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Fast and Simultaneous Determination of Urinary 8-Hydroxy-2′-deoxyguanosine and Ten Monohydroxylated Polycyclic Aromatic Hydrocarbons by Liquid Chromatography/Tandem Mass Spectrometry

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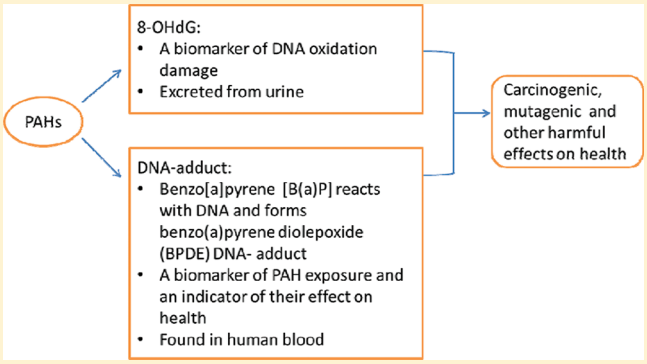
ABSTRACT: 8-Hydroxy-2′-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage, has been extensively studied to assess human exposure to carcinogenic compounds. Previous studies have associated levels of human urinary hydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) with those of 8-OHdG. However, measurements of OH-PAHs and 8-OHdG in urine are often conducted with two different analytical methods, which is both costly and time-consuming. In this study, a novel method is described to quickly and simultaneously quantify ten urinary OH-PAHs and 8-OHdG through high pressure liquid chromatography/tandem mass spectrometry (HPLC/MS/MS). Urine samples undergo solid phase extraction and concentration and then are analyzed by an optimized HPLC/MS/MS method operated in the negative electrospray ionization (ESI) and multiple reaction monitoring (MRM) mode. Deuterated, ^{15}N and ^{13}C- labeled analogues are used as internal standards. Simultaneous analysis of urinary 8-OHdG and OH-PAHs are completed within 16 min. Calibration curves of all target analytes show favorable linearity within the concentration range of 0.3−10.0 μg/L for 8-OHdG and 0.05−15 μg/L for different OH-PAHs. The method detection limits (MDLs) in pooled urine range from 0.023 μg/L to 0.625 μg/L. The method shows satisfactory accuracy and precision when we analyzed varied levels spiked in pooled urine. Recoveries for 8 of the 10 OH-PAHs were in the range of 100 ± 15% with a variation coefficient of less than 20%. Thirty-four real urine samples were analyzed for all target analytes. Except 3-OHF, most compounds could be quantified.



INTRODUCTION

Biochemical interactions between target biomolecules and reactive oxygen species can cause oxidative damage, leading to damage of cellular proteins, lipids, and DNA.[1,2] Although many types of damaged DNA lesions have been identified, 8-hydroxy-2′-deoxyguanosine (8-OHdG) has been commonly chosen as a biomarker of oxidative damage. 8-OHdG is formed from hydroxyl radical attack of deoxyguanosine residues. It is an abundant product of DNA oxidation and has been extensively studied due to its mutagenic potential and relatively high abundance. Now it is being used as a useful biomarker in the evaluation of oxidative stress caused by lifestyle, human disease, and environmental contaminant exposure.[3,4]

Polycyclic aromatic hydrocarbons (PAHs) are one of the most widespread organic pollutants in the environment. They are also present in fossil fuels or are formed by the incomplete combustion of carbon-containing fuels. Previous studies have identified PAHs as carcinogenic, mutagenic, and teratogenic.[5] Urinary monohydroxylated PAHs (OH-PAHs) have been commonly used as biomarkers to monitor the human exposure to PAHs and assess the environmental and human health risk.[6,7] Though many other physiological factors and environmental stressors may lead to DNA damage, it has been reported that higher levels of urinary 8-OHdG have been found in populations exposed to high levels of PAHs.[8−15] Positive correlations were observed between urinary OH-PAHs and 8-OHdG in those occupational populations, such as coke oven plant workers[10] and taxi drivers.[15] Such correlations were also reported in 105 healthy Korean[14] and school children in Thailand.[12]

Linking human exposure to PAHs with oxidative damage requires a large number of human urine samples collected and analyzed for 8-OHdG and OH-PAHs. In most reported studies, the measurements of urinary 8-OHdG and OH-PAHs were often carried out by using two sets of separate analytical

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methods. After sample preparation, urinary 8-OHdG was quantified by high performance liquid chromatography/electrochemical detection (HPLC/ECD),[16,17] gas chromatography/mass spectrometer (GC/MS),[18] enzyme linked immunoassay (ELISA),[19] or HPLC/tandem mass spectrometer (HPLC/MS/MS).[20,21] Urinary OH-PAHs were measured by HPLC/FD (fluorescence detection),[6,22] GC/MS,[23] or HPLC/MS/MS.[24,25] The use of these methods to measure 8-OHdG and OH-PAHs in two separate aliquots of urine samples is both costly and time-consuming.

In this study, a novel method was developed and validated for the simultaneous determination of 10 OH-PAHs and 8-OHdG in one aliquot of urine samples using HPLC/MS/MS in negative electrospray ionization (ESI) mode. The new method not only combines the analysis of compounds of different chemical structures (Figure 1) and properties in one method

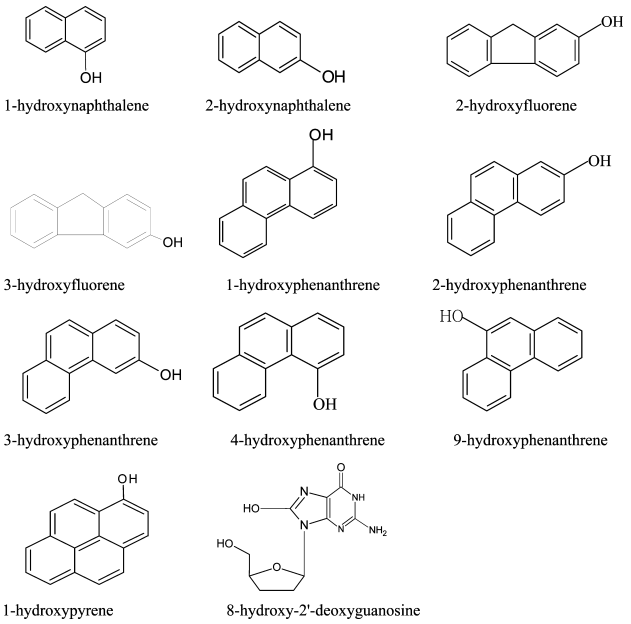

Figure 1. Chemical structures for target chemicals, OH-PAHs, and 8-OHdG.

but also provides a rapid and low cost approach to evaluate human PAH exposure and its potential health effects.

