Quantitative analysis of NNN in plasma by LC-MS/MS: a suitable alternative to urinary determination to assess the exposure to this important toxicant

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Introduction

- *N*-Nitrosonornicotine (NNN) is one of four tobacco-specific nitrosamines (TSNAs)
- NNN is classified as a group 1 carcinogen (carcinogenic to humans) by the International Agency for Research on Cancer (IARC)
- NNN usually determined in urine for the exposure assessment
- Urinary NNN excretion highly variable
- Very high concentrations were previously reported after smoking cessation which were not reflected in the urinary NNAL concentrations [1], suggesting formation despite the absence of tobacco consumption.

Mechanism of NNN formation

- Nicotine is extensively metabolized to nornicotine which accounts for approx. 5% of the nicotine amount absorbed in urine [2]
- Nitrosation of NNN from nornicotine and/or myosmine observed in vitro and in vivo [3 – 7]

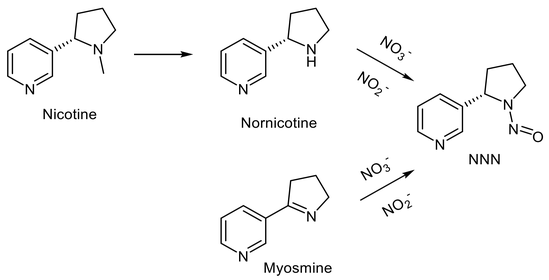


Figure 1: Metabolic formation of nornicotine from nicotine by *N*-demethylation followed by nitrosation yielding NNN (upper scheme). Nitrosation of the minor tobacco alkaloid myosmine to form NNN (lower scheme).

- Incubation experiments in non-smoker urine with nornicotine, NO_2^-/NO_3^- at room temperature
  - Incubation for 10 minutes at varying pH in triplicates
  - Concentrations: nornicotine (500 ng/mL) + NO_2^-/NO_3^- (50 µg/mL)
  - Immediate centrifugation (10 min) and further purification (SPE) according to the method (see ref [8])
  - LC-MS/MS analysis: Shimadzu Nexera UHPLC coupled with Sciex Qtrap 6500+ MS/MS
  - Method parameters: LLOQ: 0.5 pg/mL; range: 0.5-1500 pg/mL; acc. (prec.): 102 % (5.6 % CV)

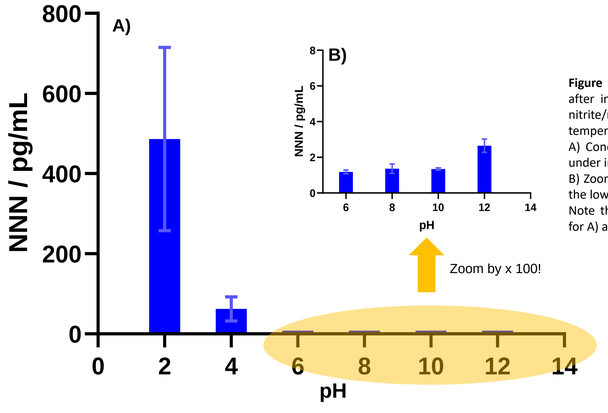


Figure 2: NNN concentration in urine after incubation with nornicotine and nitrite/nitrate for 10 minutes at room temperature (N = 3; Mean ± SD).
A) Concentrations for entire pH range under investigation (pH 2 – 14)
B) Zoom by a factor of 100 to illustrate the low concentrations at pH 6 – 14.
Note the different scales of the y-axis for A) and B).

- Rapid formation of NNN from nornicotine and nitrite at low pH (Fig. 2)
- pH ≥ 6: low conversion despite high amounts of precursors
- Further increase over time under acidic conditions (data not shown)
- Nitrosation at low pH also observed under frozen conditions over time

➢ *pH of decisive importance for NNN formation in urine*

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Drawbacks of NNN assessment in urine

- Urine pH in healthy subjects around 6 – 7
- Several diseases cause a decrease in urinary pH (pH 5.0 – 5.5)
- High variability in urine pH can lead to artefactual formation in the presence of the precursors nornicotine, myosmine, nitrite/nitrate
- NNN exposure assessment in urine highly variable
- NNN levels in urine may not necessarily reflect the product-specific exposure

Quantification of NNN in plasma – Method

- Blood pH not variable (pH 7.37 – 7.43)
- Hypothesis: NNN in plasma may be better suited to determine product-use specific exposure to NNN
- Development of a sensitive and high-throughput LC-MS/MS method in plasma based on a recently published method by the FDA [9]

LC-MS/MS method

Two step liquid-liquid extractions of 1 mL plasma with methyl *tert*-butyl ether
Reconstitution in 50 µL water/methanol (80/20 v/v)

Injection of 10 µL into the LC-MS/MS system Shimadzu Nexera UHPLC / Sciex Qtrap 6500⁺ MS

Gradient elution (see Fig. 3):
A: H_2O+ 0.1% NH_4OAc; B: ACN+0.1% FA

Column: Acquity HSS T3 1.8µ (100x2.1 mm)
Flow: 0.6 mL/min; run time: 5 min
MS/MS: ESI⁺; MRM (m/z): 178 ⟶148 (Quantifier)
178 ⟶120 (Qualifier)

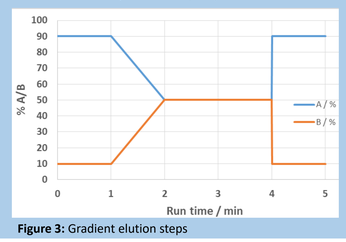


Figure 3: Gradient elution steps

Method validation

The LC-MS/MS method was fully validated according to FDA Guidance for Industry – Bioanalytical Method Validation (exemplified parameters shown in Tab. 1)

Table 1: Method validation results for precision, accuracy, and range (limits).

