative HPLC steps, and conversion of the secondary hydroxy group to a trimethylsilyl ether derivative prior to GC analysis using a TEA detector.[19] Recently, the method has been streamlined by simplifying the extraction procedure and replacing one of the HPLC purification steps with a solid-phase extraction.[20] LC−MS/MS has been used to quantify NNAL in urine of smokers.[21,22] Recently, an LC−MS/MS method having sufficient sensitivity (detection limit of 3 pg/mL) to determine NNAL in urine of SHS-exposed nonsmokers was reported. This method utilizes molecularly imprinted polymer (MIP) columns for selective extraction of NNAL and removal of interfering urinary constituents.[23]

As part of the studies on the biological consequences of SHS exposure, we required a method for determination of NNAL in urine of people with relatively low exposure levels. In addition, in some studies large volumes of urine were not available, which required a very sensitive method. In this report, we describe an LC−MS/MS method for quantitation of NNAL at subpicogram per milliliter concentrations in 5-mL urine specimens. The method involves conversion of the hydroxy group of NNAL to the hexanoate ester derivative, which facilitates the removal of interfering urinary constituents and enhances detection using electrospray ionization mass spectrometry.

EXPERIMENTAL SECTION

Instrumentation. LC−MS/MS analyses were carried out with a Surveyor HPLC interfaced to a TSQ Quantum Ultra triple-stage quadrupole mass spectrometer (Thermo-Finnigan, San Jose, CA), with a heated electrospray ion source (HESI). An HSF5 column (2.1 × 150 mm, 5 μm, Supelco, Sigma-Aldrich, Saint Louis, MO) was used for the chromatography. Solvent evaporation was carried out using a Savant SpeedVac model SC210A (Thermo-Savant, Marietta, OH).

Chemicals. NNAL-d_3 was a generous gift from Dr. Stephen Hecht, University of Minnesota. NNAL-d_0 was obtained from Toronto Research Chemicals, North York, ON, Canada. HPLC grade methanol and water from Burdick and Jackson (Muskegon, MI) were used to prepare the LC mobile phase. HPLC grade toluene, 1-butanol, pentane, and ethyl acetate from Fisher were used for extractions. β-Glucuronidase type IXA from *Escherichia coli* (3660 units/mg), hexanoic anhydride, and 4-(dimethylamino)pyridine (DMAP)were purchased from Sigma-Aldrich.

Preparation of Standards and Controls. A stock standard solution of NNAl-d_0 was prepared in 12 mM HCl and stored frozen at −20 °C. A 500 pg/mL solution of NNAl-d_3 was prepared in 10% methanol containing 12 mM HCl. Pooled urine, collected over several days, (2-L batches) from a nonsmoker with no known SHS exposure was used to prepare the standards and QCs. Standards and controls were stored frozen at −20 °C until use.

Sample Preparation. The 50 μL of 500 pg/mL NNAl-d_3 internal standard solution was added to 5-mL urine samples, standards, and QCs in 50-mL polypropylene centrifuge tubes (Fisherbrand) followed by brief vortex mixing. Sodium potassium phosphate buffer (0.5 mL of 2 M, pH 7) was added followed by the addition of 50 μL of 50 mg/mL glucuronidase dissolved in 0.1 M phosphate buffer. The samples were then incubated for 20−24 h at 37 °C to convert the NNAL glucuronides to free NNAL.