EXPERIMENTAL PROCEDURES

Materials. 8-OHdG, 2-hydroxynaphthalene (2-OHN, purity 99%), and 3-hydroxyfluorene (3-OHF) were purchased from Sigma (St. Louis, MO, USA). 2-Hydroxyfluorene (2-OHF, purity 98%), 9-hydroxyfluorene (9-OHF, purity 96%), 9-hydroxyphenanthrene (9-OHPhe), and 1-hydroxypyrene (1-OHP, purity 98%) were purchased from Aldrich (St. Louis, MO, USA). 1-Hydroxynaphthalene (1-OHN) was purchased from Fluka (purity 99%, St. Louis, MO, USA). 1-Hydroxyphenanthrene (1-OHPhe, purity 99%), 2-hydroxyphenanthrene (2-OHPhe, purity 99.6%), and 4-hydroxyphenanthrene (4-OHPhe, 50 μg/mL in acetonitrile) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). 3-Hydroxyphenanthrene (3-OHPhe, purity 98%, 50.0 μg/mL in toluene), $^{13}C_6$-3-OHPhe (purity 95%, 50.0 μg/mL in acetonitrile), and $^{15}N_5$-8-hydroxy-2′-deoxyguanosine ($^{15}N_5$-8-OHdG, $^{15}N_5$ purity 98% and 95% chemical purity) were purchased from Cambridge Isotope Lab (Andover, MA, USA). D_8-2-OHN and D_9-1-OHP were purchased from C-D-N Isotope Inc. (Quebec, Canada). D_9-2-OHF was obtained from Santa Cruz Biotech. Inc. (Santa Cruz, CA, USA). β-Glucuronidase/arylsulphatase from *Helix pomatia* was from Sigma (St. Louis, MO, USA). Methanol (LC-MS Chromasolv, ≥99.9%) was obtained from Fluka (St. Louis, MO, USA). Water (Chromosolv plus for HPLC grade) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Glacial acetic acid (HAC), sodium acetate (NaAC), and KH_2PO_4 (HPLC grade) were obtained from Fisher Scientific (Houston, TX, USA). All other reagents were of analytical grade and used without further purification. The Bond Elut C18 SPE cartridge (500 mg and 6 mL) was obtained from Varian (Santa Clara, CA, USA). Four HPLC columns were tested, including Chromolith performance RP-18e column (100 × 4.6 mm, 2 μm, Merck, Germany), Betasil phenyl C-18 (150 × 2.1 mm, 3 μm, Thermoscientific, Pittsburgh, PA, USA), and Zorbax RRHD Eclipse plus C18 (100 × 2.1 mm and 50 × 2.1 mm, 1.8 μm, Agilent, Santa Clara, CA, USA).

Standard Preparation. Stock solutions of 8-OHdG and $^{15}N_5$-8-OHdG were prepared in distilled water. Stock solutions of individual OH-PAHs and labeled OH-PAHs standards were prepared in acetonitrile with concentrations of 1.0 to 3.6 mg/mL. Calculated volumes of individual stock solutions were transferred to a 10 mL volumetric flask to make a mixture of the native standards (NSM) at concentrations of 300 μg/L for 2-OHN, 200 μg/L for 1-OHN, 2-OHF, and 3-OHF, 30 μg/L for 1-, 2-, 3-, 4-, and 9-OHPhe, and 120 μg/L for 1-OHP and 100 μg/L for 8-OHdG. Similarly, the mixture of the internal standards (ISM) was prepared in acetonitrile, which included $^{13}C_6$ -3-OHPhe at 20 μg/L, D_9-1-OHP and $^{15}N_5$-8-OHdG at 100 μg/L, D_8-2-OHN at 300 μg/L, and D_9-2-OHF at 200 μg/L. All standards were stored at −20 °C.

A pooled urine sample from 20 volunteer donors was used to validate the analytical method. Two hundred fifty milliliters of urine was mixed with 750 mL of water, and eight calibration standard solutions were prepared in 2.0 mL of the diluted urine pool. The native standard concentrations were in the range 0.012−15 μg/L, while internal standard concentrations were 15 ng/mL for D_8-2-OHN, 10 ng/mL for D_9-2-OHF, 5 ng/mL for D_9-1-OHP, and 1 ng/mL for $^{13}C_6$-3-OHPhe. HPLC grade water and urine samples without spiked analytes were used to examine whether the buffer and urine had matrix interference and background contamination. The method blank was measured with a diluted urine sample only spiked with the internal standards. All of the calibration standards and blank samples were processed with the real samples in the same batch run.

Sample Preparation. Fifty microliters (high concentration, 0.75−7.5 μg/L), 25 μL (medium concentration, 0.375−3.75 μg/L), and 10 μL (low concentration, 0.15−1.5 μg/L) NSM were added to the same diluted pooled urine to prepare the QA/QC sample. Standards (including blank and QA/QC) were prepared on the day of use. These samples were kept at −20 °C, and the stability test was done with the QC sample stored for various amounts of time. The internal standard mixture solutions (100 μL) at concentrations of 1−15 ng/mL were added to the urine sample, native standards, blank samples, and QA/QC samples.

