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Simultaneous quantitation of 7-methyl- and O^6-methylguanine adducts in DNA by liquid chromatography–positive electrospray tandem mass spectrometry

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Abstract

A methodology has been developed and validated for the simultaneous quantitation of O^6-methyl- and 7-methylguanine in DNA isolated from in vitro exposure to the model alkylating agents: *N*-methyl-*N*-nitrosourea (MNU) and methyl methane sulfonate (MMS). After exposure, DNA was isolated and directly hydrolyzed under acid conditions to hydrolytes containing DNA bases (modified and unmodified). The hydrolytes were used for direct O^6- and 7-methylguanine quantitation using a rapid and selective liquid chromatography–electrospray tandem mass spectrometry (LC/ESI-MS/MS). The lower limits of quantitation for O^6-methyl- and 7-methylguanine were 75.8 and 151.5 fmol, respectively. Linearity of the calibration curve was greater than 0.999 from 75.8 to 151,600.0 fmol for O^6-methylguanine and 0.999 from 151.5 to 303,200.0 fmol for 7-methylguanine. The intra-day assay precision relative standard deviation (R.S.D.) values for O^6-methylguanine for quality control (QC) samples were $\leq$9.2% with accuracy values ranging from 90.8 to 118%, and for 7-methylguanine the R.S.D. values for QC samples were $\leq$11%, with accuracy values ranging from 92.9 to 119%. The inter-day assay precision (R.S.D.) values for O^6-methylguanine QC samples were $\leq$7.9% with accuracy values ranging from 94.5 to 116%, and for 7-methylguanine QC samples were $\leq$7.1% with accuracy values ranging from 95.2 to 110.2%. This method was used for simultaneous determination of the levels of 7-methyl- and O^6-methylguanine in DNA acidic hydrolytes present in a series of incubations from salmon testis DNA treated with either MNU or MMS.

Keywords: Quantitation; 7-Methyl- and O^6-methylguanine adducts; Liquid chromatography–tandem mass spectrometry

1. Introduction

It has been accepted that many chemical carcinogens initiate the complex process of mutagenesis and carcinogenesis by interaction of electrophilic moieties from these agents with critical nucleophilic sites (the nitrogen and oxygen atoms in bases, and the phosphate oxygen in the sugar-phosphate backbone) in DNA [1]. Alkylating chemicals such as *N*-methyl-*N*-nitrosourea (MNU) and methyl methane sulfonate (MMS) have been widely used as model alkylating agents, capable of inducing cancer. These chemicals can react with the nucleophilic sites in DNA to form the major alkyl (methyl) adduct at the 7 atom of guanine [2]. However, other minor alkyl (methyl) DNA adducts at other positions of DNA bases (such as O^6 atom of guanine) can be formed. Among these minor alkyl (methyl) adducts, methylation at the O^6-position of guanine has been shown to induce mispairing and is associated with malignancy and mutagenicity [3]. Different agents result in different alkylation profiles and sensitive methods for detecting and quantifying these DNA adducts are indispensable for comparative analysis of data on adducts, mutation induction, and mutational spectra in cells or tissues exposed to those alkylating agents. Through such comparative analysis, information regarding the mechanisms of mutagenicity and carcinogenicity of individual methylated DNA adducts in different tissues or cells may be explored.

Early efforts to quantify these methylated DNA adducts involved the use of radioactively labeled genotoxic alkylating agents, resulting in the presence of radiolabeled adducts in DNA which, upon hydrolysis and subsequent separation by HPLC, were quantified by virtue of the radioactive label [2,4]. This kind of procedure is expensive because it requires the syn-

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thesis of radiolabelled mutagens and special precautions are necessary for proper radiochemical handling for such methods. Alternatively, these adducts can be quantified using immunological detection systems [5], however, this method requires the availability of antibodies capable of selectively recognizing the specific DNA adduct either in intact cells, in isolated DNA or in hydrolyzed DNA. The availability and selectivity of antibodies, and additional, onerous, sample handling requirements also limit this method's application. In addition, ^{32}P-postlabeling methods have been developed and used for DNA adduct quantitation [6], but this method entails use of radioactive materials, and can produce ambiguous results caused by nonspecific binding or lack of appropriate authentic standards [7].

Recent developments in mass spectrometric technology, coupled with liquid chromatography is providing a new tool for DNA adducts quantitation [7]. For example, with this technology, O^6-methyl-2′-deoxyguanosine and 7-methyl-2′-deoxyguanosine adducts were quantified from rat tissues after exposure to *N*-methyl-nitrosourea (MNU) [8]. However, this methodology still depends on slow (long time) sequential enzymatic digestion, which requires expensive phosphodiesterase (such as nuclease P1). Besides, the 7-methyl-2′-deoxyguanosine is unstable even under physiological conditions [8]. This could lead to the degradation of 7-methyl-2′-deoxyguanosine released during the DNA enzymatic hydrolysis, giving an inaccurate quantitation of 7-methyl-2′-deoxyguanosine in the DNA under study.

