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A Sensitive LC–MS/MS Method for the Quantification of 3-Hydroxybenzo[*a*]pyrene in Urine-Exposure Assessment in Smokers and Users of Potentially Reduced-Risk Products

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Abstract: Benzo[*a*]pyrene (BaP), a human carcinogen, is formed during the incomplete combustion of organic matter such as tobacco. A suitable biomarker of exposure is the monohydroxylated metabolite 3-hydroxybenzo[*a*]pyrene (3-OH-BaP). We developed a sensitive LC–MS/MS (liquid chromatography coupled with tandem mass spectrometry) method for the quantification of urinary 3-OH-BaP. The method was validated according to the US Food and Drug Administration (FDA) guideline for bioanalytical method validation and showed excellent results in terms of accuracy, precision, and sensitivity (lower limit of quantification (LLOQ): 50 pg/L). The method was applied to urine samples derived from a controlled clinical study to compare exposure from cigarette smoking to the use of potentially reduced-risk products. Urinary 3-OH-BaP concentrations were significantly higher in smokers of conventional cigarettes (149 pg/24 h) compared to users of potentially reduced-risk products as well as non-users (99% < LLOQ in these groups). In conclusion, 3-OH-BaP is a suitable biomarker to assess the exposure to BaP in non-occupationally exposed populations and to distinguish not only cigarette smokers from non-smokers but also from users of potentially reduced-risk products.

Keywords: 3-hydroxybenzo[*a*]pyrene; LC–MS/MS; urine; human biomonitoring; derivatization; potentially reduced-risk products

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed during the incomplete combustion of organic matter. High exposures are observed at special workplaces such as cookeries, steel factories, and road buildings. Exposure of the general population to PAHs is mainly caused by environmental factors such as polluted air and water, by the consumption of smoked and grilled food, and by smoking of conventional (combustible) cigarettes (CC), respectively [1–6].

Over the past decade, several new nicotine and tobacco products have been introduced as alternatives to smoking with a potentially reduced health risk compared to CC. As many PAHs are carcinogenic due to their metabolic activation of DNA reactive compounds, the measurement of specific biomarkers is of great importance to assess the exposure to PAHs from potentially reduced-risk products.

For the determination of PAH exposure, usually, respective monohydroxylated urinary metabolites are analyzed by means of LC–MS/MS (liquid chromatography coupled with tandem mass spectrometry) or GC–MS (gas chromatography–mass spectrometry). For instance, 1-hydroxypyrene, monohydroxy-fluorenes, and monohydroxy-phenanthrenes are frequently determined in urine samples in order to investigate exposure to PAHs [7–10]. Benzo[*a*]pyrene (BaP, Figure 1) is classified as a Group 1 carcinogen (carcinogenic to

humans) by the International Agency for Research on Cancer (IARC) and is by now the best-studied PAH [3,11].
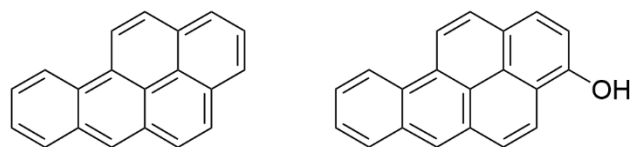
Figure 1. Chemical structure of benzo[*a*]pyrene (BaP, **left**) and its metabolite 3-hydroxy-benzo[*a*]pyrene (3-OH-BaP, **right**).

A key metabolite of BaP is (+)-anti-BaP-7,8-diol-9,10-epoxide (BPDE), which is considered as an ultimate carcinogen, reacting with cellular DNA, proteins, and glutathione. Furthermore, BPDE can react by enzymatic hydrolysis to form BaP-(7,8,9,10)-tetrol, which is excreted in the urine after conjugation with, e.g., glucuronic acid [11–15]. This biomarker, therefore, found use in studying exposure to BaP [16,17]. Very low concentration levels require laborious sample preparation to achieve sufficient sensitivity of the analytical methods, making routine analysis very challenging for this biomarker.

An alternative biomarker of BaP exposure is the monohydroxylated metabolite 3-hydroxybenzo[*a*]pyrene (3-OH-BaP, Figure 1), which is excreted in urine after conjugation. Nearly 100% of the urinary 3-OH-BaP detected in humans is excreted as glucuronide or sulfate [18].

Several methods have been developed and established for the determination of 3-OH-BaP in urine for occupationally exposed subjects [19–23]. However, those methods are limited by the lack of sensitivity to determine the burden of BaP exposure in the general population. In order to cover not only occupational but also environmental exposure, including cigarette smoking, sensitivity in the pg/L-range is required. This can be achieved by optimizing the sample preparation, including derivatization steps [24–27], purification and concentration procedures [28–30], or by application of different ionization techniques, such as atmospheric pressure laser ionization (APLI) [31]. Thus, many of these methods are hampered by complex analytical procedures and specific/expensive equipment that can only be used to a limited extent in larger cohorts of human biomonitoring campaigns and clinical studies, respectively.

The aims of the current study were to adjust and validate a sensitive and robust method for the quantitation of 3-OH-BaP in urine with a sufficiently high sample throughput. Further, the validated method was applied to urine samples collected in a controlled clinical trial [32] with 10 users per group of 5 different nicotine-containing products, including smokers of conventional cigarettes (CC), users of electronic cigarettes (EC), users of heated tobacco products (HTP), users of nicotine replacement therapy (NRT), users of oral tobacco (OT), and non-users (NU), in order to distinguish differences in the exposure from these products.