Ten microliters of β-glucuronidase/arylsulfatase enzyme and 3 mL of 0.1 mol/L HAC-NaAC buffers were added to the 2.0 mL urine sample to adjust the pH to 5.5 and then incubated at 37 °C overnight. The target analytes were extracted through solid phase extraction (SPE) cartridges. The procedures were as follows: the SPE cartridges were conditioned with 5.0 mL of methanol, 5.0 mL of water, and 6.0 mL of 25 mmol/L KH_2PO_4 buffer. Then, 2.0 mL of urine samples were loaded onto the cartridges. The cartridges were washed with 2 mL of 25 mmol/L KH_2PO_4 buffer and 10 mL of distilled water. During these procedures, the flow rate was held below 1 mL/min. Finally, the cartridges were dried using a vacuum pump, and the target analytes were eluted with 4 mL of acetonitrile. After being concentrated to 5−10 μL using a TurboVap, the residues were reconstituted with 200 μL of methanol for instrumental analysis.

Instrumental Analysis. All samples were analyzed with an Agilent 6460 LC-MS Triple Quadrupole system (Santa Clara, CA, USA), coupled with an Ultra HPLC 1290, G4220A Infinity Binary Pump, G1316C Infinity TCC, and G4226A Infinity Sampler. A Zorbax RRHD Eclipse Plus C18 (2.1 × 100 mm, 1.8 μm, Agilent, USA) column was employed to separate the compounds in the LC system. The mobile phases were 0.1% acetic acid in water (v/v, solvent A) and

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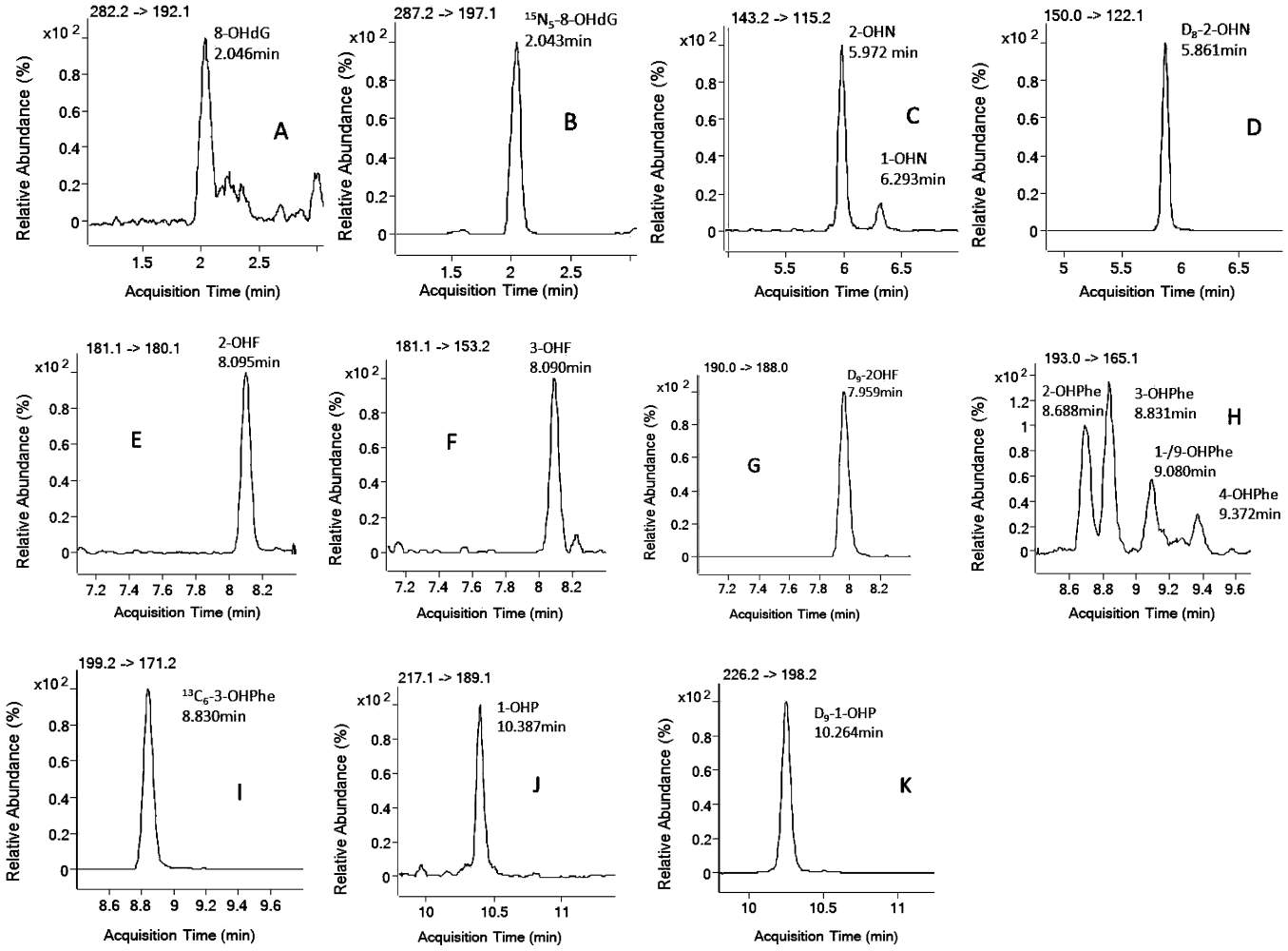

Figure 2. LC/MS/MS chromatograms from the analysis of a QC material, showing the detected OH-PAHs and corresponding internal standards. The concentrations were 0.314 μg/L for 8-OHdG (A), 0.469 μg/L for 2-OHN, 0.313 μg/L for 1-OHN (C) and 2-OHF (E), 2.5 μg/L for 3-OHF (F), 0.047 μg/L for 2-OHPhe and 3-OHPhe, 0.092 μg/L for 4-OHPhe, 0.184 μg/L for 1-/9-OHPhe (H), and 0.0.376 for 1-OHP (J). The corresponding labeled standard concentrations were 5 μg/L for $^{15}N_5$-8-OHdG (B), 15 μg/L for D_8-2-OHN (D), 10 μg/L for D_9-2-OHF (G), 1 μg/L for $^{13}C_6$-3-OHPhe (I), and 5 μg/L for D_9-1-OHP (K).

