Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 844 (2006) 60–66
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantification of O^6-methyl and O^6-ethyl deoxyguanosine adducts in C57BL/6N/$Tk^{+/-}$ mice using LC/MS/MS

Mona I. Churchwell, Frederick A. Beland, Daniel R. Doerge *

Division of Biochemical Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, United States

Received 19 April 2006; accepted 30 June 2006
Available online 22 August 2006

Abstract

The carcinogenicity of many alkylating agents is derived from their ability to form persistent DNA adducts that induce mutations. This paper presents and validates methodology, based on LC with tandem mass spectrometry, for the separate or concurrent quantification by isotope dilution of O^6-methyl-2′-deoxyguanosine (O^6Me-dG) and O^6-ethyl-2′-deoxyguanosine (O^6Et-dG) DNA adducts. The limits of quantification were estimated to be $\leq$0.2 adducts/10^8 nucleotides for either adduct. This sensitivity permitted evaluation of adduct levels in livers from separate groups of untreated adult C57BL/6N/$Tk^{+/-}$ and C57BL/6N X Sv129 mice (undetectable to 5.5 ± 6.7 O^6Me-dG/10^8 nucleotides; undetectable to 0.04 O^6Et-dG/10^8 nucleotides). Treatment of adult C57BL/6N/$Tk^{+/-}$ mice with equimolar doses (342 μmol/kg body weight) of *N*-methyl-*N*-nitrosourea and *N*-ethyl-*N*-nitrosourea produced adduct levels in liver of 1700 ± 80 O^6Me-dG/10^8 nucleotides and 260 ± 60 O^6Et-dG/10^8 nucleotides, respectively, when assessed 4 h after dosing. These methods should be useful for evaluations of DNA adducts in relation to cellular processes that modify carcinogenic and toxicological responses in experimental animals and humans.

Keywords: O^6-Methyl-2′-deoxyguanosine; O^6-Ethyl-2′-deoxyguanosine; Mass spectrometry; DNA adducts

1. Introduction

Alkylating agents, including the *N*-nitroso-*N*,*N*-dialkylamines, have been central to research on chemical carcinogenesis [1,2]. The genotoxicity associated with alkylating agents requires inherent or metabolically derived electrophilic reactivity at nucleophilic sites on DNA bases to form covalent adducts. The ability of adducts to affect the fidelity of transcription during cell division depends on their chemical structures and their ability to disrupt DNA secondary and tertiary structure. Accumulation of DNA adducts, which depends on relative rates of formation and spontaneous or enzymatic removal, can produce cytotoxic and mutagenic responses in vitro and in vivo. Because dose-response characteristics for steady state DNA adduct levels in target tissues often correlate well with tumor incidence data from chronic rodent bioassays [3], measurement of DNA adducts can be useful for reducing reliance on default assumptions in extrapolations across dose and species, which are inherent in human cancer risk assessment.

Measurement of DNA adducts in tissues of animals and humans exposed to chemical carcinogens has been done extensively by using ^{32}P-postlabeling, primarily because of the high sensitivity and the general ability to detect DNA adducts [4,5]. However, the accuracy and precision of adduct quantification is often questionable because of significant uncertainties (e.g., enzymatic efficiency, chromatographic efficiency). Immunoassays have been quite useful for DNA adduct measurements because of similarly high sensitivity compared to ^{32}P-postlabeling, although antibody-analyte cross-reactivity and analytical precision are often limitations. Mass spectrometry, when combined with chromatographic separations, has more recently made important contributions to DNA adduct quantification, particularly in the LC-tandem electrospray mass spectrometry configuration (LC-ES/MS/MS) where the analytical sensitivity can approach that of ^{32}P-postlabeling and immunoassays, while the accuracy approaches that of direct radiochemical decay measurements [4,6–8]. Mass spectrometry permits a degree of structural identification not possible with the other

* Corresponding author. Tel.: +1 870 543 7943; fax: +1 870 543 7720.
E-mail address: ddoerge@nctr.fda.gov (D.R. Doerge).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.06.042

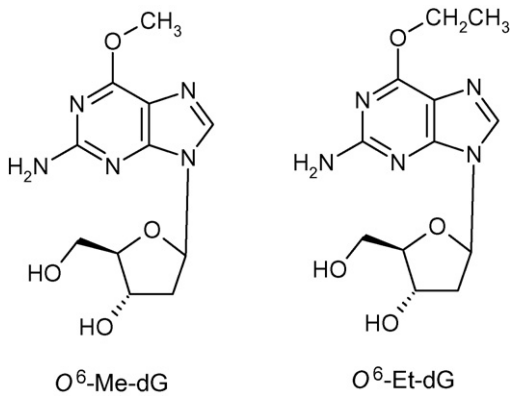

Fig. 1. Structures of O^6-methyl-2′-deoxyguanosine and O^6-ethyl-2′-deoxyguanosine.

techniques and provides the ability to analyze readily multiple adducts using stable isotope dilution, which yields significant analytical advantages in method precision and accuracy.

