Determination of 3-Hydroxybenzo[a]pyrene Glucuronide/Sulfate Conjugates in Human Urine and Their Association with 8-Hydroxydeoxyguanosine

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ABSTRACT: While 3-hydroxybenzo[a]pyrene (3-OHBaP) is a preferable biomarker to assess human exposure to benzo[a]pyrene (BaP), a sensitive and simple method is lacking. In this study, a specific and sensitive method based on liquid chromatography coupled with electrospray tandem mass spectrometry (LC–ESI-MS/MS) was developed for direct analysis of 3-OHBP glcucuronide and sulfate conjugates in human urine samples without enzymatic hydrolysis. The limits of detection (LODs) were 0.06 ng L⁻¹ for BaP-3-sulfate (BaP-3-S) and 0.16 ng L⁻¹ for BaP-3-glucuronide (BaP-3-G), which showed high sensitivity. Both compounds showed excellent linearity (r² > 0.99) in the range of 0.01–10 μg L⁻¹ in the instrumental calibration. The absolute recoveries of the target analytes spiked in human urine for the entire analytical procedure were 68.3 ± 4.96% (mean ± SD) and 63.7 ± 5.47% for BaP-3-S and BaP-3-G, respectively. This method was applied to quantify BaP-3-G and BaP-3-S in 150 urine samples collected from healthy volunteers. The mean concentration of BaP-3-S was 0.67 ng g⁻¹ creatinine (<LOD to 10.20 ng g⁻¹ creatinine), about 10-fold lower than that of BaP-3-G (6.73 ng g⁻¹ creatinine, < LOD to 52.64 ng g⁻¹ creatinine). For comparison, we also detected the concentration of free 3-OHBP in 15 randomly selected samples without enzymatic hydrolysis and found at least >98% of 3-OHBP is excreted mainly in these two conjugated forms in human urine. A statistically significant positive association was observed between urinary 3-OHBP conjugates and urinary 8-OHdG levels (p < 0.001) in the general population. This study developed a sensitive and simple method to determine urinary glucuronide/sulfate conjugated BaP metabolites and for the first time found that BaP exposure associated with 8-OHdG levels in the general population.

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a large class of ubiquitous pollutants produced by incomplete combustion of organic materials in the environment, and humans are exposed to PAHs from a wide variety of sources. Benzo[a]pyrene (BaP), one of the most carcinogenic, teratogenic, and mutagenic PAHs, has been associated with an increased risk to humans (Group 1). Recent papers also showed excellent linearity (r² > 0.99) of < 0.01–10 μg L⁻¹ in the instrumental calibration. The absolute recoveries of the target analytes spiked in human urine for the entire analytical procedure were 68.3 ± 4.96% (mean ± SD) and 63.7 ± 5.47% for BaP-3-S and BaP-3-G, respectively. This method was applied to quantify BaP-3-G and BaP-3-S in 150 urine samples collected from healthy volunteers. The mean concentration of BaP-3-S was 0.67 ng g⁻¹ creatinine (<LOD to 10.20 ng g⁻¹ creatinine), about 10-fold lower than that of BaP-3-G (6.73 ng g⁻¹ creatinine, < LOD to 52.64 ng g⁻¹ creatinine). For comparison, we also detected the concentration of free 3-OHBP in 15 randomly selected samples without enzymatic hydrolysis and found at least >98% of 3-OHBP is excreted mainly in these two conjugated forms in human urine. A statistically significant positive association was observed between urinary 3-OHBP conjugates and urinary 8-OHdG levels (p < 0.001) in the general population. This study developed a sensitive and simple method to determine urinary glucuronide/sulfate conjugated BaP metabolites and for the first time found that BaP exposure associated with 8-OHdG levels in the general population.