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(Using twice the enzyme concentration did not increase the measured amount of NNAL.) Potassium carbonate (0.5 mL of 50% w/v) was added, and the samples were extracted by vortex mixing for 5 min with 8 mL of toluene/1-butanol (70:30). The two phases were separated by centrifugation at 4000*g* for 5 min. After freezing the tubes in a dry ice−acetone bath, the organic upper layers were poured into 16 × 125 mm glass culture tubes containing 0.8 mL of 1 M H_2SO_4. The tubes were then vortexed 5 min and centrifuged as described above. The aqueous layers were frozen with a dry ice−acetone bath, and the organic layers were poured off and discarded. The acid layer was then washed with 5 mL of ethyl acetate/toluene (2:1) by vortex mixing 5 min. The tubes were centrifuged, the aqueous acid layers were frozen in a dry ice−acetone bath, and the upper wash layer was discarded. The acid phases were made basic with 0.8 mL of 50% (w/v) K_2CO_3 and extracted by vortex mixing 5 min with 4 mL of ethyl acetate/toluene (1:2). After centrifugation and freezing the aqueous layers, the organic layers were transferred to 13 × 100 mm glass culture tubes. The organic extracts were then evaporated to dryness using the SpeedVac at high heat setting. Hexanoic anhydride (50 μL) and 10 μL of 50 mg/mL DMAP in toluene were then added to the dry extracts; the tubes were then tightly capped and heated at 70 °C for 15 min. Saturated aqueous sodium bicarbonate (0.5 mL) and 4 mL of 10% ethyl acetate in pentane were added, and the derivatives were extracted by vortex mixing, centrifugation, and freezing the aqueous layers. The organic layers were transferred to 13 × 100 mm tubes containing 0.5 mL of 1 M H_2SO_4, and the organic layers were discarded after vortexing, centrifuging, and freezing. The acid layers were made basic with 0.5 mL of 50% (w/v) K_2CO_3 and then extracted with 4 mL of 10% ethyl acetate in pentane by vortex mixing, centrifugation, and freezing the aqueous layers. The organic layers were transferred to a new set of 13 × 100 mm tubes and evaporated using the SpeedVac at medium heat. The samples were reconstituted in 125 μL of 10% methanol containing 12 mM HCl and transferred to 300-μL polypropylene insert vials for analysis. Extraction efficiency was determined by spiking NNAL-d_0 and NNAL-d_3 into blank extract prior to evaporation and derivatization. A typical run consisted of 46 study samples plus 26 standards and QCs for a total of 72 samples. One analyst can carry out the enzyme incubation, extraction, and derivatization steps in 3 days, and the entire run, including LC−MS/MS analysis can be completed in 1 week.

Liquid Chromatography. The extracts (50 μL) were chromatographed with a methanol and water solvent system containing 10 mM ammonium formate at 0.333 mL/min using a linear gradient from 33 to 100% methanolic buffer over 11 min. The column was then held at 100% methanol for 1 min and then ramped back to the initial conditions over 1 min. Re-equilibration time was 7 min for a total run time of 20 min per sample.

Mass Spectrometry. The mass spectrometer was operated using electrospray ionization, in the positive ion mode. The ion source parameters were optimized by infusing a solution of the hexanoate ester derivative of NNAL into the ion source via a syringe pump. The vaporizer temperature was 372 °C, the heated capillary temperature was 265 °C, and the spray voltage was 3500 mV. Data was acquired in the selected reaction monitoring (SRM) mode. The transitions 308 to 84 and 311 to 87 at a collision energy of 20 eV were used for the hexanoate derivatives of NNAL and the internal standard, NNAL-d_3, respectively. The mass resolution (fwhm) was set at 0.5 amu for both Q1 and Q3.

Data Analysis. The Finnigan XCalibur/LC Quan software was used to generate calibration curves (linear regression, $1/X$ weighting) and calculate concentrations using peak area ratios of analyte/internal standard. Two sets of six standards, spanning the range of 0.25−50 pg/mL were included in each run of 40−45 clinical samples. For studies of low-level SHS exposure, three sets of quality control specimens at 0, 0.5, 1.0, and 2.0 pg/mL were included. Analysis of spiked urine samples indicated that the method is linear to at least 500 pg/mL.

Clinical Samples. Urine specimens were obtained from nonsmokers participating in research studies, described elsewhere,[24,25] and were stored frozen at −20 °C prior to analysis.

RESULTS AND DISCUSSION

Method Development. Using LC-ESI-MS/MS methods similar to published methods,[17,21−23] we had previously been able to achieve a detection limit of 5 pg/mL for 3-mL urine specimens.[26] However, some of our studies involved subjects with low-level exposure to SHS,[24] and a method with higher sensitivity was required. The limiting factor was not instrument sensitivity, but rather trace amounts of coeluting substances in the urine extracts. Attempts to separate these interfering substances chromatographically and with further MIP column purification[23] of the liquid/liquid solvent extracts were unsuccessful.