This paper describes the development of LC-ES/MS/MS methodology for the separate or concurrent analysis of two types of alkylated DNA adducts (Fig. 1), O^6-methyl-2′-deoxyguanosine (O^6Me-dG) and O^6-ethyl-2′-deoxyguanosine (O^6Et-dG), which have been shown to be involved in the tumorigenic responses in animals and are present in human tissues [2,3,9].

2. Experimental section

2.1. Reagents

Micrococcal nuclease, prostatic acid phosphatase, calf thymus DNA (Type I), salmon testes DNA, *N*-methyl-*N*-nitrosourea (MNU), *N*-ethyl-*N*-nitrosourea (ENU), Bis–Tris, and formic acid were purchased from Sigma Chemical (St. Louis, MO). Spleen phosphodiesterase was purchased from Worthington (Lakewood, NJ) and nuclease P1 was obtained from Roche (Indianapolis, IN). Deionized water was produced on-site with a Milli-Q water system (Millipore Corp., Bedford, MA) and the acetonitrile was HPLC grade.

2.2. Liquid chromatography

The liquid handling system consisted of a Waters 2795 (Milford, MA), an automated switching valve (TPMV, Rheodyne, Cotati, CA), and a second quaternary gradient HPLC pump (GP40, Dionex, Sunnyvale, CA). The liquid handling system was used for sample injection, cleanup, and regeneration of the trap and analytical columns; the quaternary pump, containing 90% of 0.1% aqueous formic acid and 10% acetonitrile, was used to back-flush the trap column to the analytical column during analysis and to keep a constant flow of mobile phase going into the mass spectrometer during sample loading and preparation periods as previously described [10].

For O^6Me-dG, samples were loaded onto a reverse phase trap column (Luna C18(2), 2 mm × 30 mm, 3 micron, Phenomenex, Torrance, CA) and washed to waste for 3.1 min at 0.2 mL/min with 95% of 0.1% aqueous formic acid containing 5% acetonitrile. After switching the valve, the concentrated sample zone was back-flushed from the trap column onto the analytical column (Luna C18(2), 2 mm × 150 mm, 3 micron, Phenomenex) at a flow rate of 0.2 mL/min with 91% of 0.1% aqueous formic acid containing 9% acetonitrile and sample components were eluted into the mass spectrometer for a 2-min period. During the chromatographic run, the valve was switched back and the trap column was cleaned to waste with 75% acetonitrile and 25% of 0.1% aqueous formic acid for 2 min at 0.5 mL/min and then re-equilibrated to the initial mobile phase conditions. Total run time was 13 min. The O^6Et-dG method was similar except that washing of the trap column was conducted at 0.5 mL/min for 1.5 min and the elution of analytical column was performed with 85% of 0.1% aqueous formic acid containing 15% acetonitrile. The combined method was the same as described for the O^6Me-dG method except that a step gradient from 9 (0–3 min) to 20% (3.1–10 min) acetonitrile in 0.1% aqueous formic acid was used to elute both adducts sequentially from the analytical column in a total run time of 18.5 min.

2.3. Mass spectrometry

A Quattro Ultima triple quadrupole mass spectrometer (Waters), equipped with an ES interface, was used with a capillary potential of 0.75 kV, a source temperature of 100 °C, and desolvation temperature of 400 °C. Nitrogen gas was used as desolvation gas (750 L/h) and cone gas (100 L/h). Argon was used as collision gas, at a collision cell pressure of 1.5×10^{-3} mBar. Positive ions were acquired in MRM mode (dwell time = 0.2 s and interchannel delay = 0.03 s) for the $(M+H)^+$ to BH_2^+ transitions for O^6Me-dG (*m/z* 282 → 166) using a collision energy of 16 eV. A confirmatory transition was monitored (*m/z* 282 → 149) using a collision energy of 33 eV. Similarly, the transitions monitored for O^6Et-dG were (*m/z* 296 → 180) using a collision energy of 16 eV. A confirmatory transition was monitored (*m/z* 296 → 152) using a collision energy of 28 eV. Transitions were monitored for the internal standards $^{15}N_5$-O^6Me-dG (*m/z* 287 → 171) and $^{15}N_5$-O^6Et-dG (*m/z* 301 → 185) using the same collision energies as for the unlabeled adducts. A cone voltage of 25 V was used for all transitions. Resolution was set to give peak widths at half-height of 0.9 Th for product and precursor ions.