We report here a new method using liquid chromatography–positive electrospray tandem mass spectrometry (LC/ESI-MS/MS) for the separation and direct quantitation of 7-methyl- and O^6-methylguanine adducts from acid hydrolysis of salmon testis DNA treated in vitro with MNU or MMS.

2. Experimental

2.1. Caution

The MNU and MMS were handled in accordance with NIH guidelines for Laboratory Use of Chemical Carcinogens [9].

2.2. Reagents, solvents and materials

Acetonitrile (HPLC grade), Methanol (HPLC grade), water (HPLC grade), acetic acid (HPLC grade), ammonium hydroxide, ammonium acetate, and formic acid were obtained from Fisher Scientific (Itasca, IL, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

2.3. Standards

7-Methylguanine, O^6-methylguanine, and [^{2}H$_3$]-O^6-methylguanine (Fig. 1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). [^{2}H$_3$]-7-Methylguanine (Fig. 1) was synthesized based on published references with minor modifications [10,11]. Briefly, 2′-deoxyguanosine hydrate (300 mg, 1.053 mmol) was dissolved in dimethyl sulphoxide (DMSO, 3 mL) and iodomethane-d_3 (400 μL, 6.42 mmol) was added to the mixture at room temperature. The final mixture was stirred for 2.5 h at room temperature. The mixture turned orange. Excess iodomethane-d_3 was removed by purging with nitrogen. The final solution was diluted with chloroform (30 mL) at room temperature and then cooled to 0 °C for 1 h. The resulting precipitate was isolated by vacuum filtration and washed with cold ethanol (3 mL) and ethyl ether (5 mL). Drying gave 2′-deoxy-7-methylguanosinium iodide (385 mg, Fig. 1). 2′-Deoxy-

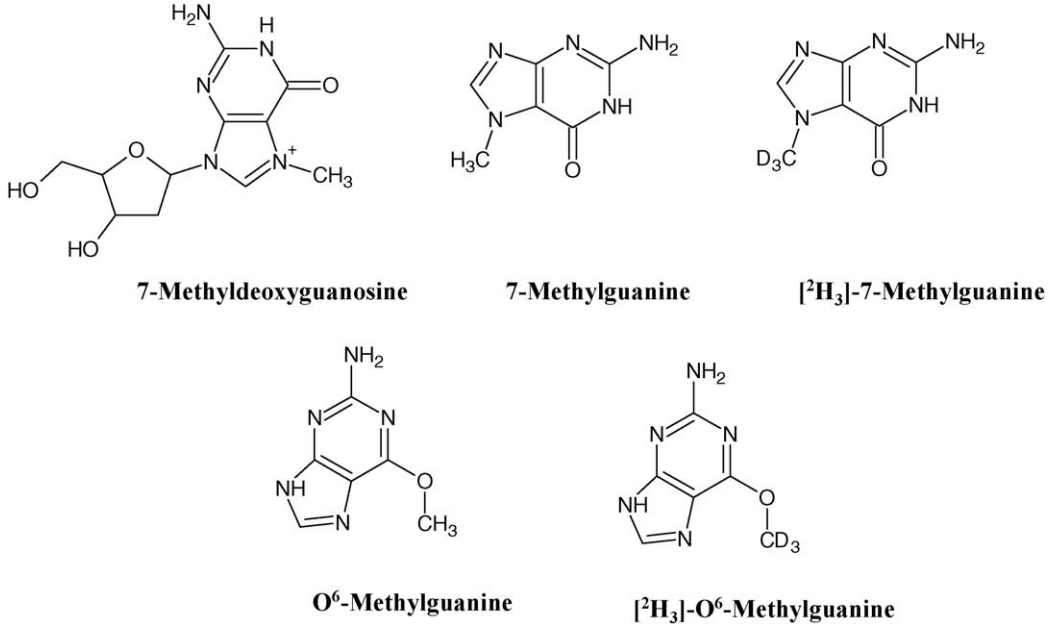

Fig. 1. Chemical structures of some DNA bases and internal standards.

7-methylguanosinium iodide (230 mg) was dissolved in HPLC water (5 mL) and concentrated hydrochloride (100 μL, 10N) was added. The mixture was heated in a water bath (85–90 °C) for 1.5 h. After being cooled to room temperature, the pH of the solution was adjusted to 8.5 with ammonium hydroxide. The precipitate was isolated by filtration and washed by HPLC water (2 × 2 mL), cold ethanol (2 × 2 mL), and ethyl ether (2 × 2 mL), respectively. Vacuum drying gave [$^2H_3$]-7-methylguanine (30 mg). MS full scan (positive ion electrospray) of the resulting material gave a major protonated molecular ion with *m/z* 169.2 (100%) $[M+H]^+$; MS/MS with CID of *m/z* 169.2 of protonated [$^2H_3$]-7-methylguanine gave a major product ion with *m/z* 152.2 (100%) $[M+H-17]^+$.

