Detection of 1,*N*²-propano-2'-deoxyguanosine adducts in genomic DNA by ultrahigh performance liquid chromatography-electrospray ionization-tandem mass spectrometry in combination with stable isotope dilution

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ABSTRACT

Crotonaldehyde (Cro) is one of widespread and genotoxic α,β -unsaturated aldehydes and can react with the exocyclic amino group of 2'-deoxyguanosine (dG) in genomic DNA to form 1,*N*²-propano-2'-deoxyguanosine (ProdG) adducts. In this study, two diastereomers of high purity were prepared, including non-isotope and stable isotope labeled ProdG adducts, and exploited stable isotope dilution-based calibration method. By taking advantage of synthesized ProdG standards, we developed a sensitive ultrahigh performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UHPLC-ESI-MS/MS) method for accurate quantification of two diastereomers of ProdG adducts. In addition to optimization of the UHPLC separation, ammonium bicarbonate (NH₄HCO₃) was used as additive in the mobile phase for enhancing the ionization efficiency to ProdG adducts and facilitating MS detection. The limits of detection (LODs, S/N = 3) and the limits of quantification (LOQs, S/N = 10) are estimated about 50 amol and 150 amol, respectively. By the use of the developed method, both diastereomers of ProdG adducts can be detected in untreated human MRC5 cells with a frequency of 2.4–3.5 adducts per 10⁸ nucleotides. Crotonaldehyde treatment dramatically increases the levels of ProdG adducts in human MRC5 in a concentration-dependent manner.

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1. Introduction

Crotonaldehyde (Cro), or 2-butenal is an environmental pollutant, found in automobile emissions, tobacco smoke and combustion of carbon-containing fuels and other materials [1–3]. Cro is also found in foods, including fish, meat, fruit, vegetable, and alcoholic beverage [4,5]. It is one of the lipid peroxidation products resulting from endogenous metabolism [4,6].

Cro has been shown to be mutagenic in both *Salmonella typhimurium* and human lymphoblasts and to induce liver tumors in rats [4,7,8]. The mutagenicity and carcinogenicity of Cro are related to Cro-DNA adducts [7]. Cro can react with the exocyclic

amino group of 2'-deoxyguanosine (dG) in genomic DNA, forming two diastereomers of 1,*N*²-propano-2'-deoxyguanosine (ProdG), (6*S*,8*S*) and (6*R*,8*R*) [9,10]. ProdG adducts can also be produced by the consecutive reaction of two molecules of acetaldehyde with dG [9,11,12]. Surprisingly, polyamines and histone can promote the formation of ProdG adducts [13]. ProdG adducts in DNA have the open and closed forms, which are present in a dynamic equilibrium. The closed form predominates in single-stranded DNA (ssDNA), while the open form predominates in double-stranded DNA (dsDNA) [14]. ProdG adducts can further cause inter-strand cross-links and DNA-protein cross linking [15,16]. In human cells, ProdG adducts mainly promote DNA miscoding through G → T transversions and inhibit DNA synthesis [17]. Miscoding is observed more frequently with (6*S*,8*S*)ProdG than with (6*R*,8*R*)ProdG [18]. ProdG adducts have been detected in genomic DNA in various tissues of animals and human even without

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any extracorporeal Cro treatment [3,9], indicating that the ProdG adducts can be generated endogenously [19]. Nevertheless, ProdG adducts displayed a higher level in human lung DNA than that in liver DNA, especially for smokers [3,18].

Since ProdG adducts play an important role in the Cro genotoxicity [20], a sensitive and accurate method for quantification of ProdG adducts is required. A number of approaches have been developed, including the commonly used but labor-intensive 32 -P post-labeling [21–24] and LC–MS [17,18,25,26]. Among them, the mass spectrometry based methodologies take the advantages of direct analysis of polar DNA adducts with structural information [26]. With the development of electrospray ionization (ESI) technique for mass spectrometry analysis [27], LC–MS has been widely applied in biological field and the detection sensitivity increased with the continuous development of MS equipment.

In this work, we developed a highly sensitive method for accurate quantification of ProdG adducts using stable isotope dilution ultrahigh performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UHPLC–ESI–MS/MS). Our previous study showed that ammonium bicarbonate (NH_4HCO_3) significantly enhanced the ESI–MS/MS detection of acrolein–DNA adducts and 5-hydroxymethylcytosine [28,29]. Interestingly, we also found that ammonium bicarbonate can enhance ESI–MS/MS detection of ProdG adducts. By the use of optimized UHPLC–ESI–MS/MS method, the two diastereomers of ProdG adducts can be efficiently separated within 6.5 min and sensitively detected at amol level.

