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Identification and Quantification of in Vivo Metabolites of 9,10-Phenanthrenequinone in Human Urine Associated with Producing Reactive Oxygen Species

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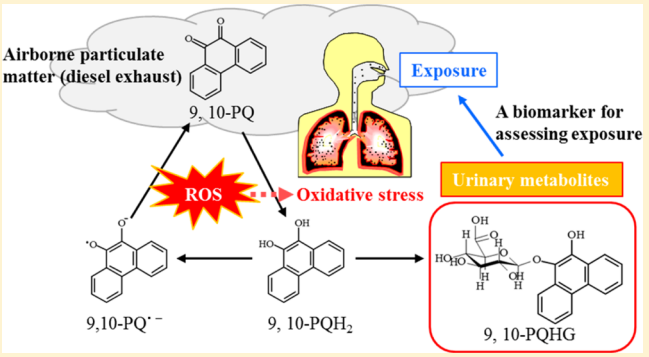
Supporting Information

ABSTRACT: Polycyclic aromatic hydrocarbon quinones (PAHQs) are components in airborne particulate matter (PM) and generate reactive oxygen species (ROS) in a redox cycling process. 9,10-Phenanthrenequinone (9,10-PQ) is a PAHQ found in diesel exhaust particulates and PM. When inhaled, it produces much more ROS than other PAHQs. We hypothesized that urinary metabolites of 9,10-PQ could serve as biomarkers of PAHQ exposure. Here, we describe methods for pretreating urine samples and analyzing 9,10-PQ metabolites by liquid chromatography with tandem mass spectrometry (LC−MS/MS). In urine from rats intraperitoneally injected with 9,10-PQ, the monoglucuronide of 9,10-dihydroxyphenanthrene (9,10-PQHG) was found to be a major metabolite of 9,10-PQ. 9,10-PQHG was also identified in the urine of a nonoccupationally exposed human by its retention time and MS/MS spectra. Furthermore, the urine contained hardly any free (unmetabolized) 9,10-PQ, but treating it with hydrolytic enzymes released 9,10-PQ from conjugated metabolites such as 9,10-PQHG. The concentrations of 9,10-PQHG in urine samples from nonoccupationally exposed subjects who lived in a suburban area were 2.04−19.08 nmol/mol creatinine. This study is the first to demonstrate the presence of 9,10-PQHG in human urine. Determination of urinary 9,10-PQHG should be useful for determining 9,10-PQ exposure.



INTRODUCTION

Airborne particulate matter (PM) released from combustion sources such as diesel exhaust is an important pollutant in urban atmospheres. PM is suspected to be a causative factor in several diseases, such as allergies,[1] respiratory diseases,[2,3] and cardiovascular diseases,[4] all of which are considered to be oxidative-stress-related disorders.[5−7] Oxidative stress in an organism arises from excessive generation of reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide, and hydroxyl radical or from depletion of antioxidants.[8] The production of ROS can be induced by both endogenous and exogenous factors.[9] Endogenous factors include physiological processes, such as oxidative phosphorylation, P-450 metabolism, peroxisomes, and inflammatory cell activation. Exogenous factors include environmental sources such as smoking, diet, and pollution.[10] ROS are mostly scavenged by antioxidative enzymes such as superoxide dismutase (SOD) or antioxidative compounds such as glutathione. However, excessive production of ROS may cause oxidative damage to nucleic acids, proteins, and lipids.[7,8] Oxidative damage is one of the causes of aging, respiratory disease, and cancer.[9,11]

Polycyclic aromatic hydrocarbon quinones (PAHQs) have been identified as a PM component and generate ROS in a redox-cycling process. One PAHQ, 9,10-phenanthrenequinone (9,10-PQ), has been identified in diesel exhaust particulates (DEP)[12,13] and PM.[12,14−17] In human A549 cells, 9,10-PQ, one of the *ortho*-PAHQs, causes massive overproduction of ROS and cytotoxicity compared to other *para*-PAHQs such as 1,4-PQ.[18] 9,10-PQ appears to be a good chemical marker for ROS production associated with PM, and exposure to PAHQs such as 9,10-PQ may cause adverse health effects by the generation of ROS,[19] although their levels in human are unknown.

9,10-PQ is formed by the combustion of fossil fuels,[13,16] by photooxidation of phenanthrene (Phe),[15] and by reactions of Phe with radicals in the atmosphere.[20] 9,10-PQ is a major PAHQ in DEP or PM. The concentrations of 9,10-PQ in DEP

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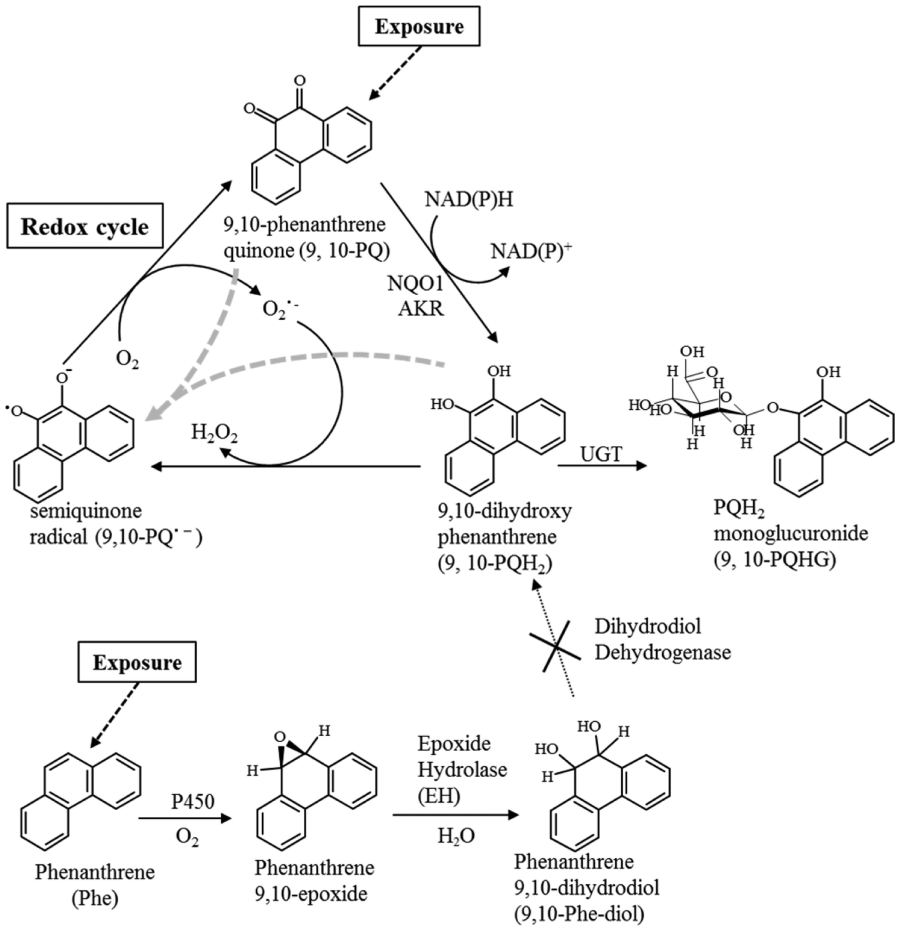

