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Article

Assessment of the Exposure to NNN in the Plasma of Smokeless Tobacco Users

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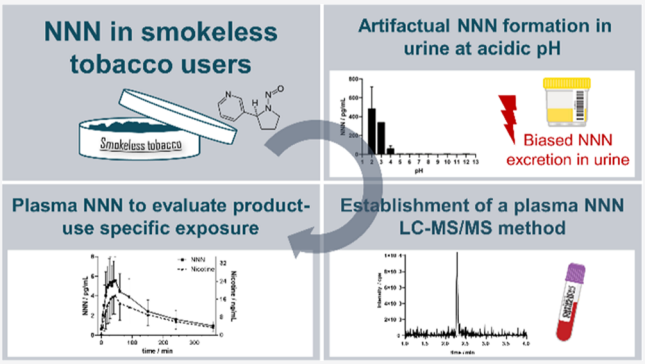
ABSTRACT: *N*-Nitrosonornicotine (NNN) is a human carcinogen present in cigarette smoke and smokeless tobacco. Urinary NNN is usually measured in order to assess the exposure to this toxicant for tobacco users. NNN excretion in urine can be highly biased due to the formation of NNN by nitrosation of nornicotine under acidic conditions, both endogenously and exogenously. Hence, urinary NNN levels may not necessarily correctly reflect the product-specific exposure. Measurement of plasma NNN may be less prone to endogenous formation due to the stable pH (7.4) of blood. We developed an LC−MS/MS method for the quantification of NNN using 1 mL of human plasma. Validation according to FDA guidelines proved that the method is selective and highly sensitive with an LLOQ of 0.3 pg/mL. Accuracy and precision averaged to 98.7 and 7.5% (CV), respectively. The assay was applied to plasma samples collected from 10 experienced moist smokeless tobacco users during and after a single use of 2 g of the product for 40 min under controlled use conditions. Blood was drawn at 15 time points over a 6 h time course. The maximum NNN concentration (C_{max}) ranged from 3.5 to 10 pg/mL (mean: 7.1 pg/mL) at a t_{max} of 32 min. Plasma NNN and nicotine were found to have similar time courses. In conclusion, the determination of NNN in plasma may be fit-for-purpose to evaluate the product-use-specific exposure to this carcinogen.



INTRODUCTION

N-Nitrosonornicotine (NNN), a class 1 carcinogen as classified by the International Agency for Research on Cancer (IARC),[1] is on US FDA's list of Hazardous and Potentially Hazardous tobacco constituents and occurs in tobacco and tobacco smoke mainly from the nitrosation of nornicotine during the fermentation and curing process.[2] The artifactual formation of NNN has been studied extensively over the past decades. Nornicotine and also myosmine may serve as precursors for NNN formation in the presence of nitrosating agents such as nitrite.[3−5] The formation rate depends on different factors such as pH, temperature, and the concentration of the nitrosating agents and the precursors.[4,5]

Carmella et al. showed for the first time in 1997 that this nitrosation reaction can also take place endogenously by treating rats with nicotine (being metabolized to nornicotine) and sodium nitrite. NNN was subsequently detectable in the urine of the rodents.[6] Furthermore, NNN was observed in the urine of a few nicotine patch users despite the absence of smoking.[7] In a cessation study with nicotine patches performed by the same research group, NNN was observed in some quitters, which was explained by endogenous formation. The increase correlated with higher urinary nitrate, presumably representing a higher nitrosating potential in these samples.[8]

We have observed implausibly high urinary NNN levels with concentrations above 200 pg/mL in some smokers, which differed significantly from all other smokers (showing an outlier range of 204−1330 pg/mL), presumably as a result of artifactual formation, either in urine endogenously or during sample storage.[9] Similar observations were reported recently for e-cigarette (EC) users in two studies.[10,11] An ex vivo study in human saliva using stable-isotope-labeled nicotine and nornicotine with and without the addition of nitrite proved that NNN is readily found after the incubation of labeled nornicotine with saliva.[10] These findings were substantiated in a recent study, where NNN was detected in the saliva of EC users, despite its absence in the e-liquid.[11] These findings show that both urinary and salivary NNN do not necessarily represent the actual NNN uptake from the product but are prone to be higher due to artifactual formation from the present precursors. Hence, urine and saliva may not be the

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matrices of choice when it comes to a robust determination of the product-related uptake of NNN which would present important data in the exposure assessment of NNN-containing tobacco products, such as cigarettes or smokeless tobacco.

Nitrosation of nornicotine occurs under acidic pH conditions.[4,12] Urinary pH is variable (pH between 5 and 8)[13] and influenced by factors such as diet and various diseases, in particular, bladder infections.[13−15] In contrast, blood pH is physiologically stabilized at pH 7.37 to 7.43. Thus, endogenous formation of NNN should be less likely to occur in blood, and therefore, plasma may be a well-suited biological matrix for the determination of NNN exposure. Following these considerations, we developed and validated a sensitive LC−MS/MS method for the determination of NNN in plasma and applied this method to plasma samples derived from smokeless tobacco users in order to explore the pharmacokinetics (PK) of plasma NNN in comparison with plasma nicotine.

