Original investigation

Quantitative Analysis of 3'-Hydroxynorcotinine in Human Urine

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Abstract

Introduction: Based on previous metabolism studies carried out in patas monkeys, we hypothesized that urinary 3'-hydroxynorcotinine could be a specific biomarker for uptake and metabolism of the carcinogen *N*'-nitrosornicotine in people who use tobacco products.

Methods: We developed a method for quantitation of 3'-hydroxynorcotinine in human urine. [Pyrrolidinone-¹³C₄]3'-hydroxynorcotinine was added to urine as an internal standard, the samples were treated with β-glucuronidase, partially purified by solid supported liquid extraction and quantified by liquid chromatography–electrospray ionization–tandem mass spectrometry.

Results: The method was accurate (average accuracy = 102%) and precise (coefficient of variation = 5.6%) in the range of measurement. 3'-Hydroxynorcotinine was detected in 48 urine samples from smokers (mean 393 ± 287 pmol/ml urine) and 12 samples from individuals who had stopped smoking and were using the nicotine patch (mean 658 ± 491 pmol/ml urine), but not in any of 10 samples from nonsmokers.

Conclusions: Since the amounts of 3'-hydroxynorcotinine found in smokers' urine were approximately 50 times greater than the anticipated daily dose of *N*'-nitrosornicotine, we concluded that it is a metabolite of nicotine or one of its metabolites, comprising perhaps 1% of nicotine intake in smokers. Therefore, it would not be suitable as a specific biomarker for uptake and metabolism of *N*'-nitrosornicotine. Since 3'-hydroxynorcotinine has never been previously reported as a constituent of human urine, further studies are required to determine its source and mode of formation.

Introduction

The purpose of this study was to develop a specific biomarker reflecting human uptake and metabolism of the tobacco-specific carcinogen *N*'-nitrosornicotine (NNN, [Figure 1](#) for structures). NNN induces tumors of the oral cavity and esophagus in rats, and of the respiratory tract in mice, hamsters, and mink.^{1,2} NNN, together with the related tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), is considered "carcinogenic to humans" by the International Agency for Research on Cancer.³ Virtually all tobacco products contain NNN, which generally occurs in microgram per gram quantities in cured tobacco and is transferred to mainstream smoke.^{3–6} The constant chronic exposure of tobacco

users to NNN represents a potentially significant risk factor for cancer development. However, quantitative aspects of variables such as dose, which could influence individual susceptibility to the carcinogenic effects of NNN are still unclear, and we cannot predict which tobacco user is at highest risk for cancer. A predictive algorithm for cancer risk could be important in prevention because those individuals at high risk might be targeted for intensive tobacco cessation interventions and increased cancer surveillance.

Urine is one of the most easily obtained and frequently stored biological fluids for biomarker studies. Presently, the only available urinary biomarkers for human uptake of NNN are NNN itself and a metabolite, NNN-*N*-glucuronide.^{7–12} Urinary NNN and NNN-*N*-glucuronide have been used in several studies of smokers and smokeless tobacco

Figure 1. Structures of some compounds discussed in the text. NNN, *N*-nitrosornicotine; hydroxy acid, 4-(3-pyridyl)-4-hydroxybutyric acid; keto acid, 4-(3-pyridyl)-4-oxobutyric acid; 3'-OH-norcot, 3'-hydroxynornicotine; POBAM, 4-(3-pyridyl)-4-oxobutyramide.

users, demonstrating elevated exposure compared to nonusers of tobacco products. In one study, levels of these biomarkers in prospectively collected urine samples were strongly related to the occurrence of esophageal cancer.¹⁰ Other investigations have demonstrated occasional formation of NNN in users of oral nicotine replacement therapy products, by analysis of NNN and NNN-*N*-glucuronide in urine.^{8,13} A significant limitation of these biomarkers is that they comprise only a small percentage of the NNN dose. Rats treated with NNN excrete the unchanged compound in urine to the extent of only 3%–5%, and the excretion of NNN in the urine of smokers has been estimated as only about 1% of the dose.^{12,14} We believe that a better understanding of NNN uptake could be achieved by quantifying urinary metabolites that comprise a greater percentage of the NNN dose.

