Detection and Quantitation of *N*′-Nitrosonornicotine in Human Toenails by Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry

Irina Stepanov and Stephen S. Hecht

The Cancer Center, University of Minnesota, Minneapolis, Minnesota

Abstract

Specific biomarkers of tobacco carcinogen uptake are critical for investigations of the role of tobacco smoke exposure in human cancers. Two new biomarkers of human exposure to tobacco-specific carcinogens have been recently developed by our research group: urinary *N*′-nitrosonornicotine (NNN) and toenail 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). In this study, we report the presence of NNN in human toenails. Toenails of 17 smokers were analyzed for total NNN. Mean total NNN level in these samples was 4.63 ± 6.48 fmol/mg toenail and correlated with previously reported total NNAL ($r = 0.96$; $P < 0.0001$), total nicotine ($r = 0.48$; $P < 0.05$), and total cotinine ($r = 0.87$; $P < 0.0001$). An interesting finding was that amounts of NNN in smokers' toenails were generally higher than those of total NNAL. The ratio of toenail NNN to NNAL averaged 2.8, whereas the previously reported ratio between these biomarkers in smokers' urine was 0.1. NNN was also found in toenail samples from 12 nonsmokers, averaging 0.35 ± 0.16 fmol/mg and positively correlating with toenail cotinine ($r = 0.58$; $P = 0.05$). The results of this study show the feasibility of quantifying NNN in human toenails, providing a potentially useful new biomarker of tobacco carcinogen exposure. (Cancer Epidemiol Biomarkers Prev 2008;17(4):945–8)

Introduction

Evaluation of tobacco carcinogen uptake and metabolism in individuals, as well as prediction of potential health risks in people who are exposed to tobacco products, is theoretically possible through the use of biomarkers of tobacco carcinogen exposure. The most practical and extensively used biomarkers that have provided important information about tobacco carcinogen dose and metabolism are urinary metabolites of a tobacco-specific lung carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (1, 2). The sum of the 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone metabolites 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides, called total NNAL, was analyzed in several studies involving smokers, smokeless tobacco users, and nonsmokers exposed to secondhand tobacco smoke (3-7). Recently, we developed a method for quantitation of total NNAL in human toenails (8, 9). Use of toenail biomarkers offers several advantages over urine analysis, with potential evaluation of long-term cumulative exposure due to slow growth being the most appealing one (10). Among other advantages are ease of collection, unlimited stability, and availability in some large-scale epidemiologic studies. We developed sensitive mass spectrometric methods for analysis of total nicotine, its major metabolite cotinine, and total NNAL in toenails (8) and, in a separate study, provided essential validation data for the use of toenail biomarkers in the investigation of the role of chronic tobacco smoke exposure in human cancer (9).

Another recent development in the field of tobacco carcinogen biomarkers was detection and quantitation of a biomarker of human exposure to *N*′-nitrosonornicotine (NNN; ref. 11). NNN, like 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, is formed during tobacco processing via nitrosation of tobacco alkaloids and is present in both unburned tobacco and cigarette smoke (12-14). Based on its occurrence in tobacco products and cigarette smoke and on its carcinogenic activity in laboratory animals, NNN is believed to be a cause of esophageal cancer in smokers (1, 15). The sum of unchanged NNN and its pyridine-*N*-glucuronide (NNN-*N*-Gluc), called total NNN, was quantified in the urine of smokers and smokeless tobacco users (11).

The purpose of this study was to detect and quantify NNN in human toenails by using a highly sensitive and selective method: liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). The structures of the biomarkers discussed here are shown in Fig. 1.

Received 10/17/07; revised 1/8/08; accepted 1/16/08.