2. Materials and Methods

2.1. Chemicals

3-Hydroxybenzo[*a*]pyrene-O-β-glucuronide (3-OH-BaP-Gluc, molecular weight (MW): 444 g/mol), 3-OH-BaP (MW: 268 g/mol), and $^{13}C_6$-3-OH-BaP-Gluc were purchased from AptoChem (Montreal, QC, Canada). $^2H_{11}$-3-OH-BaP was purchased from TRC (Toronto, ON, Canada). N,N-dimetylethylamine (DMEA) was obtained from Alfa Aesar (Karlsruhe, Germany), 2-fluoro-methylpyridinium-p-toluenesulfonate (FMPT) from TCI (EsMVchborn, Germany), formic acid 99%, ULC/MS grade from Biosolve (Valkenswaard, The Netherlands), and acetic acid, ascorbic acid, dimethyl sulfoxide, hydrochloric acid 37%, and sodium hydroxide from Merck (Darmstadt, Germany). Dichloromethane and methanol for residue analysis and LC–MS grade acetonitrile and methanol were purchased from LGC Standards (Wesel, Germany). Water was purified by means of a Sartorius arium

water system (Göttingen, Germany). The enzyme β-glucuronidase/arylsulfatase from *Helix pomatia* (4.5 and 14 U/mL) was supplied by Roche (Mannheim, Germany).

2.2. Sample Work-Up for Quantification

For sample preparation, the work-up published previously [25] was applied with major modifications. Frozen urine samples were thawed slowly at room temperature. To homogenized urine (6 mL), acetate buffer (400 µL; 1 M, pH = 5.1) was added, and the pH-value of the sample was adjusted with hydrochloric acid (1 N) to pH 5.0–5.5 if necessary. Aliquots (100 µL) of an aqueous solution of the internal standard were added, containing an absolute amount of 10 pg $^{13}C_6$-3-OH-BaP-Gluc, followed by the addition of 100 µL of ascorbic acid solution in water (150 mg/mL). For enzymatic hydrolysis, β-glucuronidase/arylsulfatase from *Helix pomatia* (20 µL) was added, and the mixture was incubated overnight (~16–18 h) at 37 °C. After incubation, samples were centrifuged (3000 rpm, 10 min), and the supernatant was decanted into a new vessel and subjected to solid-phase extraction (SPE).

The SPE cartridges (Bond Elut-LMS, 200 mg, 3 mL; Agilent, Waldbronn, Germany) were conditioned with 3 mL of dichloromethane, 2 × 3 mL of methanol, and 3 mL of water. Subsequently, the hydrolyzed urine mixture was added to the column. The tubes were washed with 3 mL of water, 3 mL of water/methanol (50/50, v/v), 1 mL of methanol, and 2 mL of methanol/acetonitrile (50/50, v/v). The target compound and internal standard were eluted with 2 × 2 mL of dichloromethane in a 4 mL glass vial. To the eluate, dimethyl sulfoxide (20 µL) was added, and dichloromethane was evaporated in a SpeedVac centrifuge (Thermo Fisher, Dreieich, Germany) without heating to a final volume of 20 µL (containing only the dimethyl sulfoxide portion).

The residue was taken up in 250 µL of FMPT solution (0.5 mg/mL in acetonitrile) and 50 µL of DMEA (0.2% in acetonitrile). The mixture was homogenized with a vortex mixer, and derivatization of the hydroxyl group was achieved by incubation of the mixture for 20 min at 45 °C. Samples were transferred to a microvial (300 µL), and the solvent was evaporated in a SpeedVac centrifuge (Thermo Fisher, Dreieich, Germany) without heating to a final volume of 20 µL (containing only the dimethyl sulfoxide portion). The residue was reconstituted in 250 µL of methanol/water/formic acid (50/49/1, v/v/v) and homogenized with a vortex mixer. The extracts were analyzed by LC–MS/MS.

2.3. LC–MS/MS

An Agilent 1200 HPLC (Agilent, Waldbronn, Germany) was equipped with an Acquity UPLC BEH C18 column, 50 × 2.1 mm i.d., 1.7 µm (Waters, Eschborn, Germany) and coupled with a triple quadrupole mass spectrometer (API 5000; Sciex, Darmstadt, Germany). The injection volume was set to 15 µL. Chromatography was performed at a column temperature of 50 °C and at a flow rate of 0.6 mL/min. Solvent A (water with 0.5% formic acid) and solvent B (acetonitrile with 0.5% formic acid) were used for elution. The gradient was 0–1 min, 20% B; 1–7 min, 20–40% B; 7–8.5 min, 40% B; 8.5–10 min, 40–90% B; 10–13 min, 90% B; 13–13.1 min, 90–20% B; 13.1–15 min, 20% B. The ion source was operated in electrospray ionization (ESI)-positive mode. Nitrogen was used as the carrier gas. Source parameters were as follows: ion spray voltage, 5500 V; source temperature, 680 °C; entrance potential, 10 V; curtain gas, 30 psi; ion source gas 1, 50 psi; and ion source gas 2, 70 psi. MS measurements were performed by multiple reaction monitoring (MRM) mode. Detailed information for the MRM transitions and MS/MS parameters are summarized in Table 1. For controlling all modules and for data analysis, Analyst 1.5.2 software (Sciex) was used.

Table 1. Retention times, mass transitions, dwell time, declustering potentials (DP), collision energies (CE), and cell exit potentials (CXP) for 3-OH-BaP and $^{13}C_6$-3-OH-BaP.

Analyte or IS	Retention Time (min)	Mass Transitions (*m/z*)	Role	Dwell Time (msec)	DP (V)	CE (V)	CXP (V)
3-OH-BaP	6.9	360 → 251	Quantifier	150	161	45	18
3-OH-BaP	6.9	360 → 267	Qualifier	150	161	45	18
$^{13}C_6$-3-OH-BaP	6.9	366 → 257	IS	150	161	45	18

2.4. Calibration

To determine the concentration of 3-OH-BaP in urine, a calibration line was generated in non-smoker urine (analyte-free) by spiking increasing amounts of 3-OH-BaP-Gluc to receive concentrations between 50 and 3321 pg/L, based on free 3-OH-BaP, while the internal standard amount remained constant (10 pg $^{13}C_6$-3-OH-BaP-Gluc). Calibrators were worked up as described above and analyzed by LC–MS/MS. The calibration line equation was obtained by linear regression ($1/y$ weighting) of the area ratio (area counts of the analyte/area counts of the internal standard) and the spiked analyte concentration. The 3-OH-BaP concentration in human urine samples was then calculated from the area counts ratios between 3-OH-BaP and $^{13}C_6$-3-OH-BaP by employing the calibration line, equation with y being the area count ratio and x being nominal the 3-OH-BaP concentration.

