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Combined analysis of *N*′-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in the urine of cigarette smokers and e-cigarette users

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ABSTRACT

A liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC–ESI$^+$–MS/MS) method for the analysis of the tobacco-specific carcinogens *N*′-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and their glucuronides (total NNN and total NNAL) in human urine was developed. The method has excellent accuracy and intra-day and inter-day precision, and limits of quantitation of 0.015 and 0.075 pmol/mL urine, respectively, for total NNN and total NNAL. A unique aspect of this method is internal assessment of possible artifactual formation of NNN by inclusion of the monitor amine [pyridine-D_4]nornicotine. We found that artifactual formation of NNN comprised only 2.5% of the measured amounts of total NNN in urine of cigarette smokers, under our conditions using ammonium sulfamate as an inhibitor of nitrosation. The method was applied to urine samples from cigarette smokers and e-cigarette users. Levels of total NNN and total NNAL in the urine of cigarette smokers averaged 0.060 ± 0.035 pmol/mL and 2.41 ± 1.41 pmol/mL urine, (N = 38), respectively, which were both significantly greater than in the urine of 27 e-cigarette users.

Introduction

The tobacco-specific nitrosamines *N*′-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Fig.1) are present in all tobacco products and are considered "carcinogenic to humans" by the International Agency for Research on Cancer [1,2]. NNN and NNK are formed during the processing and curing of tobacco. During cigarette smoking, NNN and NNK are transferred through the smoke to both the oral tissues and lungs of smokers [3]. Each cigarette typically delivers about 100–150 ng NNN and 50–100 ng NNK to the smoker [4]. NNN causes oral cavity and esophageal cancer in rats, and tumors of the respiratory tract in mice, hamsters, and mink. NNK is a powerful organoselective lung carcinogen in rats, mice, and hamsters while also inducing tumors at other sites including the pancreas and nasal mucosa [5–7]. Because of their potent carcinogenicity and tobacco-specificity, NNN and NNK are widely acknowledged as important causes of cancer in tobacco users.

Human exposure to NNN and NNK can be assessed by analysis of urine. Unchanged NNN as well as its pyridine-*N*-glucuronide are excreted in the urine [8–12]. Unchanged NNK is not generally detected in human urine. Rather, its metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL, Fig. 1) and its *O*- and *N*-glucuronides are present in the urine of all smokers [3,13,14]. Total NNN and total NNAL (the sum of the free compounds and their glucuronide metabolites) are useful biomarkers of NNN and NNK exposure. Total NNAL in particular has been quantified in thousands of urine samples from smokers [3]. Total NNN and total NNAL are also risk biomarkers; levels of urinary total NNN have been strongly related to the risk of esophageal cancer and total NNAL to the risk of lung cancer in nested case-control studies carried out within a prospective epidemiology study in Shanghai [15–17].

Accurate assessment of total NNN and total NNAL is critical in cancer prevention strategies related to tobacco products. A reliable combined assay for quantifying these metabolites in human urine would therefore be an important addition to a panel of carcinogen exposure and cancer risk biomarkers. Our group was the

Abbreviations: NNN, *N*′-nitrosonornicotine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; SLE, supported liquid extraction; SPE, solid-phase extraction.

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first to describe an assay for free NNN and its glucuronide, as well as free and glucuronidated *N*′-nitrosoanabasine (NAB) and *N*′-nitrosoanatabine (NAT) in human urine [8]. The levels of total NNN were compared to those of total NNAL, determined separately. Two research groups have subsequently described combined assays for total NNAL, total NNN, total NAB, and total NAT in human urine [10,11,18]. A method for total NNN has also been briefly summarized [19].

The two studies by Kavvadias et al. mention the problem of potential artifactual formation of NNN, but this was not addressed in the study by Xia et al. We are aware from years of experience in the trace analysis of NNN that artifact formation can present problems because all tobacco products contain nornicotine, which is readily transferred to the saliva and urine of smokers and can be easily nitrosated to form NNN [20]. We have addressed and excluded this problem in our previous studies [8,12,16,21–24]. However, one must be continually aware of the potential for artifactual formation of NNN, particularly when low levels such as those expected in the urine of e-cigarette users are being analyzed. Therefore, in the study described here for analysis of total NNN and total NNAL in urine, we carefully monitored for artifactual formation of NNN by addition of [pyridine-D_4]nornicotine to each urine sample. We have applied our method to the analysis of total NNN and total NNAL in the urine of cigarette smokers and users of e-cigarettes. The use of e-cigarettes has increased dramatically while they remain completely unregulated and little information is available regarding their toxicological effects [25–27]. It is possible that NNN could be formed endogenously in e-cigarette users by the reaction of nornicotine, a metabolite and common contaminant of nicotine, with salivary nitrite.