2.4. Alkylation of salmon testis DNA with MNU and MMS

Salmon testis DNA, which was shown to have A_{260}/A_{280} ratio of 1.8–1.9, indicating no RNA contamination in the DNA, was dissolved in HPLC grade water buffer (pH 7) at 50–60 °C to form 4 mg/mL DNA stock solution. Stock solutions of the MNU or MMS were prepared in DMSO at concentrations of 0, 5, 200, 500, 2000, and 5000 μM. These freshly prepared stock solutions were added to the corresponding DNA solutions containing a fixed amount of DNA (2 mg/mL) to form a series of in vitro DNA incubation solutions containing 0, 0.5, 20, 50, 200, and 500 μM MNU or MMS, respectively. These solutions were then incubated at 37 °C for 4 h. To precipitate the DNA, ammonium acetate water solution (3 M) was added to the corresponding incubation solution (0.1 volume) and the resulting sample was cooled on ice followed by addition of ice-cold ethanol (2.0 volumes). The final sample mixture was placed in a freezer for 4 h. The precipitated DNA was isolated and redissolved in water and precipitated two more times according to the above procedure. The final DNA was isolated by microcentrifugation for 5 min at 6000 rpm. The isolated DNA pellet was redissolved in HPLC water to form about 1 mg/mL DNA solution and stored at −80 °C for later acid hydrolysis. The final DNA concentration of each treated DNA sample was determined by calculating the ratio of absorbance (A_{260}/A_{280}); for MNU-treated DNA samples the concentrations ranged from 0.84–1.02 mg/mL and for MMS treated DNA samples the concentrations ranged from 0.80–0.92 mg/mL.

2.5. Acid hydrolysis of alkylated DNA

DNA solution (250 μL, control and treated with MNU or MMS) was mixed with 250 μL HPLC grade water and 50 μL formic acid (90% in water) was added to the mixture. After mixing, the solution was heated at 85 °C for 60 min and cooled down to room temperature. The final hydrolytes were stored frozen until used for quantitation of O^6-methylguanine and 7-methylguanine by LC/ESI-MS/MS.

2.6. LC/ESI-MS/MS conditions

Analysis was performed on a YMC^{TM}ODS-AQ 3 mm × 100 mm C_{18} column from Waters Instruments (Milford, MA, USA) using Agilent HP 1100 HPLC system (Palo Alto, CA, USA). Mobile phases consisted of HPLC-grade water containing 0.05% acetic acid (A) with pH 7.0 adjusted by ammonium hydroxide and acetonitrile (B). The gradient profile started with 1% B and linearly increased over 8 min to 95% B where it was held for 1 min. The gradient was then decreased to 5% B in 0.5 min, and a 2-min equilibration time was incorporated between runs. The flow rate was 0.5 mL/min. The entire LC flow was directed into the mass spectrometer between 3 and 10 min using a Valco valve from Valco instrument Co. (Houston, TX, USA). The eluent from the HPLC was diverted to waste for the first 3 min of the run. MS detection was done in the positive ESI mode on an Applied Biosystems Sciex API 3000 tandem mass spectrometer (Concord, Ontario, Canada) equipped with a TurboIonspray® ionization source. The orifice and ring voltages were 50 and 350 V, respectively; the source block was set at 450 °C and ion source voltage to 4500 V; nebulizer and curtain gases (nitrogen) settings, 6 and 6 (arbitrary units), respectively; heater gas (nitrogen), 8 L/min. Tandem mass spectrometric analysis was performed using nitrogen as collision gas (CAD setting 9). Quantitation was performed with multiple reaction monitoring (MRM). The collision energy was 30 eV. The mass transitions (precursor to product) monitored were 166 > 149 for O^6-methylguanine and 7-methylguanine and 169 > 152 for [$^2H_3$]-O^6-methylguanine and [$^2H_3$]-7-methylguanine. A dwell time of 200 ms was used for each transition. The quadrupoles Q1 and Q3 were set on unit resolution.

Twenty-five microliters of each sample was injected to the LC–ESI-MS/MS system.

2.7. Preparation of stock and working solutions and calibration standards

Stock solutions of O^6-methylguanine and [$^2H_3$]-O^6-methylguanine were prepared at 1.0 mg/mL in methanol and stock solutions 7-methylguanine and [$^2H_3$]-7-methylguanine were prepared at 1.0 mg in methanol containing 0.1% formic acid (volume/volume; v/v). A series of standard working solutions with concentrations in the range of 0.1–100 μg/mL were obtained by further dilution of the corresponding standard stock solution with methanol. The internal standard (IS) working solutions (1 μg/mL for [$^2H_3$]-O^6-methylguanine, 0.2 μg/mL for [$^2H_3$]-7-methylguanine) were prepared by diluting the internal standard stock solution with methanol. All solutions were stored at 4 °C. Calibration standards of O^6-methylguanine spiked with a fixed amount of IS ([$^2H_3$]-O^6-methylguanine 20 ng/mL) at ratio of O^6-methylguanine/[$^2H_3$]-O^6-methylguanine of 0.005, 0.025, 0.05, 0.25, 1.25, 2.5, 5, and 50 and 7-methylguanine spiked with a fixed amount IS ([$^2H_3$]- 7-methylguanine) at ratio of 7-methylguanine/[$^2H_3$]-7-methylguanine of 0.01, 0.05, 0.1, 0.5, 2.5, 5, 10, 50, and 100 were prepared by diluting the corresponding appropriate standard working solution and IS working solution of O^6-methylguanine and 7-methylguanine in HPLC grade water containing 5% formic acid (v/v).