2.5. Method Validation

The method was validated according to the US Food and Drug Administration (FDA) guideline [33]. The method was initially developed and validated using the free forms of both the reference and the internal standard for quantification. Hence, analyte-free non-smoker urine was spiked with free 3-OH-BaP in different concentration levels (low, medium, and high) to cover the entire calibration range. As internal standard, $^2H_{11}$-3-OH-BaP was used during the initial method validation. All working solutions of the analyte and the standard were freshly prepared before use. As a consequence of the stability investigations, the final method comprises the glucuronides 3-OH-BaP-Gluc and $^{13}C_6$-3-OH-BaP instead of the free forms, as discussed in the Section 3. Additionally, ascorbic acid was added to protect the free 3-OH-BaP formed in the urine samples during enzymatic hydrolysis.

To monitor the accuracy and the precision during study sample analysis, internal quality control samples (QCs) were prepared by spiking analyte-free non-smoker urine with known concentrations of 3-OH-BaP-Gluc. The QCs, covering the expected concentration range (QC low, QC medium, QC high), were randomly interspersed with the study samples (min. 5% of total sample size or at least two per level) during sample work-up and analysis. The acceptance criteria for the QCs were defined by accuracy of 85–115%. The target values were previously determined by analyzing six QCs per level.

Selectivity was verified for the MRM transitions of the analyte (quantifier and qualifier) and the corresponding internal standard. Samples of six different analyte-free non-smoker urines were compared with a blank sample containing only the reference compounds, prepared and analyzed under the same conditions. Each transition was screened for potential interferences that had the same retention times as the analyte or the internal standard signal. The same six samples were spiked with 400 pg/L 3-OH-BaP and analyzed for accuracy (85–115%) and precision (CV ≤ 15%).

The LLOQ (lower limit of quantification) was determined by analyzing five replicates of spiked non-smoker urine at the lowest concentration (50 pg/L), achieving a precision of at least 20% and an accuracy rate of 80–120%. The LOD (limit of detection) was then obtained by dividing the LLOQ by 3.

Accuracy and precision were determined by spiking non-smoker urine at different concentration levels (LLOQ, low, medium, and high). Inter-day accuracy and precision were determined by analyzing five spiked urine samples per level on three different

days. Intra-day accuracy and precision were obtained from the analysis of one day. The acceptance criteria for intra-day and inter-day precision were specified by the calculation of coefficients of variation (CVs), which should be below 15% and 20%, respectively, for concentrations below three times LLOQ. Accuracy rates should be in the range of 85–115% of nominal concentrations and 80–120% for concentrations below three times the LLOQ.

Recovery rates indicate analyte losses during sample work-up. The recovery rates were determined at three different concentration levels by comparing the analyte area of non-smoker urine samples spiked before sample work-up (N = 6) and after SPE extraction (N = 3) with free 3-OH-BaP. Samples spiked after SPE extraction correspond to 100% and served as reference.

The matrix effect (ME) was evaluated by comparing the signals of analyte and internal standard at two different concentration levels (low and high) of post-spiked (after SPE extraction) processed urine samples (N = 3) with a sample of the reference standards. The relative difference to the reference signals (100%) was defined as ME. Relative differences of >0% indicate a positive ME (signal enhancement), and relative differences <0% indicate a negative ME (signal suppression).

Carryover effects were analyzed by repeated injections (N = 3 × 5) of extracts spiked with high levels of the analyte (2000 pg/L) followed by the injection of a blank sample (MeOH). No carryover effects were detected when the signal of the blank sample was at or below the LOD signal.

The stability of the analyte (free 3-OH-BaP or 3-OH-BaP-Gluc) was determined at room temperature for 24 h (short-term stability), at 10 °C for 72 h in the autosampler (post-preparative stability), and below −20 °C (long-term stability). Moreover, six cycles of freeze–thaw stability and the storage stability of stock solutions were monitored. Stability monitoring was performed at two concentration levels (low and medium) in triplicates. Acceptable tolerances were 85–115% compared to the base level (time 0).

2.6. Human Study

The details of the study protocol for the controlled, single-center, and open-label clinical trial has been published previously [32]. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Medical Association Hamburg. The study population covered exclusive users of five different nicotine-containing products (CC, EC, HTP, NRT, and OT) and a control group of non-users of any nicotine-containing product (NU). Each group consisted of 10 subjects. Complete urine voids were collected over three days of inpatient stay and pooled to yield 12 h urine samples (12 h periods: from 6 p.m. to 7 a.m. and from 7 a.m. to 6 p.m.). The analysis of 3-OH-BaP comprised the 12 h urine samples on the last day (Day 3, U6 and U7 + 8) of the inpatient stay, as these samples were collected on the third day of confinement under controlled conditions (diet control, habit control), which is the longest time period of control within this study. Main characteristics (user group, sex, age, BMI, and 24 h urine volume of Day 3) of the study population are summarized in Table 2.

For data evaluation, creatinine levels were additionally determined using the Jaffé method [34]. Product use status was verified by the determination of urinary nicotine and its ten metabolites (=total nicotine equivalents, TNE) using SPE (96-well plates) and LC–MS/MS analysis (HILIC column) by modification of a previously published method [35] (Table S1).