NNAL is a moderately polar organic compound, and it occurred to us that, by esterification of the hydroxy group, it could be converted to a relatively nonpolar substance. Doing so might be expected to improve sensitivity in LC−MS/MS analysis for the following reasons: (1) A relatively nonpolar substance could be partitioned between an aqueous phase and a nonpolar organic solvent to remove many of the multitude of potentially interfering polar organic compounds present in urine. (2) The relatively nonpolar ester derivative would be better retained on reversed-phase HPLC columns than the parent NNAL, and this could be a mechanism for separation of more polar, potentially interfering substances in the sample. (3) More efficient separation from other ionizable substances in urine, by extraction and chromatography, would be expected to reduce the extent of matrix suppression of ionization often occurring in electrospray ionization (ESI) of biological extracts.[27−29] (4) Ionization efficiency of the ester derivative in an ESI source might be enhanced compared to NNAL, due to more efficient desolvation. The hydroxy group of NNAL would be expected to hydrogen bond strongly with the usual reversed-phase HPLC solvents water, methanol, and acetonitrile, but an ester would be expected to do so less avidly. (5) Increasing the mass by derivatization might be expected to

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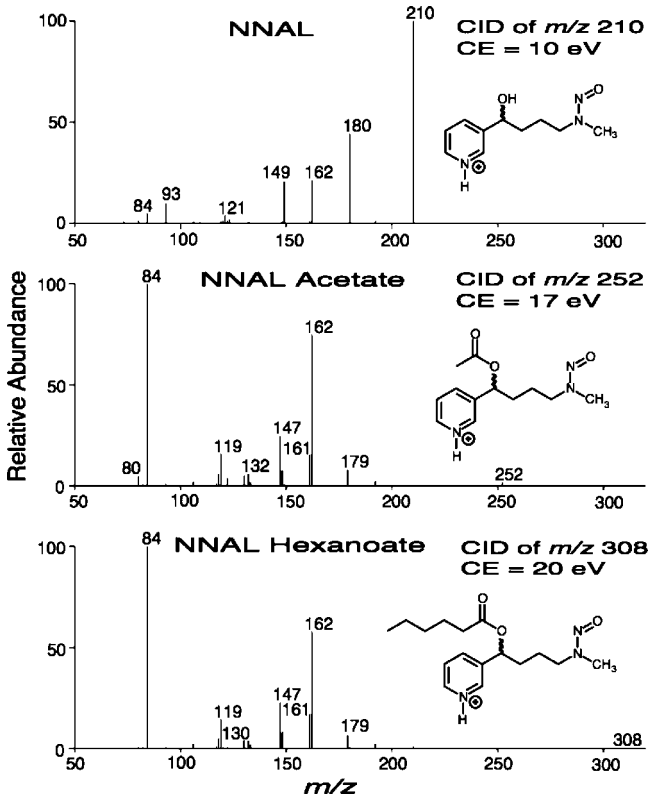

Figure 2. Product ion spectra of NNAL, NNAL acetate, and NNAL hexanoate.

increase selectivity in mass spectrometric detection, since potentially interfering substances in urine would be expected to have relatively low molecular weight. Consequently, we prepared the acetate and hexanoate derivatives and evaluated them for sensitivity and specificity of detection in urine extracts.

ESI was used, and the most abundant ion transitions of each derivative (Figure 2) were evaluated in the SRM mode. HPLC separation was carried out using a gradient of water−methanol with ammonium formate (pH ~6) buffer. Both derivatives provided better sensitivity than underivatized NNAL, but the hexanoate was by far the best, presumably owing to its much lower polarity, facilitating chromatographic separation from coextracted substances in urine, as well as more efficient desolvation in the electrospray ion source. Depending on the mobile-phase composition, the ESI response was 1.5−2.3 times greater for the hexanoate derivative than for underivatized NNAL, with the response for the acetate derivative between the two. Not surprisingly, the differences were greater under conditions of gradient elution, in which the hexanoate derivative eluted with a lower percentage of water in the mobile phase than the acetate or underivatized NNAL (Table 1). These data support the notion that these less polar derivatives bind less avidly to water and methanol, facilitating desolvation and increasing transmission of ions into the mass spectrometer. However, the most important factor affecting sensitivity was separation from interfering urinary constituents, since the intrinsic ESI response was not the factor limiting the sensitivity of the method (Figure 3). By comparing spiked urine to aqueous standards, suppression of ionization was estimated to be ~25%. Of the SRM transitions evaluated, *m/z* 308 to *m/z* 84 of the hexanoate derivative provided the best selectivity in terms of minimizing interference by urinary constituents. Although a