Figure 1. Metabolic pathways of 9,10-PQ and Phe to 9,10-PQHG.

and standard reference material (SRM) 1649a (urban dust) are 24.19 ± 2.11 and 1.18 ± 0.13 μg/g, respectively.[12] The levels of 9,10-PQ found in PM samples were 5−730 pg/m^3 in California,[12] 22.7−311 pg/m^3 in Southern California,[17] 100−1980 pg/m^3 in Los Angeles,[15] 630−1200 pg/m^3 in Athens,[14] and 138−690 pg/m^3 in Nagasaki.[16] 9,10-PQ is formed by gas-phase reactions of Phe with ozone, hydroxyl radical, and nitrate radicals.[20] In Los Angeles, 90% of the 9,10-PQ was formed by secondary reactions during transport.[15]

The existence of 9,10-PQ in the atmosphere indicates that humans inhale it. The metabolic pathway of 9,10-PQ plays an important role in the production of ROS, which are responsible for inducing DEP toxicity.[21,22] 9,10-PQ is metabolized to 9,10-dihydroxyphenanthrene (9,10-PQH$_2$) by NAD(P)H quinone oxidoreductase (NQO1) and aldo-keto reductase (AKR) through two-electron reduction (Figure 1).[23−25] 9,10-PQH$_2$ interacts with 9,10-PQ through a disproportionation reaction to yield the semiquinone radical (9,10-PQ$^{\bullet-}$), which then reacts with molecular oxygen to produce 9,10-PQ and superoxide ($O_2^{\bullet-}$). 9,10-PQH$_2$ is oxidized spontaneously or by the interaction with $O_2^{\bullet-}$ to produce 9,10-PQ$^{\bullet-}$ and hydrogen peroxide. 9,10-PQ$^{\bullet-}$ is readily converted to 9,10-PQ followed by the reduction of 9,10-PQ to 9,10-PQH$_2$. This oxidation−reduction reaction cycle (redox cycle) contributes to the toxicity of 9,10-PQ following the generation of a large amount of ROS. Because SOD eliminates ROS, SOD inhibits the oxidation of 9,10-PQH$_2$. In vitro, once 9,10-PQH$_2$ is formed by two-electron reduction, its conjugation by UGTs could terminate redox cycling of the quinone, and the monoglucuronide of 9,10-PQH$_2$ (9,10-PQHG) is transported into extracellular space.[25] When inhaled, Phe is oxidized to its epoxide by P-450s (CYP) and then metabolized to the *trans*-dihydrodiol by epoxide hydrolase. However, there is no evidence that dihydrodiol dehydrogenases oxidize phenanthrene 9,10-dihydrodiol to 9,10-PQH$_2$ in the redox cycle.[26]

Biomarkers have become a valuable tool for assessing human exposure to environmental contaminants.[27] Exposure to PAHQs through inhalation can be measured with biomarkers in biological samples such as urine or blood. Potential biomarkers of PAHQs are urinary metabolites of 9,10-PQ, especially 9,10-PQHG, although a method for detecting such metabolites has not yet been developed. The biomarker approach should result in the surveillance for exposure to 9,10-PQ.

Here, we describe a sensitive and specific analytical procedure for the determination of 9,10-PQHG in human urine using liquid chromatography with tandem mass spectrometry (LC−MS/MS). We identified and quantified 9,10-PQHG in urine samples from an intraperitoneally (i.p.) 9,10-PQ-administrated rat and from healthy and nonoccupationally exposed human subjects.

MATERIALS AND METHODS

Materials. All reagents were of analytical grade and used without any further purification. 9,10-PQ was purchased from Sigma (St. Louis, MO, USA). 9,10-PQHG and 1-hydroxypyrene glucuronide-d_9 (1-OHP-d_9-G) were synthesized enzymatically from 9,10-PQ and 1-OHP-d_9, respectively, using methods from previous studies.[25,28] 9,10-

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PQ-d_8 was synthesized from phenanthrene-d_8 using a modification of the method in the previous study.[29] β-Glucuronidase/aryl sulfatase, type H-2 from *Helix pomatia* (β-glucuronidase activity 98 000 units/mL and aryl sulfatase activity 1080 units/mL), β-glucuronidase, type VII-A from *Escherichia coli* (β-glucuronidase activity: 5 292 000 units/g), and aryl sulfatase, type V from *Patella vulgata* (aryl sulfatase activity: 37 units/mg), were from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile was obtained from Kanto Chemical (Tokyo, Japan), and water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Ammonia solution (28%, ultrapure) was from Kanto Chemical (Tokyo, Japan). Other chemicals were from Wako (Osaka, Japan).

Analysis of 9,10-PQHG by LC−MS/MS. The Agilent 1100 series LC system consisted of a G1379A degasser, a G1312A binary pump, a G1367A autosampler, and a G1316A column oven (all from Agilent Technologies; Palo Alto, CA). For identification and quantification of 9,10-PQHG in rat and human urine, the separation was performed on an XBridge C18 column (150 × 2.1 mm i.d., 3.5 μm, Waters) with a guard column of XBridge C18 (20 × 2.1 mm i.d., 5 μm, Waters). All of the column temperatures were kept at 30 °C. A gradient elution using 0.01% NH_4OH in water (eluent A) and 0.01% NH_4OH in methanol (eluent B) was carried out (B, 10−50% liner gradient for 20 min, 90% isocratic for 20−30 min) at a flow rate of 0.2 mL/min.