BaP is an indirect carcinogen and requires metabolic activation to exert its carcinogenic and other adverse effects. Phase I reactions of BaP are catalyzed primarily by CYP1A and CYP1B and produce metabolites including epoxides, dihydrodiols, phenols, and quinones. Urinary 3-OHBSA, the main monohydroxylated BaP metabolite, has been proposed as a biomarker to assess human exposure to BaP, while previous studies have only occasionally applied it due to the lack of sensitive methods. Our lab developed a dansyl chloride (DNS) derivatization method for sensitive determination of 3-OHBP in human urine by liquid chromatography–electrospray tandem mass spectrometry (LC–ESI-MS/MS), but like most of the previous similar studies, this method still needed deconjugation of the glucuronide and sulfate with β-glucuronidase and aroylsulfatase and chemical derivatization. The conjugation reactions catalyzed by phase-II enzyme including glucuronidation and sulfation are the primary important detoxification pathways of BaP, which can reduce metabolism of phenolic metabolites to form more toxic quinones and diol-epoxides. Considering that 3-OHBP is excreted mainly in conjugated forms, it is reasonable that conjugated metabolites can potentially be used to assess BaP exposure if we have a method that can directly detect the trace conjugated metabolites in urine without a lengthy enzymatic deconjugation procedure prior to analysis. High-performance liquid chromatography (HPLC) with fluorescence detector has been used to quantify the BaP glucuronide and sulfate conjugates in smokers’ urine, but this method lacked sensitivity and high specificity for low concentration levels in complex urine matrix.

PAHs can be metabolized by cytochrome P450 (mainly CYP1A1) enzymes and aldo-keto reductases (AKRs) to generate active o-semiquinone anion radical, which is
CYP1A1 enzyme mediated via aryl hydrocarbon receptor (AhR), and subsequently enhancing the formation of ROS.\textsuperscript{27,28} Chemicals such as dioxins can also promote the induction of asthma, diabetes, and cancer.\textsuperscript{29,30} Previous studies have induced oxidative DNA damage and related-diseases like naphthalene, monohydric metabolites of low molecule PAHs including near e-waste recycling facilities.\textsuperscript{40} In all these studies, on their emission sources,\textsuperscript{42} using BaP metabolites is more reasonable for evaluating BaP-induced oxidative damage. However, no papers have reported the association between urinary 8-OHdG with urinary OH-

<table>
<thead>
<tr>
<th>target compounds</th>
<th>elemental composition</th>
<th>molecular weight</th>
<th>retention time (min)</th>
<th>transition monitored (m/z)</th>
<th>cone (V)</th>
<th>collision (V)</th>
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<tr>
<td>BaP-3-S</td>
<td>C\textsubscript{29}H\textsubscript{14}S\textsubscript{3}O \textsuperscript{-}</td>
<td>347.37</td>
<td>2.55</td>
<td>347.05 &gt; 239.0347.05 &gt; 267.0</td>
<td>2323</td>
<td>6919</td>
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<tr>
<td>BaP-3-G</td>
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<td>2.50</td>
<td>443.13 &gt; 267.0443.13 &gt; 174.9</td>
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<td>2.07</td>
<td>355.18 &gt; 144.7355.18 &gt; 275.2</td>
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</table>

known free radical intermediate and can generate reactive oxygen species (ROS) via redox cycling.\textsuperscript{5,6,26} Some coexisting chemicals such as dioxins can also promote the induction of CYP1A1 enzyme mediated via aryl hydrocarbon receptor (AhR), and subsequently enhancing the formation of ROS.\textsuperscript{27,28} The ROS can then cause oxidative damage of DNA and form 8-hydroxydeoxyguanosine (8-OHdG),\textsuperscript{25,26} As one of the predominant forms of oxidative lesions in DNA, 8-OHdG is a critical biomarker for oxidative DNA damage and has been widely used as a preferred biomarker when investigating PAHs-induced oxidative DNA damage and related-diseases like asthma, diabetes, and cancer.\textsuperscript{39,30} Previous studies have reported correlations of urinary 8-OHdG with urinary OH-PAHs in coke oven workers,\textsuperscript{51,52} general populations,\textsuperscript{33–35} school children,\textsuperscript{36–38} pregnant women,\textsuperscript{39} and people living near e-waste recycling facilities.\textsuperscript{40} In all these studies, monohydric metabolites of low molecule PAHs including naphthalene, fluorene, phenanthrene, and pyrene, especially 1-hydroxypyrene (1-OHPy), were used to assess the exposure of PAHs. Since different PAHs have different potencies to cause DNA oxidation,\textsuperscript{41} and the composition of PAHs is dependent on their emission sources,\textsuperscript{42} using BaP metabolites is more reasonable for evaluating BaP-induced oxidative damage. However, no papers have reported the association between BaP metabolites with 8-OHdG in the general population.