2. Materials and methods

2.1. Materials

We purchased [pyridine-D_4]NNN and [$^{13}C_6$]NNN from Cambridge Isotope Laboratories (Andover, MA), while [pyridine-D_4]nornicotine, NNN, NNAL, and [$^{13}C_6$]NNAL (Fig. 1) were obtained from Toronto Research Chemicals (Ontario, Canada). Recombinant β-glucuronidase (catalog # G8295) was purchased from Sigma–Aldrich (Milwaukee, WI). Phosphate buffered saline was procured from Invitrogen (Grand Island, NY). All other chemicals were from Sigma–Aldrich, Fisher Scientific (Fairlawn, NJ), or Alfa Aesar (WardHill, MA). True Taper® 96-well plates were procured from Analytical Sales & Services (Pompton Plains, NJ) and silicone cap mats required to cover the 96-well plates were from Phenomenex (Torrance, CA). Five ml Biotage Isolute Supported Liquid Extraction+ (SLE+) diatomaceous earth-based liquid–liquid extraction cartridges were obtained from Biotage (Charlotte, NC) and Oasis MCX 60 mg, 60 μm solid-phase extraction 96-well plates were from Waters (Milford, MA). Strata SI-1 Silica (55 μm, 70 A, 100 mg) 96-well SPE plates were purchased from Phenomenex (Torrance, CA). A Cerex 96-well positive pressure processor (Chromtech, Apple Valley, MN) and an Eppendorf multi-channel pipette were used during sample processing.

2.2. Urine samples

The urine samples used in this work were obtained from ongoing studies of the University of Minnesota Tobacco Research Programs, approved by the University of Minnesota Institutional Review Board. The validated method was applied for analysis of total NNN and total NNAL in urine samples from 27 e-cigarette users and 38 cigarette smokers. The urine samples from the e-cigarette users have been previously analyzed and reported by our group [27]. One sample with a relatively high NNAL level of 0.953 pmol/mL was excluded from this secondary analysis. The urine samples from cigarette smokers were baseline samples from clinical studies on tobacco harm reduction.

2.3. Combined analysis of total NNN and total NNAL in urine

The urine samples, which had been kept frozen at −20 °C, were thawed by allowing them to stand at 4 °C overnight the day before the experiment. Three milliliter aliquots of urine or H_2O blanks were added to 10 mL disposable glass centrifuge tubes with screw caps (Fisher Scientific). Milli-pure water (Milli-Q Advantage A10 Ultrapure water purification system, EMD Millipore, Billerica, MA) was used for preparation of all solutions. [$^{13}C_6$]NNN (1.09 pmol) and [$^{13}C_6$]NNAL (0.93 pmol) in 50 μL 1X phosphate buffered saline (1.05 mM KH_2PO_4, 155.2 mM NaCl, 2.96 mM Na_2HPO_4, pH 7.4) were added to each of the tubes containing urine along with 50 μL of 0.1 mg/μL ammonium sulfamate, 16.9 nmol [pyridine-D_4]nornicotine, 400 μL of 10X phosphate buffer and 8000 units of β-glucuronidase in 80 μL of 1X phosphate buffered saline, pH 7.4. The tubes were incubated in a shaking water bath at 37 °C overnight. The amount of β-glucuronidase used was previously shown to be sufficient for complete hydrolysis [11].

The mixtures in the tubes were transferred onto 5 mL Biotage Isolute SLE+ cartridges. The aqueous solutions were applied, pushed past the hydrophobic frits with slight N_2 pressure, and allowed to absorb into the diatomaceous earth for 10 min. Each cartridge was eluted 3 times with 6 mL CH_2Cl_2 and once with 2 mL CH_2Cl_2 and all the eluents were collected through gravity in 15 mL glass tubes. The combined eluents in each tube were mixed with 50 μL of 0.1 mg/μL ammonium sulfamate, then dried under vacuum in a SpeedVac® at room temperature for 1.5–2 h.

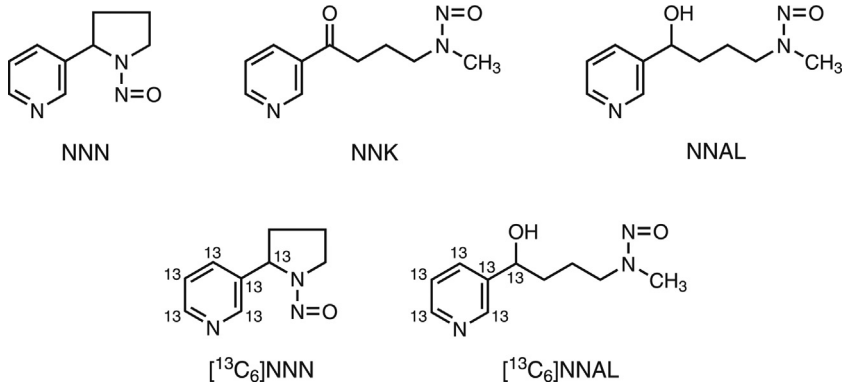

Fig. 1. Structures of NNN, NNK, NNAL, [$^{13}C_6$]NNN, and [$^{13}C_6$]NNAL.