Mass Spectrometric Quantitation of Nicotine, Cotinine, and 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanol in Human Toenails

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Abstract

Numerous studies have quantified total cotinine (the sum of cotinine and cotinine-*N*-glucuronide) and total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol [NNAL; the sum of NNAL and its *O*- and *N*-glucuronides (NNAL-Glucs)] in the urine and blood of smokers, smokeless tobacco users, and nonsmokers exposed to environmental tobacco smoke. Analysis of hair and nails has several advantages over blood and urine testing, such as accumulation of xenobiotics during long-term exposure, ease of collection, and indefinite stability of samples. We developed sensitive methods for quantitation of nicotine, cotinine, and NNAL in human toenails. Nicotine and cotinine were analyzed by gas chromatography-mass spectrometry-selected ion monitoring. NNAL was assayed using liquid chromatography-electrospray ionization-tandem mass spectrometry-selected reaction monitoring. The detection limits of the methods were 0.01 ng/mg toenail for nicotine, 0.012 ng/mg toenail for cotinine, and 0.02 pg/mg toenail for NNAL. In 35 smokers, the mean nicotine level was 5.9 $\pm$ 5.6 ng/mg toenail, mean cotinine was 1.6 $\pm$ 1.3 ng/mg toenail, and mean NNAL was 0.41 $\pm$ 0.67 pg/mg toenail. Samples collected from six nonsmokers were negative for NNAL. In smokers, NNAL correlated with cotinine ($r = 0.77$; $P < 0.0001$). The results of this study for the first time show the presence of cotinine and NNAL in human toenails. These sensitive and quantitative methods should be useful in epidemiologic studies of the role of chronic tobacco smoke exposure, including environmental tobacco smoke exposure, in human cancer. (Cancer Epidemiol Biomarkers Prev 2006; 15(12):2378–83)

Introduction

Cigarette smoking is a major cause of cancer deaths worldwide and also causes vascular and respiratory diseases (1). Nicotine and the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are two important tobacco smoke constituents representing its critical biological properties. Nicotine is the major tobacco alkaloid and the main known addictive component of tobacco smoke. NNK is a highly effective lung carcinogen in rats and also causes lung tumors in mice and hamsters (2). It also produces tumors of the pancreas, liver, and nasal mucosa (2), and administration together with another tobacco-specific nitrosamine *N*′-nitrosonornicotine caused tumors of the oral cavity in rats (3). NNK and *N*′-nitrosonornicotine have been classified recently by the IARC as carcinogenic to humans (group 1; ref. 4).

Biomarkers of tobacco smoke exposure are crucial in understanding mechanisms by which tobacco products cause cancer. Nicotine is rapidly metabolized in the human body, about 70% to 80% of the dose being converted to cotinine (5). Measurements of nicotine and cotinine levels in urine, saliva, and blood are commonly used to monitor systemic exposure to cigarette smoke in smokers and nonsmokers exposed to environmental tobacco smoke (ETS; refs. 6-9). Urinary metabolites of NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides (NNAL-Glucs), are excellent biomarkers of NNK uptake in rodents and humans (10, 11). Numerous studies have quantified total NNAL, the sum of NNAL and NNAL-Glucs, in the urine of smokers, smokeless tobacco users, and nonsmokers exposed to ETS (11-16). Total NNAL has also been quantified in the blood of smokers and smokeless tobacco users (15, 16), and a practical and sensitive method for total NNAL quantitation in plasma has been developed recently (17).