Urinary metabolites of NNN have been extensively characterized in laboratory animal models, including a primate model, the patas monkey.^{1,15} Cytochrome P450-catalyzed α -hydroxylation at the 2'- and 5'-carbons of NNN leads to the α -hydroxyNNN metabolites 2'-hydroxyNNN and 5'-hydroxyNNN (Figure 1) which are unstable and decompose to intermediates that alkylate DNA initiating the carcinogenic process. Ultimately, these α -hydroxylation pathways produce the metabolites hydroxy acid and keto acid. The 2'-hydroxylation pathway in particular has been extensively characterized in rats where it leads to the formation of DNA adducts that are believed to cause tumors.^{1,16–18} But hydroxy acid and keto acid are not suitable biomarkers for NNN exposure and metabolism in people who use tobacco products because they are also minor metabolites of nicotine, which is present in tobacco products in quantities typically 10,000 times greater than those of NNN.¹⁹ Thus, quantitation of these metabolites in smokers or smokeless tobacco users cannot provide reliable information on NNN dose. In contrast, another pathway of NNN metabolism in the patas monkey, but not reported to date in rodent models, resulted in the formation of 3'-hydroxynorcotinine (3'-OH-norcot, Figure 1) and its glucuronide accounting for approximately 22% of the urinary metabolites, while NNN itself comprised less than 1%. Based on the results of the patas monkey metabolism studies, we chose 3'-OH-norcot for further study as a potential biomarker of NNN dose. It was possible of course that 3'-OH-norcot

could be a minor metabolite of nicotine, cotinine, norcotine, or norcotinine, all of which are well established constituents of the smoker's metabolome. We are not aware of any published studies that have identified or quantified 3'-OH-norcot in human urine. Therefore, in the study reported here, we have developed an analytical method for quantitation of 3'-OH-norcot in human urine. The method was applied for the analysis of urine samples from smokers, nonsmokers, and individuals using nicotine replacement therapy.

Materials and Methods

Study Design

This study was approved by the University of Minnesota Institutional Review Board. Subjects were recruited by a member of the research staff of the University of Minnesota Tobacco Research Programs, as previously described,²⁰ and spot urine samples were collected. Inclusion criteria were as follows: current smoker of at least 10 cigarettes per day for the past year, or nonsmoker in good physical and mental health with no unstable medical condition as determined by medical history and investigator assessment. Smoking status was confirmed by determination of exhaled carbon monoxide.²¹ For nicotine patch users, the subjects were participants in a previous study examining potential ethnic differences in nicotine metabolism. The subjects were smokers dependent on nicotine who stopped smoking and used 21 mg nicotine patches for 3 days prior to urine collection.

Chemicals and Enzymes

3'-OH-norcot and [pyrrolidinone-¹³C₄]3'-OH-norcot were obtained from Toronto Research Chemicals and β -glucuronidase (*Escherichia coli* G8295) from Sigma-Aldrich.

Urine Sample Preparation

Six thousand units of β -glucuronidase in 0.1 ml of phosphate buffered saline (0.26%, w/v) and 16.4 nmol [pyrrolidinone-¹³C₄]3'-OH-norcot internal standard were added to 3 ml of urine. Following a 20 hr incubation at 37 °C, each sample was applied to a solid supported

liquid extraction Chem-Elute cartridge (5 ml, unbuffered; Agilent Technologies). The cartridge was eluted with three 8 ml aliquots of CH_2Cl_2 , and the pooled eluents were collected in a 25 ml disposable glass centrifuge tube. The CH_2Cl_2 extracts were evaporated to dryness using a Savant SpeedVac evaporator (Thermo Fisher Scientific), reconstituted to 60 μl with 15 mM ammonium acetate, transferred to 200 μl insert vials, and 8 μl were analyzed by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-MS/MS).

LC-MS/MS Analysis

Analyses were performed on a TSQ Quantum Discovery Max instrument (Thermo Fisher Scientific) in the positive ion mode with N_2 as the nebulizing and drying gas. MS parameters were as follows: spray voltage 2.8 kV; sheath gas 40; capillary temperature 230 $^\circ\text{C}$; collision energy 19 V for m/z 180 and 34 V for m/z 80; scan width 0.2 amu; Q2 gas pressure 1.0 Torr; source CID 8 V; tube lens offset 100 V. MS data were acquired and processed by Xcalibur software version 1.4 (Thermo Fisher Scientific). Eight microliters of the sample were injected from an autosampler using an Agilent 1100 capillary LC system equipped with a 5 μm , 150 mm $\times$ 0.5 mm Zorbax SB-C18 column (Agilent Technologies). The flow rate was 15 $\mu\text{l}/\text{min}$ with a gradient from 2% CH_3CN in 15 mM NH_4OAc to 8% CH_3CN in 15 min, then to 20% CH_3CN in 5 min. The mass transitions monitored were m/z 179.09 $\rightarrow$ m/z 135.05 and m/z 179.09 $\rightarrow$ m/z 80.08 for 3'-OH-norcot, (typical retention time 6.9 min) and m/z 183.09 $\rightarrow$ m/z 137.05 and m/z 183.09 $\rightarrow$ m/z 80.08 for [pyrrolidinone- $^{13}\text{C}_4$]3'-OH-norcot. A calibration curve was prepared