MATERIALS AND METHODS

Reagents and Standards. Ammonium acetate (UPLC-MS grade) and formic acid (≥99%) were purchased from Biosolve B.V. (Valkenswaard, Netherlands). Disodium phosphate (≥99.5%), potassium dihydrogenphosphate, hydrochloric acid (min. 37%), sodium chloride (p.a.), sodium hydroxide (p.a.), sodium nitrite (p.a.), and sodium nitrate (p.a.) were obtained from Merck KGaA (Darmstadt, Germany). Acetonitrile containing 0.1% of formic acid (UPLC-MS grade), dichloromethane, heptane (HPLC grade), methyl-*tert*-butylether (MTBE), and methanol (≥99.9%) were purchased from Th. Geyer GmbH (Renningen, Germany). Hexane (picograde) and toluene (picograde) were obtained from LGC Standards GmbH (Wesel, Germany). NNN and nornicotine reference standards from Toronto Research Chemicals (North York, Canada) were used. NNN-d_4 from Dr. Ehrenstorfer (Augsburg, Germany) was applied as the internal standard.

Quantification of NNN in Urine. 8 mL of urine was processed and analyzed according to Kavvadias et al.,[16] with modifications. Briefly, 20 μL of NNN-d_4 (25 ng/mL in methanol) was added to the urine sample followed by centrifugation for 10 min (3300*g*). Sample preparation by means of a two-step solid-phase extraction procedure was performed as described by Kavvadias et al.[16] The final extract was evaporated to dryness and reconstituted in 100 μL of methanol/water (1:4 (v/v)). 10 μL of the extract was injected into the LC−MS/MS system consisting of a Nexera X2 UHPLC system (Shimadzu, Neufahrn, Germany) coupled to a 6500+QTrap MS/MS from Sciex (Darmstadt, Germany). Chromatography was performed on an Acquity HSS T3 UPLC column (100 × 2.1 mm, 1.8 μm; Waters, Eschborn, Germany) at 45 °C and a flow rate of 0.6 mL/min. Elution was performed under gradient conditions with 0.1% of ammonium acetate in water (A) and 0.1% of formic acid in acetonitrile (B) as follows: 0−1 min: 10% B; 1−2 min: 10 to 50% B; 2−4 min: 50% B; 4 to 4.01 min: 50 to 10% B; and 4.01−7 min: 10% B. Positive electrospray ionization (ESI^+) was applied with an ion source temperature of 500 °C, a nebulizer gas flow of 45 psi (gas 1), a heater gas flow of 70 psi (gas 2), and an ion spray voltage of 5000 V in the multiple-reaction monitoring (MRM) mode, measuring the mass transitions 178−148 *m*/*z* as a quantifier (DP: 41 V, CE: 13 V, CXP: 14 V) and 178−120 *m*/*z* as a qualifier for the analysis of NNN. The mass transition for the internal standard (NNN-d_4) was 182−152 *m*/*z*. The samples were quantified by means of a linear calibration with $1/x$ weighting and a linear range of 0.5−1500 pg/mL.

Investigation of the NNN Formation in Urine. Pooled urine of healthy nonsmokers was fortified with nornicotine (500 ng/mL) and sodium nitrite/sodium nitrate (50 μg/mL) and incubated at varying pH values and temperatures over different time periods. For each experiment, the pH of the sample (8 mL of urine) was set to a defined value between 2 and 12 and stored frozen (−20 °C) or at room temperature for 24 h or 10 min, respectively. After incubation for the respective time period, each aliquot was processed and analyzed as described above (see the section Quantification of NNN in Urine).

Human Study. A controlled clinical study with 10 experienced users of moist smokeless tobacco (MST) was conducted in accordance with the ethical standards as set forth in the Declaration of Helsinki[17] and approved by ADVARRA Independent Review Board. All participants in this study reviewed, signed, and dated the informed consent form prior to study initiation. The 10 subjects (males between 28 and 59 years of age and BMI between 18.6 and 36.2 kg/m^2) were provided with a defined portion of 2 g of the MST product Skoal Long Cut Classic which was placed in the mouth for 40 min. 15 blood samples were drawn during and post-use of the product over a time period of 6 h for PK analysis. Samples were collected into 4 mL Vacutainer tubes containing K_2EDTA which were filled as completely as possible at the following time points after initiation of product use: 0 (5 min preuse), 5, 10, 15, 20, 25, 30, 35, 40, 45, 60, 90, 150, 240, and 360 min. Immediately after the sample was drawn, the sample tube was gently inverted five to ten times to thoroughly mix the anticoagulant and then centrifuged at room temperature at approximately 1200*g* for 15 min to achieve a clear plasma layer over the red cells. Finally, the plasma layer was transferred in equal portions of 0.5 to 1.0 mL into two polypropylene sample storage tubes, capped, and stored at −20 °C until analysis. A total of 150 plasma samples from 10 subjects (all males) were analyzed for NNN and nicotine in this study.