2.7. Data Evaluation and Statistics

The statistical parameters were evaluated with Prism (GraphPad, Version 9.0.2, La Jolla, CA, USA). All 3-OH-BaP values below the LLOQ were set to LLOQ/2 (25 pg/L). The urinary 3-OH-BaP concentrations of 12 h urine samples were referred to pg 3-OH-BaP in 24 h (pg/24 h), calculated as concentration 1 × 12 h-urine volume 1 + concentration 2 × 12 h urine volume 2. In addition, analyte concentrations were normalized based on

the creatinine concentrations and reported as pg/g creatinine (urinary 3-OH-BaP concentration in pg/L divided by the respective creatinine concentration in g/L). Mean values, standard deviations, and median values were calculated for each user group. Statistical differences between the 3-OH-BaP concentration of smokers and the five other groups were determined by applying the non-parametric Mann–Whitney *U* test (p-value < 0.05). Statistical differences between the main characteristics of the user groups were determined by applying the non-parametric, one-way ANOVA test (Kruskal–Wallis; p-value < 0.05). Correlation of 3-OH-BaP levels of smokers and smoking-dose-related variables (number of cigarettes smoked per day (CPD) and TNE) were obtained by linear regression and evaluated by calculation of the Spearman correlation coefficient.

**Table 2.** Main characteristics of the study population.

User Groups [1]	N (m/f)	Age (Years)		BMI		24 h Urine Volume (mL)	
		Mean	±SD	Mean	±SD	Mean	±SD
CC	10 (6/4)	35.1	±9.1	26.0	±3.9	2891	±828
HTP	10 (6/4)	36.1	±12	25.5	±3.2	2685	±1300
OT	10 (9/1)	28.1	±8.2	25.9	±4.2	2638	±1290
EC	10 (6/4)	38.4	±14	23.5	±2.7	1627	±664
NRT	10 (5/5)	35.3	±15	25.5	±3.5	1602	±802
NU	10 (6/4)	32.9	±8.8	24.7	±3.2	2475	±936
∑ all	60 (38/22)	34.3	±11	25.2	±3.4	2320	±1090

[1] User groups: conventional cigarettes (CC), heated tobacco products (HTP), oral tobacco (OT), electronic cigarettes (EC), nicotine replacement therapy (NRT), and non-users (NU).

3. Results

3.1. Performance of the Analytical Method

An LC–MS/MS method published by Sarkar et al. [25] was used as a starting point, further optimized, and finally validated for the quantification of urinary 3-OH-BaP. Sample preparation included enzymatic hydrolysis with glucuronidase/arylsulfatase from *Helix pomatia*, SPE extraction, and derivatization of the hydroxyl group with FMPT (Figure 2). The extracts were then analyzed by LC–MS/MS.
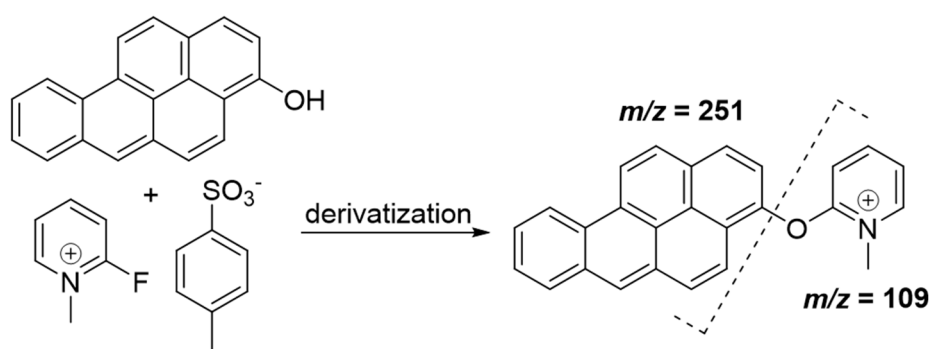
Figure 2. Derivatization of 3-OH-BaP with 2-fluoro-methylpyridinium-p-toluenesulfonate (FMPT).

The final method was validated according to FDA guidelines [33]. The method validation data are shown in Table 3.

Table 3. Method validation data for the quantification of 3-OH-BaP in urine.

Validation Parameter	Level	3-OH-BaP
LOD [1]		16.7 pg/L
LLOQ		50 pg/L
Calibration range		50–3221 pg/L
Precision, intra-day, N = 5		
	LLOQ: 50 pg/L	10.1% CV
	Low: 100 pg/L	12.0% CV
	Medium: 400 pg/L	12.3% CV
	High: 1600 pg/L	3.3% CV
Precision, inter-day, N = 3 × 5		
	LLOQ: 50 pg/L	7.9% CV
	Low: 100 pg/L	9.0% CV
	Medium: 400 pg/L	8.0% CV
	High: 1600 pg/L	5.8% CV
Accuracy, intra-day, N = 5		
	LLOQ: 50 pg/L	101.8%
	Low: 100 pg/L	105.1%
	Medium: 400 pg/L	94.0%
	High: 1600 pg/L	98.2%
Accuracy, inter-day, N = 3 × 5		
	LLOQ: 50 pg/L	105.8%
	Low: 100 pg/L	110.7%
	Medium: 400 pg/L	95.6%
	High: 1600 pg/L	99.6%
Recovery [2,3], N = 6		
	Low: 200 pg/L	121.3%
	Medium: 640 pg/L	108.9%
	High: 1600 pg/L	89.1%
Matrix effect [3], N = 3		
	Low: 200 pg/L	+31.4%
	High: 1600 pg/L	+43.3%
	Low: IS	+25.3%
	High: IS	+47.9%
Re-injection [3], N = 3 × 3		
	Low: 200 pg/L	5.0% CV
	Medium: 640 pg/L	4.6% CV

[1] LOD = LLOQ/3, [2] indicate losses during sample work-up; [3] validation experiments with initial method (cf. 2.5).

The selectivity was proven by analyzing six different analyte-free urine samples. No interfering signals at the same retention times as the analyte or internal standard MRM transitions were detected. Spiking the six samples with the analyte resulted in a mean accuracy of 86.4%.