The objective of this study is to develop a specific and sensitive LC–MS/MS method for the direct determination of glucuronide and sulfate conjugated 3-OHBAp in urine from the general population. We also determined the unconjugated free 3-OHBAp to confirm that conjugated 3-OHBAp can reflect the total 3-OHBAp in human urine. Results indicated that 3-OHBAp conjugates could be detected in the urine of the general population, and the method developed in the present paper would promote the use of conjugated 3-OHBAp in exposure assessment of BaP. We further investigated the relationship between 3-OHBAp conjugates and urinary 8-OHdG, one of the major products of DNA oxidation, to explore BaP-induced oxidative damage in the general population.

2. MATERIALS AND METHODS

2.1. Chemicals and Materials. Standards of benzo(a)pyrene-3-sulfate potassium salt (BaP-3-S) and 1-hydroxy\textsuperscript{13}C\textsubscript{12} [benz[a]anthracene \textsuperscript{13}C\textsubscript{12}-1-OHBaA] were acquired from NCI Chemical Carcinogen Reference Standard Repository (Kansas, MI, USA), and \textsuperscript{13}C\textsubscript{12}-sodium estrone 3-sulfate sodium salt (DLE1–3-S) was obtained from Toronto Research Chemicals Inc. (North York, Canada). 8-OHdG (298%) was purchased from Sigma-Aldrich (St. Louis, US). Creatinine (99%) was purchased from J&K Scientific (Beijing, China). 8-Hydroxy-2'-deoxyguanosine\textsuperscript{13}C\textsubscript{12},\textsuperscript{15}N\textsubscript{2} (\textsuperscript{13}C\textsubscript{12}-\textsuperscript{15}N\textsubscript{2}-8-OHdG: chemical purity 98%; isotopic purity 99.9%) and creatinine-d\textsubscript{1} (chemical purity 98%; isotopic purity 99.9%) were from Toronto Research Chemicals Inc. (North York, Canada). Tetrabutylammonium hydroxide sulfate (TBA\textsuperscript{H2SO\textsubscript{4}}) was purchased from J&K Chemical Technology (Beijing, China), and ammonium hydroxide (28% \textsuperscript{4}NH\textsubscript{3}) was purchased from Alfa Aesar (Tianjin, China). Dansyl chloride (DNS) was obtained from Sigma-Aldrich (US). Oasis WAX (6 cm\textsuperscript{2}, 150 mg), Sep-Pak C18 (5 cm\textsuperscript{2}, 200 mg), and silica (6 cm\textsuperscript{2}, 1 g) solid phase extraction (SPE) cartridges were purchased from Waters (Milford, MA, USA). All solvents used in this study including dichloromethane (DCM), ethyl acetate (EA), n-hexane (Hex), methyl tert-butyl ether (MTBE), acetoneitrile (ACN), and methanol (MeOH) were HPLC grade and purchased from Fisher Chemicals (New Jersey, USA). Water obtained from a Milli-Q Synthesis water purification system (Millipore, Bedford, MA, USA) was used throughout the study.

2.2. Collection of Human Urine Samples. Human urine samples (volume \textasciitilde 25 mL, n = 150) were collected from the healthy volunteers (nonsmokers without occupational exposure) and were immediately transferred into 40 mL amber glass tubes and kept at \textasciitilde 20 °C until analysis. Institutional Review Board (IRB) approvals (IRB00001052–12058) were obtained from the Peking University Institutional Review Board Office (PKUIRB) for the collection and analysis of the human urine samples.