The MST study products were characterized using analytical methods within the scope of accreditation to ISO/IEC 17025:2005. Nicotine content in the product was determined to be 12.41 mg/g (27.39 mg/g dry weight) by gas chromatography with a flame ionization detector (GC-FID)[18] and was calculated to be 24.82 mg per quid of 2 g. NNN content in the product was determined to be 1.77 μg/g (3.91 μg/g dry weight) by LC−MS/MS[19] and was calculated to be 3.54 μg per quid of 2 g. The pH of the product was determined to be 7.7 using a pH meter.[18]

Analysis of NNN in Plasma. 10 μL of sodium hydroxide (10 M), 10 μL of NNN-d_4 (25 ng/mL in water), 100 μL of saturated sodium chloride solution, and 2 mL of MTBE were added to 1 mL of plasma and stirred for 10 min. After centrifugation (10 min, 1860*g*), the organic phase was evaporated to dryness and reconstituted in 50 μL of formic acid (4 M) and 150 μL of MTBE. The extract was vortexed and centrifuged (10 min, 1860*g*). The aqueous phase was evaporated to dryness and reconstituted in 50 μL of methanol/water (1:4 (v/v)). Analysis of NNN in plasma by LC−MS/MS was performed in analogy to the analysis of NNN in urine.

Accuracy and precision of the analysis were determined according to the criteria for calibration and internal quality control (QC) samples as set forth in FDA guidelines.[20] QC samples were prepared in three different concentration levels covering the expected concentration range of the study samples. Eighteen QC samples (six per level), corresponding to 12% of study samples, were monitored. Quantification of samples and QCs was conducted by linear calibration with $1/x$ weighting and a linear range of 0.3−1000 pg/mL.

Analysis of Nicotine in Plasma. An aliquot of 100 μL plasma sample was fortified with the internal standard working solution of nicotine-d_4. The sample was then added to 100 mM ammonium bicarbonate buffer and extracted with ethyl acetate. The organic phase was evaporated to dryness under a nitrogen stream and the remaining residue was reconstituted with acetonitrile/methanol (v/v 75:25). The final extract was analyzed by LC−MS/MS consisting of a Shimadzu Nexera UHPLC coupled with a Sciex API 5000 MS/MS. Positive electrospray ionization was applied for data acquisition, and nicotine was acquired at 163−130 *m*/*z* and nicotine-d_4 was acquired at 167−134 *m*/*z*. The calibration range was 0.200−25.0 ng/mL in the linear regression model with $1/x^2$ weighting. The method was validated according to requirements in FDA Bioanalytical Method Validation—Guidance for Industry.[20]

Data Evaluation and Statistics. Analytical raw data were processed using Analyst 1.6.3 (Sciex, Darmstadt, Germany) and

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Excel 2019 (Microsoft, Unterschleißheim, Germany). PK and statistical evaluation were calculated with Prism 9.1.2 (GraphPad, LaJolla, CA, USA). Correlations between NNN and nicotine in plasma were evaluated computing the Pearson correlation coefficient (r). Half-life ($t_{1/2}$) was calculated by fitting based on a one-phase exponential decay for the mean curve of all subjects (N = 10). Area under curve $AUC_{0-\infty}$ was extrapolated as the sum of AUC_{0-360} (calculated in Prism 9.1.2) and the ratio mean $C_{360\ min}/\lambda_Z$ (λ_Z = $0.693/t_{1/2}$). Precision is expressed by the coefficient of variation (CV).

RESULTS AND DISCUSSION

NNN Formation in Urine by Nitrosation of Nornicotine. Sodium nitrite (50 μg/mL), sodium nitrate (50 μg/mL), and nornicotine (500 ng/mL) were added to urine samples and incubated at various pH levels between 2 and 12. The indicated concentrations were chosen based on a previous experiment (data not shown) in order to ensure NNN formation within a quantifiable range of our method even at low nitrosation yields. Except for the nitrite concentration, which is approximately 100-fold higher compared to published levels in human urine (around 500 ng/mL), these concentrations can be regarded as realistic for smokers and users of nicotine-containing products.[21] No NNN was detectable in controls without the addition of the precursors, regardless of the adjusted pH value. In addition, we could prove that there is no artifactual formation of NNN during the analytical procedure after the addition of nornicotine as a monitor amine as part of the method validation.[16] However, the addition of a stable-labeled monitor amine during sample work-up in analogy to Kotandeniya et al.[22] shall be considered in future studies to exclude the analytical method itself as a cause for artifactual formation. After 10 min of incubation at pH 2, 3, and 4, NNN levels of 490, 340, and 62 pg/mL, respectively, were observed. In contrast, NNN formation was only marginal at pH values ≥ 5 (1.2−2.6 pg/mL) (Figure 1).

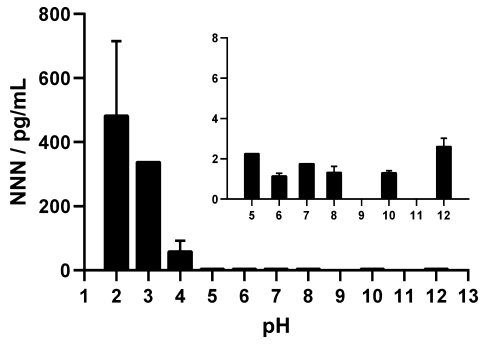

Figure 1. Formation of NNN (bars: mean; error bars: standard deviation) in urine after incubation with nornicotine (500 ng/mL), sodium nitrite (50 μg/mL), and sodium nitrate (50 μg/mL) for 10 min at room temperature. The inserted figure shows the amount of NNN formed at pH 5 to 12 with the y-axis magnified 100-fold for better illustration. Incubations were performed in triplicates except for pH 3, 5, and 7 (single value).