Quality control (QC) samples were prepared by diluting appropriate amounts of the corresponding standard working solutions with the same fixed amount of IS (described above)

in HPLC grade water containing 0.5% formic acid (v/v) at ratio of O^6-methylguanine/[$^2H_3$]-O^6-methylguanine of 0.005, 0.5, 2.5, and 25 for O^6-methylguanine and at ratio of 7-methylguanine/[$^2H_3$]-7-methylguanine of 0.05, 1, 5, and 50 for 7-methylguanine. QC samples were also stored at 4 °C.

2.8. Samples for recovery studies

A series of samples (spikes) with the same nominal concentrations as the QC samples were prepared by diluting an appropriate amount of the corresponding standard working solutions of O^6-methylguanine and 7-methylguanine and the analogous IS working solutions into control DNA (DMSO-treated DNA) acidic hydrolytes (see above). These samples were also stored at 4 °C for later use.

2.9. Method validation

The performance characteristics of the method were established by in-house validation procedures employing assays with standard solutions, sample blanks and QC samples. Linearity, matrix effects, selectivity, precision, detection, recovery, and quantitation limits were studied.

2.9.1. Linearity

Linearity of calibration was tested by analyzing the calibration standards prepared above. Calibration curves in the concentration range of 75.8–151,600.0 fmol for O^6-methylguanine and 151.5–303,200.0 fmol for 7-methylguanine were constructed by plotting the corresponding peak-area ratios of amount of injected analytes on column versus the corresponding internal standard (IS) injected on-column. Weighted ($1/x$) linear regression analysis was used to determine the slope, intercept and correlation coefficient (R^2). The concentrations of O^6-methylguanine and 7-methylguanine were determined from the peak-area ratios by using the equations of linear regression obtained from the corresponding calibration curves. The whole process was completed by using Analyst software (version 1.4) of Applied Biosystem (Concord, Ont., Canada).

2.9.2. Precision and accuracy

Intra-day accuracy and precision (each $n = 4$) were evaluated by analysis of QC samples at different times during the same day. Inter-day accuracy and precision were determined by repeated analysis of QC samples over four consecutive days ($n = 16$). The concentration of each sample was determined using calibration standards prepared on the same day. Accuracy of the method was determined by the equation: (mean of determined concentration/actual concentration) × 100%. Precision was determined by the relative standard deviation (R.S.D.%).

2.9.3. Recovery

To determine the recovery of the quantitation method, control DNA acidic hydrolytes were spiked with analytes to form the samples (spikes) for recovery studies (see above). The resulting peak-area ratios (analyte versus IS) were compared with that of the QC samples to determine the recovery values.

2.9.4. Stability

The effect of three freeze–thaw cycles and compound stability over 4 h at room temperature were evaluated by repeat analysis ($n = 4$) of QC samples. Long-term stability was also tested by assaying frozen QC samples after storage at −18 °C for 45 days. The concentrations were determined using freshly prepared calibration curves. Stability was also evaluated by means, precision, and accuracy.

2.9.5. Limits of detection and quantitation

The limit of quantitation was set on the concentration below which the method could not operate with acceptable precision and accuracy and at which the signa-to-noise ratio was greater than 10. The limit of detection was the lowest concentration that was detectable in all replicates but not necessarily quantifiable, although distinguishable from zero (signal/noise ≥3).

2.9.6. Assay application

The present quantitation method was used to directly and simultaneously determine concentrations of O^6-methylguanine and 7-methylguanine in a series of DNA hydrolytes samples from salmon testis DNA treated in vitro with MNU or MMS.

3. Results and discussion

3.1. Development of the LC/ESI-MS/MS conditions for the direct, simultaneous quantitation of O^6-methylguanine and 7-methylguanine

Recently, LC/ESI-MS/MS has been widely used for DNA adduct identification and quantitation [12–14]. However, to our knowledge, there is only one report of simultaneous quantitation of O^6-alkylated and N7-alkylated adduct from exposures of these model alkylating agents (MNU and MMS) with this approach [8]. In this method, the alkylated DNA was enzymatic hydrolyzed to deoxynucleosides (such as O^6-methylguanosine and unstable 7-methylguanosine) which were then quantified by LC/ESI-MS/MS. However, as Lawley and Brooks noted regarding 7-alkylguanine lesion in DNA that "destabilization of the alkylated deoxyguanosine moieties result(s) principally the quaternization of the N7 which ... deplete(s) the electrons from the guanine ring system" [15]. Consistent with this idea, almost all known decomposition reactions of 7-alkylguanine lesions are driven by neutralization of the formal charge that is imposed on the ring system by 7-alkylation [16]. Based on this point, the 7-methyldeoxyguanosine is unstable and can decompose, which was confirmed from previous method [8]. Therefore, the 7-methyldeoxyguanosine likely does not accurately represent total 7-methylation adducts in DNA, without considerable precautions to account for this decomposition. More recently, Ziegel et al. [17] reported a method to quantify the O^6-methyldeoxyguanosine (O^6-Me-dG) with the same approach developed by Yang et al. [8] and quantify the 7-MeG by LC–ESI-MS (single ion monitoring, SIM) sepatarely. Single ion monitoring (LC–ESI-MS) lacks selectivity compared to selective liquid chromatography tandem mass spectrometry (LC/ESI-MS/MS), therefore interferences from a complex