Grant support: National Cancer Institute grant CA-81301. LC-ESI-MS/MS was carried out in the Analytical Biochemistry Shared Resource of the Cancer Center, supported in part by the National Cancer Institute grant CA-77598.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Irina Stepanov, The Cancer Center, University of Minnesota, Mayo Mail Code 806, 420 Delaware Street Southeast, Minneapolis, MN 55455. Phone: 612-624-4998; Fax: 612-626-5135. E-mail: stepa011@umn.edu

doi:10.1158/1055-9965.EPI-07-2711

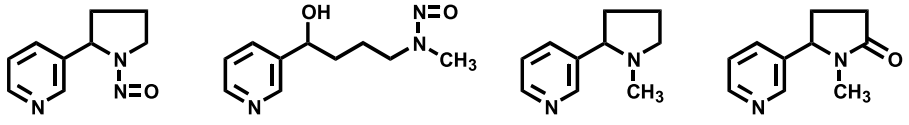

Figure 1. Structures of biomarkers discussed in the text.

Materials and Methods

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

Chemicals. NNN was purchased from Toronto Research Chemicals. [$^{13}C_6$]NNN was purchased from Cambridge Isotope Laboratories. Nicotine, [CD_3]nicotine, cotinine, and [CD_3]cotinine were obtained from Sigma.

Subjects. Toenail samples from 17 active smokers were available from our previous study (8). The smokers were originally recruited from several smoking studies conducted at the Transdisciplinary Tobacco Use Research Center as described previously (8). The entrance criteria of these studies required subjects to smoke at least 10 cigarettes per day for at least 1 year. All studies were approved by the University of Minnesota Research Subjects' Protection Programs Institutional Review Board Human Subjects Committee.

Analyses. Before digestion, toenail clippings (40-100 mg) were weighed into 5 mL polypropylene tubes and washed in CH_2Cl_2 and dried as described previously (8).

Total NNN Analysis. Toenails were digested at 50°C overnight in 2 mL 1 N NaOH. The next day, the pH of the digests was adjusted to 6 to 8 by adding 1 N HCl. [$^{13}C_6$]NNN (10 pg) was added as internal standard. The mixture was applied to a 5 mL ChemElut cartridge (Varian) and eluted with 2 × 8 mL CH_2Cl_2 into a 15 mL glass centrifuge tube, and the combined eluants were concentrated to dryness. The dry residue was redissolved in 1 mL H_2O and adjusted to pH 2 to 3 by adding 100 μL 1 N HCl, and the mixture was applied to a 60 mg Oasis MCX cartridge (Waters) activated with 5 mL CH_3OH and equilibrated with 10 mL H_2O. The cartridges were washed with 5 mL 1 N HCl, 5 mL CH_3OH, and 5 mL $H_2O/CH_3OH/NH_4OH$ (90:5:5) and these washings were discarded. Then, NNN and [$^{13}C_6$]NNN were eluted from the Oasis MCX cartridges with 5 mL $H_2O/CH_3OH/NH_4OH$ (45:50:5) and the eluant was concentrated to dryness. Residues were dissolved in 0.5 mL CH_2Cl_2 and loaded on 100 mg Bond-Elut Silica cartridges (Varian) preequilibrated with 1 mL CH_2Cl_2. The cartridges were washed with 1 mL CH_2Cl_2 and 1 mL CH_2Cl_2/ethyl acetate (50:50). NNN and [$^{13}C_6$]NNN were eluted with 2 mL ethyl acetate and the eluants were concentrated to dryness. The dry residues were transferred to autosampler vials with CH_3OH, dried, and stored at −20°C until analysis by LC-ESI-MS/MS. Before analysis, samples were redissolved in 10 μL of 2% CH_3OH in H_2O, and 5 μL were injected.

LC-ESI-MS/MS was carried out with a Finnigan TSQ Quantum Discovery Max instrument (Thermo Electron) interfaced with an Agilent Model 1100 capillary high-performance liquid chromatography system and a Model 1100 micro autosampler (Agilent). The high-performance liquid chromatograph was fitted with a 150 × 0.5 mm ZORBAX SB C18 RR 3.5 μm column (Agilent) eluted isocratically with 35% methanol in H_2O for 20 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 178 → m/z 148 ($[M + H]^+ \rightarrow [M + H - NO]^+$) for NNN and m/z 184 → m/z 154 for [$^{13}C_6$]NNN 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.