Since an excess in nitrite was used in these incubation experiments, NNN formation—if any—would presumably not be detectable under real-life conditions in healthy subjects (pH value between 5 and 8).

A profound increase could be observed at acidic pH after 24 h independent of the storage temperature (frozen or at room temperature) (illustrated for pH 2 in Figure 2). In contrast, the

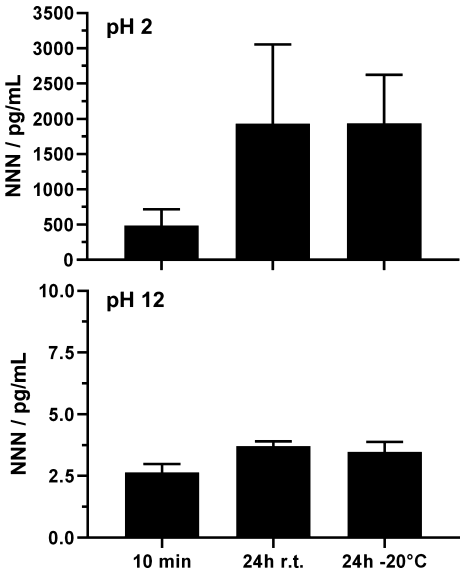

Figure 2. Formation of NNN (N = 3; bars: mean; error bars: standard deviation) in urine after incubation with nornicotine (500 ng/mL), sodium nitrite (50 μg/mL), and sodium nitrate (50 μg/mL) at pH 2 (top) and pH 12 (bottom) under various storage conditions.

increase was much less pronounced at pH 6 and above yielding 3.5 to 19.7 pg/mL (shown for pH 12 in Figure 2). The NNN amounts formed for each storage condition are summarized in the Supporting Information (Table S1).

Again, such yields are not likely to cause high NNN levels in vivo with samples derived from healthy human subjects. Interestingly, at pH 5, only 2.3 pg/mL of NNN was formed after 10 min incubation while 89 and 1100 pg/mL were found after storage for 1 day at −20 °C and at room temperature, respectively (Table S1). Apparently, the pH value is the main driver of the NNN formation in the presence of nornicotine and nitrite/nitrate in urine as expected with respect to the kinetics of the nitrosation reaction.[4,12] Endogenous NNN formation seems less likely at pH 6 and above. At and below pH 4, NNN can be formed rapidly at levels that are much higher than expected solely from NNN uptake derived from cigarette smoking or smokeless tobacco use. Acidic urinary pH with values of 5 or even lower, which may cause endogenous NNN formation in the presence of nitrite/nitrate and nornicotine in the high pg/mL range as observed in our incubation experiments and previous studies,[7−9] is associated with disease states such as bladder infections or chronic kidney disease.[15,23] In such cases, the determined NNN concentrations would not indicate a product use-related NNN uptake. Although the nitrosation was less efficient at higher pH (6 and above), implausibly high concentrations of NNN, as observed in several studies,[7−9] may occur during storage over longer time periods in the presence of sufficient amounts of the precursors nornicotine and nitrite. Generally, the probability for endogenous formation appears to increase with lower pH for subjects who are exposed to nicotine. Consequently, measurement of NNN in urine of users of tobacco and nicotine-containing products is important for the risk assessment; however, results could be severely biased by the described artifactual NNN formation under acidic pH conditions in the bladder (in vivo) or during urine sample storage (ex vivo). The ex vivo effect could be prevented by stabilization immediately after urine sample collection with

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Table 1. Precision and Accuracy Determined for NNN in Plasma[a]

concentration	intraday precision (N = 5, 3 days) (%)	interday precision (N = 15) (%)	intraday accuracy (N = 5, 3 days) (%)	interday accuracy (N = 15) (%)
LLOQ (0.3 pg/mL)	1.5−4.3	3.8	100.5−106.4	103.9
low (1.0 pg/mL)	7.9−9.8	9.9	93.5−106.0	99.5
medium (5.0 pg/mL)	3.8−9.1	6.3	93.8−97.7	95.5
high (100 pg/mL)	1.0−3.1	3.2	101.3−106.9	103.8

[a]Intraday determinations were performed on three consecutive days. Interday values express the average over all three days.

suitable inhibitors, for example, ammonium sulfamate, ascorbic acid, dihydroxyfumaric acid, caffeic acid, and ferulic acid,[22,24] or by inhibition with a strong base.[25]

Development and Validation of an LC−MS/MS Method for NNN Determination in Plasma. Since the main driver for the variability of NNN formation which cannot be directly attributed to product-derived uptake is the acidic pH of the urine, we developed a method for the quantification of NNN in plasma due to the narrow range of 7.37 to 7.43 in which the plasma pH is physiologically buffered. According to the results presented above, this pH should not lead to the artifactual NNN formation that can occur with urine analysis.