2. Experimental section

2.1. Chemicals and materials

[$^{15}\text{N}_5$]-2'-deoxyguanosine monohydrate ([$^{15}\text{N}_5$]dG·H₂O) and crotonaldehyde were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA) and Aladdin (Shanghai, China), respectively. 2'-deoxyguanosine monohydrate (dG·H₂O), venom phosphodiesterase (VPDase) and ammonium bicarbonate were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ammonium formate was ordered from Agilent Technologies Inc. (Santa Clara, CA, USA) and ammonium acetate was ordered from Signopharm Chemical Reagent Co. (Beijing, China). Deoxyribonuclease I (DNase I) and calf intestinal alkaline phosphatase (CIP) were purchased from New England Biolabs Inc. (Ipswich, MA, USA).

2.2. Synthesis of ProdG and [$^{15}\text{N}_5$]ProdG

ProdG standard was synthesized from the reaction of dG with crotonaldehyde according to previous method with a slight modification [18,30,31]. In brief, crotonaldehyde (0.045 mmol) was allowed to react with dG (5 mg) in 1.0 mL of 100 mM phosphate buffer (pH 7.0) at 37 °C for 48 h (Fig. 1a). The two diastereomeric products (6S,8S) and (6R,8R) were isolated and purified by HPLC preparation and the collected fraction was lyophilized (Fig. 1b). [$^{15}\text{N}_5$]ProdG was synthesized in the same way, but dG was replaced by [$^{15}\text{N}_5$]dG. The purified ProdG adducts were identified and characterized by UV–vis spectrophotometer and a high resolution Q-TOF LC/MS system.

2.3. HPLC–UV conditions

The HPLC–UV system was used for the preparation of ProdG and [$^{15}\text{N}_5$]ProdG adducts. This was carried out using Hitachi L-2000HPLC instruments equipped with a DAD detector and an autosampler (Hitachi, Tokyo, Japan). Venusil MP C18 column (250 mm × 4.6 mm i.d., 5.0 μm , Agela, Tianjin, China) was used for separation. Mobile phase consisted of two solvents: ultra-pure

water (solvents A) and pure methanol of HPLC grade (solvents B). The flow rate was 0.8 mL/min. The separation was conducted using isocratic elution of 25%B. The eluate was monitored at 260 nm using a DAD detector.

2.4. UHPLC–ESI–MS/MS analysis

The qualitative analysis was carried out by an Agilent 1290 UHPLC system coupled with a 6540 quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Zorbax Eclipse Plus C18 column (2.1 mm i.d. × 50 mm, 1.8 μm , Agilent Technologies, Santa Clara, CA, USA) was used for separation. Mobile phase consisted of two solvents: aqueous solution with additive (solvent A), and methanol (solvent B). Isocratic elution of 20%B was used for UHPLC separation. The flow rate was 0.25 mL/min and the column temperature was set at 25 °C. Four volatile additives, including HCOOH, HCOONH₄, CH₃COONH₄ and NH₄HCO₃ were added to solvent A and tested for optimization of the MS signals of ProdG adducts.

The Q-TOF mass spectrometer was operated in 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. Source temperature was set at 300 °C. The sheath gas temperature was set at 350 °C and the flow rate was 12 L/min. Capillary voltage was set at 3500 V. Nozzle Voltage was set at 0 V. High purity nitrogen (99.999%) was used as the collision gas. 25 eV collision energy was used in targeted MS/MS mode.

The quantitative analysis was carried out by an Agilent 1290 UHPLC system coupled with a G6410B or 6495 triple quadrupole (QQQ) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Mobile phase consisted of two solvents: 2 mM NH₄HCO₃ (solvent A), and methanol (solvent B). Gradient elution of 0.0–5.0 min, 5%B; 5.0–12.5 min, 10%B; 12.5–18 min, 15%B; 18–22 min, 90%B; 22–25 min, 5%B and isocratic elution of 20%B were used.