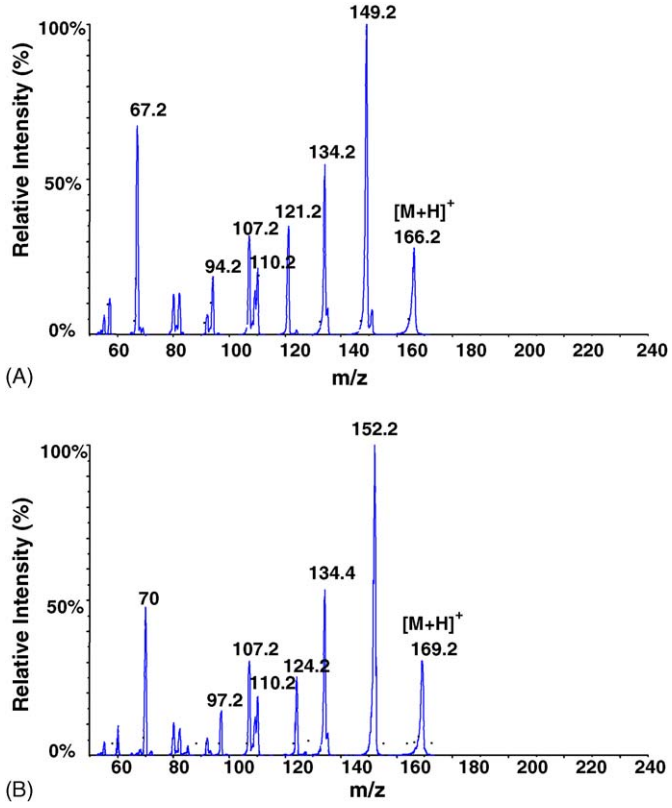

Fig. 2. (A) Positive ESI product ion spectra of $[M+H]^+$ ions of O^6-methylguanine. (B) Positive ESI product ion spectra of $[M+H]^+$ ions of $[^2H_3]$-O^6-methylguanine.

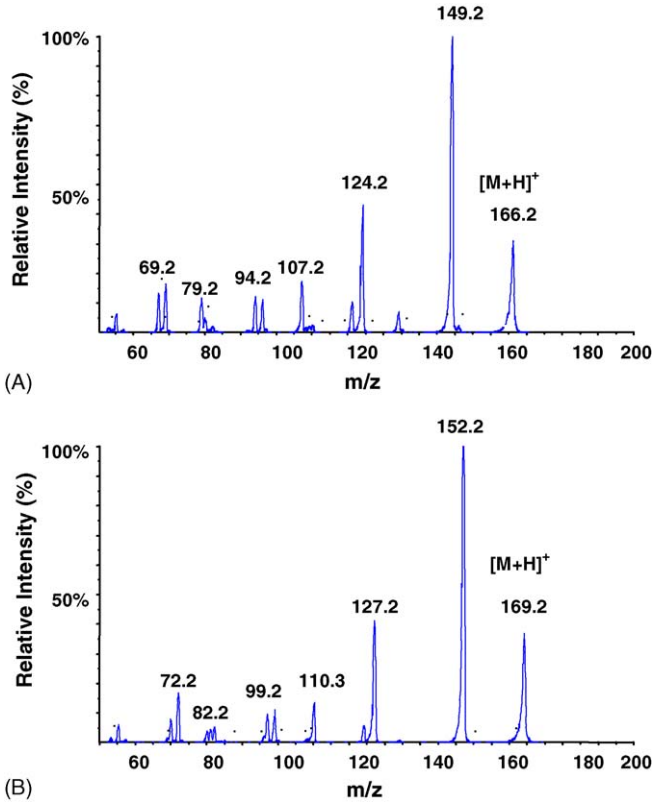

Fig. 3. (A) Positive ESI product ion spectra of $[M+H]^+$ ions of 7-methylguanine. (B) Positive ESI product ion spectra of $[M+H]^+$ ions of $[^2H_3]$-7-methylguanine.