We developed a method for the quantification of NNN in human plasma using LC−MS/MS after liquid−liquid extraction (LLE). A high-throughput method based on LLE with MTBE, recently published by the FDA, served as a starting point.[26] This method used LLE under basic conditions in the autosampler vial prior to injection of the organic phase onto the LC−MS/MS system. We improved the sensitivity by two modifications. First, we added a second LLE step with MTBE under acidic conditions leading to an improved purification from matrix components and lower chromatographic noise. Second, 1 mL instead of 20 μL of plasma was used in our method. The final extract was evaporated to dryness and reconstituted in 50 μL leading to a 20-fold enrichment of the analyte. Moreover, we found that reversed-phase chromatography using a C18 column was superior compared to the HILIC used in the original method with respect to the peak resolution. These modifications led to a significant improvement of the LLOQ by a factor of 33 (0.3 pg/mL instead of 10 pg/mL). The method was fully validated according to FDA guidelines.[20] Method performance data are shown in Table 1. Accuracy and precision were investigated in human plasma in four concentration levels, covering the LLOQ, low, medium, and high concentrations, on three different days (five independent replicates per day). Separate calibrations in plasma were prepared on each day for the quantification of the validation samples. Both intra- and interday accuracy and precision were within the acceptable range of 85−115% accuracy (80−120% accuracy at LLOQ) and 15% CV (20% CV at LLOQ) for each level. The LLOQ of 0.3 pg/mL was confirmed with a mean accuracy of 104% and a mean relative standard deviation of 3.8% on three different days. Hence, the method is characterized by its high sensitivity and broad linear range (0.3−1000 pg/mL). Selectivity was proven for five different plasma matrices. No interferences with the mass transitions of the analyte and the internal standard were observed. A mean recovery of 30% was observed over the range of 1−100 pg/mL. The recovery was consistent throughout all concentration levels. The two-step LLE improved the signal-to-noise ratio compared to the one-step LLE in the original method resulting in a gain in sensitivity despite the poor recovery of 30%. Losses during the sample preparation were fully compensated by the internal standard (NNN-d_4). The response of NNN was not affected by the matrix. There was no carryover for blank sample injections following five subsequent injections at a concentration of 100 pg/mL. Extensive stability investigations were performed, including freeze−thaw stability over six cycles, a short-term stability at room temperature for 24 h, postpreparative stability in the autosampler (10 °C) for 8 days, and a long-term stability in plasma over 18 months below −20 °C.

Plasma NNN PK in Smokeless Tobacco Users. The new method was applied to plasma samples which were originally collected in order to evaluate nicotine PK of MST users. There was only one sample (360 min after product use) below the LLOQ of 0.3 pg/mL proving the suitability of the method for plasma NNN determination. Representative chromatograms are shown in Figure 3.

Overall, NNN showed an average C_{max} of 7.1 pg/mL with peak concentrations between 3.3 and 10.1 pg/mL. The mean C_{max} for nicotine was 19.4 ng/mL (ranging from 4.3 to

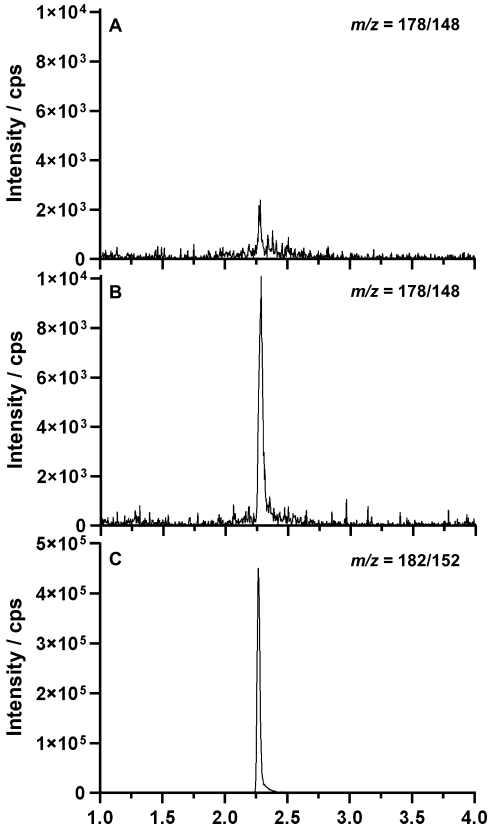

Figure 3. Multiple-reaction monitoring chromatograms of NNN in plasma. (A) QC sample at low NNN concentration (0.9 pg/mL). (B) Study sample at its C_{max} of 8.8 pg/mL. (C) Internal standard (250 pg/mL).

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Table 2. PK Results for NNN and Nicotine (N = 10)

		NNN	nicotine
$AUC_{0-360\ min}$	mean ± SD	917 ± 343 pg/mL·min	3095 ± 1507 ng/mL·min
	median	927 pg/mL·min	3006 ng/mL·min
	min−max	379−1462 pg/mL·min	811−5359 ng/mL·min
C_{max}	mean ± SD	7.1 ± 2.3 pg/mL	19.4 ± 9.1 ng/mL
	median	7.1 pg/mL	23.7 ng/mL
	min−max	3.3−10.1 pg/mL	4.3−29.0 ng/mL
t_{max}	mean ± SD	32 ± 10 min	40 ± 14 min
	median	28 min	40 min
	min−max	20−45 min	20−60 min
$t_{1/2}$	mean	98 min	122 min
$AUC_{0-\infty}$	mean	1051 pg/mL·min	3698 ng/mL·min
PK-derived dose	mean	1.20 μg[a]	
		0.60 μg[b]	4.2 mg[a]

[a]Estimated based on plasma clearance of nicotine.[27] [b]Estimated based on extraction efficiency calculated for nicotine in the product (17%). AUC: area under curve; C_{max}: maximum concentration; t_{max}: time at C_{max}; $t_{1/2}$: half-life; min: minimum; max: maximum; and SD: standard deviation.