2.3. Preparation of Human Urine Sample. For the analysis of 3-OHBAp conjugates, each human urine sample of 10 mL was mixed with 100 \mu L of 2 ng mL\textsuperscript{-1} (0.2 ng) internal standard (DLE1–3-S) and 1 mL of 1 M TBA\textsuperscript{H2SO\textsubscript{4}}, then extracted with 10 mL of MTBE. The mixture was shaken for 20 min and repeated in triplicate. Finally, a total of 30 mL of MTBE was collected and then concentrated to dryness by rotary evaporation. The residue was redissolved in 10 mL of 1 M sodium acetate buffer at pH 5. An Oasis WAX SPE cartridge was preconditioned sequentially by 5 mL of MeOH, 5 mL of H\textsubscript{2}O, and 5 mL of 1 M sodium acetate buffer (pH = 5). The urine sample was loaded onto the cartridge followed by 1 mL of H\textsubscript{2}O washing the urine tube. The cartridge was then washed with 5 mL of 1 M sodium acetate buffer (pH = 5) and 5 mL of water. 3-OHBAp conjugates were eluted with 6 mL of 2% ammonium hydroxide in a MeOH/ACN (2:8, v/v) mixture. The eluate was evaporated under a gentle nitrogen stream to around 100 \mu L, and 5 \mu L was injected into the UPLC–ESI-MS/MS system.

A method reported previously was used for the quantitation of free 3-OHBAp.\textsuperscript{37} Briefly, 15 mL of urine was mixed with 0.10 ng of \textsuperscript{13}C\textsubscript{12}-OH-BaA as internal standard and then enriched using C18 SPE cartridges. After a derivatization reaction with DNS and further cleanup with silica SPE cartridges, the dansylated 3-OH-BaP was analyzed by LC–ESI-MS/MS.

For the analysis of 8-OHdG, 0.2 mL of urine was diluted by adding 0.8 mL of Milli-Q water, 5 ng of labeled internal standard \textsuperscript{13}C\textsubscript{12}-8-OHdG was added, and then the mixture was analyzed by LC–MS/MS.\textsuperscript{43}

For the analysis of creatinine, 10 \mu L of urine was diluted 10 000-fold with Milli-Q water followed by the addition of 800 ng of creatinine-d\textsubscript{4}. Creatinine was analyzed using LC–MS/MS in electrospray positive ionization mode, and the multiple reaction monitoring (MRM) transitions monitored were 114 > 44 for creatinine and 117 > 47 for creatinine-d\textsubscript{4}.\textsuperscript{44}

2.4. UHPLC–ESI-MS/MS Analysis. The Waters ACQUITY UHPLC system (Waters, Milford, MA, USA) coupled with a Waters ACQUITY UPLC HSS C18 column (2.1 × 100 mm\textsuperscript{2}, 1.7 \mu m particle size) was used for the chromatographic separation of BaP-3-S and BaP-3-G. The column temperature was kept at 40 °C. A gradient elution was performed at a flow rate of 0.3 mL min\textsuperscript{-1} using MeOH (mobile phase A) and 0.1% ammonium hydroxide in H\textsubscript{2}O (mobile phase B). The initial composition of 10% A was increased to 50% in 1 min, then increased to 100% at 4 min and maintained for 1 min, then increased to 100% at 4 min and maintained for 1 min.
Figure 1. MS/MS spectra of 3-benzo[a]pyrene-β-D-glucopyranosiduronic acid (BaP-3-G) and benzo[a]pyrene-3-sulfate salt (BaP-3-S).

