Detection of 1,*N*²-propano-2'-deoxyguanosine in human urine by stable isotope dilution UHPLC–MS/MS analysis[☆]

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ABSTRACT

A sensitive and accurate stable isotope dilution UHPLC–MS/MS method was developed and validated for the detection and quantification of ProdG adducts in human urine, a surrogate for the ProdG adducts in genomic DNA of human. A specific solid phase extraction (SPE) approach was established for selective enrichment of urinary ProdG adducts and elimination of urinary matrix facilitating the coupled MS/MS detection. The recovery of the method is estimated about 84.8–107.2%, and the precision are about 0.8–3.6% for intraday and 2.8–10.0% for interday. Due to that the matrix effect is efficiently eliminated by SPE pretreatment, the limits of detection (LODs, *S/N* = 3) and quantification (LOQs, *S/N* = 10) are decreased to 100 and 300 amol for urinary ProdG adducts, respectively. By coupling the developed SPE pretreatment with the UHPLC–MS/MS analysis, ProdG adducts were accurately quantified in healthy human urine.

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1. Introduction

1,*N*²-propano-2'-deoxyguanosine (ProdG) adducts are genotoxic, which are the major products from the reaction of 2'-deoxyguanosine (dG) in DNA with two molecules of acetaldehyde or one molecule of crotonaldehyde [1,2]. Acetaldehyde (Ace) and crotonaldehyde (Cro) are both mutagenic and carcinogenic aldehyde [3–7]. They are present in tobacco smoke, vehicle exhaust and fuel combustion gases [2,4]. In addition, Ace is a metabolite of ethanol and can be generated by threonine catabolism [8,9]. Cro is a product resulting from lipid peroxidation and *N*-nitrosopyrrolidine metabolism [10].

Ace can react with the exocyclic amino group of 2'-deoxyguanosine in DNA to form *N*²-ethylidene-2'-deoxyguanosine primarily, an unstable Schiff base, which can react with a second molecule of acetaldehyde leading the formation of two diastereomers of 1,*N*²-propano-2'-deoxyguanosine, (6*S*,8*S*) and (6*R*,8*R*) [2,11]. Meanwhile, ProdG adducts are also major products from the reaction of dG with Cro [1,6]. ProdG adducts have been found in genomic DNA in various tissues of animals and human [3,6,12]. In vivo, ProdG adducts can inhibit DNA synthesis by promoting DNA

miscoding through G → T transversion [6,12]. In addition, ProdG adducts can lead the formation of DNA interstrand cross-links and DNA-protein cross linking [13,14].

Benefiting from its easy collection and noninvasive to provider, urine has long been considered as a preferred biological fluid for clinical diagnosis. Nucleosides once are released in the pathway of nucleic acid metabolism or repair, neither are reused nor further degraded, but are discharged to the bloodstream and excreted into urine as intact molecules [15–17]. Considering the metabolism and degradation of genomic DNA, it is reasonable to believe that ProdG adducts are present as deoxynucleosides in urine.

Various methods have been developed for the detection of urinary nucleosides, including electrochemical method [18], immunoassays [19,20], capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) [17,21–24], matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [25,26], liquid chromatography tandem mass spectrometry (LC-MS) [27–32]. In comparison to other methods, the LC-MS based methodologies take the advantages of high sensitivity and direct analysis with structural information from MS measurements [33].

In this study, we developed a solid phase extraction approach for desalting and enrichment of ProdG adducts in human urine. We further combined stable isotope dilution with ultrahigh performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS). By coupling the SPE approach with the

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UHPLC–MS/MS method, we are able to accurately quantify two diastereomers of ProdG adducts in human urine.

2. Experimental

2.1. Chemicals and materials

Creatinine and ammonium bicarbonate (NH_4HCO_3) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). ProdG adducts and [$^{15}\text{N}_5$]ProdG adducts were synthesized and prepared according to previous studies [2,11].

2.2. Quantification of creatinine in human urine

Creatinine in human urine was measured by HPLC and quantified with calibration curve [34].

2.3. Solid-phase extraction

Solid-phase extraction (SPE) was used for desalting and enriching ProdG adducts in human urine. Two types of SPE column C18 (3.0 mL, 100 mg, Agela, Tianjin, China) and HLB (3.0 mL, 60 mg, Waters, Milford, MA, USA) cartridge were tested to obtain optimized extraction. Each cartridge was preconditioned with 3.0 mL of methanol and 3.0 mL of water in sequence. Then 50 μL standard solution of ProdG adducts (final concentration of each: 100 nM) was loaded into the cartridges. The cartridges were washed with 500 μL of water followed by 500 μL of methanol/water 3:7 (v/v), 500 μL of methanol/water 5:5 (v/v) and 500 μL of methanol. The fractions were collected and evaporated with N_2 to remove methanol, lyophilized and redissolved in 50 μL of pure water. The redissolved solutions and the standard solution were analyzed by UHPLC–QTOF to evaluate the SPE protocol.