2.4. Preparation of O^6Me-dG, O^6Et-dG, $^{15}N_5$-O^6Me-dG, and $^{15}N_5$-O^6Et-dG

Diazoethane was prepared by the method of Wilds and Meader [11] and reacted with 2′-deoxyguanosine (USB Corp., Cleveland, OH) as described by Farmer et al. [12]. The O^6Et-dG was isolated by reverse phase HPLC μBondapak C18, 3.9 mm × 300 mm, Waters) by eluting with 20-min linear gradient of 100 mM ammonium acetate (pH 5.7) to 20% acetonitrile in 100 mM ammonium acetate (pH 5.7) at 2 mL/min followed by isocratic elution. The O^6Et-dG, which eluted at 21 min, was quantified using the molar extinction coefficients (9.33/mM at 248 nm and 9.12/mM at 281 nm) reported by Farmer et al.

[12]. Additional material was purified by preparative silica gel thin-layer chromatography by eluting with 10% methanol in methylene chloride and characterized by NMR spectrometry. $^{15}N_5$-O^6Et-dG was prepared, purified by HPLC, and quantified in a similar manner using $^{15}N_5$-labeled dG (>98 at.% ^{15}N) purchased from Spectra Stable Isotopes (Columbia, MD). The isotopic distribution was determined using full scan LC-ES/MS (*m/z* 150–500) and found to be 86% $^{15}N_5$; 13.5% $^{15}N_4$; and 0.5% $^{15}N_0$.

O^6Me-dG was prepared by reacting diazomethane with 2′-deoxyguanosine as described for the synthesis of O^6Et-dG. The O^6Me-dG, which was isolated by the reverse phase HPLC using the solvent conditions described above, eluted at 17 min was quantified using the molar extinction coefficients (8.13/mM at 246 nm and 8.13/mM at 278 nm) reported by Farmer et al. [12]. $^{15}N_5$-O^6Me-dG was prepared, purified, and quantified in a similar manner using $^{15}N_5$-labeled dG. The isotopic distribution, as determined using full scan LC-ES/MS, was 85.1% $^{15}N_5$; 13.5% $^{15}N_4$; and 1.5% $^{15}N_0$.

2.5. DNA modified in vitro

O^6Et-dG -modified DNA: Calf thymus DNA [7.0 mg in 4 mL of 5 mM Bis–Tris, 0.1 mM EDTA buffer (pH 7.1)] was incubated for 4 h at 37 °C with 10-fold serial dilutions (96 mg to 0.96 ng) of ENU. After incubation, the DNA was precipitated twice with ethanol and sodium chloride and re-dissolved in 5 mM Bis–Tris, 0.1 mM EDTA buffer (pH 7.1). The DNA concentrations were measured spectrophotometrically according to the relationship that a solution containing 1 mg/mL DNA gives an absorbance value at 260 nm of 50.

O^6Me-dG-modified DNA: Calf thymus DNA [6.8 mg in 4 mL of 5 mM Bis–Tris, 0.1 mM EDTA buffer (pH 7.1)] was incubated overnight at 37 °C with 10-fold serial dilutions (93 mg to 0.93 ng) of MNU. The rest of the procedure was identical to that used for O^6Et-dG-modified DNA.

2.6. DNA modified in vivo

Male adult C57BL/6N/$Tk^{+/-}$ mice (4 per group) were treated by intraperitoneal injection with 342 μmol/kg body weight MNU, 342 μmol/kg body weight ENU, or the solvent alone (100 μL of 50% aqueous DMSO). After 4 h the mice were sacrificed by CO_2 asphyxiation and their livers were removed and frozen. Hepatic nuclei were prepared from the mouse livers by the method of Basler et al. [13], and DNA was isolated from the nuclei using minor modifications of the method described by Beland et al. [14]. The DNA concentration and purity were measured spectrophotometrically.

2.7. Enzymatic hydrolysis of DNA

(1) O^6Et-modified DNA. Aliquots of DNA (~100 μg in 100 μL Bis–Tris buffer) were incubated with 4 units of micrococcal nuclease and 0.5 units of spleen phosphodiesterase overnight at 37 °C in 14 mM succinic acid, 8.5 mM calcium chloride (pH 6) buffer. Before use, the enzymes were dialyzed against water. Nuclease P1 (1.5 units in 1 mM $ZnCl_2$) was then added and the samples were incubated at 37 °C for 2 more hours. Internal standard ^{15}N-O^6Et-dG was added to the digests and the samples were centrifuged for 2 min at 12,000 rpm before analysis by LC/MS/MS. Complete hydrolysis of the DNA to nucleosides was confirmed by LC/UV analysis.