3. RESULTS AND DISCUSSION

3.1. Method Development and Optimization. It has been demonstrated that ion pairing with TBA’HSO$_4^-$ can enable the extraction of BaP-3-G and BaP-3-S from human urine by chloroform. In the present study, ion-pair liquid-liquid extraction (LLE) with TBA’HSO$_4^-$ was also adopted, and the extraction efficiency was further optimized by comparing different organic solvents including chloroform, DCM, EA, MTBE, and Hex. Since MTBE obtained the highest recoveries for both glucuronide and sulfate-conjugated metabolites of BaP, it was selected in the subsequent experiment. Since there are strong matrix effects when directly injected the LLE extracts of urine samples into LC-MS/MS, additional cleanup steps are necessary to eliminate residuary interferences. Considering that BaP-G and BaP-S are both acidic (pK$_a$ = 1 for the sulfate conjugated metabolite and 5 for the glucuronide conjugated metabolite), Waters Oasis MAX (150 mg, 6 cc) and Oasis WAX (150 mg, 6 cc) cartridges were tested for their cleanup efficiency. Formic acid (2%) in methanol and 2% NH$_4$OH in 20:80 (v/v) MeOH/ACN were used to elute the target chemicals from the polymeric sorbent MAX and WAX cartridges, respectively. We found that WAX was able to effectively reduce the interferences of urine samples. To assess potential matrix effects on urinary BaP conjugates, urine extracts from ion-pair LLE and WAX cartridge cleanup were spiked with standards of BaP-3-G (50 ng L$^{-1}$) and BaP-3-S (10 ng L$^{-1}$).

Both of the two target compounds were examined in the ESI negative ion mode. The collision-induced dissociation (CID) spectrum of the deprotonated molecule obtained from BaP-3-G was governed by cleavage of the C–O bond and loss of the sugar moiety, thus yielding the most predominant product ion at m/z 267 [M–H–C$_5$H$_8$O$_6$(176)$^-$], and the product ions at m/z 175 and 113 in the spectrum corresponded to fragment ions of glucuronide. For BaP-3-S, the most abundant product ion was m/z 267 [M–H–SO$_3$(80)$^-$] followed by the ion at m/z 239 [M–H–SO$_3$(80) – CO(28)$^-$]. Therefore, the product ion at m/z 267 was used in the quantitative selective reaction monitoring (SRM) detection of BaP-3-G and BaP-3-S, and the product ions at m/z 175 and m/z 239 were used in qualitative identification, respectively (Figure 1).

A C18 column was used to separate BaP-G and BaP-S. To promote ionization to the [M–H]$^-$ form in negative ion electrospray ionization mass spectrometry, 0.1% ammonia followed by a decrease to 10% A and held for 2 min to allow for equilibration.

Mass spectrometric analysis was performed using a Waters Xevo TQ-S quadrupole instrument detector equipped with an electrospray ionization source (negative ion mode) (Waters, USA). The two target analytes were analyzed in multiple reaction monitoring (MRM) mode in two different scan channels (Table 1). The MS/MS conditions were as follows: source temperature and desolvation temperature were maintained at 110 and 350 °C, respectively; capillary voltage was set as 3.50 kV; desolvation gas flow and cone gas flow were kept at 800 L h$^{-1}$ and 50 L h$^{-1}$; multiplier was 650 V. Masslynx V4.1 software package was used for data acquisition and integration.

2.5. Quantitation and Data Analysis. 3-OHBaP conjugates were identified by comparing the retention time (within 2%) and the ratio (within 20%) of the two selected fragment ions in MRM mode with those of standards. Since corresponding isotopically labeled standards of BaP-3-G and BaP-3-S were not commercially available, DLE1–3-S was used as surrogate internal standard in this study to automatically correct for the recovery of analytes during sample preparation as well as the matrix interference effects and the instrument fluctuation.