Table 1. Comparison of ESI Sensitivity for NNAL, NNAL Acetate, and NNAL Hexanoate with Different HPLC Mobile-Phase Compositions

	% of NNAL[a]		
analyte	isocratic[b]	fast gradient[c]	slow gradient[d]
NNAL	100	100	100
NNAL acetate	113	154	112
NNAL hexanoate	154	212	231

[a] Peak areas as percent of the peak area for NNAL. [b] 10 mM ammonium formate in 90:10 methanol/water. [c] Linear gradient of 10 mM ammonium formate, 100% aqueous changing to 100% methanolic over 2 min followed by 6 min methanolic. [d] Gradient described in Experimental Section used for urine sample analyses.

confirmation ion would be an advantage, none of the alternative product ions we investigated had enough specificity for detection in urine below ~3 pg/mL, and unfortunately, most of our study samples had concentrations below this.

A liquid−liquid extraction/derivatization procedure was devised to (1) utilize acid−base partitioning of the weakly basic NNAL, which has pyridine ring, to remove neutral and acidic substances; (2) esterify the hydroxy group with hexanoic anhydride to provide the hexanoate derivative; (3) remove excess hexanoic anhydride and other remaining neutral and acidic substances by acid−base partitioning; and (4) a final extraction with a solvent of low polarity (90−10 pentane−ethyl acetate) followed by evaporation and reconstitution in HPLC mobile phase. The efficiency of extraction was determined by spiking urine with both NNAL-d_0 and NNAL-d_3 and comparing the peak areas with urine extracts spiked prior to the derivatization step. The extraction recovery was ~60%.

Method Validation. Standard curves were linear from 0.25 to 50 pg/mL with typical r^2 values >0.99 using linear regression with $1/x$ weighting. A standard curve constructed from means of six replicates is presented in Figure 4. Precision, accuracy, and lower limit of quantitation (LLOQ) were determined by analyzing urine from a nonsmoker, who had no known exposure to SHS, spiked with NNAL. Over the range of 0.25−50 pg/mL, precision ranged from 3.2 to 15.0% (percent coefficient of variation, CV) and accuracy ranged from 88.0 to 107.1% (percent of expected) (Tables 2 and 3). Using the criteria of Shah et al., the LLOQ was determined to be 0.25 pg/mL.[30] Determining the limit of detection (LOD) in urine is complicated by the ubiquitous presence of tobacco smoke residues in the environment, and consequently, nearly everyone will have at least a minute exposure to the precursor substance NNK. Therefore, truly NNAL-free urine may be hard to verify, and it may be not be possible to distinguish NNAL from a coeluting substance with the same product ion. Consequently, we investigated the LOD by extracting, derivatizing, and analyzing standards prepared in 0.01 N HCl. The blank value was positive, as the intercept of the standard curve indicated. A 5-mL 0.0625 pg/mL standard prepared in 0.01 N HCl, following extraction, derivatization, and injection of an aliquot, gave rise to a peak with about twice the peak area of a corresponding peak in the blank. We also estimated the instrument sensitivity by injecting