(2) O^6Me-modified DNA. Aliquots of DNA (~100 μg in 100 μL Bis–Tris buffer) were incubated with 3 units of nuclease P1 (in 1 mM $ZnCl_2$) at 37 °C for 2 h. Prostatic acid phophatase (0.35 units in water) was then added and the samples incubated at 37 °C overnight. Internal standard ^{15}N-O^6Me-dG was added to the digest and the samples were centrifuged for 2 min at 12,000 rpm before analysis by LC/MS/MS. Complete hydrolysis of the DNA to nucleosides was confirmed by LC/UV analysis.

(3) Conditions for concurrent analysis of O^6Me-dG and O^6Et-dG were investigated. Micrococcal nuclease/spleen phosphodiesterase hydrolysis degraded the O^6Me-dG during hydrolysis; however, the nuclease P1/acid phophatase hydrolysis was compatible with both adducts. For this reasons, concurrent analysis of both adducts used DNA hydrolysis conditions identical to those described for O^6Me-dG.

3. Results

3.1. Method validation

3.1.1. Internal standard characterization

Calibration curves were constructed from mixtures of each $^{15}N_5$-labeled adduct at a fixed concentration (1–2 pg/injection) along with the respective unlabeled adduct present at concentrations from one tenth of the $^{15}N_5$-labeled adduct concentration up to 100-fold higher (0.2 to 200 pg/injection) for O^6Me-dG and from one-tenth of the $^{15}N_5$-labeled concentration up to 25-fold higher (0.1 to 25 pg/injection) for O^6Et-dG. In both cases, the plot of response ratio vs. concentration ratio was highly linear ($r^2 > 0.99$) and the respective slope was used to determine relative MS response factors for the predominant labeled species (i.e., $^{15}N_5$). The response factors for each $^{15}N_5$-labeled/unlabeled adduct pair were checked daily by analyzing mixtures of $^{15}N_5$-labeled and unlabeled standards.

3.1.2. Quantitative validation using DNA modified in vitro

The accuracy and precision for measuring O^6Me-dG were determined on two different days by assessing the responses for blank salmon testes DNA (20 μg) to which a known amount (2.2 pg) of unlabeled O^6Me-dG was added corresponding to 1.30 adducts in 10^7 nucleotides. The amount of DNA was selected based on a typical sample size expected in our proposed studies. On day 1, the determined value was 1.26 ± 0.096 adducts in 10^7 nucleotides ($n = 4$; accuracy = 97% of that added; relative standard deviation = 7.6%); On day 2, the determined value was 1.29 ± 0.058 adducts in 10^7 nucleotides ($n = 4$; accuracy = 99% of that added; relative standard deviation = 4.5%). In addition, a sample of calf thymus DNA (20 μg) that had been modified in vitro using MNU (6.8 mg DNA + 0.93 μg MNU) was

analyzed on two different days. On day 1, the determined value was 28 ± 0.87 adducts in 10^7 nucleotides (n = 4; relative standard deviation = 3.1%). On day 2, the determined value was 28 ± 0.58 adducts in 10^7 nucleotides (n = 4; relative standard deviation = 2.1%). The method was also validated using DNA modified with MNU at a higher level (6.8 mg DNA + 9.3 μg MNU, 250 adducts in 10^7 nucleotides) with similar performance (data not shown). The determined value for blank calf thymus and salmon testes DNA (100 μg containing 2 pg internal standard) was approximately 0.07 adducts in 10^8 nucleotides. The same response was observed for the enzyme blank (i.e., no DNA) and internal standard blank (i.e., no DNA and no enzymes), and was attributed to the amount of unlabeled O^6Me-dG present in the internal standard (1.5%). Using 100 μg samples of blank DNA containing 1 pg of $^{15}N_5$-labeled internal standard, the LOD (signal/noise = 3) was estimated at 0.03 O^6Me-dG/10^8 nucleotides and the LOQ (signal/noise = 10) was estimated at 0.1 O^6Me-dG/10^8 nucleotides.

The accuracy and precision for measuring O^6Et-dG were determined on two different days by assessing the responses for blank salmon testes DNA (100 μg) to which a known amount of unlabeled O^6Et-dG was added (20 pg) corresponding to 2.2 adducts in 10^7 nucleotides. On day 1, the determined value was 2.2 ± 0.018 adducts in 10^7 nucleotides (n = 4; accuracy = 102% of that added; relative standard deviation = 0.78%); on day 2, the determined value was 2.2 ± 0.054 adducts in 10^7 nucleotides (n = 4; accuracy = 99% of that added; relative standard deviation = 2.5%). In addition, a sample of calf thymus DNA (100 μg) that had been modified in vitro using ENU (7.0 mg DNA + 96 ng ENU) was analyzed on two different days. On day 1, the determined value was 1.6 ± 0.055 adducts in 10^8 nucleotides (n = 4; relative standard deviation = 3.5%); on day 2, the determined value was 1.6 ± 0.066 adducts in 10^8 nucleotides (n = 4; relative standard deviation = 4.1%; cf. Fig. 3C). The method was also validated using ENU-modified DNA at a higher level (70 adducts in 10^7 nucleotides) with similar performance (data not shown). The determined value for blank salmon testes DNA (100 μg) was approximately 0.5 adducts in 10^9 nucleotides. The same response was observed for the enzyme blank (i.e., no DNA) and internal standard blank (i.e., no DNA and no enzymes), and was attributed to the amount of unlabeled O^6Me-dG present in the internal standard (0.5%). Using 100 μg samples of blank calf thymus and salmon testes DNA containing 1 pg of $^{15}N_5$-labeled internal standard, the LOD (signal/noise = 3) was estimated at 0.05 O^6Et-dG/10^8 nucleotides and the LOQ (signal/noise = 10) was estimated at 0.2 O^6Et-dG/10^8 nucleotides.