Table 1. MS/MS Parameters, Retention Time, Calibration Range, IDLs, and MDLs[a]

analyte names	analyte abbreviations	MRM transitions	fragmentor (eV)	CE (eV)	RT (min)	calibration ranges (μg/L)	IDLs (μg/L)	MDLs (μg/L)
8-hydroxy-2′-deoxyguanosine	8-OHdG	282.1−192.1	135	20	2.046	0.310−10.0	0.040	0.310
$^{15}N_5$ - 8-hydroxy-2′-deoxyguanosine	$^{15}N_5$-8-OHdG	287.2−197.1	171	14	2.043			
2-hydroxynaphthalene	2-OHN	143.2−115.2	140	25	5.972	0.117−15.0	0.006	0.078
1-hydroxynaphthalene	1-OHN	143.2−115.2	120	25	6.293	0.313−10.0	0.010	0.208
D_8-2-hydroxynaphthalene	$D_8$2-OHN	150.0−122.1	80	20	5.861			
2-hydroxyfluorene	2-OHF	181.1−180.1	148	25	8.095	0.078−10.0	0.010	0.078
3-hydroxyfluorene	3-OHF	181.1−153.2	137	25	8.090	0.625−10.0	0.024	0.625
D_9-2-hydroxyfluorene	D_9-2-OHF	190.2−188.2	90	30	7.959	0.125−3.0	0.016	0.125
1-/9-hydroxyphenanthrene	1-/9-OHPhe	192.9−165.1	163	30	9.080	0.047−1.5	0.008	0.038
2-hydroxyphenanthrene	2-OHPhe	192.9−165.1	163	30	8.688			
3-hydroxyphenanthrene	3-OHPhe	192.9−165.1	163	30	8.831	0.047−1.5	0.008	0.023
4-hydroxyphenanthrene	4-OHPhe	192.9−165.1	163	30	9.372	0.093−1.5	0.016	0.085
$^{13}C_6$-3-hydroxyphenanthrene	$^{13}C_6$-3-OHPhe	199.2−171.2	160	45	8.830			
1-hydroxypyrene	1-OHP	217.1−189.1	160	35	10.387	0.188−6.0	0.016	0.136
D_9-1-hydroxypyrene	D_9-1-OHP	226.2−198.2	195	50	10.264			

[a]CE, collision energy; RT, retention time; IDLs, instrumental detection limits; MDLs, method detection limits.

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methanol (solvent B). The flow rate was set at 0.4 mL/min, and the column temperature was held at 40 °C. The gradient elution program was set as follows: 0−2 min, 5%−15% solvent B; 2−2.1 min, 15−40% solvent B; 2.1−12 min, 40−80% solvent B; and 12−13 min, 80−99% solvent B. The LC column was then equilibrated for three more minutes.

Negative ESI in multiple reaction monitoring (MRM) mode was used to record the signals of analytes and their isotope-labeled ISs. The ion source and other MS/MS parameters were optimized by injecting approximately 100 μg/L of standard solution by an injector program or by using Automation Optimizer Software supplied by the vendor (Agilent, USA). Methanol and water (v/v, 50:50) were selected as the mobile phases at a flow rate of 0.4 mL/min. To achieve the maximum sensitivity, the mass spectrometer parameters for the labeled standards were also optimized. The nitrogen gas temperature was held at 300 °C at a flow rate of 10 L/min. The nebulizer was 45 psi, and the sheath gas temperature was kept at 300 °C at a flow rate of 11 L/min. The nozzle voltages were set at 500 eV in negative ESI mode. The capillary was set at 2000 V. The mass spectrometer parameters for each of the analytes are shown in Table 1, as are the deuterated, ^{15}N-, and ^{13}C-labeled analytes. 1- and 9-OHPhe could not be separated chromatographically and were consequently quantified together (and referred to as "1/9-OHPhe"). Data analysis was carried out using the MassHunter Workstation Software B 03.01(Santa Clara, CA, USA).

Validation of the Method. The pooled urine sample was used to validate the analytical method, matrix effects, calibration curve linearity, instrumental detection limit (IDL), method detection limit (MDL), method precision and accuracy, and sample storage stability. Synthetic urine, which was prepared according to the ref 26, and 4-fold diluted urine samples were employed to test the matrix effect. The calibration standard solutions with widely varied concentrations were prepared separately by using the pooled urine or the synthetic urine to quantify urinary 8-OHdG and OH-PAH levels in general and occupational populations. A good linearity was assessed by checking the regression coefficients greater than 0.990. The IDLs of 8-OHdG and OH-PAHs were evaluated as concentrations generating the signal-to-noise ratio (S/N) equal to or greater than 3 from analyzing the lowest standard concentration prepared in methanol. The MDLs were calculated in the same way by analyzing the lowest concentrations of standard solution prepared in diluted pooled urine (Table 1).

The method precision was evaluated by calculating the relative standard deviations (RSDs) of repetitive measurement of the QC materials at three different concentrations (0.75−7.5 μg/L, 0.375−3.75 μg/L and 0.15−1.5 μg/L). More than 20 runs were completed to evaluate the inter- and intraday deviation. Prior to each batch analysis, a standard solution with a concentration close to the MDL was run first to compare the instrument responses with those from the previous run. If the deviation of responses from both runs was below 15%, it indicates that the instrument sensitivity was acceptable for the entire batch analysis. Then the analysis was started. The relative recovery and accuracy were evaluated by dividing concentrations of the target analytes by the spiked concentration. Accuracy from 80% to 120% was acceptable after analyzing low, medium, and high concentrations spiked into the urine matrixes. The sample storage stabilities were studied in a freezing and thawing cycle and shelf lives up to 8 weeks under −20 °C. All the sample storage stability studies were performed by comparing the measured concentration values from the high concentration QC samples. The post-preparation stabilities were examined by using extracted samples with concentrations close to the lowest calibration point and by storing these samples at 4 °C for 6 days. In order to fully confirm the practicality of this method, 34 urine samples donated from 34 volunteers were analyzed for the target analytes.

