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High-Performance Liquid Chromatographic and Tandem Mass Spectrometric Quantitation of *N7*-Methyldeoxyguanosine in Methylated Calf Thymus DNA

Whi-Gun Chae*

Department of Biochemistry, School of Medicine, Catholic University of Taegu, Taegu 705-034, Korea

Abstract Quantitation of *N7*-methyldeoxyguanosine (*N7*-MedG) produced in the *in vitro N*-methyl-*N* -nitrosourea (NMU) action on calf thymus DNA has been achieved by enzymatic degradation, liquid chromatographic separation and desorption chemical ionization tandem mass spectrometry. In conjunction with the resolving power of HPLC in the separation of isomers, desorption chemical ionization tandem mass spectrometry has been utilized in determining modified nucleosides at low levels using a stable-isotope labeled compound as an internal reference. The quantitative estimation of *N7*-methyldeoxyguanosine was previously established by an independent HPLC analysis of methylated calf thymus DNA. A sensitive and specific methodology for the quantitation of *N7*-MedG at the picomole level using HPLC combined with tandem mass spectrometry without radioisotope labeling process is presented. The potential of the liquid chromatographic tandem mass spectrometric analysis shows the detection of *N7*-MedG as a possible marker for human exposure to methylating agents *in vitro*.

Keywords *N7*-methyldeoxyguanosine, *N*-methyl-*N*-nitrosourea, tandem mass spectrometry, high-performance liquid chromatography

INTRODUCTION

The mutagenic and carcinogenic activities of alkylating agents have been correlated with their interaction with nucleic acids. Alkylation of DNA has been considered as a primary basis for the biological activity of these agents [1,2]. Alkylating agents show somewhat different selectivity toward the various nucleophilic DNA sites. Exposure of DNA to methylating agents results in the formation of DNA adducts. Most attention has been paid to reactions occurring at the *N7* position of guanine, the O^6-position of guanine, and the O^4-position of thymidine for those positions are the principal sites of reaction with the bases of DNA toward methylating agents [3-6].

The guanine base at *N7* position is often taken as the most useful marker site of DNA alkylation compared with other sites, since it is the most nucleophilic site in DNA, with methylating agents, more than 60% of all DNA substitution occurs at this site [3,4,6]. In addition, the *N7* of guanine is in the major DNA groove [7], and relative to most other nucleophilic sites, its reactivity is somewhat insensitive to the size of the alkyl group transferred. In addition, guanine alkylated at *N7* can undergo hydrolytic fission of the imidazole ring. The resulting product has been proposed as a significant contributor to carcinogenesis [8]. However, alkylated product of guanine at the *N7* position does not interfere with DNA replication and does not correlate with oncogenic response [9] and appears to be of minor harm to the cell in comparison to some of the other methylated adducts. Therefore, *N7*-methyldeoxyguanosine has been suggested as a possible marker for human exposure to methylating agents because of its low repair rate and insensitive reactivity as well as it being the major adduct formed.

^{13}C NMR has been utilized in determining the sites of reaction of methylating agents with nucleic acids without prior degradation or separation processes [10], the prospects for employing this method in the detection of *N7*-methyldeoxyguanosine are very limited due to its low sensitivity and the low level of modification. Various methods have been developed to detect *N7*-methyldeoxyguanosine, including high performance liquid chromatographic (HPLC) analysis with UV [11], fluorescence [12], electrochemical [13] and radiochemical detection [4], and ^{32}P-postlabeling method [14]. The main difficulty in the detection of DNA damage is the analytical obstacle involved in measuring very low concentrations of adducts in the presence of large amounts of the normal deoxyribonucleosides and isomeric modified nucleosides. HPLC often suffers from a lack of structural specificity and a single HPLC peak cannot guarantee molecular homogeneity. It is therefore essential to develop more sensitive methods for qualitative and quan-

* Corresponding author
Tel: +82-53-650-4480 Fax. +82-53-626-3262
e-mail: wgchae@cuth.cataegu.ac.kr

titative analysis with high sensitivity of the closely related deoxyribonucleoside derivatives, some of which are involatile and thermally unstable and all of which occur in particular complex matrices. Fortunately, recent progress in liquid chromatography and mass spectrometry offers capabilities to match these requirements.