by plotting MS peak area ratios against concentration ratios using standard mixes containing constant levels of [pyrrolidinone- $^{13}\text{C}_4$]3'-OH-norcot and varying levels of 3'-OH-norcot. The peak area ratios were determined by LC-MS/MS analysis of each sample and quantitation was accomplished using the calibration curve and the known amount of [pyrrolidinone- $^{13}\text{C}_4$]3'-OH-norcot added to each sample.

Results

The internal standard for this analysis, [pyrrolidinone- $^{13}\text{C}_4$]3'-OH-norcot, was added to each urine sample. The urine samples were treated with β -glucuronidase, subjected to extraction on diatomaceous earth columns, and analyzed directly by LC-MS/MS using the analyte transitions m/z 179.09 $\rightarrow$ m/z 80.08 and m/z 179.09 $\rightarrow$ m/z 135.05 and the internal standard transitions m/z 183.09 $\rightarrow$ m/z 80.08 and m/z 183.09 $\rightarrow$ m/z 137.05. The transition m/z 179.09 $\rightarrow$ m/z 80.08 corresponds to a pyridinium ion while m/z 179.09 $\rightarrow$ m/z 135.05 results from loss of CH_2CHOH from the pyrrolidinone ring of 3'-OH-norcot. Quantitation was based on the m/z 179.09 $\rightarrow$ m/z 135.05 and m/z 183.09 $\rightarrow$ m/z 137.05 transitions, while the m/z 179.09 $\rightarrow$ m/z 80.08 transition was used as a qualifier. A typical LC-MS/MS chromatogram obtained upon analysis of a smokers' urine is illustrated in Figure 2. The *cis*- and *trans*- isomers of 3'-OH-norcot and the internal standard are clearly evident, with the *cis*-isomer comprising an average of about 8% of total 3'-OH-norcot. We did not detect 3'-OH-norcot upon analysis of 10 urine samples from nonsmokers who were not using nicotine replacement therapy.

Figure 2. Liquid chromatography–electrospray ionization–tandem mass spectrometry chromatograms obtained upon analysis of 3'-hydroxynorcotinine in a smokers' urine sample. Sample flow was diverted to waste for the first 5 min of each chromatogram. A, trace used for quantitation (m/z 179.09 $\rightarrow$ m/z 135.05); B, qualifying trace (m/z 179.09 $\rightarrow$ m/z 80.08); C and D, internal standard [pyrrolidinone- $^{13}\text{C}_4$]3'-hydroxynorcotinine traces (m/z 183.09 $\rightarrow$ m/z 137.05 and m/z 183.09 $\rightarrow$ m/z 80.08). The small shaded peaks are *cis*-3'-hydroxynorcotinine and the large shaded peaks are *trans*-3'-hydroxynorcotinine.

The response of the MS/MS system was linear in the range 40–1,400 pmol/ml. Accuracy was determined by adding varying amounts of 3'-OH-norcot and the internal standard to duplicate 3 ml aliquots of a nonsmokers' urine and carrying out the analysis. The results which are summarized in Table 1 demonstrate that accuracy averaged 102% (range, 100%–109%) in the concentration range of 70–2,400 pmol/ml. Precision was determined by four replicate analyses of a nonsmokers' urine sample to which 3'-OH-norcot had been added to achieve a concentration of 596 pmol/ml. Analysis produced a value of 594 ± 34 pmol/ml (coefficient of variation = 5.6%). The assay limit of quantitation was 120 fmol/ml urine and the limit of detection was 30 fmol/ml urine. Recovery of 3'-OH-norcot was approximately 20%.