The precision was evaluated by calculation of the relative standard deviation expressed as CVs, which should not exceed 15% CV (20% CV at LLOQ). Intra-day precision ranged from 3.3% to 12.3% for the different concentration levels. The CVs for the inter-day precision were between 5.8% and 9.0%. The determined intra-day accuracy rates for the LLOQ (101.8%), the low (105.1%), the medium (94.0%), and the high (98.2%) concentration level were within the acceptable range. Inter-day accuracy was also within the range.

For the quantification method, an LLOQ of 50 pg/L was confirmed by the analysis of five independent spiked urine samples on three consecutive days. The LOD was defined as LLOQ/3 and amounted to 16.7 pg/L, showing a signal-to-noise ratio of approximately five. A linear response was found for the calibration range of 50–3221 pg/L.

High recovery rates (89 to 121%) were obtained despite numerous steps in the sample work-up, including enzymatic hydrolysis, SPE extraction, and derivatization. A positive ME in the urine of +31% to +43% was observed for the derivate of 3-OH-BaP. The MEs were fully compensated by the IS. There was no significant carryover, evaluated by a blank sample injected after five consecutive injections of samples with high concentrations. The

post-preparative stability of the final extracts was proven in the autosampler at 10 °C for at least 72 h. The reproducibility of re-injection was analyzed by measuring samples with low and medium analyte concentrations in triplicates at three different time points, resulting in CVs of 5.0% and 4.6%, respectively.

Urine samples spiked at two concentration levels with free 3-OH-BaP were stored below −20 °C and analyzed after 1, 3, and 7 days. The accuracy decreased gradually from 106% and 74% on day 1 to 93% and 58% on day 7 for 200 pg/L and 640 pg/L, respectively. Apparently, free 3-OH-BaP was not stable in urine. Degradation was also observed for standard solutions of the analyte and the internal standard in their free form, and thus, fresh solutions needed to be prepared on the day of use. As an alternative for the less stable free 3-OH-BaP, the stability of the glucuronide (3-OH-BaP-Gluc) was investigated as well. 3-OH-BaP-Gluc proved to be stable in urine for at least 30 h at room temperature (short-term stability) and for at least 15 months when stored below −20 °C (long-term stability). The analyte in its conjugated form was stable through six freeze/thaw cycles in urine samples stored below −20 °C. The stock solution of the 3-OH-BaP-Gluc in water (c = 50 μg/mL) was stable for 3.3 years when stored below −20 °C. Consequently, 3-OH-BaP-Gluc was established for the preparation of QC material and for calibration. In analogy, the glucuronide $^{13}C_6$-3-OH-BaP-Gluc was used as an internal standard. Since no interferences were found in the MRM transition, $^{13}C_6$-3-OH-BaP-Gluc was established as IS in the final method to compensate losses during sample work-up. Exemplary MRM chromatograms of non-smoker urine, a QC sample at low concentration, and a smoker urine sample are illustrated in Figure 3.
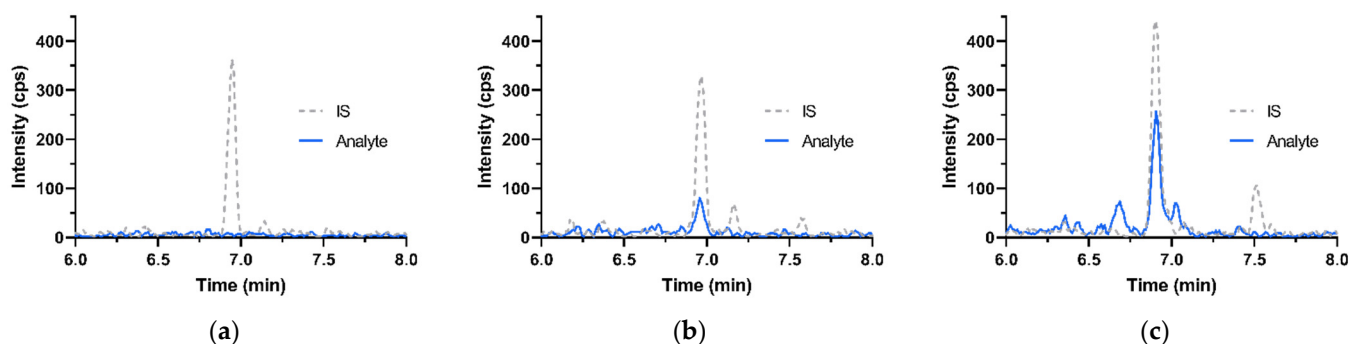

Figure 3. MRM chromatograms of the analyte 3-OH-BaP (*m/z* 360 → 251) and the internal standard $^{13}C_6$-3-OH-BaP (*m/z* 366 → 257). (**a**): Non-smoker urine sample (<LOD); (**b**): quality control sample with low concentration (c = 162 pg/L); (**c**): smoker urine sample (c = 470 pg/L).