The task of separating isomeric alkylated nucleosides utilizes HPLC. Satisfactory separations of various methylated compounds in reasonable amounts of time can be achieved by a procedure that uses an ion-paired reverse-phase column developed by the author and others [4,11]. The technique of tandem mass spectrometry [5, 6,15,16] also provides separation capabilities as well as molecular structural specific, sensitive detection.

The desorption chemical ionization tandem mass spectrometry and HPLC methodology used in this study has been previously applied in the quantitative analysis of O^6-methyldeoxyguanosine and O^4-methythymidine in several modified calf thymus DNA [5,6]. In this present study, a quantitative determination of *N7*-methyldeoxyguanosine produced by the exposure of calf thymus DNA with *N*-methyl-*N*-nitrosourea (NMU) by tandem mass spectrometry (MS/MS) coupled with HPLC as an extremely sensitive method for structural identification, separation and quantitative determination under optimized conditions has been reported. This part of the study extends the earlier works on tandem mass spectrometry [5,6,16] and lays the ground work for future *in vivo* determinations of *N7*-MedG as a possible marker for human exposure to methylating agents

MATERIALS AND METHODS

General

All reagents and solvents were of analytical reagent quality. *N7*-Methylguanine (*N7*-MeGua), and *N7*-methyldeoxyguanosine (*N7*-MedG) were purchased from Sigma Chemical Co. (St Louis, MO). [CD_3-methyl] *N7*-methyldeoxyguanosine (CD_3-*N7*-MedG) was previously prepared according to the procedure of Chang *et al.* for the ^{13}C NMR analysis of methylated DNA [10]. All enzymes utilized (bovine pancreatic ribonuclease A, deoxyribonuclease I, snake venom phosphodiesterase, alkaline phosphatase, and purine nucleosie phosphorylase from calf spleen) were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Methyl-*N*-nitrosourea (NMU) was purchased from ICN Pharmaceuticals. Inc. (Costa Mesa, CA) and recystallized in water. DNA from calf thymus (Type 1, D-1501, Sigma Chemical Co., St. Louis, MO) was purified prior to alkylation by conventional enzymatic digestion (bovine pancreatic ribonuclease A), chloroform-isoamyl alcohol (24:1) extraction, and ethanol precipitation [5].

Methylation of Calf Thymus DNA with *N*-Methyl-*N*-nitrosourea

To a pH-stat reaction vessel, 4.3×10^{-2} mmol (15 mg, as determined by UV analysis, $\varepsilon_{260} = 11{,}653$ liter $\cdot$ cm^{-1} $\cdot$ mole^{-1}, the molecular weight of the DNA taken as the sodium salt of the average molecular weight of the four major mononucleotides is 348.97) of purified calf thymus DNA was added. The DNA was then dissolved in 4.0 mL of double distilled water. To this solution, 5.0 mL of *N*-methyl-*N*-nitrosourea (NMU, 108 mg in 5.0 mL water, 1.05 mmol) was added a drop at a time on the pH-stat and the reaction was allowed to proceed for 3 hours at room temperature. The pH of the solution was maintained at 7.0 ± 0.1 by adding 0.05N NaOH. After 3 h the modified DNA was precipitated out by two ethanol treatments. After decanting the supernatant, the sample was vacuum dried at room temperature and subjected to further degradation into the nucleoside level using a mixed enzyme system.

Enzymatic Degradation and HPLC Estimation of *N7*-MedG in Modified CT-DNA

To 1.0 mg of modified calf thymus DNA, 0.85 mL of 0.1 M Tris buffer, pH 7.3 was added in a 5 mL screw top vial. Thirty units of DNase I (type III), 0.03 units of snake venom phosphodiesterase (type III) and 1.8 units of alkaline phosphatase (type III from *E. coli.*) were added to make a total volume of 1.0 mL. Enzymatic degradation was allowed to continue for 12 h at 37°C in a shake bath. The degraded sample was then filtered through Centrifree Micropartition system (Amicon Division, W. R. Grace & Co., Beverly, MA) to remove the enzymes. This sample was then analyzed by the HPLC.