29 ng/mL; Table 2). This difference by 3 orders of magnitude reflects the difference in the amounts of both compounds in the product.

The time courses of NNN and nicotine in plasma are very similar in shape (Figure 4). The controlled use over 40 min

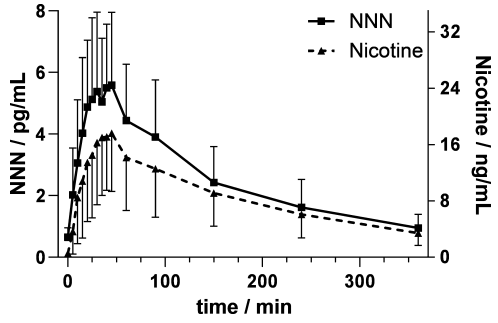

Figure 4. PK profiles of NNN and nicotine in plasma (mean ± SD; N = 10).

resulted in a concentration plateau between 20 and 40 min of use. A half-life of 122 min was calculated for nicotine, while the NNN half-life appeared to be lower with 98 min. In consideration of the high variabilities for t_{max} and the small sample size, calculations of the half-lives shall be regarded as rough estimates.

The amount of nicotine absorbed from the product was calculated as the percentage of the actual intake (systemically absorbed dose) and the amount of nicotine in the product. An actual intake of 4.22 mg was determined ($AUC_{0-\infty}$ (3698 ng/mL·min) × plasma clearance (1140 mL/min[27])), under the assumption that oral bioavailability is approximately 1.0. Hence, 17% of the amount of nicotine in the product (24.82 mg/per 2 g quid) was systemically absorbed. This is in reasonable agreement to Digard et al., who determined an extraction efficiency from smokeless tobacco products in the range between 20 and 35%.[28] Assuming a similar extraction efficiency of 20−35% for NNN, between 0.71 and 1.24 μg of NNN would be systemically absorbed (NNN content in the product: 3.54 μg/per 2 g quid), which is in good agreement with the expected range according to the literature.[28]

Individual PK variables correlated very well, with significant (p < 0.005) Pearson correlation coefficients of r > 0.8 for the area under curve (AUC, r = 0.887) and C_{max} (r = 0.803). PK is routinely assessed to characterize the efficiency of the nicotine delivery of tobacco and nicotine-containing products.[29] The high correlation between nicotine and NNN indicates that plasma NNN reflects the product-use-specific uptake in this study. The findings from this study need to be further substantiated with respect to the next generation of smokeless tobacco products and other product categories. These data will add important information to assess potential NNN uptake from these products.

CONCLUSIONS

NNN determination in urine is important in terms of risk assessment in nicotine-containing products, even in the absence of NNN in the investigated product itself, due to the risk of endogenous formation from the nicotine metabolite nornicotine and nitrite under acidic conditions. Nitrosation appeared much slower at a pH of 6 and above. Thus, implausibly high NNN levels, especially in nonexposed subjects, may also occur due to nitrosation during storage of the samples. In light of these findings, investigators should determine the pH of the collected urine in order to estimate the likelihood of artifactual NNN formation. A robust determination of the product-specific NNN uptake is important in evaluating the role of different tobacco products in the exposure to this human carcinogen. The method presented here is proved to be fit for purpose to evaluate the PK of NNN in the plasma of smokeless tobacco users. Plasma NNN correlated significantly with nicotine. Regarding the high correlation between nicotine and NNN and the stable blood pH of 7.40 which inhibits the nitrosation reaction, plasma NNN may be considered as a biomarker to determine the product-specific exposure to NNN in future clinical exposure studies with tobacco products.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.1c00431.

Results for the NNN formation from its precursors in urine (PDF)

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ABBREVIATIONS

AUC, area under curve; CV, coefficient of variation; FDA, (U.S.) Food and Drug Administration; LC−MS/MS, liquid chromatography coupled with tandem mass spectrometry; LLE, liquid−liquid extraction; LLOQ, lower limit of quantification; LOD, limit of detection; MTBE, methyl-*tert*-butylether; Nic, nicotine; NNN, *N*-nitrosonornicotine; SD, standard deviation; ULOQ, upper limit of quantification

REFERENCES

(1) International Agency for Research on Cancer (IARC). Tobacco Smoking. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*; IARC, 1986.

(2) Hoffmann, D.; Hecht, S. S.; Ornaf, R. M.; Wynder, E. L. N′-nitrosonornicotine in tobacco. *Science* **1974**, *186*, 265−267. Hecht, S. S.; Chen, C.-h. B.; Hirota, N.; Ornaf, R. M.; Tso, T. C.; Hoffmann, D. Tobacco-specific nitrosamines: formation from nicotine in vitro and during tobacco curing and carcinogenicity in strain A mice. *J. Natl. Cancer Inst.* **1978**, *60*, 819−824. Brunnemann, K. D.; Hoffmann, D. Analytical studies on tobacco-specific N-nitrosamines in tobacco and tobacco smoke. *Crit. Rev. Toxicol.* **1991**, *21*, 235−240.