matrix may more greatly affect the quantitation result by single ion monitoring. Besides, this method is not fully validated. In consideration of drawbacks of the previous methods, the O^6-methylguanine and 7-methylguanine and their corresponding stable internal standards were purchased or synthesized and used for the development of LC/ESI-MS/MS quantitation of methylated adducts at O^6 and 7 positions of guanine at DNA through the acid hydrolysis of DNA [2,18,19]. Originally, both positive and negative LC–ESI-MS/MS analyses of the two compounds were tested for sensitivity, positive mode LC–ESI-MS/MS was found to give more sensitivity than the negative mode (data not shown). The tandem mass spectra of O^6-methylguanine and its IS ($[^2H_3]$-O^6-methylguanine) and of 7-methylguanine and its IS ($[^2H_3]$-7-methylguanine) were shown in Figs. 2 and 3, respectively. The protonated $[M+H]^+$ ion of O^6-methylguanine (*m/z* 166.2) provided a major product ion (*m/z* 149.2), and the protonated $[M+H]^+$ ion of $[^2H_3]$-O^6-methylguanine (*m/z* 169.2) provided a major product ion (*m/z* 152.2) (Fig. 2). In the same pattern, 7-methylguanine and $[^2H_3]$-7-methylguanine formed their corresponding product ions (Fig. 3). The precursor-product ion multiple-reaction monitoring (MRM) transition (*m/z* 166.2 > 149.2) of O^6-methylguanine or 7-methylguanine and the transition (*m/z* 169.2 > 152.2) of $[^2H_3]$-O^6-methylguanine or $[^2H_3]$-7-methylguanine were therefore optimized for intensity using direct infusion of O^6-methylguanine or 7-methylguanine.

Various combination of organic solvents (such as acetonitrile and methanol), organic solvents containing adequate amounts of acid or basic compounds (such as acetic acid, formic acid, ammonium hydroxide, or ammonium acetate), and HPLC-grade water or HPLC-grade water containing acid or basic compounds, were investigated with a view to optimizing the mobile phase for sensitivity, speed, and peak shape. The best sensitivity and selectivity was achieved with HPLC-grade water containing 0.05% acetic acid, adjusted to pH 7.0 with ammonium hydroxide as the inorganic mobile phase and acetonitrile as the organic mobile phase. When the sample contained 0.5% formic acid (v/v), the peak shape was also improved. This sample treatment with the mobile phase system was finally used for the optimization of the electrospray source parameter settings. The final combined optimized conditions were used for the quantitation of O^6-methylguanine and 7-methylguanine in DNA hydrolytes.

3.2. Chromatography and selectivity

The typical multiple reaction monitoring (MRM) ion chromatogram was shown in Fig. 4. The overall chromatography run times were within 13 min. Under the LC/ESI-MS/MS conditions used here, the analytes of O^6-methylguanine and 7-methylguanine have a retention time 5.6 and 6.1 min, respectively (Fig. 4A), and the corresponding internal standards have the same retention times as their corresponding analytes (Fig. 4B). As shown in Fig. 4, ion chromatograms were free from interference and peak shapes were sharp and essentially

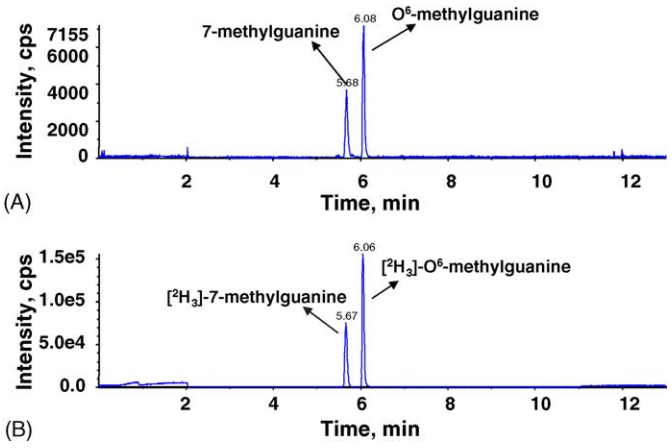

Fig. 4. Analysis of LLOQ sample by LC/ESI-MS/MS in MRM mode: MRM chromatograms of O^6-methylguanine (RT 6.1 min) and 7-methylguanine (RT 5.7 min). (B) MRM chromatograms of [$^2H_3$]-O^6-methylguanine (RT 6.1 min) and [$^2H_3$]-7-methylguanine (RT 5.7 min).

indistinguishable in profile from standards of comparable concentration.

3.3. Calibration curve, linearity, limit of detection (LOD), and lower limit of quantitation (LLOQ)

A series of calibrated standards for O^6-methylguanine and 7-methylguanine were prepared at various concentrations with fixed concentrations of the corresponding internal standards. These solutions were analyzed by LC/ESI-MS/MS (see Section 2). The calibration curves obtained on the four days were linear over the concentration ranges of 75.8–151,600.0 fmol with the correlation coefficient R^2 over 0.999 (Fig. 5B) and accuracy in the range of 94–114% (data not shown) each day for O^6-methylguanine. For 7-methylguanine, the calibration curves obtained on the 4 days were also linear over the concentration ranges of 151.5–303,200.0 fmol with the correlation coefficient R^2 over 0.999 (Fig. 5A) and accuracy in the range of 90–113% (data not shown) each day.