Additional validation was performed by modifying a constant amount of calf thymus DNA with serial 10-fold dilutions of either MNU or ENU to produce DNA modified at different levels. Table 1 shows the results for O^6Me-dG and Table 2 shows the results for O^6Et-dG. Selected modified DNA samples were analyzed on separate days to determine method precision. An MNU sample was found to contain 280 ± 8.7 O^6Me-dG/10^8 nucleotides on day 1 and 280 ± 5.8 O^6Me-dG/10^8 nucleotides on day 2. A more highly modified sample was found to contain 2700 ± 37 O^6Me-dG/10^8 nucleotides on day 1, 2800 ± 37 O^6Me-dG/10^8 nucleotides on day 2, 2300 ± 29 O^6Me-dG/10^8 nucleotides on day 3, and 2300 ± 5.9 O^6Me-dG/10^8 nucleotides on day 4. Similarly, two ENU-modified DNA samples modified at different levels were analyzed were found to contain 1.6 ± 0.065 and 700 ± 7.5 O^6Et-dG/10^8 nucleotides on day 1 and 1.6 ± 0.058 and 680 ± 2.3 O^6Et-dG/10^8 nucleotides on day 2.

Table 1
Levels of O^6Me-dG in calf thymus DNA modified in vitro with MNU

MNU (mg)	O^6Me-dG/10^8 nucleotides
0	1.5
9.3×10^{-7}	5.5
9.3×10^{-6}	76
9.3×10^{-5}	55
9.3×10^{-4}	270
9.3×10^{-3}	2.5×10^3
9.3×10^{-2}	2.9×10^4
0.93	2.1×10^5
9.3	7.2×10^5
93	4.0×10^5

A constant amount of DNA (6.8 mg) was reacted with the indicated amount of MNU at 37 °C overnight before analysis for O^6Me-dG levels.

Concurrent analysis of both O^6Me-dG and O^6Et-dG adducts in calf thymus DNA was accomplished by combining 10 μg of MNU-modified DNA with 10 μg of ENU-modified DNA and 80 μg of untreated DNA. The determined adduct levels (average, n = 2; 7.4 O^6Me-dG/10^8 nucleotides and 10.7 O^6Et-dG/10^8 nucleotides; Fig. 4) were comparable to those predicted from the respective 10-fold dilutions (Table 1, 55 O^6Me-dG/10^8 nucleotides and Table 2, 94 O^6Et-dG/10^8 nucleotides).

3.1.3. Quantitation of DNA modified in vivo

Livers from C57BL/6N/$Tk^{+/-}$ mice treated with either a single intraperitoneal injection of equimolar doses of MNU, ENU, or the vehicle were analyzed for O^6Me-dG and O^6Et-dG adducts. Untreated mouse livers contained detectable levels of O^6Me-dG (range 0.11–14, with an average of 5.5 ± 6.7 O^6Me-dG/10^8 nucleotides; data not shown) and MNU-treated mouse livers contained 1700 ± 80 O^6Me-dG/10^8 nucleotides (Fig. 2B). In all cases, the validity of the O^6Me-dG assignments was confirmed by monitoring an additional MRM transition (m/z

Table 2
Levels of O^6Et-dG in calf thymus DNA modified in vitro with ENU

ENU (mg)	O^6Et-dG/10^8 nucleotides
0	0.60
9.6×10^{-7}	0.40
9.6×10^{-6}	0.30
9.6×10^{-5}	1.2
9.6×10^{-4}	8.6
9.6×10^{-3}	94
9.6×10^{-2}	800
0.96	8.3×10^3
9.6	4.9×10^4
96	9.9×10^4

A constant amount of DNA (7.0 mg) was reacted with the indicated amount of ENU at 37 °C for 4 h before analysis for O^6Et-dG levels.