(3) Andersen, R. A.; Burton, H. R.; Fleming, P. D.; Hamilton-Kemp, T. R. Effect of storage conditions on nitrosated, acylated, and oxidized pyridine alkaloid derivatives in smokeless tobacco products. *Cancer Res.* **1989**, *49*, 5895−900. Hecht, S. S.; Chen, C.-H. B.; Ornaf, R. M.; Jacobs, E.; Adams, J. D.; Hoffmann, D. Reaction of nicotine and sodium nitrite: Formation of nitrosamines and fragmentation of the pyrrolidine ring. *J. Org. Chem.* **1978**, *43*, 72−76. Hecht, S. S.; Hoffmann, D. Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. *Carcinogenesis* **1988**, *9*, 875−884. Zwickenpflug, W. N-nitrosation of myosimine yields HPB (4-hydroxy-1-(3-pyridyl)-1-butanone) and NNN (N-nitrosonornicotine). *J. Agric. Food Chem.* **2000**, *48*, 392−394.

(4) Mirvish, S. S.; Sams, J.; Hecht, S. S. Kinetics of Nornicotine and Anabasine Nitrosation in Relation to N′-Nitrosonornicotine Occurrence in Tobacco and to Tobacco-Induced Cancer2. *JNCI, J. Natl. Cancer Inst.* **1977**, *59*, 1211−1213.

(5) Shi, H.; Wang, R.; Bush, L. P.; Zhou, J.; Yang, H.; Fannin, N.; Bai, R. Changes in TSNA Contents during Tobacco Storage and the Effect of Temperature and Nitrate Level on TSNA Formation. *J. Agric. Food Chem.* **2013**, *61*, 11588.

(6) Carmella, S.; Borukhova, A.; Desai, D.; Hecht, S. S. Evidence for endogenous formation of tobacco-specific nitrosamines in rats treated with tobacco alkaloids and sodium nitrite. *Carcinogenesis* **1997**, *18*, 587−592.

(7) Stepanov, I.; Carmella, S. G.; Han, S.; Pinto, A.; Strasser, A. A.; Lerman, C.; Hecht, S. S. Evidence for endogenous formation of *N*′-nitrosonornicotine in some long-term nicotine patch users. *Nicotine Tob. Res.* **2009**, *11*, 99−105.

(8) Stepanov, I.; Carmella, S. G.; Briggs, A.; Hertsgaard, L.; Lindgren, B.; Hatsukami, D.; Hecht, S. S. Presence of the carcinogen *N*′-nitrosonornicotine in the urine of some users of oral nicotine replacement therapy products. *Cancer Res.* **2009**, *69*, 8236−8240.

(9) Kavvadias, D.; Scherer, G.; Cheung, F.; Errington, G.; Shepperd, J.; McEwan, M. Determination of tobacco-specific N-nitrosamines in urine of smokers and non-smokers. *Biomarkers* **2009**, *14*, 547−553.

(10) Knezevich, A.; Muzic, J.; Hatsukami, D. K.; Hecht, S. S.; Stepanov, I. Nornicotine nitrosation in saliva and its relation to endogenous synthesis of *N*′-nitrosonornicotine in humans. *Nicotine Tob. Res.* **2012**, *15*, 591−595.

(11) Bustamante, G.; Ma, B.; Yakovlev, G.; Yershova, K.; Le, C.; Jensen, J.; Hatsukami, D. K.; Stepanov, I. Presence of the carcinogen *N*′-nitrosonornicotine in saliva of e-cigarette users. *Chem. Res. Toxicol.* **2018**, *31*, 731−738.

(12) Tricker, A. R.; Haubner, R.; Spiegelhalder, B.; Preussmann, R. The occurrence of tobacco-specific nitrosamines in oral tobacco products and their potential formation under simulated gastric conditions. *Food Chem. Toxicol.* **1988**, *26*, 861−865.

(13) Harlass, F. E.; Duff, P.; Herd, M. The evaluation of urine pH in screening for asymptomatic bacteriuria in pregnancy. *Mil. Med.* **1990**, *155*, 49−51.

(14) Eisner, B. H.; Porten, S. P.; Bechis, S. K.; Stoller, M. L. Diabetic kidney stone formers excrete more oxalate and have lower urine pH than nondiabetic stone formers. *J. Urol.* **2010**, *183*, 2244−2248. Frassetto, L.; Kohlstadt, I. Treatment and prevention of kidney stones: an update. *Am. Fam. Physician* **2011**, *84*, 1234−1242. Maalouf, N. M.; Cameron, M. A.; Moe, O. W.; Sakhaee, K. Metabolic basis for low urine pH in type 2 diabetes. *Clin. J. Am. Soc. Nephrol.* **2010**, *5*, 1277−1281.