The mass spectrometric analyses were performed using an API 4000 Q-Trap tandem mass spectrometer (Applied Biosystems; Foster City, CA) equipped with an electrospray ionization (ESI) interface and operated in a negative ion mode. The spray voltage was maintained at −4.5 kV. Nitrogen gas was used as the collision gas and curtain gas, whereas zero grade air was used as the nebulizer gas and heater gas, with the optimum values set, respectively, at 7, 20, 30, and 70 (arbitrary values). The source temperature was set at 600 °C. The mass spectrometer was operated under selective reaction monitoring (SRM) mode, and the monitored precursor (Q1) and product (Q3) ions were m/z 385 → 209 for 9,10-PQHG and m/z 402 → 226 for 1-OHP-d_9-G (an internal standard for 9,10-PQHG analysis) with dwell times of 1000 ms. The collision energy and the declustering potential were set at −45 eV and −75 V, respectively. The unit mass resolution was used for both Q1 and Q3 mass analyzers. The structures of 9,10-PQHG in human urine were elucidated using the enhanced product ion (EPI) scan mode in which the product ions are trapped in Q3 (in trap mode) before mass analysis. The EPI scan rate was 1000 amu/s, and the scan range was 100−400 amu. Analyst software (version 1.4, Applied Biosystems) was used to control the LC−MS/MS system and to acquire and process the data.

Analysis of 9,10-PQ by LC−MS/MS. Chromatographic separation of 9,10-PQ in the urine samples was performed on an Inertsil ODS-P (250 mm × 4.6 mm i.d., 5 μm, GL Sciences, Japan). The column temperature was kept at 30 °C. A gradient elution using 5 mM ammonium acetate in water (eluent A) and methanol (eluent B) was carried out (B, 60−90% liner gradient for 30 min) at a flow rate of 0.5 mL/min.

The mass spectrometric analyses were performed using the tandem mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) interface and operated in a positive ion mode. The spray voltage was maintained at −4.5 kV. Nitrogen gas was used as the collision gas and curtain gas, whereas zero grade air was used as the nebulizer gas and heater gas, with the optimum values set, respectively, at 7, 20, 30, and 70 (arbitrary values). The source temperature was set at 600 °C. The mass spectrometer was operated under SRM mode, and the monitored precursor (Q1) and product (Q3) ions were m/z 209 → 181 for 9,10-PQ and m/z 217 → 189 for 9,10-PQ-d_8 (an internal standard for 9,10-PQ analysis) with dwell times of 1000 ms. The collision energy and the declustering potential were set at 35 eV and 60 V, respectively. The unit mass resolution was used for both Q1 and Q3 mass analyzers. The structures of 9,10-PQ in human urine were elucidated using the EPI scan mode under the same conditions as 9,10-PQHG.

Pretreatment of Urine Samples for 9,10-PQHG Analysis. Human urine (50 mL) was adjusted to pH 2.0 with phosphoric acid, spiked with internal standard (1-OHP-d_9-G), and loaded onto an OASIS MAX Plus cartridge (225 mg, 60 μm Waters) that was primed with 5 mL of methanol and 10 mL of water. The cartridge was sequentially washed with 10 mL of 4.8% NH_4OH, 10 mL of methanol, 10 mL of methanol/water (60:20, v/v) containing 3.8% HCOOH, and 10 mL of acetonitrile/water (20:80, v/v) containing 3.8% HCOOH. The trapped metabolite was eluted with 10 mL of methanol containing 3.8% HCOOH, and the extract was evaporated to dryness. The residue was redissolved in 50 μL of methanol, and an aliquot (5 μL) of the solution was injected into the LC−MS/MS system. To obtain complete mass spectra to confirm identification of the metabolites in human urine, 1.8 L of a pooled urine sample from a smoking subject was treated with the same above procedure on a large scale. Finally, the residue from the 1.8 L of urine was redissolved in 360 μL of methanol.

Determination of 9,10-PQ in Hydrolyzed Urine Samples. Fifty milliliters of human urine was adjusted to pH 5.0 with 2.5 mL of 4 M acetate buffer (pH 5.0) and 1 M hydrochloric acid solution. To hydrolyze the conjugated metabolites, the solution was incubated with β-glucuronidase (4720 units)/aryl sulfatase (54 units), type H-2 from *Helix pomatia*, at 37 °C for 4 h. β-glucuronidase (4851 units), type VII-A from *Escherichia coli*, and aryl sulfatase (49 units), type V from *Patella vulgata*, were also used for the identification of the conjugate type. After the deuterated internal standard corresponding to 2.2 ng of 9,10-PQ-d_8 was added to the hydrolyzed urine samples, the solution was then loaded onto an Oasis HLB cartridge (225 mg, Waters) that was primed with 5 mL of methanol and 10 mL of water. The cartridge was sequentially washed with 10 mL water and 10 mL of acetonitrile/water (20:80, v/v). The trapped metabolite was eluted with 8 mL of ethyl acetate and was evaporated to dryness. The residue was redissolved in 250 μL of dichloromethane. The analytes were isolated using a normal-phase HPLC (Inertsil SIL 100A, 250 mm × 10 mm i.d., 5 μm, GL Sciences, eluted with dichloromethane at 4.0 mL/min), and the fraction was evaporated to dryness. The residue was redissolved in 50 μL of methanol, and an aliquot (5 μL) of the solution was injected into the LC−MS/MS system. To identify 9,10-PQ derived from 9,10-PQHG in human urine samples, the urine extract obtained from the pretreatment for 9,10-PQHG analysis was dissolved in 3 mL of 4 M acetate buffer (pH 5.0) and was hydrolyzed with β-glucuronidase (472 units)/aryl sulfatase (5.4 units) at 37 °C for 4 h. The reactant was treated according to the pretreatment method for 9,10-PQ analysis.

Quality Assurance/Quality Control (QA/QC). The two analytical methods for 9,10-PQHG and 9,10-PQ in human urine were examined for QA/QC. Intraday (within day) precision and accuracy were determined by replicate analysis of a pooled urine sample without enzymatic hydrolysis. The unhydrolyzed pooled urine gave concentrations of 9,10-PQ below the quantification limits. The urine samples were spiked with 40, 100, and 200 pM 9,10-PQHG or 10, 21, and 52 pM 9,10-PQ. The sample concentrations were quantified from the peak area ratio of the analytes to the corresponding deuterated internal standards.

Animal Study. Male SD rats weighing about 190 g were adapted to the environment for 1 week, and blank urine samples were collected using metabolic cages prior to the intraperitoneal administration of 9,10-PQ. A rat were intraperitoneally administered with 50 mg/kg (body weight) 9,10-PQ suspended in corn oil. Another control rat was dosed only with 1.86 mL of corn oil. Food and water were provided ad libitum. Urine was collected using metabolic cages at the following periods: 0 to 24 h and 24 to 48 h after administration. The distilled water to wash the collecting route for urine in the cages was also pooled together with each sample. These pooled samples were stored at −20 °C until analysis. The urine samples were diluted with water to 20 mL, and 1 mL of the diluted samples was pretreated for 9,10-PQHG analysis as described above. To measure the total of conjugated 9,10-PQ (the sum of 9,10-PQHG and the sulfate of 9,10-PQH_2), 1 mL of diluted urine was adjusted to pH 5.0 with acetate buffer, hydrolyzed with β-glucuronidase/aryl sulfatase, type H-2, and concentrated by the SPE method. The unconjugated (unmetabolized) 9,10-PQ was quantified in the unhydrolyzed urine.