All glass apparatus were disposable or rinsed with MeOH to avoid cross contamination between samples, and water blanks were analyzed along with the real samples as negative control to monitor potential sample contamination. Since the target analytes can be detected in the urine samples, we calculated the recoveries (n = 3) by subtracting the background concentrations in nonspiked samples from standard spiked samples at a spiking gradient (1, 5, and 10 ng L$^{-1}$) for BaP-3-G and 5, 25, and 50 ng L$^{-1}$ for BaP-3-S). The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the peak signal-to-noise ratio of real samples and on a minimal value of 3 and 10, respectively. The matrix interference will affect signal chromatographic separation and mass spectrum signal, so we evaluated the extent of signal suppression or enhancement by spiking standards of BaP-3-S (10 ng L$^{-1}$) and BaP-3-G (50 ng L$^{-1}$) into the concentrated urine extracts. The matrix effect observed for BaP-3-G/S was calculated using the percentage of signal intensity change between spiked and unspiked samples versus the signal of the same concentration standard in MeOH.

2.6. Statistics. For correlation and regression analysis, the R$^2$ value was obtained, and a p-value < 0.05 was considered to be statistically significant. If the target analytes were not detected in all samples, the 1/2 LOD was applied as a substitute value for statistical analysis when necessary. SPSS software package 17.0 (SPSS Inc., Chicago, IL, CA) and Excel 2013 were used for all the statistical analyses in this study.
hydroxide in H$_2$O was used as the aqueous mobile phase. MeOH and acetonitrile were tested as organic mobile phases. MeOH was finally selected for chromatographic separation since higher signal-to-noise ratios and better peak shape were achieved at the same concentration.

### 3.2. Method Validation

3-OHBaP conjugates were identified by comparing the retention time (within 2%) and the ratio (within 20%) of the two selected fragment ions (347.05 > 267.0, 239.0 for BaP-3-S and 443.13 > 267.0, 174.9 for BaP-3-G) in MRM mode with those of standards. MeOH was injected as blank sample after the highest concentration standard in calibration curve. The carry-over was lower than 5% of the low limit of quantification for the target compounds and the internal standard. Procedural blanks containing pure water were analyzed along with the urine samples as negative controls to check potential interferences between samples or laboratory contamination. None of the target analytes was found in the water blanks.

The method LODs and LOQs calculated by signal-to-noise ratio were 0.16 ng L$^{-1}$ and 0.53 ng L$^{-1}$ for BaP-3-G, and 0.06 ng L$^{-1}$ and 0.19 ng L$^{-1}$ for BaP-3-S, showing similar sensitivity to that obtained (0.1 ng L$^{-1}$ for LOD and 0.3 ng L$^{-1}$ for LOQ) by DNS derivatization of 3-OHBP combined with LC–MS/MS. However, the newly developed method in this study can directly determine BaP metabolites without hydrolysis and derivatization, which provides a chance to assess human BaP exposure in large-scale studies. Standard curves containing 0.005 or 0.01 to 10 μg L$^{-1}$ standards of the two target compounds were made to verify the instrumental calibration, which showed excellent linearity ($r^2 = 0.9994$ for BaP-3-S and $r^2 = 0.9985$ for BaP-3-G).

Accuracy was assessed on samples spiked with known amounts of analytes (5, 25, and 50 ng L$^{-1}$ for BaP-3-G and 1, 5, and 10 ng L$^{-1}$ for BaP-3-S). The percents of determined value to the nominal concentration were 84.9%, 82.2%, and 91.2% for BaP-3-G at 5, 25, and 50 ng L$^{-1}$, and 80.2%, 87.7%, and 106.9% for BaP-3-S at 1, 5, and 10 ng L$^{-1}$. The repeatability (in terms of intravial and intervial) was investigated to evaluate the precision of the method. The intravial repeatability was determined by repeated quantitation ($n = 6$) of one spiked sample (1 ng L$^{-1}$ for BaP-3-S and 5 ng L$^{-1}$ for BaP-3-G). The interval comparisons were determined by simultaneously measuring the same spiked sample (1 ng L$^{-1}$ for BaP-3-S and 5 ng L$^{-1}$ for BaP-3-G) in six different vials. The intravial relative standard deviations (RSDs) of the signal intensity for BaP-3-S and BaP-3-G were 7.83% and 5.61%, and the interval RSDs were 9.17% and 11.32%, respectively, which indicated an acceptable precision.