Analysis of keratinic matrices, such as hair and nails, has several advantages over urine, blood, and saliva testing. Thus, growth rates are ~1 cm monthly for hair (18), ~0.3 cm monthly for fingernails, and ~0.1 cm monthly for toenails (19); therefore, biomarker levels in hair and nails reflect exposure over a longer period. Other advantages include relative ease of sample collection and storage and seemingly indefinite stability of the collected sample caused by incorporation of the analytes of interest into the keratinic matrix (19). In recent years, hair was most commonly used for analysis of drugs of abuse for forensic purposes (19-21). Hair analysis has also been widely used to assess fetal exposure to nicotine through maternal smoking during pregnancy (21-23) and childhood exposure to ETS (18, 24, 25). Fingernails and toenails have been used for the detection of drugs of abuse (26) and in studies of arsenic intoxication (27, 28), occupational exposures (29, 30), and environmental exposure of children to trace elements (31-33). Unlike hair, nails grow in two directions, length and thickness, thus leading to dual mechanisms of xenobiotic incorporation, via the nail matrix and nail bed (19, 34). Due to the contribution of the nail bed to the incorporation process, the content of analytes in nail clippings reflects cumulative exposure over a relatively long period (19). Thus, nails seem to be more suitable in studies of chronic exposure, whereas hair is useful in monitoring of exposure during discrete periods by the axial distribution of analytes. Toenails grow more slowly than fingernails and are less likely to be environmentally contaminated with the analyte of interest than fingernails or hair (19).

It is biologically plausible that cotinine and NNAL are present in human toenails and could serve as biomarkers of chronic human exposure to nicotine and NNK. Verghese et al.

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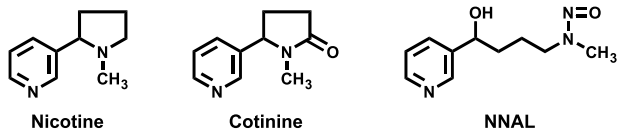

Figure 1. Structures of compounds analyzed in human toenails.

(35) reported an interesting physical sign related to smoking cessation, which they termed ''harlequin nail.'' They observed a distinct demarcation line in toenails of patients showing when they had stopped smoking. In a recent study, Al-Delaimy et al. (36) quantified nicotine in toenails using high-performance liquid chromatography (HPLC) with electrochemical detection. It was found that toenail nicotine levels differ significantly according to tobacco smoke exposure, being ~6-fold higher in smokers compared with nonsmokers exposed to ETS. In his study, Al-Delaimy et al. used toenail clippings collected as a part of the Nurses' Health Study, which was established in 1976 and involved 121,700 U.S. female registered nurses. The same study provided toenail clippings for investigation of toenail selenium concentrations and bladder cancer risk (37). Toenail clippings are often collected and stored in large-scale epidemiologic studies and could be used for investigations of tobacco smoke constituent uptake and development of cancer later in life.

The purpose of our study was to develop sensitive methods for quantitation of cotinine and NNAL in human toenails. Because HPLC with electrochemical detection is not as specific as mass spectrometry (MS), we developed a gas chromatography-MS (GC-MS) method for quantitation of nicotine and cotinine in toenails. NNAL was quantified by liquid chromatography-electrospray ionization-tandem MS (LC-ESI-MS/MS). Structures of the biomarkers analyzed here are shown in Fig. 1.

Materials and Methods

Caution. NNAL is carcinogenic and mutagenic and should be handled with extreme care, using appropriate protective clothing and ventilation at all times.

Chemicals and Enzymes. NNAL was purchased from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). [Pyridine-D_4]NNAL was synthesized from [pyridine-D_4]ethyl nicotinate (Cambridge Isotope Laborotories, Cambridge, MA) as described previously (38, 39). [$^{13}C_6$]NNAL was synthesized by $NaBH_4$ reduction of [$^{13}C_6$]NNK (Cambridge Isotope Laborotories). Nicotine, [CD_3]nicotine, cotinine, [CD_3]cotinine, and β-glucuronidase (type IX-A from *Escherichia coli*) were purchased from Sigma Chemical Co. (St. Louis, MO).

Apparatus. Analysis of nicotine and cotinine by GC-MS-selected ion monitoring was carried out with a model 6890 GC equipped with an autosampler and interfaced with a model 5973 mass-selective detector (Agilent Technologies, Palo Alto, CA) as described previously (40, 41).

LC-ESI-MS/MS was carried out on a Finnigan TSQ Quantum Discovery Max instrument (Thermo Electron Corp., Waltham, MA) interfaced with an Agilent model 1100 capillary HPLC system and a model 1100 micro autosampler (Agilent Technologies) by using a slight modification of a method described previously (17). The HPLC was fitted with a 150 × 0.5 mm ZORBAX SB C18 RR 3.5-μm column (Agilent Technologies) eluted in isocratic mode with 35% methanol in H_2O for 15 min at a flow rate of 10 μL/min. 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 → 180 for NNAL, *m/z* 214 → 184 for [pyridine-D_4]NNAL, and *m/z* 216 → 186 for [$^{13}C_6$]NNAL, at 1.0 a.m.u. 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 a.m.u.