HPLC separations were carried out on a Waters system (Millipore CO., Bedford, MA) consisting of a U6K injector, two 6000A solvent delivery system interfaced by a model 680 solvent programmer, and a 440 dual wavelength detector. An Alltech (Deerfield, IL) Econosphere C-18-3μ column was eluted at 1 mL/min with 0-20% CH_3CN, #7 gradient for 60 min in 50 mM ammonium formate, 3 mM tetraethylammonium chloride at pH 6.9 for quantitative analysis of *N7*-methyldeoxyguanosine (Rt=23 min) in modified calf thymus DNA. The mobile phase was prepared, filtered through 0.45 μm Millipore filters and degassed prior to use.

Tandem Mass Spectrometric Analysis of *N7*-methylguanine

Mass spectral sample was prepared according to the above procedure for the HPLC analysis of *N7*-MedG except that the known amount of [CD_3-methyl] *N7*-methyldeoxyguanosine was added prior to enzymatic degradation. The fraction containing *N7*-MedG (both ladeled and unlabled) from the HPLC separation were pooled, lyophilized, and then reconstituted with double distilled water and then further subdivided for direct analysis of MS/MS. Tandem mass spectra of *N7*-methylguanine were obtained using a 4500 Finnigan (San Jose, CA) triple quadrupole mass spectrometer. To lower the limits of detection further in MS/MS experiments, different experimental conditions were investi-

gated for both the merged daughter scan mode and multiple reaction monitoring scan mode Optimum experimental conditions of multiple reaction monitoring (MRM) scan mode for *N7*-methyldeoxyguanosine in modified calf thymus DNA are as follows: ammonia as reagent gas at a pressure of approximately 0.65 Torr, argon collision gas at a pressure of approximately 0.8 mTorr and a collision energy of approximately 15 eV. The direct evaporation probe (DEP) was heated to 600°C at a rate of 200°C/s. One scan was recorded every 0.09 s as the probe was heated. An average of 14 scan was summed during an average desorption time of 1.26 s

RESULTS AND DISCUSSION

The approach used in this study was first to separate isomeric methylated nucleosides by HPLC using an ion-paired reverse-phase column, and then to determine their molecular identities quantitatively by tandem mass spectrometry. Excellent separation of the structural isomers of nucleosides was achieved as reported earlier [5] *N7*-MedG was independently quantitated by HPLC. The quantitation of *N7*-MedG estimated was calculated from the HPLC results based on the amount of *N7*-MeGua after the treatment with nucleoside phosphorylase which catalyze the conversion of *N7*-MedG into *N7*-MeGua. The lyophilized fraction of NMU-treated CT-DNA (200 μg) was estimated by HPLC using UV absorbance at 254 nm to have approximately 12.6 μg *N7*-MedG. The corresponding amount of CD_3-labeled reference was added to the DNA before enzymatic hydrolysis. The hydrolysate was then separated, collected, and freeze dried for MS/MS analysis. Attempts to obtain the daughter spectrum of intact nucleoside (*N7*-MedG) instead of base (*N7*-MeGua) from both standards and NMU treated calf thymus DNA resulted in a complete degradation of the molecular ion within the source. Therefore, further optimization of base level analysis was carried out.

Merged Daughter Experiment of *N7*-Methyldeoxyguanosine in NMU-CT DNA

Samples were prepared in the following manner. A 1.0 mg sample of NMU-treated CT DNA was spiked with 63 μg of CD_3-*N7*-MedG as the internal standard in a total volume of 1.0 mL. After enzymatic degradation, an aliquot corresponding to 200 μg of the product was subjected to HPLC separation using ion-paired reagent and ammonium formate buffer. The fraction (1.5 mL) containing both unlabeled and labeled *N7*-MedG from the HPLC separation was collected, freeze-dried and then treated with the nucleoside phosphorylase for 1 h to convert all *N7*-MedG into *N7*-MeGua. After Amicon filtration, the sample was reinjected in an ammonium formate mobile phase to remove the ion-pairing reagent, which has been found to interfere in the mass spectral analysis. The fraction was collected and 20 μL of the reconstituted sample was analyzed by MS/MS (merged daughter scan) after reconstitution with double-distilled water in a total volume of 3.15 mL.