2.4. Pretreatment of human urine samples

Urine samples were provided by thirteen healthy volunteers who were not smoking and hard-drinking. Urine sample of 200 μL was mixed with 1 nM each of [$^{15}\text{N}_5$]ProdG (final concentration) and treated by the optimized SPE protocol.

2.5. UHPLC–MS/MS analysis

The UHPLC–QTOF analysis was carried out with an Agilent 1290 UHPLC system coupled with a 6530 quadrupole tandem time-of-flight (Q–TOF) mass spectrometer (Santa Clara, CA, USA). Zorbax Eclipse Plus C18 column (2.1 mm i.d. $\times$ 50 mm, 1.8 μm , Santa Clara, CA, USA) was used for the separation. Mobile phase consisted of two solvents: 2 mM NH_4HCO_3 (solvent A), and methanol (solvent B). 20%B isocratic elution was used for UHPLC separation. The flow rate was 0.25 mL/min and the column temperature was set at 25 $^\circ\text{C}$.

The mass spectrometer was operated in the positive ionization mode. Nitrogen was used for both nebulization and desolvation. The nebulization gas was set at 35 psi, the flow rate of desolvation gas was 10 L/min, and source temperature was set at 300 $^\circ\text{C}$. The sheath gas temperature was set at 350 $^\circ\text{C}$ and the flow rate was 12 L/min. Capillary voltage was set at 3500 V. Nozzle Voltage was set at 2000 V.

The UHPLC–MS/MS analysis was carried out with an Agilent 1290 UHPLC system coupled with a G6410B triple quadrupole (QQQ) mass spectrometer (Santa Clara, CA, USA). Zorbax Eclipse Plus C18 column was used for separation. The optimized mobile phase consisted of two solvents: 2 mM NH_4HCO_3 (solvent A), and methanol (solvent B). Gradient elution: 0–5.0 min, 5%B; 5.0–12.5 min, 10%B; 12.5–18 min, 15%B; 18–22 min, 90%B; 22–28 min, 5%B. The flow rate was 0.25 mL/min with the column

Fig. 1. Extracted ion chromatograms (EICs) of each SPE fraction obtained by UHPLC–QTOF. a, C18; b, HLB. EICs of ProdG adducts were extracted by its theoretical value of 338.1459 with mass error equal to 50 ppm.

temperature set at 25 $^\circ\text{C}$. Each sample was analyzed for three times with an injection volume of 5.0 μL .

The mass spectrometer was operated in the positive ionization mode. A multiple reaction monitoring (MRM) mode was adopted for selective detection of the ProdG adducts: m/z 338.1 $\rightarrow$ 178.0 (collision energy, 25 eV) and m/z 338.1 $\rightarrow$ 222.1 (collision energy, 5 eV) for ProdG; m/z 343.1 $\rightarrow$ 227.1 (collision energy, 5 eV) for [$^{15}\text{N}_5$] ProdG. Nitrogen was used for both nebulization and desolvation. The nebulization gas was set at 40 psi, the flow rate of desolvation gas was 9 L/min, and the source temperature was set at 300 $^\circ\text{C}$. Capillary voltage was set at 3500 V. High purity nitrogen (99.999%) was used as the collision gas.

2.6. Method validation

Standard solutions with varying amounts of ProdG containing 1 nM each of [$^{15}\text{N}_5$]ProdG were prepared and analyzed by UHPLC–MS/MS. Calibration curves were obtained by linearly plotting the peak area ratios of ProdG/[$^{15}\text{N}_5$]ProdG vs the corresponding ProdG. The recovery was measured by spiking human urine with the known amounts of ProdG and 1 nM each of [$^{15}\text{N}_5$]ProdG. The prepared urine samples were treated by SPE protocol and then analyzed by UHPLC–MS/MS. The recovery was calculated according to the formula: $\text{Recovery}\% = (\text{the measured amount} - \text{the background amount}) / \text{added amount} \times 100\%$. The precision of intraday and interday were estimated by triplicate

quantification of ProdG in human urine on a single day and on three consecutive days, respectively.

3. Results and discussion

3.1. Optimization of solid-phase extraction protocol

More than three thousand chemicals have been found in human urine. Among them, salts, which can suppress ionization efficiency and contaminate ion source are present with high abundance [30]. In order to clean up salts and selectively enrich urinary ProdG adducts, C18 and HLB cartridges were used to obtain the optimized extraction. We observed that C18 cartridges can not completely absorb ProdG adducts during loading the standard solution into the cartridge and most of ProdG adducts were washed out by pure

water (Fig. 1a). In comparison to C18, HLB cartridges were observed to absorb the ProdG adducts completely. In addition, ProdG adducts were not washed away when HLB cartridge was washed by pure water. Consequently, salts can be eliminated with ProdG adducts being absorbed in cartridge. By adjusting the ratio of H₂O and MeOH, ProdG adducts were concentrated on 30% MeOH and 50% MeOH (Fig. 1b). By the use of HLB cartridges, salts (Na⁺, Cl⁻, K⁺ and urea) which cause ionization suppression and ion source contamination can be effectively eliminated and ProdG adducts were selectively enriched.