Nicotine, Cotinine, and NNAL Analysis. Nicotine, cotinine, and NNAL in toenail clippings were analyzed as described previously (8).

Results

Method Development. The accuracy of the assay was determined by spiking toenail samples from two nonsmokers with 2, 5, and 10 pg NNN. Mean recovered NNN in these samples was 2.5, 5.8, and 12.3 pg, respectively ($r = 0.99$). Analysis of NNN in four aliquots of a smoker's digested toenail sample produced a coefficient of variation of 2.7%. A typical chromatogram of a toenail sample from a smoker is illustrated in Fig. 2B. The detection limit of the method is 0.02 pg/mg toenail in a 50 mg sample or 5 fmol/sample.

Analysis of Smokers' Toenails. Toenails of 17 smokers from our previous study (8) were analyzed for total NNN. The results of these analyses as well as NNAL data for these samples from our previous study are summarized in Table 1. Mean total NNN level in these samples was 4.63 ± 6.48 (SD) fmol/mg toenail and correlated with the previously reported total NNAL ($r = 0.96$; $P < 0.0001$), total nicotine ($r = 0.48$; $P < 0.05$), and total cotinine ($r = 0.87$; $P < 0.0001$; ref. 8).

Analysis of Nonsmokers' Toenails. NNN levels found in nonsmokers' toenails were significantly lower than those in smokers ($P = 0.03$) and averaged 0.35 ± 0.16 fmol/mg. Also, toenails of all nonsmokers analyzed here contained nicotine and cotinine, averaging 0.76 ± 0.22 and 0.044 ± 0.020 pmol/mg toenail, respectively. Toenail NNN positively correlated with cotinine in these subjects ($r = 0.58$; $P = 0.05$) but not nicotine ($r = 0.34$; $P = 0.29$).

Discussion

In this study, we report the presence of a tobacco-specific carcinogen, NNN, in human toenails and establish a LC-ESI-MS/MS method for its quantitation. Two recent previous studies conducted by our research group led to the discovery of two new biomarkers of human tobacco carcinogen exposure, that is, urinary NNN (11)

and toenail NNAL (8). These findings, along with the availability of highly effective sample preparation techniques and highly sensitive and selective LC-ESI-MS/MS instrumentation, encouraged us to investigate the possibility of analyzing NNN in human toenails.

The method described here was developed based on our reported procedure for NNAL analysis in toenails (8, 9) and on a modified procedure for NNN analysis in urine (16). In addition to extraction of the digested and neutralized toenail sample on ChemElut and MCX cartridges, as reported for the toenail NNAL assay (8), we introduced an additional step (normal phase extraction) in the sample preparation procedure to produce cleaner samples and thus increase our chance of detecting and quantifying NNN. The method is characterized by a low detection limit for NNN and excellent accuracy and precision. Similar to the NNAL analysis in toenails, a disadvantage of the proposed method is that we are not able to distinguish between free NNN and NNN-*N*-Gluc. Because the analysis involves digestion in 1 N NaOH at the initial step, any NNN-*N*-Gluc, if present in human toenails, would be converted to its aglycone; thus, total NNN is measured. However, previous experiments with β-glucuronidase indicated that NNAL-*O*-Gluc was not present in human toenails (8). Moreover, for the purpose of general evaluation of human NNN uptake, total NNN would be sufficient.