To assess the short-term and long-term freeze and thaw stability of the 3-OHBP conjugates, we re-injected the samples after 1 week and 1 year storage at −20 °C, and the peak area of each compound was compared with the original injection. The relative variations of signal intensities of BaP-3-G and BaP-3-S after freezer storage and thaw were within 10% ($n = 6$), which indicated that monohydroxy conjugates of BaP were comparatively stable. The stabilities of the stock solution and working solutions of the analytes and internal standard were also assessed by re-injecting the chemical standard solutions in MeOH, which were stored at −20 °C. The RSDs of the signal intensity between the stock solution and working solutions were lower than 6% ($n = 6$).

The matrix effects observed for each analyte were calculated using the percentage of peak area change in the urine matrix spiked with chemical standard versus the peak area of the same concentration in MeOH, and the signal suppression was less than 12%.

Prior to the analysis of real samples, a recovery test was conducted by spiking of each target analyte at a concentration gradient into the urine matrices, with subsequent passage through the entire analytical procedure. After subtracting the background concentrations in nonspinked urine from those in spiked urine, the absolute recoveries (mean ± SD) in the urine samples were 63.7 ± 5.47%, 69.1 ± 7.04%, and 70.5 ± 3.44% for BaP-3-G at 5, 25, and 50 ng L$^{-1}$, and 68.3 ± 4.96%, 75.3 ± 13.1%, and 74.3 ± 5.94% for BaP-3-S at 1, 5, and 10 ng L$^{-1}$ (Table 2), respectively. The recovery of the surrogate standard (DLE1–3-S) was 73.3 ± 6.1% ($n = 3$) at the concentration of 20 ng L$^{-1}$ in urine. Thus, the newly developed analytical procedure was more rapid, highly sensitive, and specific for the analysis of 3-OHBP conjugates in human urine.

3.3. Urine Concentrations of BaP Conjugated Metabolites. The detection frequency was 92% (138/150) for BaP-3-G and 83% (124/150) for BaP-3-S. Figure 2 shows the typical LC–MS/MS chromatograms of BaP-3-G and BaP-3-S in human urine samples. The mean concentration of BaP-3-G was 6.73 ng g$^{-1}$ creatinine (<LOD: 52.64 ng g$^{-1}$ creatinine), which was about 10-fold higher than that (0.67 ng g$^{-1}$ creatinine, < LOD: 10.20 ng g$^{-1}$ creatinine) of BaP-3-S. This paper for the first time reported the concentration of BaP-3-G in human urine. Only one paper reported the mass–volume concentrations of BaP-3-S in two urine samples of smokers, and the mean concentration of BaP-3-S detected in the general population in this study (1.69 ng L$^{-1}$) was much lower than those (67.3 and 42.1 ng L$^{-1}$) in the urine of smokers. In the 15 randomly selected samples, the concentrations of BaP-3-G were <LOD: 7.91 ng L$^{-1}$, which was about nine-fold higher than those (<LOD: 7.69 ng L$^{-1}$) of BaP-3-S (Table 3). Glucuronide- or sulfate-conjugated OH-BaP are excreted from the human body through urine and feces together with some amount of free OH-BaPs. To understand the distribution of BaP-3-G, BaP-3-S, and unconjugated free 3-OHBP in original human urine, we also analyzed the unconjugated free 3-OHBP in these 15 urine samples without enzyme hydrolysis using the DNS derivatization LC–MS/MS method reported previously. The mean concentration of free 3-OHBP was 0.19 ng L$^{-1}$ (0.0007 nmol L$^{-1}$), which accounted for only 1.16% of the total concentration of the three forms of total 3-OHBP (Figure 4). At least >98% of 3-OHBP is excreted mainly in conjugated forms in human urine, indicating that BaP-3-G plus BaP-3-S can well indicate the total 3-OHBP.