Human Studies. Urine samples were obtained from 16 non-occupationally exposed subjects (age range, 22−40; gender, 13 males

and 3 females) who were students or staff of Kanazawa University and who lived in the suburban area of Kanazawa. Thirteen were nonsmokers, and three were smokers. Human urine samples were collected in a polyethylene bottle and stored at −20 °C until analysis. To compensate for fluctuations resulting from diuresis, the urinary concentrations of 9,10-PQHG were normalized to the urinary creatinine concentration (nmol/mol creatinine). The concentration of urinary creatinine was determined for each sample using a colorimetric method by an autoanalyzer.

RESULTS

Development of LC−MS/MS Method and Urine Sample Preparation. To optimize the detection of 9,10-PQHG, ESI mass spectrum of the standard compound was obtained in negative ion mode. The major peak in the mass spectra of 9,10-PQHG corresponded to the deprotonated molecular ions $[M - H]^-$, m/z 385.1 (data not shown). An EPI scan gave information about the fragmentation pattern of the $[M - H]^-$ ion, and the main product ions of 9,10-PQHG were m/z 209.2 $[M - H - 176]^-$ (Figure 2A). 1-OHP-d_9-G as an internal standard indicated the same fragmentation pattern as 9,10-PQHG. To optimize the detection of 9,10-PQ, APCI mass spectrum of the standard compound was obtained in positive ion mode. The major peak in the mass spectrum of 9,10-PQ corresponded to the protonated molecular ions $[M + H]^+$, m/z 209.1 (data not shown). The $[M - H]^+$ ion of 9,10-PQ was fragmented to m/z 181.1 $[M - CO]^+$ and m/z 153.0 $[M - CO_2]^+$ as product ions (Figure 2B). According to the results, the observed precursor and product ions were chosen for SRM modes for 9,10-PQHG and 9,10-PQ analyses.

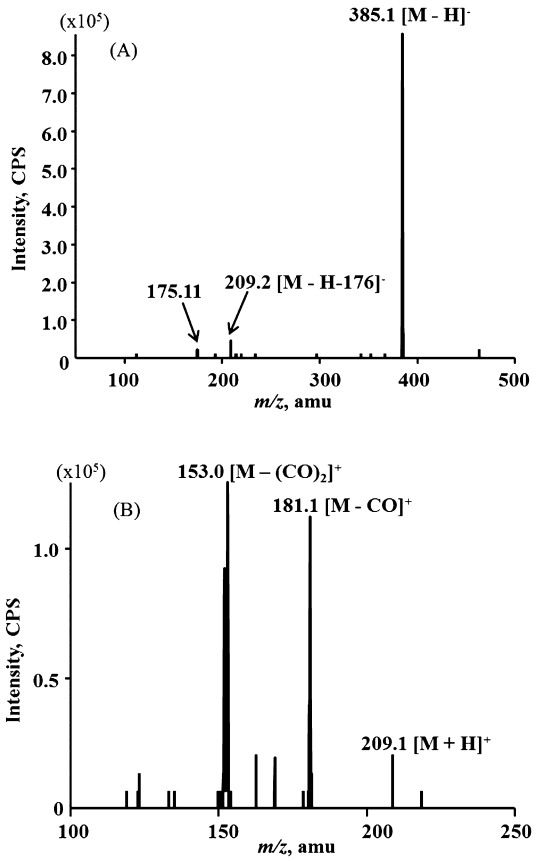

Figure 2. MS/MS (EPI) spectra of the (A) $[M - H]^-$ ion of standard 9,10-PQHG and (B) $[M + H]^+$ ion of standard 9,10-PQ. The spectra of (A) 9,10-PQHG and (B) 9,10-PQ obtained from the standards correspond to 50 pmol/injection, respectively.

Identification and Quantitation of 9,10-PQHG and 9,10-PQ in Rat Urine after i.p. Administration of 9,10-PQ. Typical SRM chromatograms and MS/MS spectra of $[M - H]^-$ ions for 9,10-PQHG and 9,10-PQ excreted in a rat urine at 0−24 h after i.p. administration of 9,10-PQ are shown in Figures S1 and S2 in the Supporting Information. The retention time of the peak from the rat urine was consistent with that of the 9,10-PQHG standard (Figure S1A,C). The peaks corresponding to 9,10-PQ were observed in the hydrolyzed and unhydrolyzed rat urine samples (Figure S2C,E). The hydrolysis of the metabolite conjugates increased the intensity of the 9,10-PQ peak. The fragmentations on MS/MS spectra for both peaks observed in the rat urine samples were also consistent with those for the standards (Figures S1B,D and S2B,D). These results indicate that 9,10-PQHG and unmetabolized 9,10-PQ were present in the rat urine. The levels of 9,10-PQHG and 9,10-PQ in the urine samples of the control were below the detection limits. The amount of 9,10-PQHG excreted into the urine during 0−48 h after the administration was 0.174 mg (Table 1), namely, 1.9% of the administrated 9,10-PQ was metabolized to 9,10-PQHG via 9,10-PQH_2 and then excreted into urine. The sum of unmetabolized 9,10-PQ and the conjugates in the urine was 0.294 mg, accounting for 3.2% of administered 9,10-PQ. Conjugated 9,10-PQ (defined as the sum of the glucuronide and sulfate of 9,10-PQH_2) was taken as the increase of 9,10-PQ following enzymatic hydrolysis and was found to be 0.204 mg. 9,10-PQHG (0.174 mg) accounted for 85% of the total conjugate, whereas the sulfate accounted for the remaining 15%. Consequently, the results indicate that 9,10-PQHG was a major conjugate in the urine.