3.2. Method validation

Excellent linearity for each ProdG adducts ((6S,8S)ProdG: $y = 1.077x - 0.002$, (6R,8R)ProdG: $y = 1.015x - 0.002$, $R^2 \geq 0.999$) is

Fig. 2. Identification of ProdG adducts in human urine by UHPLC-MS/MS (no.1 urine sample). a, urinary ProdG (338.1 → 222.1) vs [¹⁵N₅] ProdG (343.1 → 227.1); b, urinary ProdG (338.1 → 178.0) vs [¹⁵N₅]ProdG (343.1 → 227.1); c, urinary ProdG (338.1 → 222.1) vs urinary ProdG (338.1 → 178.0); d, standard ProdG (338.1 → 222.1) vs standard ProdG (338.1 → 178.0).

Table 1
Quantification of ProdG adducts in human urine.

Sample number	Gender	ProdG (fmol/L) (mean ± SD)		ProdG/creatinine (nmol/mol) (mean ± SD)	
		(6S,8S)	(6R,8R)	(6S,8S)	(6R,8R)
1	Male	8.7 ± 0.2	4.8 ± 0.1	0.77 ± 0.02	0.43 ± 0.01
2	Female	9.4 ± 0.1	6.7 ± 0.2	0.84 ± 0.09	0.60 ± 0.02
3	Male	45.9 ± 1.4	35.3 ± 1.2	3.47 ± 0.11	2.67 ± 0.09
4	Female	7.1 ± 1.0	7.4 ± 1.3	0.58 ± 0.08	0.61 ± 0.11
5	Female	9.8 ± 0.5	11.2 ± 0.9	1.19 ± 0.06	1.36 ± 0.11
6	Female	11.5 ± 1.2	10.0 ± 1.8	0.94 ± 0.10	0.82 ± 0.15
7	Male	9.3 ± 0.3	8.0 ± 0.1	1.33 ± 0.04	1.14 ± 0.02
8	Female	3.2 ± 0.4	3.6 ± 0.3	0.31 ± 0.04	0.35 ± 0.03
9	Male	16.7 ± 0.6	16.1 ± 0.7	1.69 ± 0.06	1.63 ± 0.07
10	Female	5.2 ± 0.3	4.1 ± 0.2	0.49 ± 0.03	0.39 ± 0.02
11	Female	6.8 ± 0.4	6.1 ± 0.7	0.67 ± 0.04	0.60 ± 0.07
12	Male	4.2 ± 0.2	4.3 ± 0.3	0.38 ± 0.02	0.39 ± 0.03
13	Female	3.6 ± 0.3	5.5 ± 0.5	0.34 ± 0.03	0.52 ± 0.05
Average		10.9 ± 11.2	9.5 ± 8.5	1.01 ± 0.85	0.89 ± 0.67

achieved with a dynamic range of 0.5–50 fmol. The limit of detection (LOD, $S/N = 3$) is estimated to be about 50 amol and the limit of quantity (LOQ, $S/N = 10$) is about 150 amol in standard solution. The estimated recoveries are about 85.4–107.2% for (6S,8S)ProdG and 84.8–106.9% for (6R,8R)ProdG, respectively. The intraday precision values represented by relative standard deviations (RSDs) were 0.8–3.0%, and the interday precision values differ from 2.0–10.0%.

The matrix effect was evaluated by comparing the peak areas of spiked 1 nM [$^{15}\text{N}_5$]ProdG in SPE treated urine sample with that in standard solution. The ratios are from 0.35–0.60 for the tested 13 urine samples. In consideration of matrix effect, the LOD and LOQ are estimated about 100 and 300 amol, respectively.

3.3. Identification and quantification of ProdG in human urine

Two characteristic ion pairs were selected for the identification of the urinary ProdG adducts. Both the primary peak (338.1 $\rightarrow$ 222.1) and the secondary peak (338.1 $\rightarrow$ 178.0) were completely overlapped to that of the added [$^{15}\text{N}_5$]ProdG (343.1 $\rightarrow$ 227.1) (Fig. 2a and b). Furthermore, the area of the primary peak is 2.3 times bigger than that of the secondary peak in human urine. Similar result was observed for ProdG standards (Fig. 2c and d). These results demonstrate that the detected peaks in human urine have the same properties to that of the ProdG standard in chromatographic retention and MS fragmentation pattern, providing substantial evidence for the presence of ProdG adducts in human urine.

Urine samples provided by thirteen healthy people were collected for ProdG examination using the developed method. The results are summarized in Table 1.

4. Conclusions

An SPE coupled stable isotope dilution UHPLC–MS/MS method was developed for sensitive detection and accurate quantification of ProdG adducts in human urine. A specific SPE approach was developed for selective enrichment of urinary ProdG adducts and effective elimination of urinary matrix. By the use of the developed method, two diastereomers of urinary ProdG adducts can be sensitively detected and accurately quantified with only 200 μL of human urine. The results of this work provide compelling evidence supporting the presence of ProdG adducts in healthy human urine.

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