The QQQ mass spectrometer was operated in positive ionization mode. A multiple reaction monitoring (MRM) mode was adopted for selective detection of the dG adducts: m/z 268.1 → 110.1 for dG (collision energy, 5 eV); m/z 338.1 → 222.1 for ProdG (collision energy, 5 eV); m/z 343.1 → 227.1 for [$^{15}\text{N}_5$] ProdG (collision energy, 5 eV). 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. Source temperature was set at 300 °C. Capillary voltage was set at 3500 V. High purity nitrogen (99.999%) was used as the collision gas. Each sample was analyzed for three times with an injection volume of 2 μL .

2.5. Calibration curves and recovery

The standard solutions containing 1 nM each of [$^{15}\text{N}_5$]ProdG and varying concentrations of ProdG (0.1, 0.5, 1, 5 and 10 nM) were prepared and analyzed by UHPLC–MS/MS. Calibration curves were obtained by linearly plotting the peak area ratios of non-isotopic ProdG adducts to stable isotopic standards versus the amount of the added non-isotopic ProdG adducts.

To evaluate the recovery of the UHPLC–MS/MS method, MRC5 genomic DNA spiked with the known amounts of non-isotopic ProdG adducts and unspiked sample were separately measured. The recovery was calculated according to the formula: Recovery% = (the measured level – the background level)/added amount × 100%.

2.6. MRC5 cells culture, DNA extraction and enzymatic digestion

Human embryonic lung fibroblast MRC5 cells were seeded in 6.0 cm plastic dishes and cultured in 5.0 mL DMEM growth medium

Fig. 1. (a) The formation of 1,N²-propano-2'-deoxyguanosine from the reaction of crotonaldehyde with 2'-deoxyguanosine in DNA; (b) synthesis and preparation of ProdG or [¹⁵N₅]ProdG adducts; (c) identification and detection of ProdG adducts in MRC5 cells.

supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin G and 100 U/mL streptomycin sulfate. The dishes were put in a humidified 37 °C incubator supplied with 5% CO₂.

The genomic DNA of MRC5 cells was extracted with the Promega Wizard genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The extracted DNA was redissolved in 50 µL of water. The amount of DNA was determined at 260 nm using Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA) and estimated by assuming that 1.0 OD equals to 50 µg/mL. For the detection of ProdG adducts, the DNA samples (5 µg each) were mixed with 1.0 nM (final concentration) of [¹⁵N₅](6S,8S)ProdG and [¹⁵N₅](6R,8R)ProdG each (Fig. 1c). The samples were enzymatically digested with 2.5 units of DNase I, 1.5 units of CIP, and 0.1 mg of VPDase at 37 °C for 12 h. The total volume was 50 µL. Then the samples were filtered by ultrafiltration tubes (MW cutoff: 3 KDa, Pall, Port Washington, NY, USA) to remove the enzymes and subjected to UHPLC-MS/MS analysis. Each sample was analyzed at least three times.

3. Results and discussion

3.1. Synthesis, preparation and characterization of standards

ProdG (or [¹⁵N₅]ProdG) standards were synthesized from the reaction of dG (or [¹⁵N₅]dG) with crotonaldehyde. The two diastereomeric products (6S,8S) and (6R,8R) were prepared by conventional HPLC. Under the optimization condition, the baseline separation (*R* > 1.5) of two diastereomer was achieved (Fig. 2a). The

two fractions corresponding to the peaks at 13.9 min and 16.0 min were collected, respectively. Then the elution fractions were evaporated with nitrogen and lyophilized.

The characteristic molar absorptivity at 260 nm ($\epsilon_{\text{max}}(260\text{nm})$) of the two purified ProdG adducts were measured by UV spectrophotometer and calculated with Lambert-Beer law. By testing 0.3 mM (6S,8S)ProdG and 0.31 mM (6R,8R)ProdG, we obtained UV absorbance 0.467 and 0.487, respectively. Consequently, the estimated $\epsilon_{\text{max}}(260\text{nm})$ are 15567 for (6S,8S)ProdG and 15710 for (6R,8R)ProdG which are consistent with previous studies (UV $\epsilon_{\text{max}}(260\text{nm})$ = 15600 for (6S,8S)ProdG; UV $\epsilon_{\text{max}}(260\text{nm})$ = 15700 for (6R,8R)ProdG) [14,18].