Identification of 9,10-PQHG in Human Urine. SRM chromatograms of a sample and a spiked sample prepared from 50 mL of human urine are shown in Figure 3B, and SRM chromatograms of 1-OHP-d_9-G (internal standard) are shown in Figure 3E. The spiked standard to the urine sample increased the intensity of the peak at the retention time of 9,10-PQHG (Figure 3C). The physiological components of the urine did not interfere with the identification and quantification of the analyte in the chromatograms. To obtain complete MS/MS spectra with adequate sensitivity, we extracted the analyte from a large volume (1.8 L) of human urine. MS/MS spectra from the urine sample are shown in Figure 4. The fragment ions m/z 385.1 $[M - H]^-$ and 209.1 $[M - H - 176]^-$ for 9,10-PQHG were consistent with those for the standard (Figures 2A and 4A). The concentrated 9,10-PQHG from human urine was enzymatically hydrolyzed to 9,10-PQH_2. The 9,10-PQH_2 was then autoxidized to 9,10-PQ, as shown by the MS/MS spectrum (Figure 4B) and its retention time. The fragment ions m/z 181.1 $[M - CO]^+$ and 153.0 $[M - CO_2]^+$ from the protonated 9,10-PQ ion were consistent with the fragment ions from the standard (Figures 2B). 9,10-PQ in the unhydrolyzed urine samples was not observed in the chromatogram. These results indicate that 9,10-PQHG was present in the urine of healthy control subjects and that 9,10-PQHG was generated via metabolism of 9,10-PQ.

Calibration Curve and Validation. The calibration curve for the 9,10-PQHG standard was linear at concentrations of 5 fmol to 1 pmol/injection (correlation coefficient, $r^2 > 0.999$). The slopes of these calibration curves were almost identical to those of the working curves obtained by adding standards of

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Table 1. Concentrations of 9,10-PQHG and 9,10-PQ in a Rat Urine Sample after i.p. Administration of 9,10-PQ

	9,10-PQHG (mg)	unconjugated 9,10-PQ (mg)[a]	conjugated 9,10-PQ (mg)[b]
collection interval 0−24 h	0.142	0.063	0.176
collection interval 24−48 h	0.032	0.027	0.028
total	0.174	0.090	0.204

[a]Unconjugated 9,10-PQ is defined as the unmetabolized 9,10-PQ excreted in the urine. [b]Conjugated 9,10-PQ is defined as the increased 9,10-PQ amount after the enzymatic hydrolysis, that is, it is equal to the difference between the amount of 9,10-PQ in the hydrolyzed urine and the unconjugated 9,10-PQ.

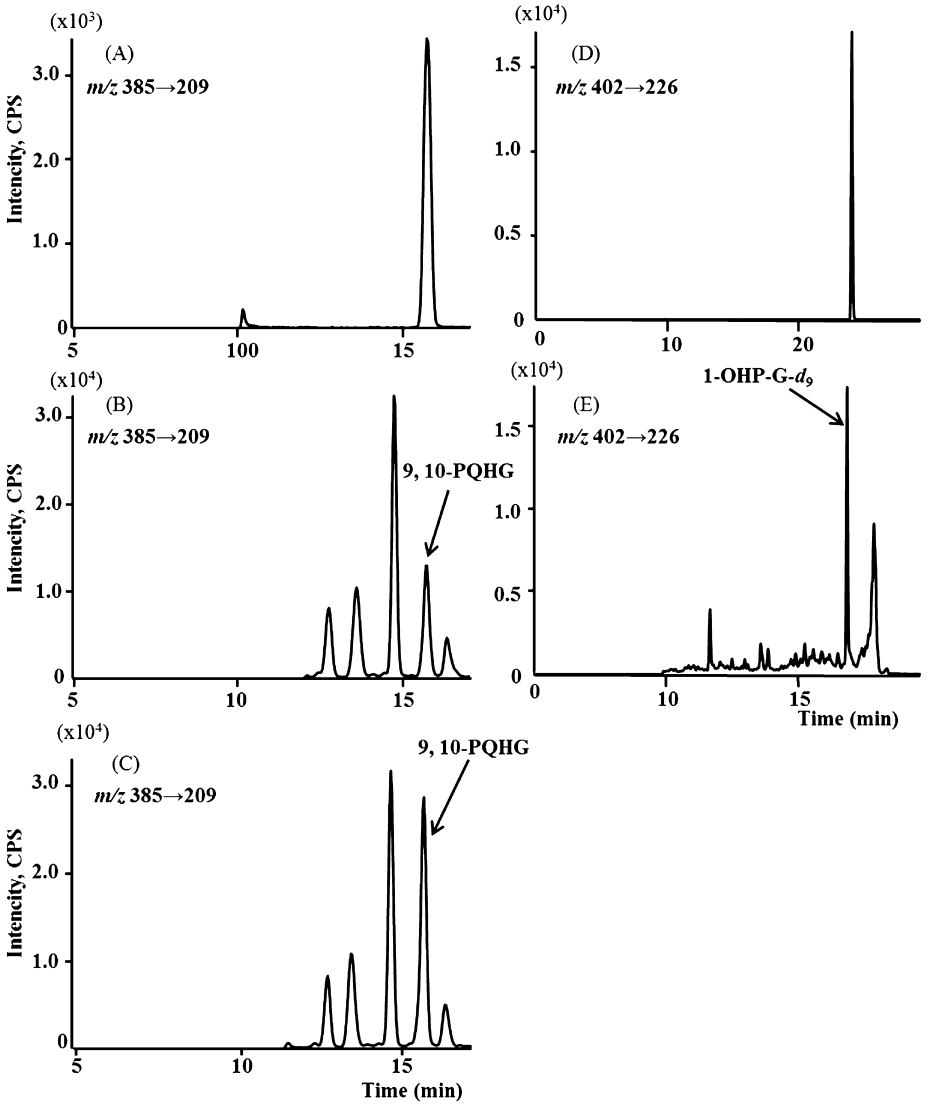

Figure 3. Representative SRM chromatograms of standard and human urine samples. (A) 9,10-PQHG standard, (B) human urine, (C) human urine spiked with 4 pmol 9,10-PQHG, (D) 1-OHP-d_9-G (internal standard), and (E) 1-OHP-d_9-G in the urine sample.

9,10-PQHG into human urine. The slopes of the working curves were 0.0233 ± 0.0019 (mean ± SD, n = 6). The instrumental detection limit of 9,10-PQHG was 1.2 fmol/injection (S/N = 3). The precision and accuracy of urinary 9,10-PQHG determination with the developed system were examined by adding three different known amounts of 9,10-PQHG to a human urine sample. The relative standard deviations (RSD, %) of the precision study (n = 5) were in the range of 1.7−6.2 for the urine samples spiked at the concentrations of 40, 100, and 200 pM 9,10-PQHG (Table 2). The accuracy values of the study (n = 5) were in the range of 92−106%. These values indicate that the proposed method is satisfactory for determining 9,10-PQHG in human urine.