Data Analysis. SPSS program, version 10, was used for statistical analysis. Pearson's correlations (one tailed) were used to test the associations between 8-OHdG and individual OH-PAHs. The concentration of analyte was viewed as the zero value for statistical processing if lower than the method detection limits.

RESULTS

Table 1 lists analytical parameters for all analytes. The validation results of the analysis of 8-OHdG and OH-PAHs are shown in Tables 1 and 2. From reported toxicological studies, we selected monohydroxylated PAH metabolites. OHN, OHF, OHPhe, and 1-OHP are the widely used biomarkers due to their toxicity or potential negative effect on human health.[5,6,9] HPLC could not completely resolve 2-OHF and 3-OHF, but MS/MS differentiated them by different MRM transitions. We observed that both 2-OHF and 3-OHF generated similar MRM transitions: m/z, 181−180 and m/z, 181−153. However, the sensitivity of 2-OHF by using 181−180 as a MRM transition is ten times higher than that of 3-OHF by using 181−180 as a MRM transition. Thus, the contribution of the 3-OHF to 2-OHF's transition was

Table 2. Method Precision and Accuracy (n = 20) in Pooled Urine Spiked at Various Concentrations

analytes	added amount (μg/L)	expected amount (μg/L)	measured mean values (μg/L)	accuracy (% of expected)	precision CV (%)
8-OHdG	0.0		0.011		
	0.5	0.511	0.555	108.6	14.61
	1.25	1.261	1.222	96.9	14.60
	2.5	2.511	2.422	96.5	8.58
2-OHN	0.0		0.049		
	1.5	1.549	1.731	111.7	9.55
	3.75	3.799	3.566	93.9	9.57
	7.5	7.549	7.478	99.1	6.61
1-OHN	0.0		0.015		
	1.0	1.015	1.174	115.7	15.92
	2.5	2.515	2.356	93.7	9.13
	5.0	5.015	4.750	93.8	10.45
2-OHF	0.0		0.055		
	1.0	1.055	1.071	101.5	14.51
	2.5	2.555	2.312	90.5	14.43
	5.0	5.055	4.670	92.8	10.07
3-OHF	0.0		0		
	1.0	1.000	1.150	115	15.86
	2.5	2.500	2.436	97.4	12.26
	5.0	5.000	4.631	92.6	11.89
1/9 -OHPhe	0.0		0.009		
	0.30	0.309	0.324	104.9	19.07
	0.75	0.759	0.703	92.6	16.48
	1.5	1.509	1.459	96.7	12.23
2-OHPhe	0.0		0.005		
	0.15	0.155	0.146	94.2	16.28
	0.375	0.380	0.325	85.5	16.39
	0.75	0.755	0.636	84.2	15.75
3-OHPhe	0.0		0.005		
	0.15	0.155	0.152	98.1	14.94
	0.375	0.380	0.331	87.1	9.93
	0.75	0.755	0.698	92.5	9.18
4-OHPhe	0.0		0.009		
	0.15	0.159	0.161	101.3	16.82
	0.375	0.384	0.357	93.0	11.13
	0.75	0.759	0.743	97.9	13.02
1-OHP	0.0		0.004		
	0.6	0.604	0.657	108.8	6.95
	1.5	1.504	1.442	95.9	12.84
	3.0	3.004	3.097	103.1	11.44

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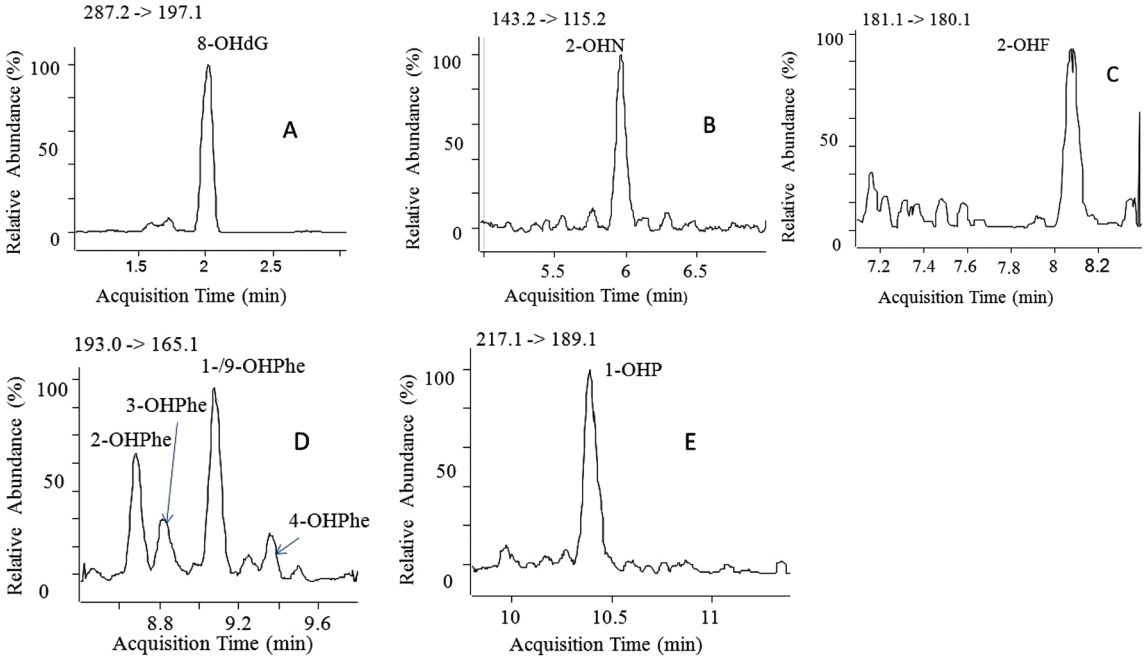

Figure 3. Chromatograms from the analysis of a real urine sample. A, 8-OHdG; B, 2-OHN and 1-OHN; C, 2-OHF; D, OH-Phe; and E, 1-OHP.

