Analysis of 3-hydroxybenzo[*a*]pyrene in urine as a biomarker of exposure for benzo[*a*]pyrene in smokers and users of potentially reduced risk products

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3-Hydroxybenzo[*a*]pyrene – Biomarker of Exposure to Benzo[*a*]pyrene in Urine

- Benzo[*a*]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH), formed during the incomplete combustion of organic materials
- BaP is classified as a human carcinogen (IARC Class 1)
- BaP is included in the established list of harmful and potentially harmful constituents in tobacco products and smoke
- Urinary 3-Hydroxybenzo[*a*]pyrene (3-OH-BaP) is frequently determined as a biomarker of BaP exposure
- High 3-OH-BaP concentrations were found in occupationally exposed populations (higher than 200 ng/g creatinine)[1-3]

Development of a highly sensitive method for human biomonitoring in non-occupationally exposed populations based on a previously published method[4]

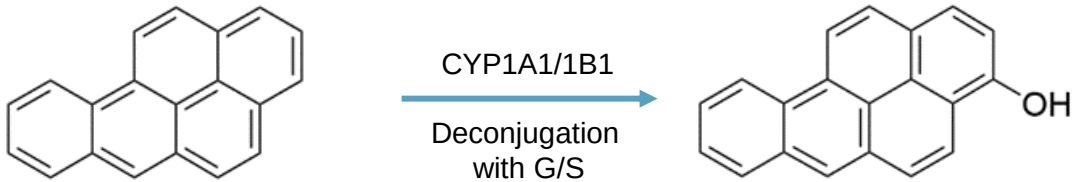

Figure 1: Metabolism of BaP

[1] Förster, K. et al. 3-Hydroxybenzo[*a*]pyrene in the urine of workers with occupational exposure to polycyclic aromatic hydrocarbons in different industries. Occup. Environ. Med. 2008, **65**, 224-229.
[2] Gündel, J. et al. High-performance liquid chromatographic method with fluorescence detection for the determination of 3-hydroxybenzo[a]pyrene and 3-hydroxybenz[a]anthracene in the urine of polycyclic aromatic hydrocarbon-exposed workers. J. Chromatogr. B. Biomed. Sci. Appl. 2000, **738**, 47-55.
[3] Barbeau, D. et al., Relevance of Urinary 3-Hydroxybenzo(*a*)pyrene and 1-Hydroxypyrene to Assess Exposure to Carcinogenic Polycyclic Aromatic Hydrocarbon Mixtures in Metallurgy Workers. Ann. Occup. Hyg. 2014, **58**, 579-590.
[4] Sarkar, M. et al. Evaluation of biomarkers of exposure in adult cigarette smokers using Marlboro Snus. Nicotine Tob. Res. 2010, **12**, 105-116.

Quantification of 3-OH-BaP in Urine – Sample Preparation

1. Enzymatic Hydrolysis

- 6 mL urine
- 10 pg IS ($^{13}C_6$-3-OH-BaP-Gluc)
- Acetate buffer (pH 5.1)
- β-Glucuronidase/ arylsulfatase
- Ascorbic acid solution
- Incubation over night, 37°C

2. SPE

- SPE tubes: Bond Elut-LMS, 200mg, 3 mL (Agilent)
- Conditioning
- Application of urine sample
- Washing
- Elution: 2x2 mL DCM

3. Derivatization

- Add 20 µL DMSO
- Evaporation of the solvent
- Derivatization: 2-fluoro-methyl-pyridinium p-toluene-sulfonate, 20 min, 45°C
- Evaporation of the solvent
- Reconstitution: 250 µL of MeOH/H_2O/FA (50/49/1)

Quantification of 3-OH-BaP in Urine – LC-MS/MS Analysis

4. LC-MS/MS

- System: Agilent 1200 HPLC and Sciex MS/MS API 5000
- Gradient elution:
  A: H_2O + 0.5 % FA
  B: ACN + 0.5 % FA
- Column: Acquity UPLC BEH C18, 50x2.1mm, 1.7µm
- Flow: 0.6 mL/min
- Run time: 15 min
- MS/MS: ESI^+