The results of the quantitative MS/MS analysis (daughter scan mode) of *N7*-MedG from NMU modified CT DNA in Table 1 showed poor precision (average ratio of CH_3/CD_3 = 1.70) and poor reproducibility (relative standard deviation = 73%). Therefore, multiple reaction monitoring (MRM) would be used for the analysis of *N7*-MedG.

MRM Experiment of *N7*-Methyldeoxyguanosine in NMU-CT DNA

The merged daughter experiments constructed for the *N7*-MedG in NMU-CT DNA were scanned from 120 to 175 amu in 0.14 sec. The scan rate for all MRM experiments was 0.03 sec/amu. Each parent ion, each characteristic fragment ion and one background ion were monitored. In MRM mode, all of the analysis time was spent monitoring the reaction of interest. Therefore, this technique was markedly more sensitive but was less specific.

The precision increased with the number of scans available for summation. The number of scans acquired in an experiment was strongly dependent on the rate of heating of the direct evaporation probe filament Slower heating rates promote thermal degradation rather than desorption of neutral species. A higher heating rate of 200°C/s and a fast scanning rate were also selected and used for many multiple reaction monitoring experiments of *N7*-MedG analysis to increase precision and sensitivity.

Quantitation of *N7*-MedG, based on the measured 166+/169+ ion ratio could be performed using calibration curves. Generation of calibration curves for isotope dilution assays usually proceeded by the measurement of the ion intensity ratio in a series of standards, each containing a fixed amount of internal standard and a variable amount of analyte. The average of three experiments, at variable amounts (2-20 ng) of CH_3-*N7*-MedG and fixed amount (10 ng) of CD_3-*N7*-MedG on the column background, was obtained. Linear regression analysis of the isotope ratios obtained from the spectra upon the mole ratios of the labeled and unlabeled compounds was performed.

Multiple reaction monitoring spectra from the NMU treated calf thymus DNA sample containing 35 picomole (10 ng) of CD_3-*N7*-MedG internal standard, corresponded to approximately 2.5 μL of reconstituted sample, and an expected amount (10 ng) of CH_3-*N7*-MedG was obtained in Fig. 1. Each parent ion (m/z at 166 and 169), each characteristic fragment ion (m/z at 149, 152, 124 and 127) and one background ion (m/z at 141) were monitored. The blank HPLC samples corresponding to the same amount for each nucleoside were analyzed immediately before the analysis of real sample to prevent carry-over possibility and to confirm that there was no significant interference from the blank.

The results of the quantitation of *N7*-MedG in the NMU-CTDNA samples with the multiple reaction

Table 1. Quantitative MS/MS analysis of *N7*-MedG from NMU-treated CT DNA (Merged daughter scan mode, base level analysis)

Trial	Estimated ($^1H_3$)methyl nucleoside (HPLC)(mole)	($^2H_3$)Methyl nucleoside added (mole)	Relative ratio of ($^1H_3$) methyl/($^2H_3$) methyl nucleosides	Calculated ($^1H_3$)methyl nucleoside (MS/MS)(mole)
1	2.8×10^{-10}	2.8×10^{-10}	1.35	3.8×10^{-10}
2	2.8×10^{-10}	2.8×10^{-10}	0.70	2.0×10^{-10}
3	2.8×10^{-10}	2.8×10^{-10}	3.51	9.8×10^{-10}
4	2.8×10^{-10}	2.8×10^{-10}	1.25	3.5×10^{-10}
	2.8×10^{-10}		Average 1.70 S.D. 1.24 (RSD=73%)	4.8×10^{-10}