We analyzed urine samples from 48 smokers, 31 of whom were male (Table 2). Mean ages were 44.7 ± 9.17 (SD) years for males and 39.7 ± 11.8 years for females. The males smoked an average of 24.4 ± 6.0 cigarettes per day and the females 22.4 ± 7.6 . Levels of 3'-OH-norcot ranged from 32 to 1,158 pmol/ml urine, with a mean of 393 ± 287 (SD) pmol/ml urine and a median of 353 pmol/ml. There was no correlation between cigarettes per day and 3'-OH-norcot. We also analyzed 3'-OH-norcot in urine samples from 12 former smokers who were using the 21 mg nicotine patch (Table 3). It was detected and quantified in all samples, ranging from 199 to 1,687 pmol/ml urine with a mean of 658 ± 491 (SD) pmol/ml urine and a median of 432 pmol/ml.

Discussion

We developed an LC-MS/MS method for quantitation of 3'-OH-norcot in human urine using [pyrrolidinone- $^{13}\text{C}_4$]3'-OH-norcot as an internal standard. Application of this method provided the first evidence for the presence of 3'-OH-norcot in the urine of both smokers and ex-smokers using nicotine replacement therapy, but not in nonsmokers. However, these data, while important in their own regard, do not support our original proposal that 3'-OH-norcot could be a biomarker of NNN uptake and metabolism. Considering that the amount of NNN in mainstream cigarette smoke is about 100 ng per cigarette,²² a smoker of 20 cigarettes per day would be exposed to about 2 μg , or 11.3 nmol of NNN per day. In our study, excretion of 3'-OH-norcot amounted to an average of about 600 nmol per day, based on an estimated daily excretion of 1.5 L of urine. This amount is about 50 times higher than the likely exposure to NNN, even if one considers the possibility of endogenous formation of this carcinogen from nornicotine and nitrogen oxides, which would not likely reach such levels under normal circumstances. Furthermore, we detected 3'-OH-norcot in the urine of ex-smokers using the nicotine patch, in which NNN exposure is expected to be relatively low and intermittent.¹³

The simplest explanation for our results, which is also consistent with our data from individuals using the nicotine patch, is that

3'-OH-norcot is a previously unidentified minor metabolite of nicotine or one of its metabolites. Our mean value of 393 pmol/ml urine 3'-OH-norcot (0.393 nmol/ml) in 50 smokers compares to a median value of total nicotine equivalents of 36 nmol/ml in a recent study of 437 Caucasian smokers using a median of 20 cigarettes per day.²³ Thus, we estimate that 3'-OH-norcot comprises about 1% of nicotine metabolism in smokers.

Norcotinine (Figure 1) is the most likely nicotine metabolite precursor to 3'-OH-norcot. McKennis et al.²⁴ were the first

Table 2. Levels of 3'-Hydroxynorcotinine in Smokers' Urine

ID	Sex	Age	CPD	3'-OH-norcot (pmol/ml)
1	M	51	25	528
2	M	58	30	623
3	M	43	25	353
4	M	49	30	77
5	M	51	30	322
6	F	19	30	107
7	F	53	30	464
8	M	36	35	440
9	M	51	40	32
10	M	40	35	635
11	M	41	23	1,158
12	M	38	20	142
13	F	50	30	273
14	M	50	20	539
15	M	42	20	813
16	M	31	20	423
17	F	40	20	97
18	M	50	20	819
19	F	19	20	67
20	F	58	40	416
21	M	29	20	155
22	F	46	20	443
23	M	33	20	107
24	M	36	20	400
25	F	31	20	277
26	M	40	19	174
27	M	31	20	710
28	F	41	18	467
29	F	23	10	562
30	F	36	20	183
31	F	34	18	352
32	M	47	20	602
33	F	49	20	64
34	F	38	20	360
35	M	57	20	445
36	M	41	20	659
37	M	61	20	133
38	M	48	20	146
39	M	59	20	704
40	F	45	25	658
41	M	58	30	120
42	F	53	30	169
43	M	41	23	84
44	M	31	20	1,052
45	F	40	10	223
46	M	51	25	137
47	M	51	30	115
48	M	40	35	1,056
Average				393 ± 287

Note. CPD = cigarettes per day.

Table 1. Accuracy of the Assay for 3'-Hydroxynorcotinine

3'-OH-norcot added ^a		3'-OH-norcot found ^b		Accuracy (%)
ng	pmol	ng	pmol	
40	220	43	240	109
160	890	164	920	103
320	1,790	322	1,800	100
640	3,580	640	3,580	100
1,280	7,150	1,281	7,160	100

^aAdded to 3 ml of a nonsmoker's urine.