The limit of detection (LOD) of O^6-methylguanine and 7-methylguanine with a signal-to-noise ratio ≥3:1, was determined to be 22.7 and 75.8 fmol, respectively. The lower limit of quantitation (LLOQ) of O^6-methylguanine and 7-methylguanine with a signal-to-noise ratio ≥8:1 and accuracy

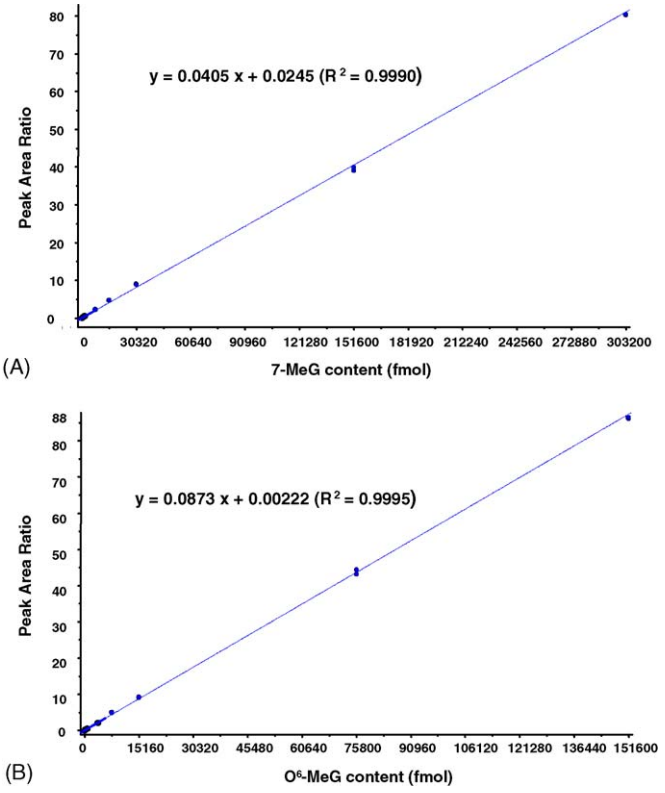

Fig. 5. (A) Linear response of 7-methylguanine ranging from 151.5 to 303,200.0 fmol. (B) Linear response of O^6-methylguanine ranging from 75.8 to 151,600.0 fmol.

of 85–110%, was determined to be 75.8 and 151.5 fmol, respectively (data not shown).

3.4. Precision and accuracy

The intra-day and inter-day precision and accuracy values for O^6-methylguanine and 7-methylguanine are shown in Table 1. The intra-day assay precision R.S.D. values for O^6-methylguanine QC samples were ≤9.2% with accuracy values ranging from 90.8 to 118%, and for 7-methylguanine QC samples were ≤11.0% with accuracy values ranging from 92.9 to 119%. The inter-day assay precision R.S.D. values of O^6-methylguanine QC samples were ≤7.9% with accuracy values ranging from 94.5 to 116%, and for 7-methylguanine QC sam-

Table 1
Intra- and inter-assay precision and accuracy for O^6-methylguanine (O^6-MeG) and 7-methylguanine (7-MeG)

Analytes	Injected content (fmol)	Intra-assay precision and accuracy			Inter-assay precision and accuracy		
		Mean (n = 4) (fmol)	R.S.D. (%)	Mean accuracy (%, n = 4)	Mean (n = 16) (fmol)	R.S.D. (%)	Mean accuracy (%, n = 16)
O^6-MeG	75.8	77.3	9.2	101.4	71.2	7.93	94.5
	1515.1	1545.4	3.9	100.2	1560.5	4.19	102.5
	7580.1	8372.1	3.8	115.0	8777.6	2.72	115.8
	75801.0	76558.0	8.21	101.0	76512.5	5.89	101.7
7-MeG	151.5	181.8	11.0	119.0	166.6	7.1	106.5
	3030.2	3181.7	3.2	105.1	3181.7	3.5	105.1
	15160.0	16676.0	4.5	110.0	16706.3	4.1	110.2
	151600.0	146445.6	3.68	96.6	144596.1	3.5	95.4

Table 2
Stability of O^6-methylguanine and 7-methylguanine

	Nominal injected content (fmol)							
	O^6-Methylguanine				7-Methylguanine			
	75.8	1515.1	7580.1	75801.0	151.5	3030.2	15160.0	151600.0
Stability after three cycles of freeze/thaw ($n=4$)								
Mean	68.2	1590.9	9005.1	78741.1	175.7	3272.6	16964.0	145384.4
R.S.D. (%)	3.5	4.4	3.8	5.6	3.3	3.4	4.02	2.9
Accuracy (%)	90.0	105.0	118.9	103.8	116.5	108.2	111.9	95.9
Stability after storage at −20 °C for 45 days ($n=4$)								
Mean	65.2	1575.7	8959.7	78043.7	165.1	3212.0	16645.7	147506.8
R.S.D. (%)	8.8	4.3	2.4	4.5	11.2	4.2	3.83	3.19
Accuracy (%)	86.0	103.8	118.2	102.9	109.3	106.1	109.8	97.3

ples were ≤7.1% with accuracy values ranging from 95.2 to 110.2%.