Analysis of samples collected from smokers showed that amounts of NNN in smokers' toenails are generally higher than those of NNAL (Table 1). The ratio of toenail NNN to NNAL averaged 2.8, whereas the ratio between the levels of these biomarkers in smokers' urine was 0.1 (11). This could be due to differences in NNN and NNAL pharmacokinetics in humans. The biological half-life of NNAL in laboratory animals is generally slightly longer than that of NNN (17-21); however, there are no published data on NNN pharmacokinetics in humans. Differences in the chemical structure and polarity of the NNN and NNAL molecules, with NNAL being the more polar, should be considered. Despite the large number of

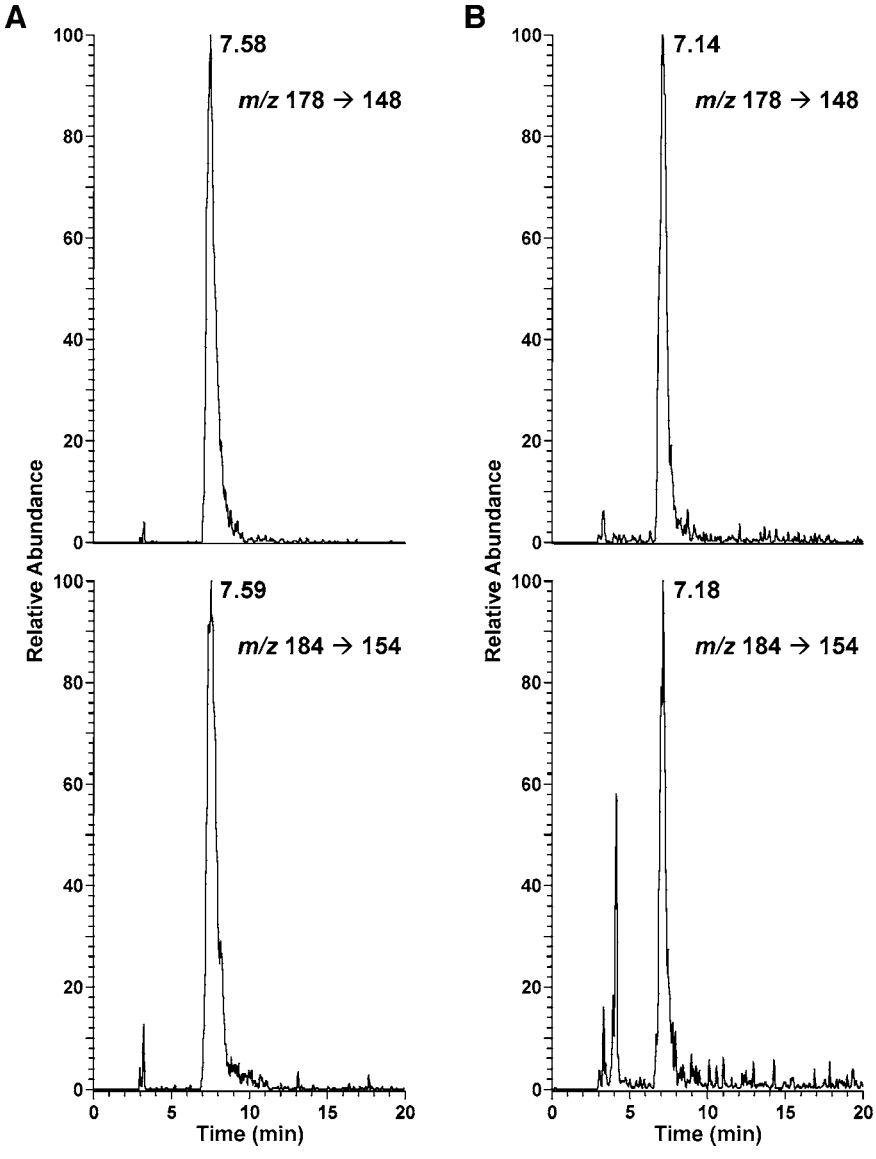

Figure 2. Chromatograms obtained on LC-ESI-MS/MS analysis of total NNN in toenails from a smoker. Transitions *m/z* 178 → *m/z* 148 (NNN) and *m/z* 184 → *m/z* 154 ([$^{13}C_6$]NNN, internal standard) are shown for (**A**) standard mix and (**B**) a smoker's toenail sample.