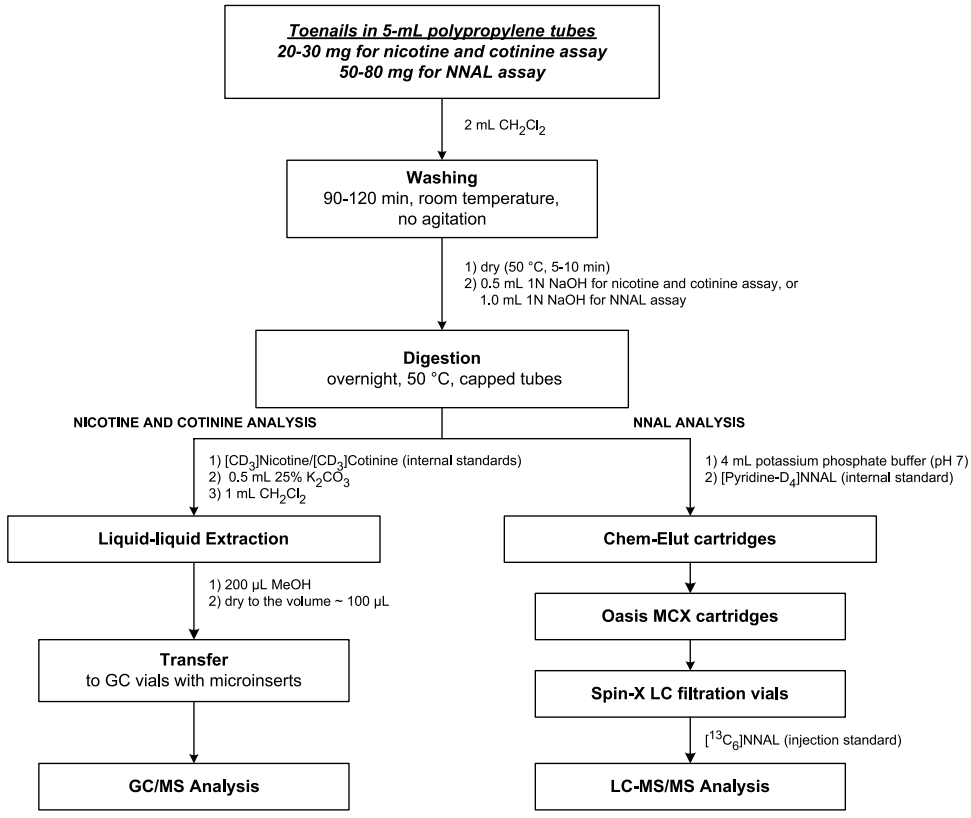

Figure 2. Analytic procedure for nicotine, cotinine, and NNAL analysis in human toenails.

Table 1. Nicotine and cotinine in subjects with different exposures

Group	No. subjects*	Mean nicotine (ng/mg)	Mean cotinine (ng/mg)
Nonsmokers, not exposed to ETS	4	0.11	0.012
Nonsmokers, exposed to ETS	2	0.37	0.036
Smokers	2	1.2	0.33

*Samples from each subject were analyzed in duplicate.

Subjects. Thirty-five active smokers were recruited from a longitudinal study of tobacco biomarkers and several smoking cessation studies conducted at the Transdisciplinary Tobacco Use Research Center (Minneapolis, MN). The entrance criteria of these studies required subjects to smoke at least 10 cigarettes daily for at least 1 year. Subjects were offered the opportunity to participate in this addendum study for additional payment. All studies were approved by the University of Minnesota Research Subjects' Protection Programs Institutional Review Board Human Subjects Committee.