3.5. Stability studies

The freeze/thaw stability of O^6-methylguanine and 7-methylguanine was evaluated. The measured concentrations of O^6-methylguanine and 7-methylguanine were compared to the nominal values, with accuracy ranging from 91 to 119% for O^6-methylguanine and 96 to 116% for 7-methylguanine. The stability of the QC samples stored at −20 °C for 45 days was also evaluated with the same quantitation procedure as the freeze/thaw stability study. The final accuracy was from 87.3 to 118.2% for O^6-methylguanine and from 97.3 to 109.8% for 7-methylguanine (Table 2).

These results show that no significant degradation occurs after 3 freeze/thaw cycles or after storage −20 °C for 45 days, indicating that O^6-methylguanine and 7-methylguanine are stable under the method conditions described above.

3.6. Recovery of analytes

The recoveries of O^6-methylguanine and 7-methylguanine were estimated by analyzing lower limit of quantitation, low, medium, and high spike samples and the QC samples under the same concentrations. The determined concentrations of spike samples were compared with corresponding concentrations from the QC samples. Good recoveries were obtained at all those concentration ranges for O^6-methylguanine (Table 3). For 7-methylguanine, at the lower concentrations (151.5 and 3030 fmol), slightly higher recoveries were obtained (Table 3). This could be a result of matrix effects due to the DNA hydrolytes.

3.7. Quantitation of O^6-methylguanine and 7-methylguanine in series of MNU or MMS treated DNA samples

The method was applied to quantify the levels of O^6-methylguanine and 7-methylguanine from a series of MNU- or MMS-treated DNA samples and the quantitation results are shown in Table 4. These results show that both MNU and MMS form more 7-methylguanine adducts than the O^6-methylguanine adduct for all the concentration treatments investigated here. These results confirmed the previous report [2]. At equimolar concentrations, MNU forms more total adducts than MMS. This difference could be due to the recognized reactivity differences between these two alkylating agents. It is known that MNU and MMS are both direct-acting methylating agents and that their chemical reaction mechanisms are different. MNU reacts via an S_N1 (nucleophilic unimolecular substitution) mechanism, whereas MMS reacts through an S_N2 (nucleophilic bimolecular substitution) mechanism [20]. Based on the results from Table 4, the ratios of 7-MeG to O^6-MeG are about 200 for MMS and 6.5 for MNU. These ratios are a little bit lower than that from previous publication result done by Beranek et al. [2]. Their results showed that the ratios of 7-MeG to O^6-MeG were about 262 for MMS and 11.2 for MNU. The small ratio difference may result from the large difference in the ratio of substrate concentration to the DNA concentration. In Beranek's paper [2], the ratio of substrate to DNA was 1 mmol alkylating agent to 1 mol DNA, however, in the present study the ratio with the highest concen-

Table 3
Recoveries of O^6-methylguanine and 7-methylguanine[a]

	Nominal injected content (fmol)							
	O^6-Methylguanine				7-Methylguanine			
	75.8	1515.1	7580.1	75801.0	151.5	3030.2	15160.0	151600.0
Mean recoveries (%)	100	107	107.7	100.5	117	117	103	103

[a] Each recovery value represents the average of four determinations ($n=4$) and is expressed as a percentage of the measured concentration of spike at certain concentrations divided by the measured concentration of the corresponding QC sample.

Table 4
Quantitation of O^6-methylguanine (O^6-MeG) and 7-methylguanine (7-MeG) in DNA hydrolytes from MNU- or MNU-treated salmon testis DNA[a]

Sample name (DNA with MNU or MMS)	O^6-MeG adducts per 10^6 bases			7-MeG adducts per 10^6 bases		
	Mean	S.D.	R.S.D. (%)	Mean	S.D.	R.S.D. (%)
Control	ND			ND		
0.5 μM MNU	<LLOQ			<LLOQ		
20 μM MNU	69.4	0.73	1.06	493.0	9.30	1.89
50 μM MNU	312.3	3.89	1.25	2002.9	14.19	0.71
200 μM MNU	1019.1	12.5	1.22	6633.5	179.83	2.71
500 μM MNU	1164.5	8.71	0.75	7513.0	109.08	1.45
0.5 μM MMS	ND			5.85	0.11	1.96
20 μM MMS	<LLOQ			229.6	6.04	2.63
50 μM MMS	3.0	0.12	4.13	611.22	14.17	2.32
200 μM MMS	13.6	0.67	4.91	2828.7	76.48	2.70
500 μM MMS	29.8	0.20	0.68	5732.3	55.47	0.97

S.D.: standard deviation; R.S.D.: relative standard deviation; ND: not detectable.
[a] About 11 μg DNA was injected to the mass spectrometer.

tration of MNU or MMS (500 μM) is 1 mmol alkylating agent to 200 mol DNA, in an attempt to more closely represent in vivo conditions.

4. Conclusion

A new LC/ESI-MS/MS analytical method for the simultaneous determination of O^6-methylguanine and 7-methylguanine in DNA treated with MNU and MMS has been established. This method shows satisfactory sensitivity, precision, and accuracy. This method may be useful in future mechanistic studies evaluating the relationship between macromolecular alkylation and biological response from alkylating agents.

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