However, the quantitative evaluation for the analysis of 9,10-PQ in the hydrolyzed urine samples was also examined. The calibration curve for the 9,10-PQ standard was linear at concentrations of 50 fmol to 250 pmol/injection (r^2 > 0.997). The slopes of these calibration curves were almost identical to those of the working curves obtained by adding standards of 9,10-PQ into human urine. The slopes of the working curves were 0.0064 ± 0.0024 (mean ± SD, n = 4). The instrumental detection limit of 9,10-PQ was 5.8 fmol/injection (S/N = 3). The precision and accuracy of urinary 9,10-PQ determination

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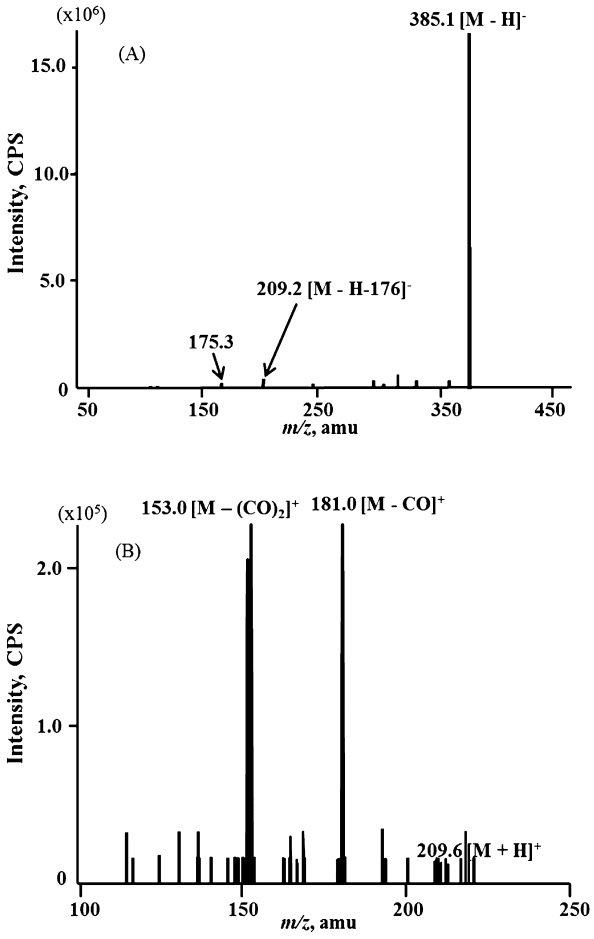

Figure 4. MS/MS (EPI) spectra of the $[M - H]^-$ ion in the same analysis triggered by the SRM peaks of (A) 9,10-PQHG from 1.8 L of a human urine and (B) 9,10-PQ from the hydrolyzed urine.

with the developed system were examined by adding three different known amounts of 9,10-PQHG to a human urine sample. The results are summarized in Table S1. The relative standard deviations (RSD, %) of the precision study (n = 5) were in the range of 2.7−5.8 for the urine samples spiked at the concentrations of 50, 100, and 250 pM 9,10-PQ. The accuracy values of the study (n = 5) were in the range of 106−113%. These values indicate that the proposed method is satisfactory for determining 9,10-PQ in human urine.

Quantification of 9,10-PQHG in Human Urine Sample. Figure 5 shows the distribution of glucuronide and sulfate of the 9,10-PQ metabolite in human urine (n = 5). The amount of 9,10-PQ detected after the hydrolysis of the urine samples with the enzyme (type H-2), that is, the sum of the glucuronide and sulfate, was taken as 100%. β-glucuronidase-labile metabolites accounted for 73 ± 10% (mean ± SD), whereas sulfates contributed 26 ± 7% of the total. This proportion of the

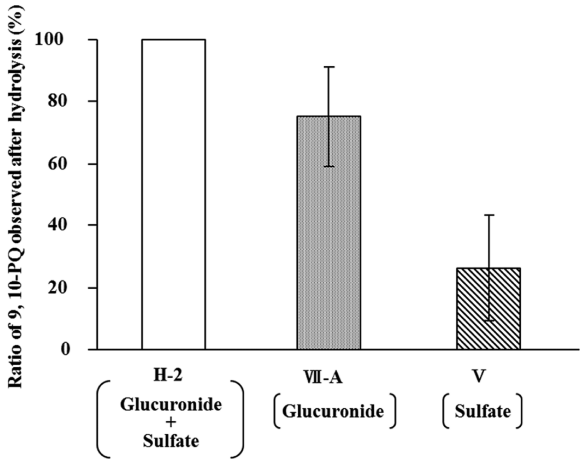

Figure 5. Distribution of the conjugates of the 9,10-PQ metabolite in human urine based on urinary 9,10-PQ obtained after hydrolysis by different types of enzymes. H-2: β-glucuronidase (4720 units)/aryl sulfatase (54 units), type H-2, from *Helix pomatia*. The ratio of the observed 9,10-PQ concentration after the hydrolysis by the H-2 enzyme was taken as 100%. VII-A: β-glucuronidase (4851 units), type VII-A, from *Escherichia coli*. V: aryl sulfatase (49 units), type V, from *Patella vulgate*.

conjugates was similar to that in the rat urine sample. The distribution data indicate that 9,10-PQHG is a major metabolite of 9,10-PQ in human urine. 9,10-PQHG was found in spot urine samples of 16 subjects, with total levels of 9,10-PQHG metabolites in the range of 2.04−19.08 nmol/mol creatinine (Table 3). The 9,10-PQHG concentrations of smokers tended to be higher than those of nonsmokers, although the sample size was small.

Table 3. Urinary Concentrations of 9,10-PQHG in the Human Study Subjects (n = 16)

	9,10-PQHG concentration	
	nonsmoker (n = 13)	smoker (n = 3)
mean ± SD (nmol/mol of creatinine)	4.70 ± 1.94	13.7 ± 5.94
range (nmol/mol of creatinine)	2.04−7.86	7.32−19.08
mean ± SD (pM)	39.7 ± 22.6	128.2 ± 66.7
range (pM)	10.6−74.5	35.4−187.6

DISCUSSION

Our results demonstrate that a 9,10-PQ metabolite, 9,10-PQHG, is excreted into urine of human subjects exposed to environmental levels of 9,10-PQ. Urinary 9,10-PQHG was selected as a biomarker of low-level 9,10-PQ exposure in humans. Therefore, we developed direct and indirect LC−MS/MS methods for measuring urinary 9,10-PQHG and 9,10-PQ derived from the hydrolysis of 9,10-PQHG. The direct method

Table 2. Precision and Accuracy in the Determination of Urinary 9,10-PQHG[a]

spiked concentration (pM)	0	40	100	200
found ± SD (pM)	63.6 ± 2.7	103.9 ± 6.4	169.2 ± 2.8	247.0 ± 6.5
RSD (%)	4.2	6.2	1.7	2.6
accuracy (%)		101	106	92

[a]Precision is expressed as the percentage of relative standard deviation (RSD,%). Accuracy is expressed as the percentage of accuracy [(mean observed concentration/spiked concentration)100].