Analyses. Toenail clippings (20-30 mg for nicotine/cotinine assay and 50-80 mg for NNAL assay) were weighed into 5-mL polypropylene tubes and washed without agitation for 1.5 to 2 h in 2 mL CH_2Cl_2 at room temperature. Then, the tubes were vortexed, CH_2Cl_2 was removed by aspiration, and the toenails were vortexed one more time with 1 mL CH_2Cl_2. After washing, the toenails were dried in a heating block at 50°C (5-10 min).

Nicotine and Cotinine Analysis. To analyze nicotine and cotinine, toenails were digested at 50°C overnight in 0.5 mL 1 N NaOH. The next day, 5 μL of a mixture containing 5 ng/μL each of [CD_3]nicotine and [CD_3]cotinine as internal standards were added to a 5-mL glass centrifuge tube (Kimble, Vineland, NJ) containing 0.5 mL of 25% aqueous K_2CO_3 and 1 mL CH_2Cl_2. The digested toenail sample was added to the tube, and the mixture was treated and analyzed as described previously (40, 41).

NNAL Analysis. To analyze NNAL, toenails were digested at 50°C overnight in 1 mL 1 N NaOH. The next day, the digests were added to a 15-mL glass centrifuge tube containing 10 pg [pyridine-D_4]NNAL as internal standard in 4 mL potassium phosphate buffer (prepared from 0.1 mol/L KH_2PO_4 adjusted to pH 7). The mixture was applied to a 5-mL ChemElut cartridge (Varian, Harbor City, CA) and eluted with 2 × 8 mL CH_2Cl_2 into a 15-mL glass centrifuge tube. The combined eluants were concentrated to dryness (Speedvac concentrator). The dry residue was redissolved in 1 mL H_2O, adjusted to pH 2 to 3, further purified on Oasis MCX cartridges (Waters Corp., Milford, MA), and transferred to autosampler vials as described previously (12). Dry samples were stored at −20°C. Before analysis by LC-ESI-MS/MS, samples were redissolved in 10 μL of 2% methanol in H_2O containing 2.5 pg/μL [$^{13}C_6$]NNAL as injection standard, and 5 μL were injected.

Statistical Analyses. Pearson product moment correlation coefficients and statistical significance of the correlations were determined using SigmaPlot 2001, version 7.101 (SPSS, Inc., Chicago, IL).

Results

The procedures for nicotine, cotinine, and NNAL analysis in toenails are summarized in the scheme shown in Fig. 2.

Nicotine and Cotinine Assay. We initially analyzed only a few samples collected from nonsmokers not exposed to ETS, two nonsmokers exposed to ETS, and two smokers. The results of this first experiment summarized in Table 1 were in good agreement with the results reported by Al-Delaimy et al. (36) in terms of nicotine levels in different exposure categories. A typical GC-MS chromatogram of cotinine in human toenails is shown in Fig. 3.

Precision of the developed nicotine and cotinine assay was determined by dividing a toenail digest from a single smoker into six aliquots and analyzing each for nicotine and cotinine. Relative SDs were 10.1% for nicotine and 4.8% for cotinine. The accuracy of the assay was determined by spiking an ETS-exposed nonsmoker's toenail digest with 0.5, 1, 2.5, and 5 ng cotinine/mg toenail. Analysis gave 0.42, 0.84, 2.3, and 4.4 ng cotinine/mg toenail, respectively, producing good correlation between spiked and measured cotinine (r = 0.99). The detection limits of the method were 0.01 ng/mg toenail for nicotine and 0.012 ng/mg toenail for cotinine.

NNAL Assay. The aqueous toenail digest was enriched by partitioning with CH_2Cl_2 on a ChemElut liquid-liquid extraction cartridge (Fig. 2). Final enrichment was accomplished by a mixed mode cation exchange extraction on an Oasis MCX solid-phase extraction cartridge. The fraction