3.2. Human Study—Urinary Excretion of 3-OH-BaP

The validated method was applied to urine samples from a controlled clinical study [32]. Each group consisted of 10 subjects, resulting in a total number of 60 subjects stratified by product use. The study population was assigned to one of the five groups of users of different nicotine-containing products (CC, EC, HTP, NRT, and OT) based on their product use or to the control group of NU. The main characteristics of the subjects are summarized in Table 2. The confined and diet-controlled clinical study was chosen to ascertain similar (low) exposure to BaP from sources other than product use such as diet or ambient air. Therefore, 3-OH-BaP was quantitated in the 12 h urine samples of the last study day, as this was the longest time period under controlled conditions. Group comparisons were performed based on the total amount of urinary 3-OH-BaP excreted over 24 h (pg/24 h), as summarized in Figure 4 and Table 4.
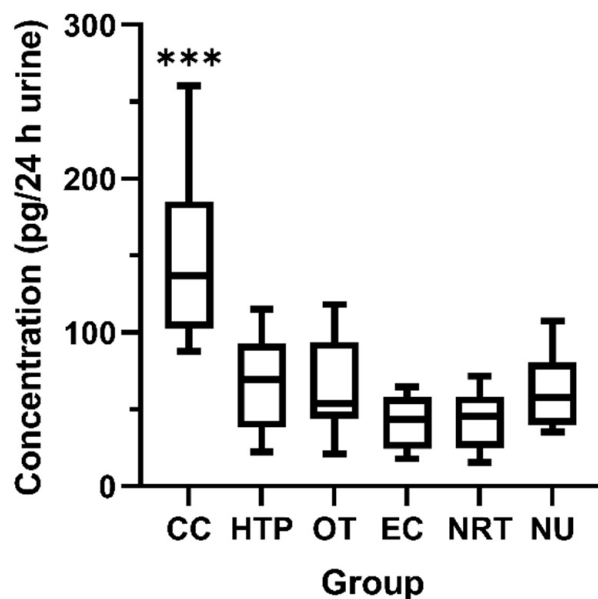
Figure 4. Box plots for urinary 3-OH-BaP excretion (pg/24 h) of six different user groups on Day 3. Boxes and lines represent the range of twenty-fifth/seventy-fifth percentile and the median value. The whiskers illustrate the minimum and maximum concentration. Differences between the smoking (CC) and all other groups were found to be significant when using the non-parametric Mann–Whitney *U* test (***: *p*-value < 0.002).

Table 4. Descriptive statistics for urinary 3-OH-BaP excretion (pg/24 h) of six different user groups.

| | 3-OH-BaP (pg/24 h) [1] | | | | | |
	CC	HTP	OT	EC	NRT	NU
Mean ± SD	149.0 ± 57.0	67.14 ± 32.6	65.96 ± 32.3	40.68 ± 16.6	43.31 ± 19.2	61.88 ± 23.4
Median	136.9	69.40	53.75	43.35	45.40	57.60
Min–max	87.70–260.3	22.30–115.1	21.10–118.0	17.90–64.60	15.30–71.50	35.40–107.3
<LLOQ, N (%) [2]	8 (40%)	20 (100%)	20 (100%)	20 (100%)	19 (95%)	20 (100%)

[1] Levels of 3-OH-BaP excreted within 24 h (N = 10 per group). Concentrations <LLOQ were set to LLOQ/2 (25 pg/L) for calculation of 12 h and 24 h excretion; [2] referred to the concentration in pg/L of 12 h urine samples (N = 20 per group).

The highest mean concentration of 149 pg/24 h was determined for smokers, with 60% of samples above the LLOQ in this group. In contrast, all samples from the other groups, including the NU, were not quantifiable, except for one 12 h urine sample in the NRT group. Mean values varied between groups due to differences in 12 h urine volumes (cf. Table 1). Urinary 3-OH-BaP excretion was significantly higher (*p*-value < 0.002) in smokers compared to all other groups (Figure 4).

3.3. Correlation of 3-OH-BaP with Smoking Specific Parameters

To investigate the specificity of 3-OH-BaP as biomarker of tobacco smoke exposure, urinary 3-OH-BaP levels were plotted against the smoking dose, as indicated by the number of cigarettes smoked per day (CPD) (Figure 5a) and the total nicotine equivalents (TNE) excreted in urine (Figure 5b). Linear regression showed a moderate correlation (of borderline significance) between the 3-OH-BaP concentrations and CPD (Spearman's *r* = 0.63) and a weak (statistically not significant) correlation between 3-OH-BaP concentrations and urinary TNE (Spearman's *r* = 0.52). A reason for the only moderate or weak correlation could be the relatively small sample number. Nevertheless, the positive correlation of 3-OH-BaP with these smoking dose parameters indicates that urinary 3-OH-BaP is a suitable biomarker to assess BaP exposure by cigarette smoking.

(**a**)

(**b**)
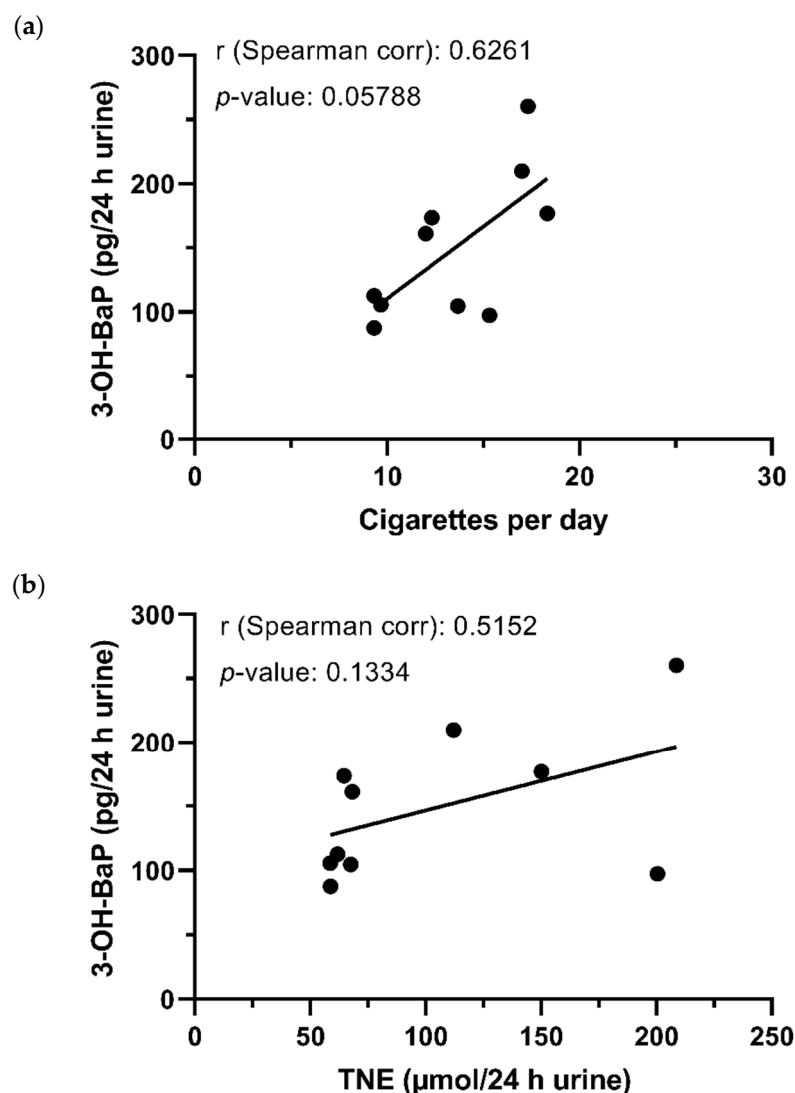
Figure 5. Spearman's correlation between urinary 3-OH-BaP concentrations and (**a**) number of cigarettes smoked per day (CPD) and (**b**) urinary total nicotine equivalents (TNE) of CC users on Day 3 (N = 10).