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for 9,10-PQHG was developed using a specific fragmentation of *O*-glucuronide conjugates in tandem mass spectrometry. Previously, conjugate metabolites of pyrene and pharmaceuticals were directly analyzed using LC−MS/MS with ESI mode in which *O*-glucuronides showed a characteristic fragmentation pattern that allowed them to be sensitively detected.[28,30−32] The product ion from 9,10-PQHG at m/z 209.1 $[M - H - 176]^-$ represents the loss of the sugar moiety, and the ion at m/z 175.1 in the spectrum corresponds to fragment ions of glucuronide. Urinary 9,10-PQHG was purified by a solid-phase extraction cartridge, which is a mixed-mode anion exchange and reversed-phase sorbent for acidic compounds. The adsorption of 9,10-PQHG on the sorbent is dominated by hydrophobic interaction with the aromatic moiety of the analyte and anion-exchange interaction with its glucuronic acid moiety.[28,30]

For the indirect method, 9,10-PQ produced by the autoxidation of 9,10-PQH_2, resulting from the enzymatic hydrolysis of 9,10-PQHG, was determined by an LC−MS/MS method with APCI in positive mode. Because the APCI mode is more effective than the ESI mode for ionization of PAHQs,[33−35] we selected the positive APCI mode. The LOQ of 9,10-PQ (5.8 fmol/injection, S/N = 3) was reconstructed with a sensitivity as high as that in a previous report,[33] and the method was applied to identify 9,10-PQ derived from 9,10-PQHG hydrolysis.

We developed the two methods for the definitive identification of 9,10-PQHG. The direct method for measuring urinary 9,10-PQHG is more acceptable for the routine quantification than the indirect method for measuring 9,10-PQ. The detection limit of 9,10-PQHG (1.2 fmol/injection) was lower than that of 9,10-PQ (5.8 fmol/injection). The MS/MS fragmentation pattern and the SPE pretreatment specific to glucuronic acid moiety of 9,10-PQHG provide distinct advantages in detection specificity and purification efficiency as compared with 9,10-PQ without a characteristic substructure. We treated 50 mL of urine samples from nonoccupationally exposed human subjects to obtain enough intensity of the analyte peaks for the quantification. In the case of the subjects who are expected to be exposed to high amounts of 9,10-PQ from diesel exhaust or other combustion sources, the precise quantification of 9,10-PQHG can be performed with less than 10 mL of urine.

As described above, 9,10-PQHG is a detoxification metabolite of 9,10-PQ, which terminates the redox cycle and is exported to the extracellular space.[25] Our results suggest that 9,10-PQ inhaled from the environment is reduced to 9,10-PQH_2 and metabolized to its conjugates, which are then excreted into the urine. Urine from a 9,10-PQ-administered rat was found to contain 9,10-PQHG (the glucuronide of 9,10-PQH_2), the sulfate of 9,10-PQH_2, and unmetabolized free 9,10-PQ. Urine from nonoccupationally exposed humans also had 9,10-PQHG and sulfate conjugate of 9,10-PQH_2 but hardly any free 9,10-PQ. Furthermore, the detection of 9,10-PQ produced by the autoxidation of 9,10-PQH_2, resulting from the enzymatic hydrolysis of 9,10-PQHG, indicated that 9,10-PQHG was present in the urine.[25] Our results demonstrate that 9,10-PQ is metabolized in the human body in the same way that it is metabolized in the rat after i.p. administration of 9,10-PQ.

In the rats, 3.2% of the intraperitoneally administered 9,10-PQ was excreted into the urine as conjugates or free 9,10-PQ after 48 h. A fraction of 9,10-PQ was probably excreted into the bile. In rats intravenously administrated pyrene, the excreted amount of 1-hydroxypyrene, a major pyrene metabolite, was 3−5-fold higher in the bile than in the urine, and the sum of cumulative urinary and biliary excretion amounts represented less than 15% of the dose.[36] In this study, the percentage excreted as free (unconjugated) 9,10-PQ in the rat urine was 31% of the total amount of metabolites, whereas free 9,10-PQ was hardly quantitated in the unhydrolyzed urine from a human who was exposed to environmental sources. NQO1, AKR, and/or phase II conjugating enzymes may be saturated under the high dose tested in the rat.

The high percentages of 9,10-PQHG in the conjugated metabolites in human urine (Figure 5) and in the in vivo study (Table 1) suggest that 9,10-PQHG is a major 9,10-PQ conjugate metabolite in human urine. In humans, PAH metabolites are excreted in the urine as glucuronide and sulfate conjugates, with the former being the predominant form.[37] The 1-hydroxypyrene-glucuronide (1-OHP-G) levels account for more than 80% of total pyrene metabolites in human urine, and the concentration of urinary 1-hydroxypyrene after deconjugation (sum of its glucuronide and sulfate) is correlated with the concentration of 1-OHP-G concentration in human urine.[28,38,39] The predominant excretion of 9,10-PQHG in urine is similar to that of the PAH metabolites, which have been used as biomarkers, and the monitoring of 9,10-PQHG should be a promising candidate to assess the total 9,10-PQ metabolites in urine.

The phase II conjugating enzymes, uridine 5′-diphosphate (UDP) glucuronosyltransferases (UGTs), catalyze the *O*-glucuronidation of phenols, quinols, and dihydrodiols, and the glucuronidation represents an important pathway in the elimination of PAHs.[40] A variety of UGT isoforms are responsible for glucuronidation of 9,10-PQ via 9,10-PQH_2. UGT 1A6 and 1A10 among eight tested recombinant UGTs exhibited high activity for 9,10-PQHG formation, whereas 1A4 and 2B17 were poor catalysts for the glucuronidation.[25] The formation of 9,10-PQHG, resulting in detoxification and elimination of 9,10-PQ, should depend on enzymatic activities associated with two-electron reduction of 9,10-PQ and the glucuronidation of 9,10-PQH_2. NQO1 is markedly overexpressed in mammalian cells compared with UGTs;[41] therefore, the redox-cycling reactions might predominate over glucuronidation. Cell lines stably expressing NQO1 and UGTs readily metabolize quinones to their glucuronides and are resistant to quinone-induced cytotoxicity.[42] However, a transcription factor, Nrf2, activated by ROS may be involved in the excretion of 9,10-PQHG. It upregulates the genes responsible for NQO1, AKR, and UGTs[43,44] and accelerates 9,10-PQHG formation and detoxification of PQ.[25] However, we could not estimate kinetic parameters of the metabolism using only the concentrations of 9,10-PQHG in human spot urine.