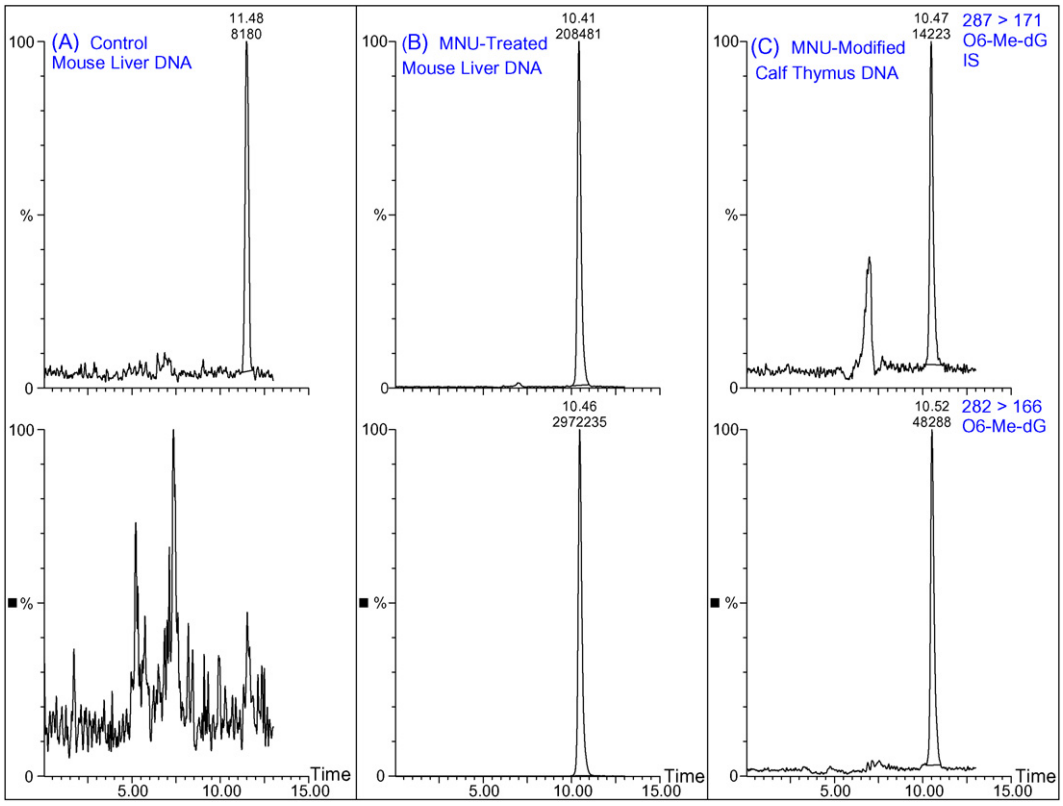

Fig. 2. MRM chromatograms of O^6-methyl-2′-deoxyguanosine and its labeled internal standard (IS) in (A) control mouse DNA (100 μg) containing <LOQ (0.2 adducts/10^8 nucleotides; 0.5 pg IS); (B) liver DNA (17.4 μg) from a mouse treated with MNU (342 μmol/kg body weight) containing 1610 adducts/10^8 nucleotides (20 pg IS); (C) calf thymus DNA (70.3 μg) modified in vitro with MNU containing 4.9 adducts/10^8 nucleotides (1 pg IS).