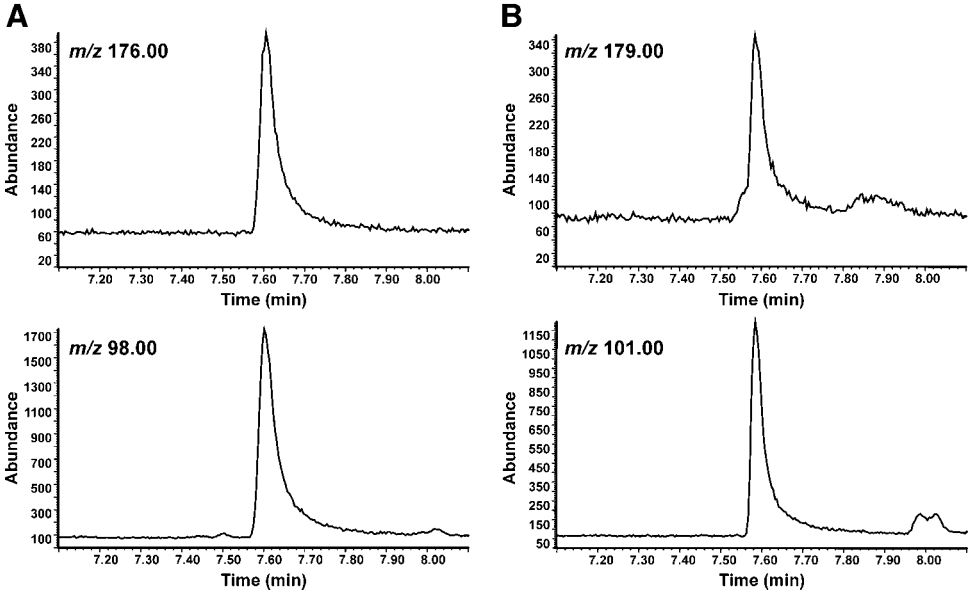

Figure 3. GC-MS chromatograms obtained on analysis of cotinine in toenails from a smoker. **A.** Cotinine, *m/z* 176 (molecular ion) and *m/z* 98 (used for quantitation). **B.** [CD_3]cotinine (internal standard), *m/z* 179 (molecular ion), and *m/z* 101 (used for quantitation).

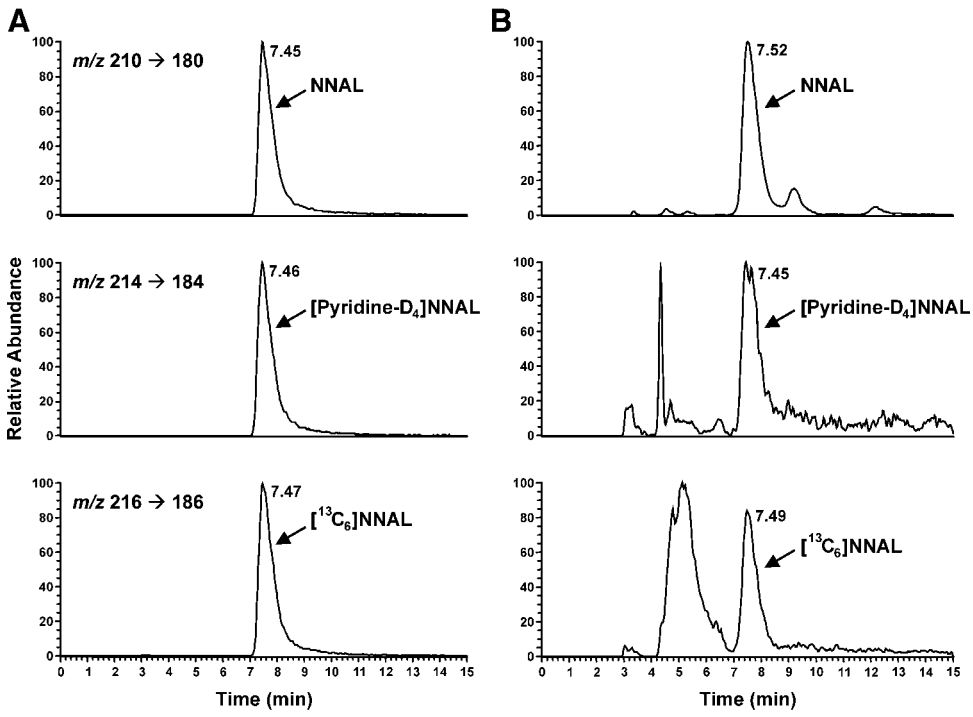

Figure 4. Chromatograms obtained on LC-ESI-MS/MS analysis of NNAL, [pyridine-D_4]NNAL (internal standard), and [$^{13}C_6$]NNAL (injection standard). **A.** standard mix. **B.** a smoker's toenail sample.

containing NNAL was then directly analyzed by LC-ESI-MS/MS. A typical chromatogram is shown on Fig. 4.