Table 1. Total NNN and total NNAL in smokers' toenails

Subject	fmol/mg toenail		NNN/NNAL ratio
	NNN	NNAL*	
1	26.9	17.8	1.5
2	2.70	2.01	1.3
3	3.51	0.88	4.0
4	5.60	2.81	2.0
5	2.68	1.10	2.4
6	0.60	1.13	0.5
7	6.97	5.28	1.3
8	2.57	1.41	1.8
9	0.36	0.45	0.8
10	0.10	0.50	0.2
11	2.20	1.30	1.7
12	9.83	4.18	2.4
13	3.70	0.18	21
14	0.00	0.51	0.0
15	8.90	2.21	4.0
16	0.68	1.38	0.5
17	1.36	0.64	2.1
Mean (SD)	4.63 (6.48)	2.58 (4.16)	2.8 (4.7)

*Molar amounts of total NNAL in toenails of these smokers were calculated from the data obtained in our previous study (8).

studies on drug incorporation into nails (reviewed in ref. 10), the mechanism of this process has not been established yet, and it is unclear how the physicochemical characteristics of different compounds affect their interaction with the nail matrix. Studies involving the antifungal drug fluconazole, for instance, have shown that low oral doses of this drug produce high nail concentrations compared with other antifungal preparations probably due to the hydrophilicity of fluconazole, which is responsible for its high bioavailability and its high plasma concentrations (22). On the other hand, nail concentrations of cocaine generally exceed those of its metabolites, suggesting that lipophilicity is an important factor that influences xenobiotic affinity for keratin (23).

The strong positive correlation of toenail NNN with toenail NNAL and cotinine observed in smokers is similar to that reported for urinary biomarkers (11). Moreover, in our previous study, we showed that toenail NNAL and cotinine positively correlated with the levels of these biomarkers in plasma and urine (9). These considerations indicate that toenail NNN promises to be a useful biomarker of human chronic exposure to NNN. In fact, our long-term goal is to use toenail biomarkers in epidemiologic studies of the role of secondhand smoke exposure in human cancer. In this study, all nonsmoker toenails had low but detectable levels of total NNN as well as nicotine and cotinine. The presence of nicotine and cotinine in toenails of nonsmokers who report no exposure to secondhand smoke was shown in other studies (8, 24). Further investigations are necessary to evaluate the applicability of toenail NNN as a biomarker of tobacco carcinogen exposure in nonsmokers.

In summary, we have developed an assay for total NNN in human toenails. This new biomarker may be useful in epidemiologic studies of the role of smoking in esophageal cancer. Investigation of the role of secondhand smoke exposure in human cancer is another potential application. Further studies will be focused on the development of a method that will allow simultaneous determination of total NNAL and total NNN in human toenails.

Acknowledgments

We thank Bradley Lieberman for outstanding technical assistance, Dr. Peter W. Villalta for help with mass spectrometry techniques, and Bob Carlson for editorial assistance.