Both the theoretical and measured *m/z* values of purified ProdG adducts are shown in Table 1. The measured mass values are very close to the theoretical mass values, and the relative mass errors are from 0.59–1.75 ppm. Full scan MS mode for the detection of molecular ion and targeted MS/MS mode for the detection of the fragment ions were operated synchronously. We observed *m/z* 338.1459 for [M + H]⁺ (M = ProdG), 222.1070 for [M + H-dR]⁺ (dR = Ribodesose) and 178.0722 for [M + H-dR-C₂H₄O]⁺ (Fig. 2b). Similar MS fragmentation patterns for [¹⁵N₅]ProdG were also observed. These results confirmed that the ProdG was successfully prepared.

3.2. Optimization of separation and MS detection of the diastereomers of ProdG adducts

Previously, we showed that ammonium bicarbonate can improve the MS signals of ArcdG adducts and 5-hydroxymethylcytosine [28,29]. We speculated that, like ArcdG,

Fig. 2. Preparation and characterization of ProdG standards. (a) Chromatograms obtained upon HPLC preparation of ProdG (UV wavelength at 260 nm); (b) typical mass spectrograms obtained upon UHPLC-QTOF in full scan MS mode and targeted MS/MS mode (CID 25 eV).

Table 1

The relative deviation of m/z between theoretical and measured of ProdG adducts. The theoretical values are calculated with software according to the relative abundance of elements.

dG adducts	Molecular formula	Theoretical m/z	Measured m/z	Relative mass error (ppm, $\times 10^{-6}$)
ProdG	$C_{14}H_{19}N_5O_5$	338.1459	338.1461	0.59
$[^{15}N_5]ProdG$	$C_{14}H_{19}[^{15}N_5]O_5$	343.1311	343.1317	1.75

ammonium bicarbonate can enhance MS detection of ProdG adducts because they also contain dG moiety.

We tested four additives, which was added to the mobile phase one by one, including three volatile ammonium salts (2–10 mM) and formic acid (0.01–0.1% v/v). By the use of optimized mobile phase (Fig. 3b), two diastereomers of ProdG adducts were well separated regardless of additives. As demonstrated in Fig. 3, ammonium bicarbonate can enhance the MS signals of ProdG adducts by 2.15–3.23 folds and 1.49–1.63 folds comparing to the same concentration of ammonium formate and ammonium acetate, respectively. In comparison to formic acid, ammonium bicarbonate improves the MS signals by 2.17–3.40 times for ProdG adducts (Fig. 3a and b).

We further examined the ionization complexes of ProdG adducts using UHPLC-QTOF under full scan mode. The acquisition range of m/z was set from 100 to 1000. As shown in Fig. 3c, $[M + Na]^+$ and $[M + K]^+$ are the major metal complexes formed during ionization. When $HCOOH$, $HCOONH_4$ and CH_3COONH_4 were

used as additives, the abundance of $[M + Na]^+$ complexes accounted for 1.05–3.03% and $[M + K]^+$ was about 0.08–1.38%, compared with $[M + H]^+$ (100%). Interestingly, when NH_4HCO_3 was used, the $[M + Na]^+$ and $[M + K]^+$ complexes were reduced to 0.14% and 0.02%, respectively. In a word, NH_4HCO_3 can enhance the protonation of ProdG and suppress the formation of unwanted ProdG-metal complexes so that the MS signal increases. On the basis of the results above, 2 mM NH_4HCO_3 displayed an optimum effect and was selected for the detection of ProdG adducts.

3.3. Calibration curves, and recovery

The calibration curves were obtained by Agilent G6410B triple quadrupole mass spectrometer. Excellent linearity for each ProdG adducts (correlation coefficient: $R^2 \geq 0.999$) was achieved with a dynamic range of 0.2–20 fmol. The limits of detection (LODs, $S/N = 3$) are about 50 amol and the limits of quantification (LOQs, $S/N = 10$) are about 150 amol (Table 2). This was achieved using G6410B MS. When the latest series of Agilent triple quadrupole mass spectrometer (G6495) is used, the sensitivity further increases at least 10 times.

The recovery was determined by spiking MRC5 DNA with the known amounts of ProdG adducts (1.0, 5.0 and 10.0 fmol). The recoveries varies from 79.9% to 105.4% for (6S,8S)ProdG, and 80.7% to 114.4% for (6R,8R)ProdG (see Table 3).

Table 2

Dynamic range, linearity, and LOD of ProdG adducts by UHPLC-MS/MS.