Each trial represents an aliquot from the reconstituted HPLC peak. Amount treated DNA to yield aliquot for MS/MS analysis is 3.7×10^{-9} mole

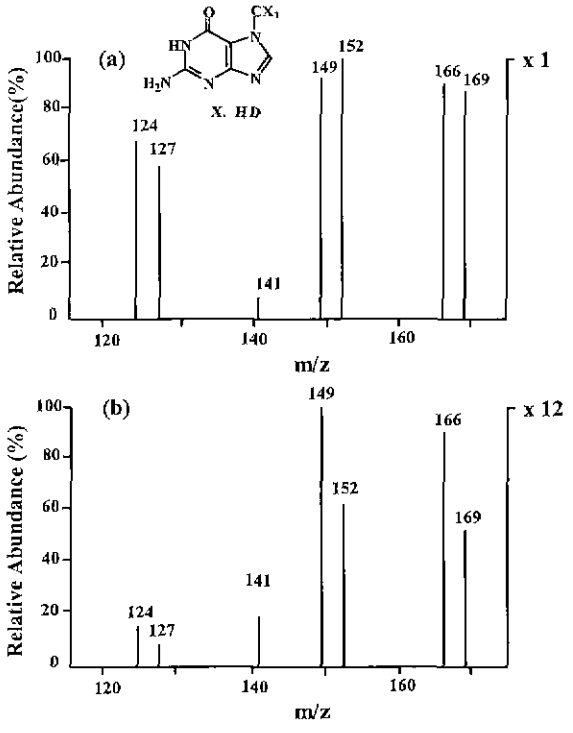

Fig. 1. Typical MS/MS multiple reacton monitoring spectra from (a) the NMU-treated CT-DNA sample containing 35 picomole of (CD_3)*N7*-MedG as the internal standard and (b) the HPLC blank sample which corresponds to the same amount of each nucleoside analyzed. Note that the vertical scale of plot (b) has been expanded 12 times The relative abundances of characteristic parent ions (protonated methylguanine base, m z 166, and its d_3 analog 169) were measured for quantitation

monitoring experiment are summarized in Table 2. Four replicate samples of NMU-*N7*-MedG at the level of 10 ng yielded an average 166+/169+ ratio of 1.04 with a

Table 2. Quantitative MS/MS analysis of *N7*-MedG from NMU-treated CT DNA [Multiple reaction monitoring scan mode, base level analysis]

Trial	Estimated ($^1H_3$)methyl nucleoside (HPLC)(mole)	($^2H_3$)Methyl nucleoside added (mole)	Relative ratio of ($^1H_3$) methyl/($^2H_3$) methyl nucleosides	Calculated ($^1H_3$)methyl nucleoside (MS/MS)(mole)
1	3.5×10^{-11}	3.5×10^{-11}	1.03	3.6×10^{-11}
2	3.5×10^{-11}	3.5×10^{-11}	1.01	3.5×10^{-11}
3	3.5×10^{-11}	3.5×10^{-11}	1.00	3.5×10^{-11}
4	3.5×10^{-11}	3.5×10^{-11}	1.14	3.9×10^{-11}
	3.5×10^{-11}		Average 1.04 S.D. 0.06 (RSD=5.8%)	3.6×10^{-11}

Each trial represents an aliquot from the reconstituted HPLC peak. Amount treated DNA to yield aliquot for MS/MS analysis is 4.6×10^{-10} mole.

relative standard deviation of 5.8%. Each spectrum provided at least 100,000 counts for the base peak. The results from above experiment show impressive sensitivity and precision for the analysis of *N7*-MedG in NMU-CT DNA using multiple reaction monitoring.

When used in conjunction with the resolving power of HPLC in the separation of isomeric nucleosides, MS/MS allows for the determination of *N7*-MedG in picomole level using stable-isotope labeled compound as the internal standard. These results demonstrate that the methodology used in this study should be adequate for the detection and quantitation of *N7*-MedG as the marker for possible human exposure to methylating agents and using this method does not require pre- or post-radioisotope or chemical labeling as in the case of other very sensitive methods.

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