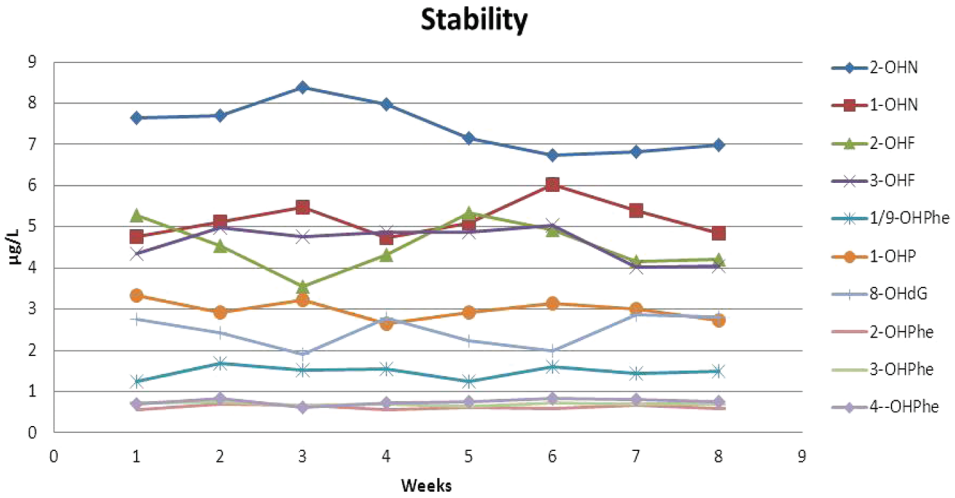

Figure 4. Storage stability of 8-OHdG and OH-PAHs at −20 °C for 8 weeks.

negligible. All of the calibration curves had favorable linearity (regression coefficients >0.990). The method showed good linearity for 8-OHdG in the range of 0.310−10.0 μg/L. A good linearity was also shown in narrow concentration ranges (0.313−10.0 μg/L) for 1-OHN and 0.625−10.0 μg/L for 3-OHF. Particularly high sensitivity was found for 2-OHF, 1-+9-OHPhe, and 1-OHP, as well as with a wider linearity range covering 3−4 orders of magnitude. The slope deviation is an important parameter to evaluate method precision. Each calibration curve's slope deviation from the 20 runs was evaluated and was less than 15%. The MDLs ranged from 0.023 to 0.625 μg/L. The overall imprecision ranged from 9.6% to 25.3% (2 values above 20%). The recovery and accuracy ranged from 84% to 115%, while mean relative recoveries were in the range 94.7% to 103.6% (Table 2). Since the spiked amounts were fairly low, the results were within an acceptable error range.[25,27]

Figure 4 shows the stability of 8-OHdG and OH-PAHs spiked in a pooled urine sample over eight weeks. The mean relative recoveries were 99.4% for 8-OHdG, 99.4% for 2-OHN, 103.6% for 1-OHN, 92.1% for 2-OHF, 92.6% for 3-OHF, 85.2% for 2-OHPhe, 94.7% for 3-OHPhe, 98.5 for 1- and 9-OHPhe, 101.3% for 4-OHPhe, and 99.8% for 1-OHP. Urinary 8-OHdG and OH-PAHs were stable for at least 8 weeks by storing them at −20 °C. The coefficient of variation (%CV) of short-term storage was in the range of 12 to 28%; all except 4-OHPhe and 8-OHdG were within the acceptable between-run precision (20%) of an analytical method.[25]

The results of QC samples run with real urine samples met the established acceptable criteria. No contamination was found in the water and urine blank. 8-OHdG, 2-OHN, 1-OHN, and 2-OHF could be detected in most samples, and 2-OHPhe, 3-OHPhe 1-/9-OHPhe, 4-OHPhe, and 1-OHP could be detected in a large number of samples. Because of its low sensitivity and/or low concentration in real urine samples,[28] 3-OHF could not

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Table 3. Mean Concentrations and Relative Recoveries Using Synthetic Urine Calibration Standards (Unit: μg/L)

	spiked conc.	mean measured conc. (n = 4)	relative recoveries (%)	spiked conc.	mean measured conc. (n = 6)	relative recoveries (%)
8-OHdG	3.750	3.116	83.10	1.250	1.107	88.59
2-OHN	11.250	7.524	66.88	3.750	2.886	76.95
1-OHN	7.500	8.656	115.42	2.500	1.832	73.29
2-OHF	7.500	7.121	94.95	2.500	2.236	94.52
3-OHF	7.500	7.715	102.87	2.500	2.215	88.61
2-OHPhe	1.500	2.481	165.41	0.375	0.310	82.73
3-OHPhe	1.500	1.432	95.49	0.375	0.358	95.40
4-OHPhe	1.500	2.117	141.10	0.375	0.490	130.73
1/9-OHPhe	3.000	4.267	142.20	0.750	1.165	155.30
1-OHP	6.000	1.118	18.64	1.500	0.878	58.53

be detected and quantitated accurately in our study. The detailed results are shown in Table 4, and typical chromatograms from a real urine sample are shown in Figure 3. From these pilot studies, only 2-OHN has significant correlation with 8-OHdG (R^2 = 0.375, p = 0.038). Except for 2-OHN, there were no correlations between other individual target analytes and 8-OHdG.