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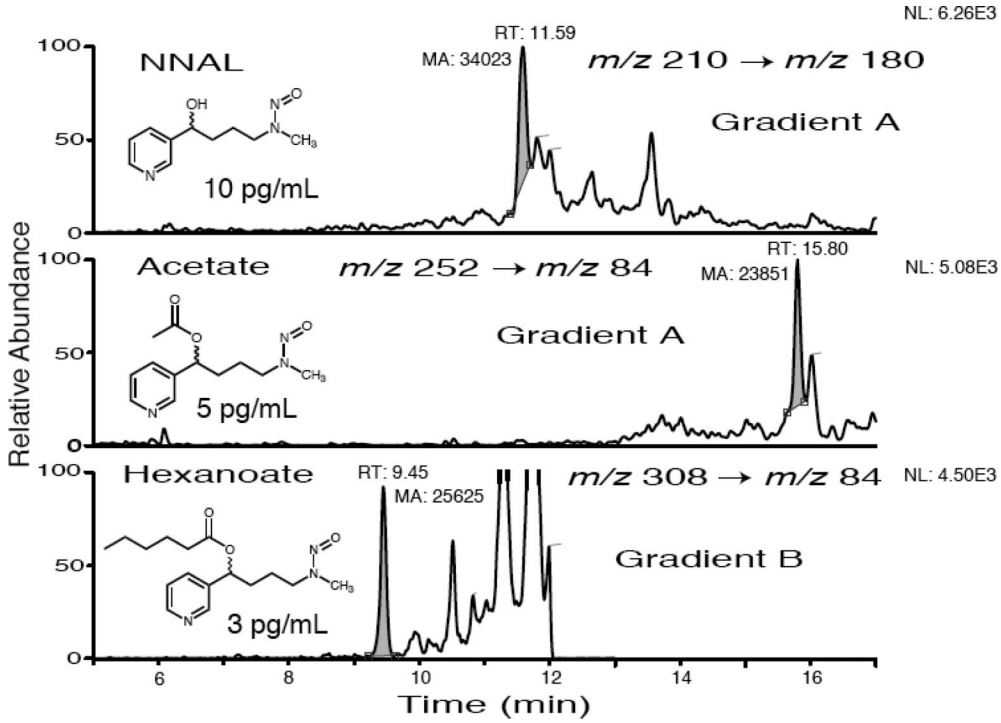

Figure 3. Chromatograms of urine extracts containing NNAL, NNAL acetate, and NNAL hexanoate. Mobile phase (10 mM ammonium formate in methanol−water), column, and flow rate as described in experimental section. A was a linear gradient from 10% methanol to 70% methanol over 15 min; B was a linear gradient from 33% methanol to 100% methanol over 11 min.

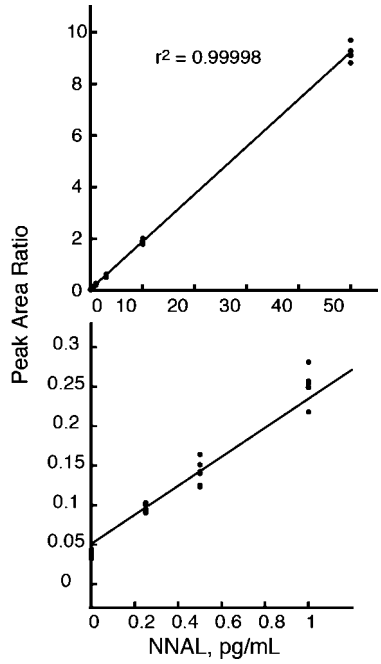

Figure 4. Calibration curve for NNAL in urine. Six replicates at each concentration, linear regression, 1/*x* weighting. Lower panel is expanded scale for range of 0−1 pg/mL.

Table 2. Within-Run Precision and Accuracy for Determination of NNAL in Urine[a]

added amount (pg/mL)	measured mean (pg/mL)	accuracy (% of expected)	precision CV (%)
0	<LLOQ		12.3[b]
0.25	0.24	95.2	12.6
0.50	0.47	94.1	12.5
1.00	1.08	107.9	10.2
3.00	2.87	95.8	8.3
10.00	10.10	101.0	4.2
50.00	49.99	100.0	3.2

[a] Six replicate analyses of urine from a nonsmoker with no known SHS exposure spiked with NNAL. [b] For blank urine, precision is based on the area ratios of peak corresponding to analyte/peak of internal standard.