(15) Nakanishi, N.; Fukui, M.; Tanaka, M.; Toda, H.; Imai, S.; Yamazaki, M.; Hasegawa, G.; Oda, Y.; Nakamura, N. Low urine pH Is

a predictor of chronic kidney disease. *Kidney Blood Pressure Res.* **2012**, *35*, 77−81.
(16) Kavvadias, D.; Scherer, G.; Urban, M.; Shepperd, J.; Cheung, F.; Errington, G.; McEwan, M. Simultaneous determination of four tobacco-specific *N*-nitrosamines (TSNA) in human urine. *J. Chromatogr. B* **2009**, *877*, 1185−1192.
(17) World Medical Association (WMA). World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *JAMA, J. Am. Med. Assoc.* **2013**, *310*, 2191.
(18) Centers for Disease Control and Prevention (CDC). Revised Protocol for Analysis of Nicotine, Total Moisture, and pH in Smokeless Tobacco Products. *Fed. Regist.* **2009**, *74*, 712−719.
(19) International Organization for Standardization (ISO). *ISO 21766:2018—Tobacco and Tobacco Products—Determination of Tobacco-specific Nitrosamines in Tobacco Products—Method Using LC-MS/MS*, 2018.
(20) Food and Drug Administration (FDA). Bioanalytical Method Validation—Guidance for Industry. https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf, 2018.
(21) Tsikas, D.; Böger, R. H.; Bode-Böger, S. M.; Gutzki, F.-M.; Frölich, J. C. Quantification of nitrite and nitrate in human urine and plasma as pentafluorobenzyl derivatives by gas chromatography-mass spectrometry uinsg their 15N-labelled analogs. *J. Chromatogr.* **1994**, *661*, 185−191. Piller, M.; Gilch, G.; Scherer, G.; Scherer, M. Simple, fast and sensitive LC-MS/MS analysis for the simultaneous quantification of nicotine and 10 of its major metabolites. *J. Chromatogr. B* **2014**, *951*−*952*, 7−15.
(22) Kotandeniya, D.; Carmella, S. G.; Pillsbury, M. E.; Hecht, S. S. Combined analysis of N'-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in the urine of cigarette smokers and e-cigarette users. *J. Chromatogr. B* **2015**, *1007*, 121−126.
(23) Demirbas, A.; Sarici, H.; Kilinc, M. F.; Telli, O.; Ozgur, B. C.; Doluoglu, O. G.; Bozkurt, S. The Relationship between Acidic Urinary pH and Overactive Bladder; Alkalization of Urine Improves the Symptoms of Overactive Bladder. *Urol. Int.* **2015**, *95*, 223−226.
(24) Porubin, D.; Hecht, S. S.; Li, Z.-z.; Gonta, M.; Stepanov, I. Endogenous formation of N'-nitrosonornicotine in F344 rats in the presence of some antioxidants and grape seed extract. *J. Agric. Food Chem.* **2007**, *55*, 7199−7204. Rundlöf, T.; Olsson, E.; Wiernik, A.; Back, S.; Aune, M.; Johansson, L.; Wahlberg, I. Potential nitrite scavengers as inhibitors of the formation of N-nitrosamines in solution and tobacco matrix systems. *J. Agric. Food Chem.* **2000**, *48*, 4381−4388.
(25) Carmella, S. G.; Akerkar, S. A.; Richie, j.; Hecht, S. S. 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*, 635−642. Michael, M.; Meger-Kossien, I.; Riedel, K.; Scherer, G. Biomonitoring of environmental tobacco smoke (ETS)-related exposure to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). *Biomarkers* **2000**, *5*, 33−45. Urban, M.; Scherer, G.; Kavvadias, D.; Hagedorn, H.-W.; Feng, S.; Serafin, R.; Kapur, S.; Muhammad, R.; Jin, Y.; Mendes, P.; et al. Quantitation of *N*'-Nitrosonornicotine (NNN) in Smokers'Urine by Liquid Chromatography-Tandem Mass Spectrometry. *J. Anal. Toxicol.* **2009**, *33*, 260−265.
(26) Loukotková, L.; VonTungeln, L. S.; Vanlandingham, M.; da Costa, G. G. A simple and highly sensitive UPLC-ESI-MS/MS method for the simultaneous quantification of nicotine, cotinine, and the tobacco-specific carcinogens N'-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in serum samples. *J. Chromatogr. B* **2018**, *1072*, 229−234.
(27) Benowitz, N. L.; Jacob, P., 3rd; Denaro, C.; Jenkins, R. Stable isotope studies of nicotine kinetics and bioavailability. *Clin. Pharmacol. Ther.* **1991**, *49*, 270−277.
(28) Digard, H.; Proctor, C.; Kulasekaran, A.; Malmqvist, U.; Richter, A. Determination of nicotine absorption from multiple tobacco products and nicotine gum. *Nicotine Tob. Res.* **2013**, *15*, 255−261.
(29) Benowitz, N. L. Systemic absorption and effects of nicotine from smokeless tobacco. *Adv. Dent. Res.* **1997**, *11*, 336−341. Fearon, I. M.; Gale, N.; McEwan, M.; Stiles, M. F.; Round, E. K. Nicotine pharmacokinetics of electronic cigarettes: A review of the literature. *Regul. Toxicol. Pharmacol.* **2018**, *100*, 25−34. Voos, N.; Goniewicz, M. L.; Eissenberg, T. What is the nicotine delivery profile of electronic cigarettes? *Expert Opin. Drug Delivery* **2019**, *16*, 1193−1203.

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