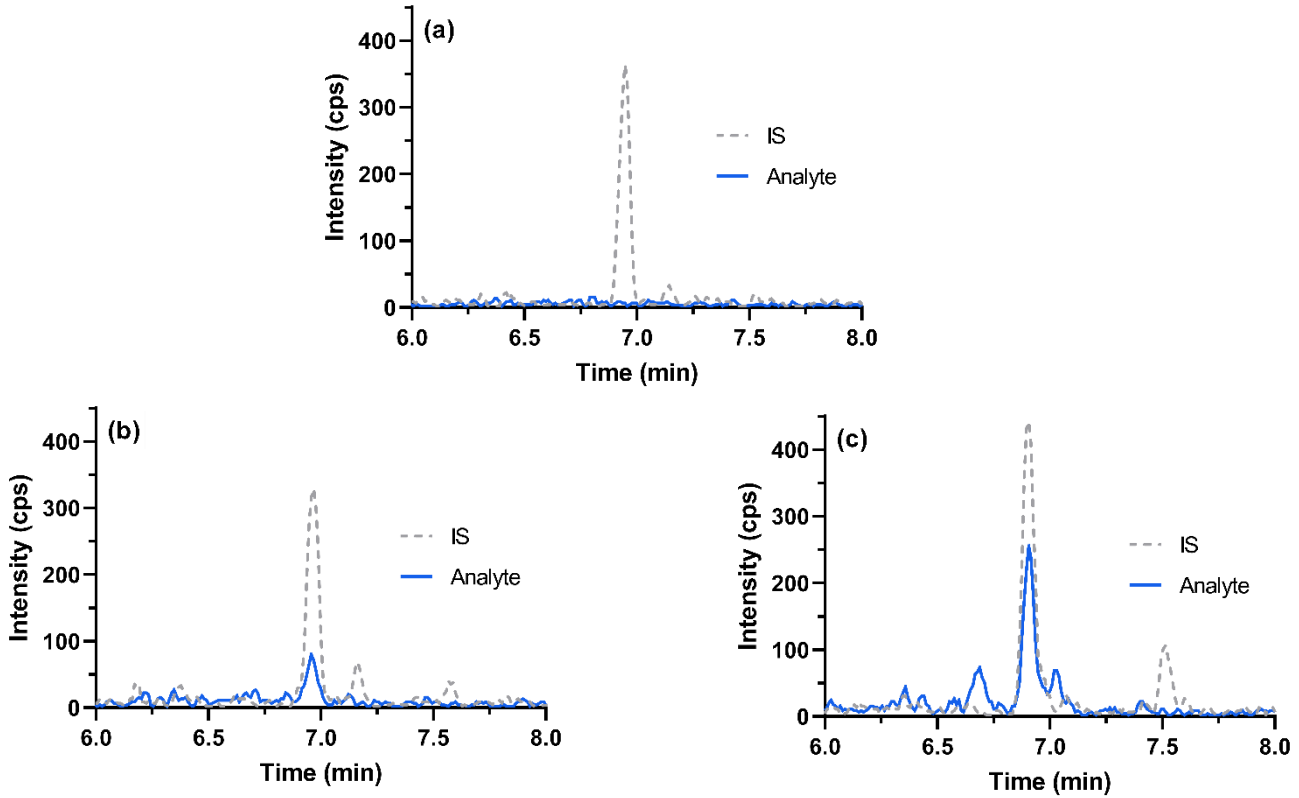

Figure 2: MRM chromatograms of 3-OH-BaP (*m/z* 360 → 251) and the IS $^{13}C_6$-3-OH-BaP (*m/z* 366 → 257) of (a) non-smoker urine, (b) QC low, and (c) smoker urine

Quantification of 3-OH-BaP in Urine – Method Validation

- Validation according to FDA guidelines
- LOD: 16.7 pg/L
- LLOQ: 50 pg/L
- Calibration range: 50 − 3321 pg/L
- Matrix effect: low (200 pg/L) +31 %; +25 % (IS)
  high (1600 pg/L) +43 %; +48 % (IS)

Table 1: Method validation results for precision and accuracy

	LLOQ (50 pg/L)	Low (100 pg/L)	Medium (400 pg/L)	High (1600 pg/L)
Precision, intra-day	10.1% CV	12.0% CV	12.3% CV	3.3% CV
Precision, inter-day	7.9% CV	9.0% CV	8.0% CV	5.8% CV
Accuracy, intra-day	101.8%	105.1%	94.0%	98.2%
Accuracy, inter-day	105.8%	110.7%	95.6%	99.6%

Method application - Clinical Study[1]

- Aim: Exposure assessment to BaP in smokers and users of potentially reduced risk products in non-occupationally exposed, healthy subjects compared to non-users
- Confinement over three days and diet-controlled clinical study
- Collection of all urine voids on each study day and preparation of 12h-urines for analysis