Table 3. Between-Run Precision and Accuracy for Determination of NNAL in Urine[a]

added amount (pg/mL)	measured mean (pg/mL)	accuracy (% of expected)	precision CV (%)
0.50	0.487	97.4	15.0
1.00	0.950	95.0	8.8
2.00	1.76	88.0	4.4

[a] Seven replicate analyses (three different runs) of urine from a nonsmoker with no known SHS exposure spiked with NNAL.

known amounts of NNAL hexanoate. Injecting an aliquot of a methanol solution containing 62.5 amol of NNAL hexanoate (which would correspond to ~13 fg of NNAL) resulted in a peak of about twice the area of a corresponding peak in the methanol blank.

Specificity was determined by analyzing urine samples from eight nonsmokers with no known SHS exposure. All had NNAL concentrations below the limit of quantitation of 0.25 pg/mL. A urine specimen from a person reporting a brief exposure to SHS 17 h prior to the sample collection had a concentration of 0.99 pg/mL. Chromatograms of urine extracts from three people with varying levels of SHS exposure are presented in Figure 5.

Application of the Method. Urine specimens from 73 nonsmoking research subjects who suffered from chronic obstructive pulmonary disease[24] were analyzed for NNAL. Concentrations

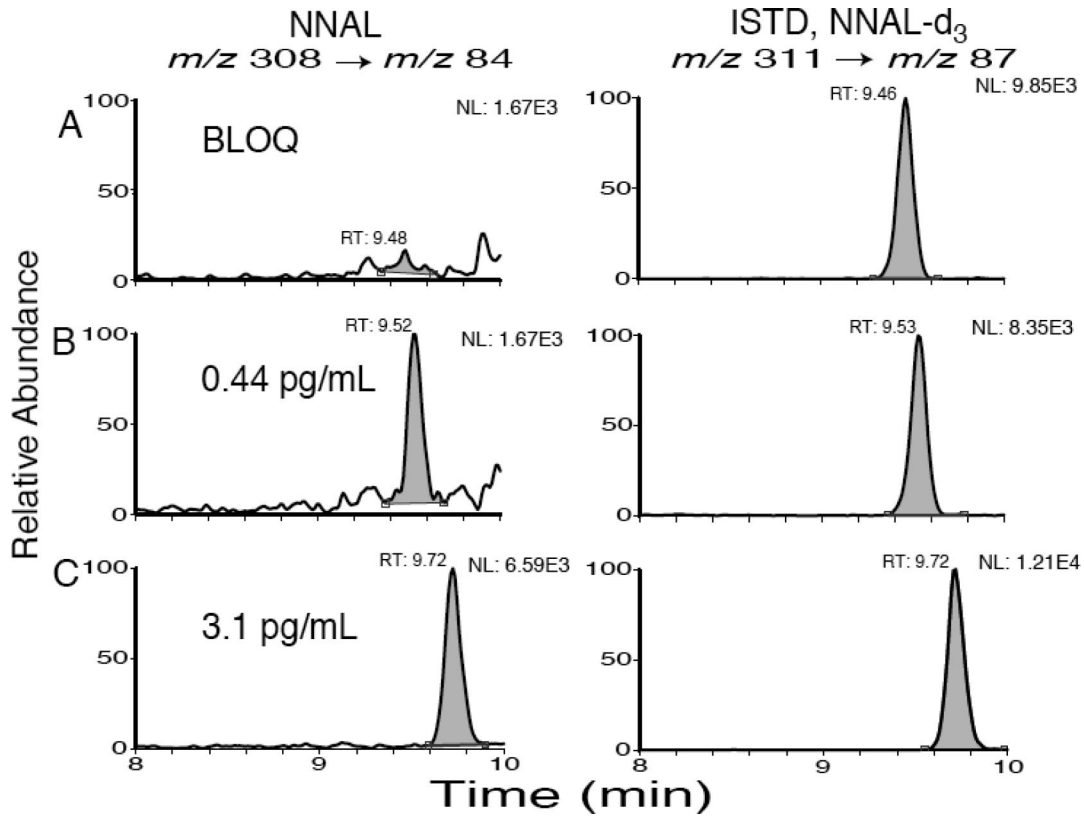

Figure 5. Chromatograms of nonsmokers' urine extracts. (A) Person with little or no SHS exposure; (B) subject in study of SHS exposure in people with chronic obstructive pulmonary disease;[24] (C) college student after SHS exposure in a bar.[25]