4. Discussion

4.1. Analytical Method

BaP exposure is most frequently investigated by analyzing the urinary metabolite 3-OH-BaP. Numerous methods have been reported for the quantification of this biomarker in occupationally exposed workers [19–22]. However, these methods generally lack sensitivity for the quantification of 3-OH-BaP in the non-occupationally exposed population. The purpose of the current work was to develop and validate a sensitive method for the quantification of trace amounts of urinary 3-OH-BaP in cohorts of non-occupationally exposed subjects, i.e., in clinical and epidemiological studies. The procedure described by Sarkar et al. [25] was used as a starting point and further modified with respect to the extraction procedure and use of conjugated standards in order to achieve the required sensitivity along with a sufficient sample throughput and robustness. One important improvement in our method in terms of repeatability and accuracy was the implementation of $^{13}C_6$-3-OH-BaP-Gluc as an internal standard. Using the glucuronides as standard and IS material proved to be superior to the unstable free analyte during method validation, calibration, and quality control procedures. In native urine samples, 3-OH-BaP was found to be present in its conjugated form in urine at almost 100% [18]; thus, instability of the

analyte would not present an issue in real samples. Another advantage would be that $^{13}C_6$-3-OH-BaP-Gluc could also compensate for losses during enzymatic hydrolysis.

Thus far, only a few methods have been described in the literature that are capable of quantifying BaP exposures in the low pg/L range besides Sarkar et al. [25] and our method. One other method has been published with a similar LLOQ of 50 pg/L by application of liquid chromatography–fluorescence detection (FD) and automated off-line solid-phase extraction [28,36]. While fluorescence detection achieved comparable sensitivity, our method is more specific and selective, applying MRM detection of several analyte-specific mass transitions. Further, LC–MS/MS methods were developed for the quantification of 3-OH-BaP, yielding higher LLOQs. Simon et al. published an automated column-switching high-performance liquid chromatography method for the determination of 3-OH-BaP in urine, yielding quantification limits of approximately 400 pg/L [22,29,30,37]. Several groups analyzed 3-OH-BaP by employing derivatization of the hydroxyl group with dansyl chloride and subsequent analysis by LC–MS/MS, resulting in LLOQs of 250 pg/L [26], 300 pg/L [24], and 580 pg/L [27]. Richter-Brockmann et al. followed a different approach by means of GC–MS using atmospheric pressure laser ionization (APLI). A higher sensitivity compared to our method was reported by the use of APLI, which apparently improved the ionization yields for the methyl ether of 3-OH-BaP with an LLOQ of 1.8 pg/L [31]. Additionally, ascorbic acid was added as an antioxidant before enzymatic cleavage to protect the resulting free 3-OH-BaP from oxidative decomposition during the following work-up and analysis—a procedure that has been established for other PAHs before [38]. Hence, we investigated the addition of ascorbic acid in our method as well. QC samples were analyzed with and without the addition of ascorbic acid, showing no differences in terms of accuracy, sensitivity, and specificity (data not shown). Since the overall variability appeared to be slightly improved by the addition of ascorbic acid, this procedure was implemented into the final method for validation of accuracy, precision, calibration range, and LLOQ and for analysis of the clinical study samples.

4.2. Human Study

The validated method was applied to urine samples of NUs and users of five different nicotine-containing products (CC, EC, NRT, HTP, and OT) who participated in a controlled clinical trial [32]. The aim was not only to investigate whether cigarette smokers and non-smokers differ but also whether cigarette smokers differ from other users of potentially reduced-risk products in terms of exposure to various toxicants, among them BaP. No significant differences were found in terms of the general study group characteristics for age, BMI, and urine volume between the different groups.

The group of CC smokers and NU differ significantly in terms of urinary excretion of 3-OH-BaP. With mean values of 149.0 pg/24 h urine (225.9 pg/g creatinine, Table S2) and 61.88 pg/24 h urine (90.73 pg/g creatinine, Table S2) in CC smokers and NUs, respectively, the determined values were in the same range as reported in the literature [25,28,30].

Barbeau et al. [28] analyzed urinary 3-OH-BaP in non-occupationally exposed non-smokers and smokers. They found an average concentration of 0.009 nmol/mol creatinine for non-smokers and 0.023 nmol/mol creatinine for smokers. These concentrations equaled 45 pg/24 h urine and 155 pg/24 h urine, respectively, assuming a mean 24 h urine volume of 1.5 L and a mean urinary creatinine concentration of 1.5 g/L [28]. The mean concentrations for non-smokers and smokers of 59 pg/24 h urine and 131 pg/24 h urine determined by Lafontaine et al. [30] were in the same range. Sarkar et al. [25] showed, through the analysis of various smoking-specific biomarkers, including 3-OH-BaP, a significant reduction in biomarker concentrations in the group that stopped consuming conventional cigarettes after the baseline of the study. The values at post-baseline were 155 pg/24 h urine for the continuous smoking group and 56 pg/24 h urine for the group that had completely quit using any tobacco product for eight days, which was comparable to non-smokers. In other recent studies analyzing 3-OH-BaP in the urine of non-smokers and smokers, higher concentrations were found in both groups. Concentrations in non-occupationally

subjects (N = 4–7) were found in a range of <LOD to 820 pg/L for non-smokers and of 320 to 2150 pg/L for smokers [26,27,31]. Richter-Brockmann et al. speculated that the addition of ascorbic acid may have led to higher concentrations due to improved stability of the analyte. However, our studies using QCs could not prove this hypothesis since comparable concentrations of 3-OH-BaP were observed for identical samples worked-up with and without the addition of ascorbic acid.