DISCUSSION

In the previous reports, due to different polarities between 8-OHdG and OH-PAHs, urinary 8-OHdG and OH-PAHs were extracted and measured separately with various columns and analytical methods. Harri et al.[21] separated and determined urinary 8-OHdG using an online LC/MS/MS method with an analytical anion exchange column. Hu et al.[10] measured 8-OHdG using a LC/MS/MS with a polymine-II end-capped HPLC column after SPE pretreatment. The LC/MS/MS was operated in ESI positive mode for both methods. However, urinary OH-PAHs were concentrated by liquid−liquid extraction or SPE.[24,30−34] Although different SPE methods could be used for the analysis of both 8-OHdG and OH-PAHs, the SPE pretreatment procedures were completely different for 8-OHdG and OH-PAHs. The published procedures required two different methods of pretreatment and analysis of samples in two aliquots of urine samples, taking a fairly long time.[9]

In this study, 8-OHdG and OH-PAHs were extracted and analyzed using a single analytical method and in one aliquot of urine. The SPE procedure was optimized using different conditioning and elution solvents. Following the routine SPE operation procedure, 5.0 mL of methanol and water was first used to condition the cartridge. After loading the urine sample, it was washed with 2.0 mL of water. The target analytes were eluted with 1 mL of methanol and acetonitrile five times. Five fractions were collected for the qualitative analysis.

We found that 8-OHdG was eluted with the urine sample during the sample loading step, while OH-PAHs were only detected in the methanol and acetonitrile elution fractions. This indicated that 8-OHdG was not retained in the SPE cartridge due to its higher polarity. In order to improve SPE efficiency for both 8-OHdG and OH-PAHs, 6.0 mL of KH_2PO_4 buffer (pH 5.5) was used to precondition the cartridge before loading the urine sample. Under such a weak acid condition, the SPE cartridge could retain 8-OHdG, which prevented the 8-OHdG coeluting with the urine samples during loading. In the elution step, we found that 4.0 mL of acetonitrile could completely elute all the target analytes, while 4.0 mL of methanol could not. Therefore, acetonitrile was selected as the optimal elution solvent.

In the LC system, 5% methanol was used as the initial mobile phase to separate the targets; however, we found that the peak of 8-OHdG widened and tailed significantly. Glacial acetic acid (1%), added in water as the mobile phase modifier, resulted in a sharp peak for 8-OHdG. Each parent PAH compound typically has several isomeric metabolites with an OH group substituted in various positions on the benzene rings. For example, there are two isomers for hydroxylated naphthalene, two isomers for hydroxylated fluorene, and five isomers for hydroxylated phenanthrene. It is difficult to obtain baseline separation for the five isomers of OH-Phe even by using GC/MS.[33] This study completely separated the isomeric metabolites in a reasonably short chromatographic time.

The HPLC separation of the isomeric metabolites of PAHs has been identified as a challenging task in previous published studies.[24,25] To achieve separation, four HPLC columns were evaluated. Of the four, we determined Zorbax RRHD Eclipse Plus C18 (100 × 2.1 mm, 1.8 μm, Agilent, USA) as the best analysis column because its high resolution and rapid separation enabled us to complete a sample analysis in less than 16 min, as shown in a LC/MS/MS chromatogram (Figure 2). While 2-OHN and 1-OHN were completely separated, 2-OHF and 3-OHF could not be separated even under optimized chromatographic conditions. However, they could be quantified by using different MRM transitions (Table 1). The five OH-Phe isomers had to be measured with identical optimal MRM transitions, and the quantitation of each isomer depended on the complete LC separation for these five isomers. In this study, 2-, 3-, and 4-OHPhe were separated, but 1- and 9-OHPhe could not reach baseline separation despite using different mobile phases and LC gradients. Consequently, 1- and 9-OHPhe were quantified together by integrating the coelution peak of 1- and 9-OHPhe. Unfortunately, neither the injector program nor Automation Optimizer Software could generate efficient MRM transitions and mass spectrometer parameters for sensitive quantitative analysis of 9-OHF. The possible reason was that 9-OHF was too stable and had a symmetrical chemical structure, which prevented it from efficient collision-induced dissociation (CID) fragmentation in the collision cell of the MS system. Thus, the analysis of this compound was not conducted in this study. Interestingly, both fragmentor and collision energy varied with the chemical structure of OH-PAHs and tended to increase with the number of benzene rings in the OH-PAHs (Table 1).

Considering that the pooled urine sample contains PAH metabolites and might affect the determination of the MDL and calibration curve, we initially utilized synthetic urine as an alternative matrix instead of pooled urine to prepare calibration standards. We spiked the pooled urine with native standard mixtures at levels between 0.375 and 11.250 ng/mL and ISs

between 1.5 and 15 ng/mL. We then quantified target analytes in this spiked urine samples by using a set of calibration standards prepared in the synthetic urine. The measured concentrations of 8-OHdG, 2-OHF, 3-OHF, and 3-OHPhe were close to the spiked levels with recoveries of 80−120%, but the measured values of all other target analytes deviated far from the spiked amounts. The recoveries varied from 18.6% to 165.4% (Table 3). This suggested that synthetic urine as an alternative matrix could not compensate for the matrix effects of authentic urine samples. We also found that the recoveries of a few target analytes, such as 1-OHP, varied in tested matrixes, including water and diluted urine with varied ratios. Very often, the matrix effects were displayed by enhanced or reduced responses of the target analytes, which caused significantly varied recoveries (Table 3). Matrix effects might be influenced by sample extraction methods, chromatographic separation conditions, use of labeled IS or surrogates, as well as mass spectrometry ion source settings.[27,29]

Since "blank" urine, where all target analytes were below the MDL, was not available, the four times diluted pooled urine was selected as the alternative matrix to prepare calibration standards. We found that the diluted urine matrix compensated for the responses of all analytes and produced accurate quantification results. Moreover, in the diluted urine matrix the lowest concentration points in the calibration standard curves were 3−5 times higher than blank urine, and therefore, MDL can be lowered. All calibration curves showed good linearity with a regression coefficient greater than 0.993. A few of the lowest concentration points could not fit the curve, so they were excluded from the calibration curve if the accuracy was beyond 80%−120%. For the analysis of urinary OH-PAHs, the CV % from 20 runs was acceptable as some of the spiked concentrations were lower than 1 μg/L, and the results were comparable with those from other LC/MS/MS methods.[25,32] In previously reported LC/MS/MS methods, the recoveries and precisions varied. Onyemauwa et al. reported that the recoveries were from 71% to 94% and that the precisions of 5 out of 14 target analytes were above 15% and below 20%. Moreover, the precisions of 2-OHF and 9-hydroxybenzo(a)-pyrene were even higher than 20%.[25] Another study showed good precision (<15%) and accuracy (±15%), except for 3-OHF with a precision of over 20%.[32]

The IDLs were comparable with those in some GC-MS reports.[23,33] The MDLs increased 5−10 times compared with IDLs due to the matrix effect and noise. Because of the lower sensitivity, it was difficult to detect 3-OHF in authentic urine samples. Compared with 2-OHF, 3-OHF appears to be a minor metabolite of fluorene in low abundance.[35] Thus, we did not report the analysis of this compound in this study.