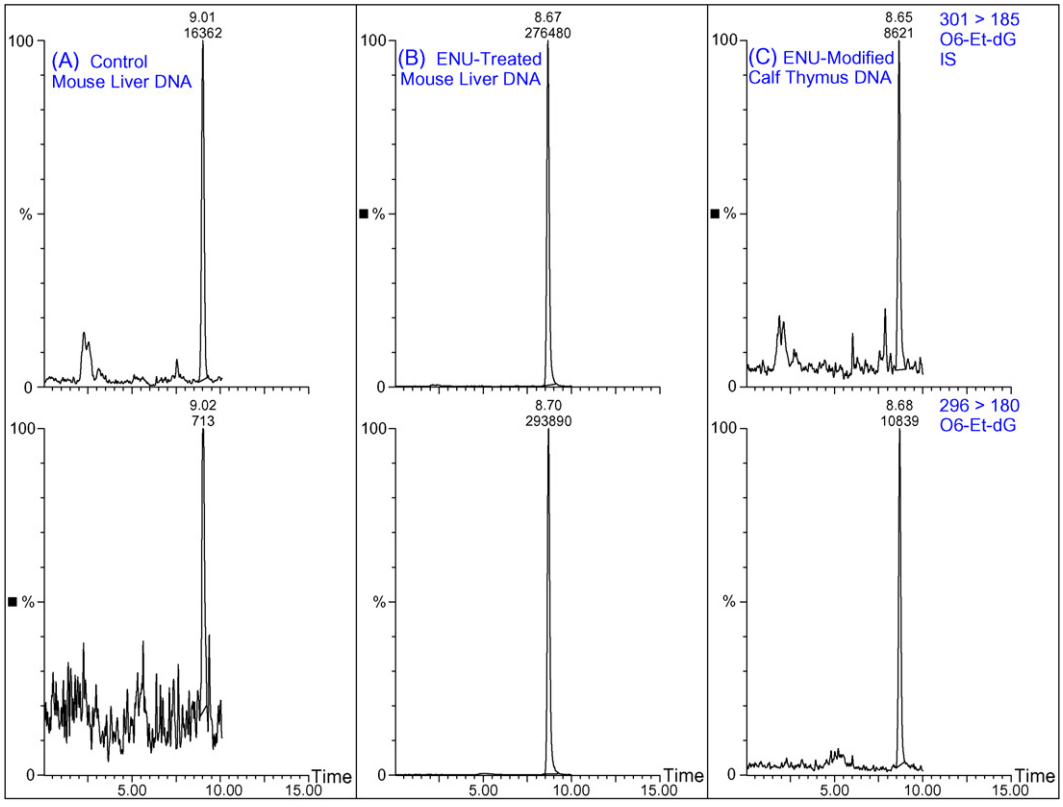

Fig. 3. MRM chromatograms of O^6-ethyl-2′-deoxyguanosine and its labeled internal standard (IS) in (A) control mouse DNA (100 μg) containing 0.5 adducts/10^8 nucleotides; 0.5 pg IS); (B) liver DNA (14.3 μg) from a mouse treated with ENU (342 μmol/kg body weight) containing 172 adducts/10^8 nucleotides (25 pg IS); (C) calf thymus DNA (75.8 μg) modified in vitro with ENU containing 1.5 adducts/10^8 nucleotides (1 pg IS).

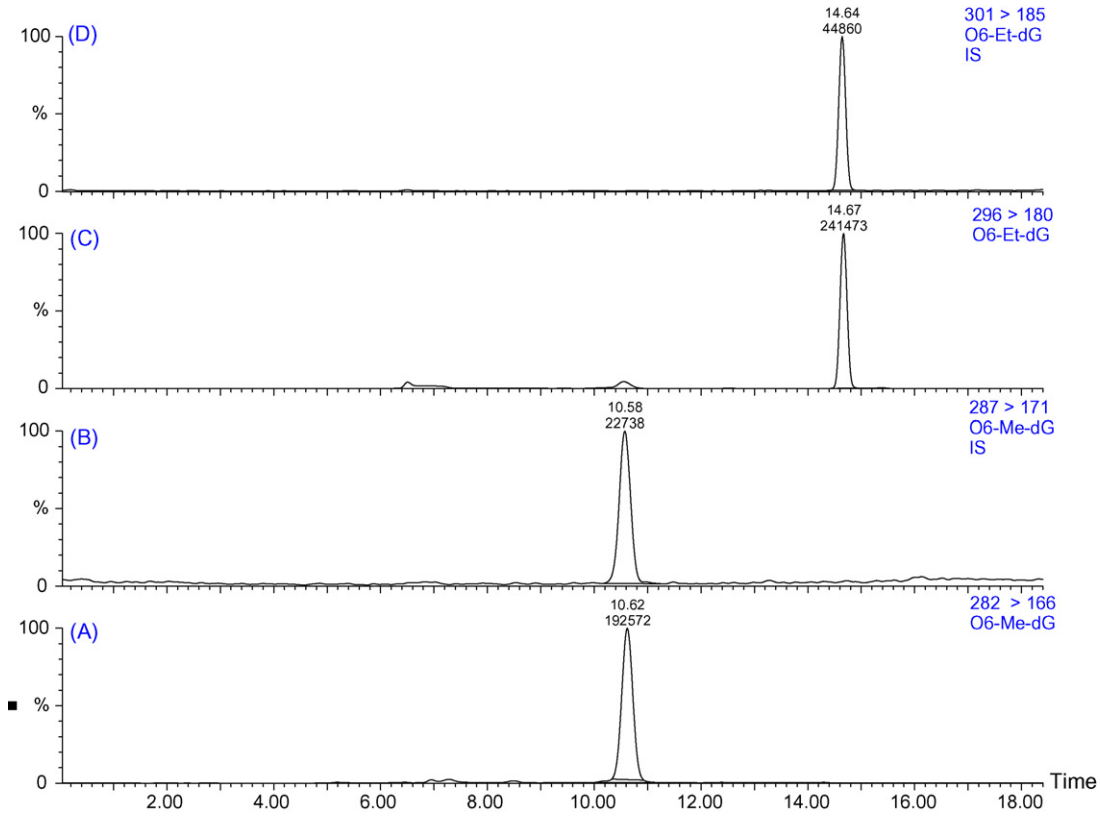

Fig. 4. Concurrent analysis of O^6-methyl-2′-deoxyguanosine (A, B) and O^6-ethyl-2′-deoxyguanosine (C, D) in a calf thymus DNA sample containing 7.8 O^6-methyl-2′-deoxyguanosine/10^8 nucleotides (1 pg IS) and 10.3 O^6-ethyl-2′-deoxyguanosine/10^8 nucleotides (2 pg IS) combined from calf thymus DNA modified in vitro with either MNU or ENU and diluted with untreated DNA to a total of 100 μg.