In contrast to most studies that investigated exposure in smokers and non-smokers only, Sarkar et al. studied 3-OH-BaP in subjects switching from CC to a snus product. They observed a reduction of about 45% in smokers eight days after switching to snus use, which was in the range of the cessation arm in this study, with a decrease of about 56% [25]. To the best of our knowledge, our clinical study was the first to analyze urinary 3-OH-BaP concentrations to distinguish between smokers and non-smokers, and additionally included four other groups of users of new generation nicotine/tobacco products, such as HTP, OT, EC, and NRT. The four other nicotine user groups could be clearly distinguished from the smokers in terms of their urinary 3-OH-BaP levels, which were indistinguishable from those of NU (99% < LLOQ). The somewhat lower concentrations of EC and NRT users can be explained by the normalization with the 24 h urine volume. It is important to emphasize that the urine volumes collected did not differ significantly between the different user groups (Table 2).

In addition to the number of CPD, urinary cotinine concentration or TNE are commonly used as a biomarker of exposure to nicotine products and have been used for the classification of smoking status. Richter-Brockmann et al. showed a positive correlation of urinary 3-OH-BaP concentration to CPD and cotinine, respectively ($R^2 = 0.88$ each) [31]. We could confirm the positive correlation between urinary 3-OH-BaP and smoking dose, measured as CPD or TNE in our study (Figure 5).

This study was performed under confined and diet-controlled conditions to reduce the influence of other sources for BaP exposure and to also ascertain compliance of single product use during the inpatient stay. In the clinical study, urine voids were collected over three days, which is regarded as a sufficient time period for the washout of 3-OH-BaP. This was evident, for example, when looking at the progression from Day 1 to Day 3 of a non-compliant NRT user (Figure 6), as identified by the observed CEMA (*N*-acetyl-S-(2-cyanoethyl)-L-cysteine) concentration (a biomarker of exposure to acrylonitrile), although reporting exclusive NRT use for the last three months. Smoking was identified as a major source of acrylonitrile exposure in several studies [39–41]. A CEMA cut-off between 0.4 and 0.7 μg/L was recently suggested [42]. The non-compliant subject in the NRT user group showed a CEMA concentration of 86 μg/L on Day 1 (U0) equal to 84 μg/12 h urine fraction, strongly indicating cigarette smoking before the study started.

The high 3-OH-BaP concentration (>200 pg/12 h urine) in the U0 fraction supports these findings. A downward trend was observed from Day 1 to Day 3, indicating that non-compliant behavior and other sources of BaP exposure could be excluded during the course of the study. Despite possible non-compliance and other BaP exposure sources, NUs and all other nicotine user groups were significantly distinguishable from CC smokers in the urine samples collected before the study started (U0) (*p*-value < 0.05, Table S3). These findings show that 3-OH-BaP is significantly elevated in smokers in an uncontrolled setting as well, emphasizing the suitability of 3-OH-BaP as a biomarker to discriminate cigarette smoking from other nicotine-containing products such as e-cigarettes, smokeless and oral tobacco, or heated tobacco products. Since only a small sample size of each user group (N = 10) was used here, these findings would need to be confirmed in larger cohorts.
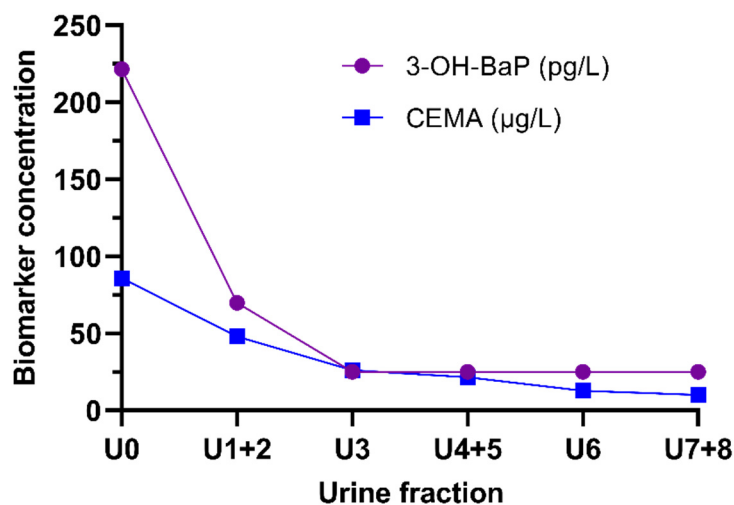
Figure 6. Progression of 3-OH-BaP (pg/L) and CEMA (μg/L) concentration of an NRT user over the three study days.

5. Conclusions

The new LC–MS/MS method is highly sensitive and allows for quantification of urinary 3-OH-BaP in cohorts of non-occupationally exposed subjects due to high throughput. Covering a broad, linear calibration range and an LLOQ of 50 pg/L, the actual method is suitable for the quantification of occupationally and non-occupationally exposed populations. Smokers can be differentiated from non-smokers as well as from users of new generation tobacco/nicotine and oral tobacco products. A moderate correlation between urinary 3-OH-BaP and the smoking dose was observed. Hence, 3-OH-BaP is a suitable biomarker to discriminate smokers from users of potentially reduced-risk products. The method is also suitable for assessing low exposures to BaP originating from diet and ambient air.

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