In the human urine samples from healthy and nonoccupationally exposed subjects, 9,10-PQHG levels were in the range of 2.04−19.08 nmol/mol creatinine. The concentrations are lower than those of 1-OHP-G, which has been used as a biomarker. The urinary concentrations of 1-OHP-G ranged from 15.3 to 99.7 nmol/mol creatinine in a similar subject group.[28] It is anticipated that the exposure level of 9,10-PQ should be lower than that of PAHs such as pyrene. The exposure essentially depends on the inhalation of 9,10-PQ from the atmosphere. To our knowledge, there have been no reports of the dietary intake of 9,10-PQ. The concentrations of 9,10-PQ in the atmospheric particulate matter samples collected in a site along a busy arterial road and a suburban site in Kanazawa city were 1.17 ± 0.77 (0.28−2.27 pmol/m^3, n = 7) and 0.97 ±

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0.77 pmol/m^3 (0.29−2.10 pmol/m^3, n = 7), respectively (see the Supporting Information for the analysis of 9,10-PQ in the atmosphere). The 9,10-PQ levels in Kanazawa are similar to those in a site with heavy traffic in Nagasaki city (0.66−3.31 pmol/m^3),[16] whereas the 9,10-PQ levels in Los Angeles (0.48−9.51 pmol/m^3)[15] and Athens (8.26 pmol/m^3)[14] are higher than those in Kanazawa. The concentrations of 9,10-PQ in Kanazawa should be comparable to the atmospheric benzo[*a*]-pyrene levels (1.3 ± 0.6 pmol/m^3) and lower than those of pyrene (gas-phase, 8.7 ± 8.7 pmol/m^3; particle-phase, 2.6 ± 1.1 pmol/m^3).[45] It is likely that the human subjects in this study were exposed to lower levels of atmospheric 9,10-PQ than the residents in other cities.

It is predicted that the exposure level of Phe is much higher than that of 9,10-PQ because of their atmospheric levels.[15,45] The major Phe metabolites that have been identified in human urine are the conjugates of hydroxyphenanthrenes (OH-Phe),[46−49] phenanthrene dihydrodiols (Phe-diol),[48] and *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (TPhe).[50−52] A large amount of the urinary metabolites is accounted for by the metabolites derived from 1,2- and 3,4-epoxides resulting from the epoxidation of Phe by P-450, whereas 9,10-Phe-diol and 9-OH-Phe from 9,10-epoxide take up no more than 20% of Phe-diols and 5% of OH-Phes, respectively.[48,49,51] This indicates that the metabolic pathway from Phe to 9,10-Phe-diol should be a minor route. Furthermore, dihydrodiol dehydrogenases, members of AKR superfamily, regioselectively oxidize non-K region *trans*-dihydrodiols but are inactive on K region *trans*-dihydrodiols such as 9,10-Phe-diol.[26,53,54] The indirect exposure to 9,10-PQ introduced into the redox cycle through 9,10-Phe-diol from the metabolism of Phe is negligible (Figure 1); therefore, urinary 9,10-PQHG originates from only direct exposure to atmospheric 9,10-PQ.

The concentration of urinary 9,10-PQHG of the smoker appeared to be higher than those of the nonsmokers (Table 3), although a larger sample size and comparison with other biomarkers such as urinary nicotine and cotinine specific to smoking status[55] are required to confirm this. Although the existence of 9,10-PQ in tobacco smoke has been reported,[56] its precise concentration is still unknown. However, the concentrations of Phe in mainstream smoke from 30 domestic cigarettes were higher than the other PAHs except for fluorene,[57] and the urinary excretion of OH-Phes in smokers was significantly higher than that in nonsmokers.[46] Smoking definitely increases the exposure amount of Phe, whereas Phe should not be a factor in the rise in urinary 9,10-PQHG concentration because of the metabolic pathways (Figure 1). Future studies are needed to quantify urinary 9,10-PQHG concentrations in the general population and to validate the risk of oxidative stress resulting from 9,10-PQ exposure increased by contact with combustion sources such as cigarette smoke.

In conclusion, we successfully developed an LC−MS/MS method for determining urinary 9,10-PQHG. A large volume urine sample was effectively enriched, and the analyte was specifically purified using the developed pretreatment method. This method enabled us to identify and quantify 9,10-PQHG in rat urine after i.p. administration of 9,10-PQ and in urine samples from healthy human subjects. Human exposure to this new type of hazardous chemical that is related to oxidative stress has not been previously assessed. Therefore, a biomarker for PAHQ exposure and investigations of PAHQ concentration in the atmosphere are needed. We are presently examining the correlation between urinary 9,10-PQHG and personal exposure to atmospheric fine PM containing PAHQs in variously exposed subjects. Finally, our demonstration that 9,10-PQHG is present in human urine and our development of a method for detecting the metabolite should be useful for the surveillance of the health effects of ROS through 9,10-PQ exposure and for accelerating studies of the relationship between exposure to PAHQs and oxidative-stress-related disorders.

■ ASSOCIATED CONTENT

Supporting Information

SRM chromatograms and MS/MS spectra of the metabolites in urine from rats intraperitoneally injected with 9,10-PQ, validation data for the determination of urinary 9,10-PQ, and analysis of atmospheric 9,10-PQ. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

PM, airborne particulate matter; LC−MS/MS, liquid chromatography with tandem mass spectrometry; PAHs, polycyclic aromatic hydrocarbons; DEP, diesel exhaust particulate matter; ESI, electrospray ionization; SRM, selected reaction monitoring; EPI, enhanced product ion; ROS, reactive oxygen species; SOD, superoxide dismutase; PAHQs, polycyclic aromatic hydrocarbon quinones; 9,10-PQ, 9,10-phenanthrenequinone; Phe, phenanthrene; 9,10-PQH$_2$, 9,10-dihydroxyphenanthrene; 9,10-PQ$^{\bullet-}$, 9,10-phenanthrenequinone semiquinone radical; NQO1, NAD(P)H quinone oxidoreductase; AKR, aldo-keto reductase; 9,10-PQHG, monoglucuronide of 9,10-PQH$_2$; CYP, cytochrome P-450; APCI, atmospheric pressure chemical ionization; 1-OHP-G, 1-hydroxypyrene glucuronide; GC−MS/MS, gas chromatography with tandem mass spectrometry; UGT, uridine 5′-diphosphate (UDP) glucuronosyltransferase

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