Analyte	Dynamic range (fmol)	Linearity	R^2	LOD (amol)
(6S,8S)ProdG	0.2–20	$y = 1.018x - 0.045$	0.999	50
(6R,8R)ProdG	0.2–20	$y = 1.005x - 0.022$	0.999	50

Fig. 3. The optimization of separation and MS detection of ProdG adducts. (a) MS signals of (6S,8S)ProdG and (6R,8R)ProdG obtained upon UHPLC-QTOF using different concentration of HCOOH, HCOONH₄, CH₃COONH₄ or NH₄HCO₃ as additives to the mobile phase ($n=3$); (b) extracted ion chromatograms (EICs) of (6S,8S)ProdG and (6R,8R)ProdG; (c) typical mass spectrum of ProdG (take (6S,8S) as a model). The additive in solvent A for b and c was 2.0 mM ammonium salts or 0.1% (v/v) formic acid. Isocratic elution of 20%B was used for separation of two diastereomers of ProdG adducts. The final concentration of each standard ProdG was 100 nM and the injection volume was 5.0 μ L. [B+H]⁺ corresponds to the molecular ion by losing a molecule of ribose group and EICs were extracted by their theoretical values (m/z 338.1495).

Table 3
Recovery of ProdG adducts by UHPLC–MS/MS.

Analyte Adding amount (fmol)	(6S,8S)ProdG (%)	(6R,8R)ProdG (%)
1.0	103.6 ± 8.1	98.3 ± 2.2
5.0	79.9 ± 5.2	80.7 ± 3.9
10.0	105.9 ± 5.4	114.4 ± 11.4

Fig. 4. Identification of ProdG adducts in cultured MRC5 cells by UHPLC–MS/MS. Mobile phase conditions: (a) 0.0–5.0 min, 5%B; 5.0–12.5 min, 10%B; 12.5–18 min, 15%B; 18–22 min, 90%B; 22–25 min, 5%B; (b) 20%B. Details are described in Experimental Section. Since the work was completed by three mass spectrometers (G6540, G6410B and G6495) and guard column was selectively used (G6410B use, G6540 nonuse and G6495 use), the retention time of ProdG adducts may shift under the same elution condition.

3.4. Identification and detection of ProdG adducts in untreated MRC5 cells

MRC5 cells are human embryonic lung fibroblasts without cancerization [32–34]. To determine whether ProdG is present in genomic DNA of untreated MRC5 cells, we tested two elution procedures (one gradient elution and the other isocratic elution) for UHPLC separation (Fig. 4). We observed that the two diastere-

Fig. 5. The induction of ProdG adducts in Cro treated MRC5 cells.

omers of the ProdG adducts (black line, m/z 338.1 → 222.1) are completely overlapped to corresponding diastereomers of the stable isotopic labeled standards ($[^{15}\text{N}_5]\text{ProdG}$ adducts, red line, m/z 343.1 → 227.1), although their retention times varied with the changed elution. Moreover, even with varied retention times, the peak area ratios of ProdG adducts to $[^{15}\text{N}_5]\text{ProdG}$ adducts do not change (0.06 for (6S,8S) and 0.04 for (6R,8R)). These results provide compelling evidence supporting the presence of ProdG adducts in untreated MRC5 cells. The mean frequencies of (6S,8S)ProdG and (6R,8R)ProdG are 3.5 ± 0.2 and 2.4 ± 0.1 adducts per 10^8 nts (nucleotides), respectively. The results indicate that trace ProdG adducts may be spontaneously generated in cultured MRC5 cells.

3.5. The induction of ProdG adducts in treated MRC5 cells

By exploiting the developed method, we further examined the induction of ProdG adducts in MRC5 cells treated with varying concentration of Cro (1, 5, 10, 50 and 100 μM). The cells were treated for 24 h. As shown in Fig. 5, we observed that the levels of ProdG adducts were significantly increased when MRC5 cells were treated with Cro. Furthermore, higher concentration of Cro (50–100 μM) tends to induce higher levels of ProdG adducts.

4. Conclusions

In this work, we developed a sensitive approach for accurate quantification of ProdG adducts in cultured MRC5 cells using stable isotope dilution UHPLC–MS/MS analysis. Ammonium bicarbonate was used as additive to the LC mobile phase for the enhancement of MS detection. By the use of this method, two diastereomers of ProdG adducts were satisfactorily separated and accurately quantified in cultured MRC5 cells. This work proved the presence of ProdG in cultured human normal cells. In addition, the levels of ProdG adducts in human MRC5 can be apparently elevated by the exposure of crotonaldehyde in a concentration-dependent manner.

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