^bMean of duplicate determinations.

to identify norcotinine as a metabolite of nicotine and cotinine. Norcotinine has been identified in various metabolic studies and in the urine of smokers.^{25,26} The McKennis group also demonstrated that norcotinine was a metabolite of nornicotine.^{26,27} Evidence exists for the formation of norcotinine from nornicotine and cotinine in humans, the latter catalyzed by cytochrome P450 2A6.^{26,28} Thus norcotinine, as a metabolite of nicotine, cotinine, and nornicotine, would be a central and potentially attractive metabolic precursor to 3'-OH-norcot. Schwartz and McKennis²⁹ examined the metabolism of norcotinine in rats and identified hydroxy acid, keto acid, and 3-pyridylacetic acid as urinary metabolites. We also observed keto acid as a urinary metabolite of norcotinine in the rat.³⁰ Neither our study in rats nor those of McKennis identified 3'-OH-norcot as a metabolite of norcotinine, but we did not specifically look for it and it is unlikely that McKennis did either. Gorrod et al. studied the in vitro metabolism of norcotinine by rat hepatic

microsomal preparations and identified 4-(3-pyridyl)-4-oxobut-ynamide (POBAM) (Figure 1) and nicotinamide as metabolites; POBAM was further metabolized to hydroxy acid.³¹ Collectively, these experiments although performed under different conditions and with varied purposes form a fairly cohesive if somewhat limited description of norcotinine metabolism. In what is apparently the only report specifically focused on formation of 3'-OH-norcot from norcotinine, we looked for this compound as a metabolite of norcotinine in patas monkey liver microsomes, but failed to detect it.¹⁵ It is also possible that 3'-OH-norcot is produced by metabolic demethylation of 3'-OH-cotinine, a major nicotine metabolite in humans. Comprehensive reviews of nicotine metabolism do not mention 3'-OH-norcot as a metabolite^{25,26} and we were unable to find any references to this compound in addition to those cited above using various internet search methods.

Another possible source of 3'-OH-norcot in urine is from tobacco or cigarette smoke, but this would not explain its presence in nicotine patch users. We were unable to find any reference to identification of 3'-OH-norcot in tobacco products.³²

While the amounts of 3'-OH-norcot in urine preclude its use as a biomarker of NNN exposure in smokers not participating in specialized studies, it does not invalidate the concept that 3'-OH-norcot is a metabolite of NNN in humans. Our proposed metabolic routes from NNN to 3'-OH-norcot in the patas monkey are summarized in Figure 3.¹⁵ Published studies of NNN metabolism catalyzed by human enzymes and human tissues or microsomal preparations focus mainly on the well-known α -hydroxylation pathways of NNN metabolism and have not specifically looked for 3'-OH-norcot as a metabolite.^{1,33} Clinical studies using specifically labelled NNN molecules, as reported for NNK,³⁴ would be another way to further investigate the extent of formation and potential utility of 3'-OH-norcot as an NNN biomarker in humans.

While the quantitation of 3'-OH-norcot as a new metabolite in the urine of people exposed to nicotine is of interest, our study did

Table 3. Levels of 3'-Hydroxynorcotinine in the Urine of Subjects Who Stopped Smoking and Were Using the 21-mg Nicotine Patch

Subject no.	Sex	Age	3'-OH-norcot (pmol/ml)
1	M	41	199
2	M	19	1,344
3	M	46	340
4	M	49	245
5	F	23	369
6	F	18	1,687
7	F	42	749
8	M	41	450
9	F	40	413
10	F	21	388
11	M	46	472
12	M	44	1,241
Average			658 $\pm$ 491

Figure 3. Proposed pathways of metabolism of NNN to 3'-hydroxynorcotinine as observed in the patas monkey. NNN, N-nitrososornicotine; 3'-OH-norcot, 3'-hydroxynorcotinine.

not successfully achieve its major goal which was identification of a new biomarker for uptake and metabolism of NNN. The characterization and quantitation of metabolites or adducts that are unique to NNN metabolism, as opposed to other related tobacco constituents, is challenging. Currently, we are investigating the possible applicability of DNA adducts resulting from 5'-hydroxylation of NNN as potential specific biomarkers.

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Declaration of Interests

None declared.

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