The detection limit of the assay for NNAL in toenails from smokers was 0.02 pg/mg toenail, starting with a 50 mg sample. Precision of the assay was determined by analyzing six aliquots of a smoker's digested toenail sample. The results were 1.4 ± 0.16 fmol NNAL/mg toenail (relative SD, 11.4%). NNAL in the toenails from the same smoker was also determined by a modified method, in which β-glucuronidase treatment (37°C, overnight) was added after digestion and pH adjustment to 6 to 7. This modification did not affect the measured NNAL. The accuracy of the assay was determined by spiking aliquots of pooled nonsmokers' toenail digest with 0.5, 1, 2, 5, and 10 pg NNAL. Analysis gave 0.51, 1.1, 2.0, 5.3, and 9.2 pg NNAL, respectively, producing high correlation (r = 0.99) between spiked and measured NNAL. Recoveries of internal standard averaged 22 ± 10% (n = 28).

Analysis of Toenail Samples from Smokers. The assays were applied to toenail samples from 35 smokers and 6 nonsmokers. Data for nicotine, cotinine, and NNAL in toenails from smokers are summarized in Fig. 5. All nonsmoker samples were negative for NNAL; nicotine and cotinine levels in these subjects averaged 0.09 and 0.01 ng/mg, respectively. In smokers, mean nicotine level was 5.9 ± 5.6 ng/mg toenail, mean cotinine was 1.6 ± 1.3 ng/mg toenail, and mean NNAL was 0.41 ± 0.67 pg/mg toenail. NNAL correlated with cotinine (r = 0.77; P < 0.0001; Fig. 6).

Discussion

We developed sensitive methods for quantitation of nicotine, cotinine, and NNAL in human toenails. Previous studies on cotinine and total NNAL in biological fluids, such as urine and blood, provided information on short-term exposure to nicotine and NNK. Thus, cotinine has a half-life of about 15 to 17 h, and the distribution half-life for urinary NNAL is 3 to 4 days. Toenail cotinine and NNAL levels integrate tobacco smoke exposure over a relatively long period, about 3 to 5 months, thus being potentially useful biomarkers in studies of the role of chronic tobacco smoke exposure in human cancer. For the purposes of this study, the measurements were carried out mainly in smokers; cotinine was also quantified in two nonsmokers exposed to ETS. The limit of detection of these assays reached into the femtomol range for nicotine and cotinine and subfemtomol range for NNAL.

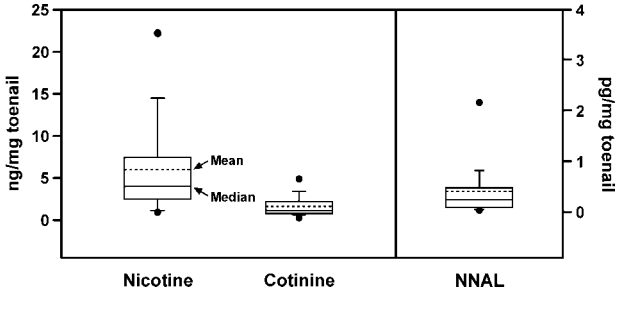

Figure 5. Nicotine, cotinine, and NNAL in toenails of smokers. *Boxes,* 25th and 75th percentiles; *lines outside boxes,* 10th and 90th percentiles; *dots,* outliers.

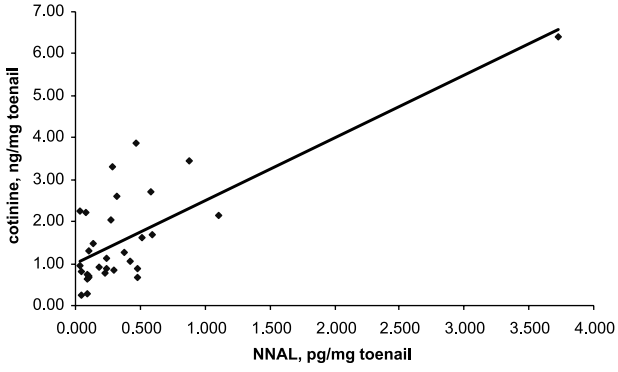

Figure 6. Relationship between cotinine and NNAL in the toenails of 31 smokers (r = 0.77; P < 0.0001).