Table 2. Method Limits of Detection (LODs), Limits of Quantification (LOQs), and Absolute Recoveries ($n = 3$) Spiked with Standards at Low Concentration Levels

<table>
<thead>
<tr>
<th>chemicals</th>
<th>LOD (ng L$^{-1}$)</th>
<th>LOQ (ng L$^{-1}$)</th>
<th>recoveries</th>
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<tr>
<td>BaP-3-S</td>
<td>0.06</td>
<td>0.19</td>
<td>68.3 ± 4.96%</td>
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<td>BaP-3-G</td>
<td>0.16</td>
<td>0.53</td>
<td>63.7 ± 5.47%</td>
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<tr>
<td>DLE1–3-S</td>
<td>73.3 ± 6.10%</td>
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</table>

*Data were presented as mean ± standard deviation.

(DLE1–3-S) was 73.3 ± 6.1% ($n = 3$) at the concentration of 20 ng L$^{-1}$ in urine. Thus, the newly developed analytical procedure was more rapid, highly sensitive, and specific for the analysis of 3-OHBP conjugates in human urine.
confirmed that >98% of total 3-OHB[a]P detected in human urine came from 3-OHB[a]P conjugates, and therefore, 3-OHB[a]P conjugates should have the function to indicate BaP exposure.

BaP-3-G and BaP-3-S were all derivatives of 3-OHB[a]P, and 3-OHB[a]P was their common precursor. Theoretically, one subject with high 3-OHB[a]P level should have high levels of BaP-3-G and BaP-3-S accordingly. Considering glucuronidation and sulfation may have individual differences, we investigated correlation between them. The result showed that BaP-3-G concentration linearly correlated with that of BaP-3-S ($r^2 = 0.645, p < 0.005$) due to their inevitable inherent relationship (Figure 3).

### 3.4. Association of Conjugated Metabolites of BaP with 8-OHdG.

The mean and median urinary concentrations of 8-OHdG in 150 healthy volunteers were 2.06 and 1.21 μg

<table>
<thead>
<tr>
<th>samples</th>
<th>BaP-3-G</th>
<th>BaP-3-S</th>
<th>3-OH-BaP</th>
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<td>0.08</td>
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Figure 3. Scatter plot of correlation between urinary glucuronide- and sulfate-conjugated metabolites levels in the general population ($n = 150$).
g⁻¹ creatinine, respectively, which were lower than the mean concentration (6.02 µg g⁻¹ creatinine) and median concentration (3.13 µg g⁻¹ creatinine) of 8-OHdG in the general U.S. population in Michigan and Texas during 2005–2009 and the median concentration in the Japanese preschool children (4.45 µg g⁻¹ creatinine). A statistically significant positive correlation was observed between conjugated metabolites of BaP and in-transformed urinary 8-OHdG levels (p < .0001) (Figure 5), suggesting that BaP exposure would associate with oxidative DNA damage. Although in vitro studies have confirmed that exposure to BaP could significantly increase 8-OHdG in human cells⁴⁷,⁴⁸ and it was well reported that metabolites of the other PAHs like 1-OHPy associated with 8-OHdG in urine samples, this paper for the first time observed the significant relationship between 3-OHBaP conjugates and 8-OHdG and filled the gaps in the research of BaP-induced oxidative damage in the general human population.

4. CONCLUSION

Overall, we developed a LC–MS/MS method for the direct and sensitive determination of both BaP-3-G and BaP-3-S in urine without time-consuming hydrolysis and derivatization. BaP-3-G and BaP-3-S were for the first time detected in general human urine samples and accounted for >98% of total 3-OHBaP excreted in human urine. A statistically significant positive association was observed between urinary 3-OHBaP conjugates and 8-OHdG levels. This study provided a method to use BaP monohydroxy conjugates to assess BaP exposure in general population and made it possible to understand the BaP-induced adverse effect.

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Notes
The authors declare no competing financial interest.

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