For the ESI in LC/MS/MS, the ion suppression and enhancement caused by salts and biomolecules in the urine sample affected the method accuracy. Stable isotope labeled internal standards had the ability to compensate for such matrix effects. ^{13}C-labeled compounds have physical and chemical properties similar to those of their corresponding unlabeled compounds and are often used to compensate for the loss of native target analytes in sample preparation and variations in instrument sensitivity. Since most ^{13}C-labeled OH-PAHs were not commercially available, deuterated compounds were used to quantify the target analytes. The deuterated compounds often differ from their native compounds in chromatographic characteristics, such as retention times. For example, in this study, 3-OHPhe and $^{13}C_6$-3-OHPhe coeluted at the same retention time of 9.053 min from the column, but the deuterated 2-OHN, 2-OHF, and 1-OHP were eluted 0.1−0.2 min earlier than their native compounds (Figure 2). Therefore, deuterated compounds and unlabeled compounds might be subject to different ionization suppression and could be the reason why we were unable to determine the target analytes accurately with deuterated standards in QC urine samples using synthetic urine as the calibration matrix. Use of the pooled and diluted urine could compensate for the matrix effects and give the accurate quantification. Application of such partially matrix-matched urine samples to prepare calibration standards for the LC/MS/MS method might be an approach to compensate matrix effects and lower MDL when non-^{13}C-labeled OH-PAHs are used as ISs.

Because of its much lower sensitivity and lower concentration in real urine samples, 3-OHF was detected in only one sample. The data are not listed in Table 4. As observed in Table 4, four compounds including 8-OHdG, 2-OHN, 1-OHN, and 2-OHF could be quantified in most real samples, and the detection frequencies of 2-OHPhe, 3-OHPhe, 1-/9-OHPhe, and 4-OHPhe in these urine samples were 85%, 85%, 80%, and 59%, respectively. But for 1-OHP, the detection frequency was comparatively lower.

Table 4. Urinary Levels of the Selected OH-PAHs and 8-OHdG from a US Urban Population (μg/L, n = 34)

compounds	mean	SD	ranges	numbers of B.L.Q	numbers of N.D.
8-OHdG	3.27	3.01	B.L.Q-11.64	6	0
2-OHN	7.65	8.3	0.38−41.80	0	0
1-OHN	1.59	2.36	N.D-8.71	0	1
2-OHF	0.87	1.21	B.L.Q-6.53	0	2
1-/9-OHPhe	0.47	0.49	N.D-2.84	0	5
2-OHPhe	0.20	0.19	N.D-0.80	0	5
3-OHPhe	0.51	0.53	N.D-2.26	0	7
4-OHPhe	0.58	0.63	N.D-2.40	0	14
1-OHP	0.99	0.74	N.D-2.36	12	6

The Center for Disease Control and Prevention reported that geometric mean (95% Conf. interval) of OH-PAHs was in the range of 2.360−3.050 μg/L for 1-OHN, 2.110−2.890 μg/L for 2-OHN, 0.262−0.354 μg/L for 2-OHF, 0.096−0.126 μg/L for 3-OHF, 0.046−0.0635 μg/L for 2-OHPhe, 0.0874−0.110 μg/L for 3-OHPhe, 0.0226−0.0279 μg/L for 4-OHPhe, and 0.0799−0.0997 μg/L for 1-OHP in general populations in USA.[36] For the occupational exposure and general population in some developing countries, the concentrations of urinary OH-PAHs are even higher.[6,24] Thus, the developed method in this study could quantify the exposure of general and occupational populations. The previous studies reported that the levels of urinary 8-OHdG in general population varied from 0.16 to 16.5 μg/L (n = 246)[20] and were 10.0 ± 7.9 μg/L in cancer patients and 2.82 ± 2.24 μg/L in healthy persons.[37] A calibration range of 0.310−5.0 μg/L for 8-OHdG in the method could quantify this biomarkers. The method enables us to further explore the associations of human exposure to air PAHs with DNA oxidation damage in megacities, based on our preliminary study of children's exposure to PAHs and its association with 8-hydroxy-2′-deoxyguanosine in Guangzhou, China.[9]

CONCLUSIONS

A new method was developed and validated for the rapid, accurate, and simultaneous determination of 8-OHdG and 10 OH-PAHs in a single aliquot of urine sample. All targets could be extracted with a single SPE method and analyzed in less than 16 min by LC/MS/MS in negative ESI mode. The results show that different matrixes (synthetic urine vs diluted pooled urine) lead to different accuracies. Four-fold diluted pooled urine appears to counteract the matrix effect. The method covers wide calibration ranges and is robust and sensitive enough to measure the target PAH exposure and the relevant effect biomarker (8-OHdG) in real urine samples from nonoccupational as well as occupational populations. The simultaneous measurement of these two chemicals is of great importance to establish the associations between human PAH exposure and health effects.

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Notes

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ABBREVIATIONS

PAH, Polycyclic aromatic hydrocarbons; ELISA, enzyme linked immunoassay; GC/MS, gas chromatography/mass spectrometer; HPLC/MS/MS, high performance liquid chromatography/tandem mass spectrometer; HPLC/ECD, high performance liquid chromatography/electrochemical detection; FD, fluorescence detection; ESI, electrospray ionization; SPE, solid phase extraction; MDL, method detection limit; CDC, Centers for Disease Control and Prevention; MRM, multiple reaction monitoring; RSDs, relative standard deviations; CID, collision-induced dissociation.

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