Table 2: Main characteristics of the study population

User group	N (m/f)	Age (years) Mean ± SD	BMI Mean ± SD	24h-urine[2] (mL) Mean ± SD
Cigarette (CC)	10 (6/4)	35.1 ± 9.1	26.0 ± 3.9	2891 ± 828
Heated tobacco products (HTP)	10 (6/4)	36.1 ± 12	25.5 ± 3.2	2685 ± 1300
Oral tobacco (OT)	10 (9/1)	28.1 ± 8.2	25.9 ± 4.2	2638 ± 1290
E-cigarette (EC)	10 (6/4)	38.4 ± 14	23.5 ± 2.7	1627 ± 664
Nicotine replacement therapy (NRT)	10 (5/5)	35.3 ± 15	25.5 ± 3.5	1602 ± 802
Non-users (NU, control)	10 (6/4)	32.9 ± 8.8	24.7 ± 3.2	2475 ± 936

[1] Sibul, F. et al. Identification of biomarkers specific to five different nicotine product user groups: Study protocol of a controlled clinical trial. Contemporary Clinical Trials Communications 2021, **22**, 100794.
[2] Urine voids collected on the last study day (6 pm until 6 pm)

Clinical Study – Results

- Analysis of 12h-urines on day 3 (longest time of confinement/control in the study)[1]
- CC: 60 % of study samples with concentrations >LLOQ
- HTP, OT, EC, NRT, and NU: 99 % of the study samples showed concentrations <LLOQ
- Mean value of CC smokers[2]: 149 pg/24 h
- Mean values of the other groups[2]: 40.7−67.1 pg/24 h
- Significantly higher concentrations in CC smokers

Application of the method allowed differentiation between smokers of CCs and users of other nicotine products as well as NUs

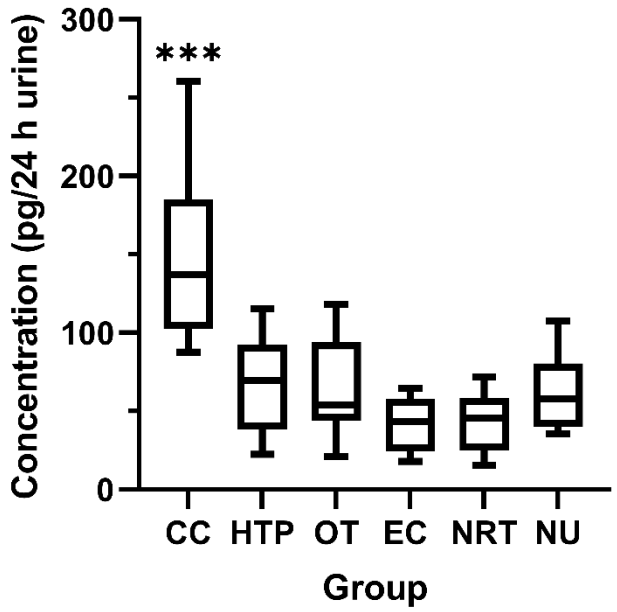

Figure 3: Box plots for urinary 3-OH-BaP excretion (pg/24h). Significant differences between the cigarette smokers (CC), the other user groups (HTP, OT, EC, and NRT), and the non-users (NU) were evaluated using the non-parametric Mann-Whitney U test (***: *p*-value < 0.002)

[1] 12 h periods: from 6 pm to 7 am and from 7 am to 6 pm
[2] All values below the LLOQ were set to LLOQ/2 (25 pg/L).

Clinical Study – Results

- Moderate correlation (Spearman) of 3-OH-BaP excretion with cigarettes per day and with total nicotine equivalents (TNE) in smokers