References

1. Hecht SS. Tobacco carcinogens, their biomarkers, and tobacco-induced cancer. Nat Rev Cancer 2003;3:733–44.
2. Hecht SS. Human urinary carcinogen metabolites: biomarkers for investigating tobacco and cancer. Carcinogenesis 2002;23:907–22.
3. Carmella SG, Akerkar S, Richie JP, Jr., et al. Intraindividual and interindividual differences in metabolites of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in smokers' urine. Cancer Epidemiol Biomarkers Prev 1995;4:635–42.
4. Anderson KE, Carmella SG, Ye M, et al. Metabolites of a tobacco-specific lung carcinogen in nonsmoking women exposed to environmental tobacco smoke in their homes. J Natl Cancer Inst 2001;93:378–81.
5. Hecht SS, Ye M, Carmella SG, et al. Metabolites of a tobacco-specific lung carcinogen in the urine of elementary school-aged children. Cancer Epidemiol Biomarkers Prev 2001;10:1109–16.
6. Hecht SS, Carmella SG, Chen M, et al. Quantitation of urinary metabolites of a tobacco-specific lung carcinogen after smoking cessation. Cancer Res 1999;59:590–6.
7. Hecht SS, Carmella SG, Ye M, et al. Quantitation of metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone after cessation of smokeless tobacco use. Cancer Res 2002;62:129–34.
8. Stepanov I, Feuer R, Jensen J, et al. Mass spectrometric quantitation of nicotine, cotinine, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in human toenails. Cancer Epidemiol Biomarkers Prev 2006;15:2378–83.
9. Stepanov I, Hecht SS, Lindgren B, et al. Relationship of human toenail nicotine, cotinine, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol to levels of these biomarkers in plasma and urine. Cancer Epidemiol Biomarkers Prev 2007;16:1382–6.
10. Palmeri A, Pichini S, Pacifici R, et al. Drugs in nails. Physiology, pharmacokinetics and forensic toxicology. Clin Pharmacokinet 2000; 38:95–110.
11. Stepanov I, Hecht SS. Tobacco-specific nitrosamines and their pyridine-*N*-glucuronides in the urine of smokers and smokeless tobacco users. Cancer Epidemiol Biomarkers Prev 2005;14:885–91.
12. Hecht SS, Hoffmann D. Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. Carcinogenesis 1988;9:875–84.
13. Hecht SS. Biochemistry, biology, and carcinogenicity of tobacco-specific *N*-nitrosamines. Chem Res Toxicol 1998;11:559–603.
14. Spiegelhalder B, Bartsch H. Tobacco-specific nitrosamines. Eur J Cancer Prev 1996;5:33–8.
15. Hecht SS, Hoffmann D. The relevance of tobacco-specific nitrosamines to human cancer. Cancer Surv 1989;8:273–94.
16. Porubin D, Hecht SS, Li Z, et al. Endogenous formation of *N'*-nitrosonornicotine in F344 rats in the presence of some antioxidants and grape seed extract. J Agric Food Chem 2007;55:7199–204.
17. Adams JD, LaVoie EJ, O'Donnell M, et al. Pharmacokinetics of tobacco-specific *N*-nitrosamines. IARC Sci Publ 1984;57:779–85.
18. Adams JD, LaVoie EJ, Hoffmann D. On the pharmacokinetics of tobacco-specific *N*-nitrosamines in Fischer rats. Carcinogenesis 1985; 6:509–11.
19. Adams JD, LaVoie EJ, O'Mara-Adams KJ, et al. A study of tobacco carcinogenesis XXIX. Pharmacokinetics of *N'*-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in laboratory animals. Cancer Let 1985;28:195–201.
20. Upadhyaya P, Zimmerman CL, Hecht SS. Metabolism and pharmacokinetics of *N'*-nitrosonornicotine in the patas monkey. Drug Metab Dispos 2002;30:1115–22.
21. Hecht SS, Trushin N, Reid-Quinn CA, et al. Metabolism of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in the patas monkey: pharmacokinetics and characterization of glucuronide metabolites. Carcinogenesis 1993;14:229–36.
22. Faergemann J, Laufen H. Levels of fluconazole in normal and diseased nails during and after treatment of onychomycoses in toenails with fluconazole 150mg once weekly. Acta Derm Venereol 1996; 76:219–21.
23. Garside D, Ropero-Miller JD, Goldberger BA, et al. Identification of cocaine analytes in fingernail and toenail specimens. J Forensic Sci 1998;43:974–9.
24. Al-Delaimy WK, Mahoney GN, Speizer FE, et al. Toenail nicotine levels as biomarker of tobacco smoke exposure. Cancer Epidemiol Biomarkers Prev 2002;11:1400–4.