	LLOQ (0.5 pg/mL)	Low (1.0 pg/mL)	Medium (5.0 pg/mL)	High (100 pg/mL)
Intraday precision	5.7 – 15.4 % CV	7.9 – 9.8 % CV	3.8 – 9.1 % CV	1.0 – 3.1 % CV
Intraday accuracy	92.8 – 101.4 %	93.5 – 106.0 %	93.8 – 97.7 %	101.3 – 106.9 %
Interday precision	10.7 % CV	9.9 % CV	6.3 % CV	3.2 % CV
Interday accuracy	96.0 %	99.5 %	95.5 %	103.8 %
Limits:	LOD: 0.1 pg/mL	LLOQ: 0.5 pg/mL	ULOQ: 1,000 pg/mL	

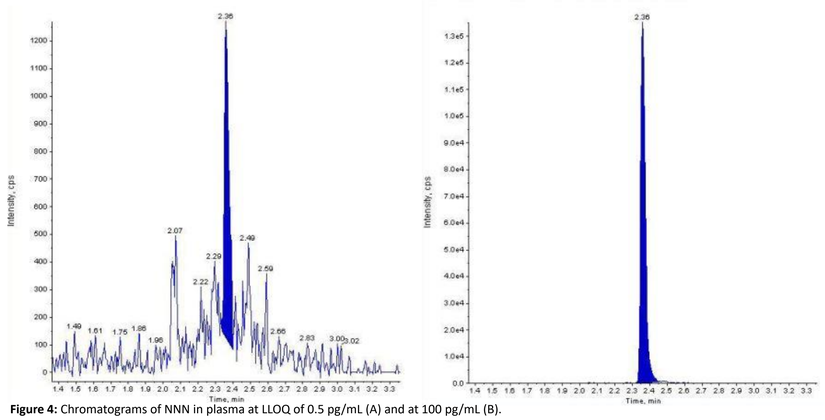


Figure 4: Chromatograms of NNN in plasma at LLOQ of 0.5 pg/mL (A) and at 100 pg/mL (B).

Quantification of NNN in plasma – Results

Clinical pilot study

- Human plasma samples collected from 10 subjects using moist snuff tobacco (MST)
- Product use under controlled conditions: 2g for 40 min
- Sample collection: 15 samples per subject within 6 hours to evaluate pharmacokinetics of NNN and nicotine

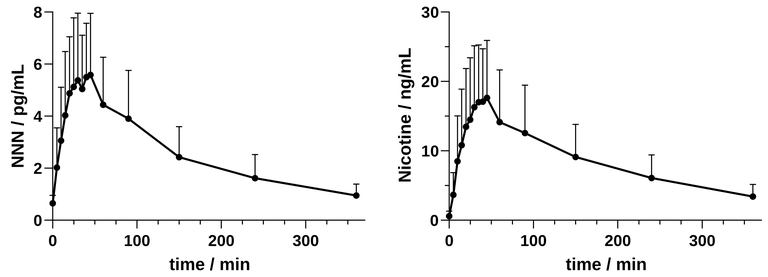


Figure 5: Pharmacokinetic profiles of NNN (left) and nicotine (right) in MST users (N = 10; Mean ± SD). Product was used for the first 40 minutes.

Table 2: Individual PK results for the ten subjects (area under curve (AUC) and maximum concentration (C_{max})) for NNN and nicotine (Nic)

AUC NNN / (pg/mL)*min	C_{max} NNN / pg/mL	AUC Nic / (ng/mL)*min	C_{max} Nic / ng/mL
548.4	5.80	1423	8.56
889.9	6.65	4000	24.6
975.8	7.57	4572	26.5
1426	10.1	4383	23.8
1462	8.08	5359	29.0
379.0	3.33	810.7	4.27
697.7	4.18	1487	7.05
1021	9.83	2908	23.6
964.0	8.75	2950	24.2
803.5	6.23	3061	22.4

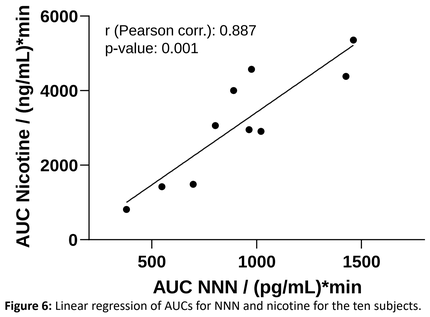


Figure 6: Linear regression of AUCs for NNN and nicotine for the ten subjects.

- NNN quantifiable in 97% of study samples
- Method sensitivity (LLOQ: 0.5 pg/mL) sufficient to quantify NNN in plasma
- Peak concentrations shortly after end of product use (40 min)
- Mean C_{max}: 7.1 pg/mL (3.3-10.1 pg/mL)
- Excellent correlation (Pearson correlation) between NNN and nicotine (AUC r: 0.887; C_{max} r: 0.803)

Conclusion

- Acidic conditions increase nitrosation rate of NNN from nornicotine and myosmine in urine

➢ ***Urinary NNN measurements may not indicate product-specific exposure to NNN***

- No variability in blood pH (7.37 – 7.43)

➢ ***Development of a highly sensitive LC-MS/MS method for the determination of NNN in plasma and application in a pilot study with 10 MST users***

- Method fit-for-purpose to quantify NNN in plasma, e.g. in PK studies
- Plasma levels of NNN and nicotine correlated significantly

➢ ***plasma NNN may be a more robust biomarker of NNN exposure to assess the product-specific NNN uptake***

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