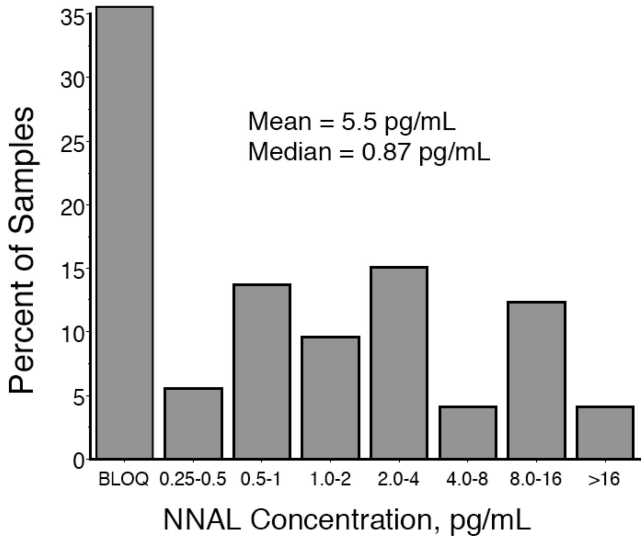

Figure 6. Distribution of NNAL concentrations in urine of 73 research subjects with chronic obstructive pulmonary disease.[24] BLOQ = below limit of quantitation.

ranged from below the LOQ (26 subjects) to 153 pg/mL, with a mean of 5.5 pg/mL, and median of 0.87 pg/mL. (Figure 6). The mean is similar to that reported by other investigators for nonsmokers.[18] We also analyzed urine samples from eight nonsmoking college students before and after they spent 6 h in smoking-allowed bars. In all eight subjects, concentrations of NNAL in urine collected ~16 h after entering the bar were higher than those from samples collected prior to entering the bar (Figure 7).

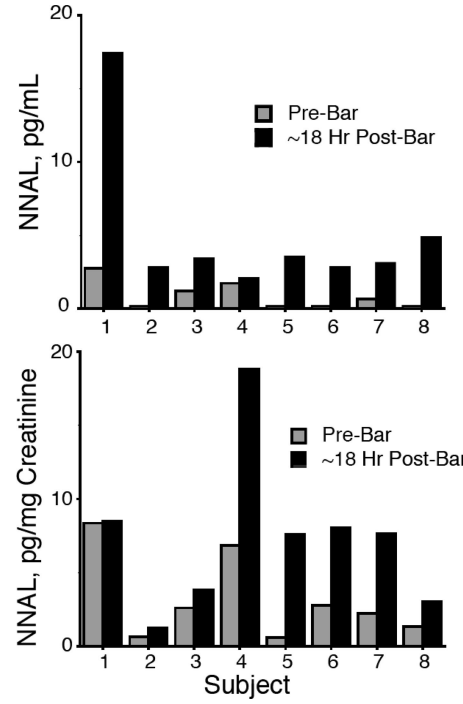

Figure 7. Concentrations of NNAL in urine of eight college students prior to and after spending 6 h in smoking-allowed bars.[25] In the lower panel, concentrations are normalized to creatinine to adjust for differences in urinary flow.

CONCLUSION

A method for determination of the tobacco-specific carcinogen biomarker NNAL in human urine at subpicogram per milliliter

concentrations has been developed. Key to the success of this method was conversion of the analyte to a relatively nonpolar derivative, which facilitates removal of interfering substances in the sample matrix by chromatography and by the extraction procedure, and enhances detection by electrospray ionization mass spectrometry. This method appears to be the most sensitive method yet reported for determination of NNAL in urine. Applicability of the method to determination of NNAL in urine of humans exposed to low levels of secondhand tobacco smoke has been demonstrated. Although derivatization adds extra steps to the sample preparation procedure, conversion of hydrophilic analytes to more hydrophobic derivatives has the potential to greatly improve the sensitivity and specificity of ESI mass spectrometric analyses.

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