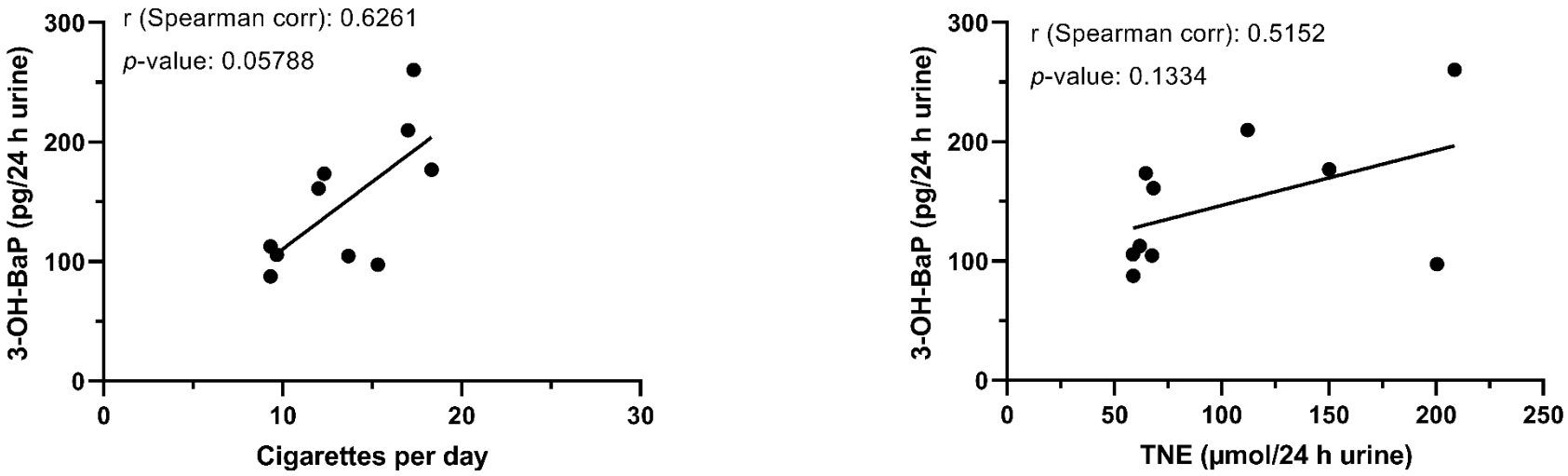

Figure 4: Linear regression for 3-OH-BaP and cigarette consumption per day (left), and for 3-OH-BaP and TNE (right) of smokers on Day 3 (10 subjects).

Clinical Study – Results

- CEMA (*N*-acetyl-S-(2-cyanoethyl)-L-cysteine): biomarker of exposure to acrylonitrile, a major source is smoking
- Progression of 3-OH-BaP and CEMA concentration of an NRT user over three days
- High concentrations of both smoking specific biomarkers indicate non-compliance

Three days were considered sufficient time for washout

- NUs and all other nicotine user groups were also significantly distinguishable from CC smokers in the urine samples collected before study start (U0) (*p*-value <0.05)

3-OH-BaP is considered a suitable biomarker in uncontrolled studies

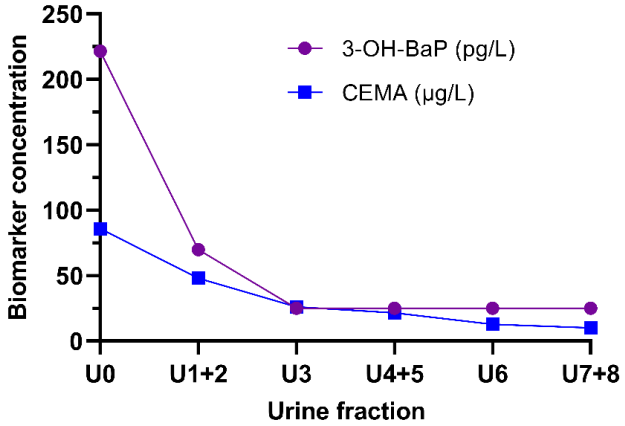

Figure 5: Progression of 3-OH-BaP (pg/L) and CEMA (µg/L) concentration of an NRT user over the three study days.

Conclusion

- Developement of a highly sensitive LC-MS/MS method for the quantification of 3-OH-BaP in urine suitable for the determination in non-occupationally exposed populations
- Method validation according to FDA guidelines revealed excellent accuracy/precision with a very low LLOQ of 50 pg/L
- Application of the method to 12h-urine samples collected in a controlled clinical study with smokers of CCs, users of ECs, HTPs, OT, NRT, and NUs
- 3-OH-BaP mostly not quantifiable in users of potentially reduced risk products (similar to NU) under the confined conditions
- Smokers of CCs showed significantly higher 3-OH-BaP excretion as compared to all other user groups and the NUs and moderate correlation to cigarette consumption and urinary TNE levels

3-OH-BaP is a suitable biomarker to assess the exposure
to the carcinogen BaP by cigarette smoke exposure
(Manuscript currently under review)

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