For nicotine and cotinine analysis in toenails, we used GC-MS-selected ion monitoring, which is characterized by high sensitivity and specificity. The method also allows using deuterium-labeled nicotine and cotinine as internal standards, providing high accuracy of nicotine and cotinine quantitation in toenails. Overall, our results are in good agreement with those reported by Al-Delaimy et al. (Table 1; ref. 36). Small sample sizes limit our ability to compare these two methods, and future studies are necessary to assess toenail nicotine and cotinine levels in different exposure categories.

The method for NNAL analysis in toenails (Fig. 4) consists of a few simple steps and resembles our recently published method for total NNAL analysis in blood (17). Isocratic elution of the LC column considerably reduced the time needed for LC-MS-ESI analysis of a single sample. This modification did not result in appearance of coeluting peaks, which would interfere with quantitation (Fig. 5). It is unknown whether NNAL-Glucs are present in toenails. NNAL-*N*-Gluc would be hydrolyzed to NNAL at the initial step of the procedure, when the toenails are digested in 1 N NaOH (42). NNAL-*O*-Gluc is not hydrolyzed to NNAL by base treatment (42), but it is converted to NNAL by treatment with β-glucuronidase (12, 17, 42). Overnight treatment with β-glucuronidase of a smoker's toenail digest adjusted to pH 6 to 7 did not alter the measured NNAL, thus indicating that there is no NNAL-*O*-Gluc present in toenails, or its amount is extremely low.

Although toenail nicotine levels were assessed previously (36) and nicotine and cotinine were analyzed in another keratinic matrix, hair (reviewed in ref. 43), no other study has reported the presence of cotinine and NNAL in toenails. To validate our new methods and investigate the relationship between the analytes, we analyzed toenail clippings collected from active smokers. The interindividual variability and the ranges of toenail nicotine and cotinine levels observed here are remarkably similar to those observed on hair analysis. Thus, Kintz (44) reported 0.91 to 38.27 ng nicotine/mg hair and 0.09 to 4.99 ng cotinine/mg hair in 56 smokers. In our study, nicotine ranged from 0.79 to 22.67 ng/mg toenail, and cotinine ranged from 0.23 to 6.38 ng/mg toenail. This similarity is not surprising because the growth of both hair and nail is slow, and nicotine and cotinine are brought into these keratinic matrices by similar mechanisms (through the blood circulation). However, in our opinion, toenail analysis has several advantages over hair testing for studies of chronic exposure. Toenails are considered to be relatively free of external contamination and grow more slowly than hair, and the content of biomarkers in nail clippings reflects cumulative rather than discrete exposure, thus eliminating the issue of irregular growth and nonuniform axial distribution of biomarkers present in hair. Moreover, the limit of detection of nicotine in toenails (0.01 ng/mg toenail) is lower than that reported for mass spectrometric analysis of nicotine in hair (0.24 ng/mg hair; ref. 45).

The ratio between cotinine and NNAL in smokers' toenails was found to be ~4,600 and is similar to that observed in the urine of smokers (15). The correlation of toenail cotinine with toenail NNAL observed in this study was also similar to that reported for the urine of smokers (r = 0.68; ref. 46) and ETS-exposed nonsmokers (r = 0.71; ref. 14).

Although this study provides accurate and sensitive methods for nicotine, cotinine, and NNAL analysis in human toenails, additional studies, such as a longitudinal study in smokers and investigation of the kinetics of cotinine and NNAL disappearance from quitters' toenails, are necessary to validate toenail cotinine and NNAL as useful biomarkers of human chronic exposure to tobacco smoke.

In summary, the results of this study show for the first time that cotinine and NNAL are present in human toenails. The methods developed here should be useful in epidemiologic studies of the role of chronic tobacco smoke exposure, including ETS exposure, in human cancer.

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