282 → 149) for the expected chromatographic retention time and the ratio of MRM intensities when compared to authentic standards.

By contrast, untreated mouse livers contained levels of O^6Et-dG at the LOD (Fig. 3A) and ENU-treated mouse livers contained 260 ± 60 O^6Et-dG/10^8 nucleotides (Fig. 3B). To understand better the endogenous levels of O^6Me-dG and O^6Et-dG adducts in untreated mice, 3 male and 3 female C57BL/6N X Sv129 mouse liver samples that had been collected as part of a previous bioassay were analyzed on a separate occasion. In this group of mice, no detectable levels of O^6Me-dG were observed (<0.03 O^6Me-dG/10^8 nucleotides; Fig. 2A) and O^6Et-dG was consistently observed at levels near the LOD (0.04 O^6Et-dG/10^8 nucleotides; data not shown). The validity of O^6Et-dG assignments was similarly confirmed by monitoring an additional MRM transition (*m/z* 296 → 152).

4. Discussion

This paper focuses on the analysis of two O^6-alkylated dG adducts that have demonstrable miscoding potential, are persistent in vivo, and have been consistently associated with mutations and carcinogenesis in vivo [2,3,9]. Using ^{32}P-postlabeling Blömeke et al. [15] concurrently measured O^6Me-dG and O^6Et-dG adducts in human lung in the range of 3–120/10^8 nucleotides and <1–70/10^8 nucleotides, respectively. Clearly, the current LC-ES/MS/MS method for concurrent analysis of O^6Me-dG and O^6Et-dG adducts has sensitivity comparable to ^{32}P-postlabeling in addition to the advantages described above.

Two previous publications described the isotope dilution LC-ES/MS/MS quantification of Me-dG adducts in rodent liver with good reported method precision and accuracy. Yang et al. [16] described the concurrent analysis in rat liver by direct injection of O^6Me-dG and N7-Me-dG, an isomeric adduct whose formation has been shown to be unrelated to mutations or cancer [9,17]. The LOQ and LOD values reported for O^6Me-dG standards were 130 fmol and 85 fmol, respectively, corresponding to approximately 40 and 30 O^6Me-dG/10^8 nucleotides for the analysis of 100 μg aliquots of DNA. The level of O^6Me-dG reported in vehicle-treated female Sprague–Dawley rat liver was approximately 2400 adducts/10^8 nucleotides and that after treatment with MNU (50 mg/kg IP) was approximately 59,000 adducts/10^8 nucleotides. Brink et al. [18] measured O^6Me-dG using on-line sample cleanup. The LOQ reported for O^6Me-dG was 7.5 adducts/10^8 nucleotides and adduct levels in untreated female F344 rat liver adduct levels were reported to be near the method detection limit (approximately 4 adducts/10^8 nucleotides) and in rat livers following treatment by oral gavage with 50 μg/kg *N*,*N*-dimethyl-*N*-nitrosamine were 20 adducts/10^8 nucleotides. The method of Brink et al. [18] did include 8-oxo-dG and 1N^6-etheno-dA adducts in the separation, but only the isotopically labeled internal standard for O^6Me-dG was used for quantification. Similarly, Zhang et al. [19] simultaneously quantified N7-Me-guanine and O^6Me-guanine in calf

thymus DNA modified in vitro by MNU or MMS with LOQs of 200/10^8 nucleotides and 400/10^8 nucleotides, respectively.

The method sensitivity reported here for O^6Me-dG adducts is several orders of magnitude greater when compared with the method of either Yang et al. [16] or Zhang et al. [19]. Furthermore, the reported O^6Me-dG levels in untreated rat liver (2400 adducts/10^8 nucleotides [16]) appear to be several orders of magnitude higher than the untreated mouse liver results presented here (<LOD to 5.5 ± 6.7 adducts/10^8 nucleotides) and the previously reported untreated rat liver results (4/10^8 nucleotides) of Brink et al. [18]. The method sensitivity reported here for O^6Me-dG is approximately two orders of magnitude greater when compared with the method of Brink et al. [18] for a comparable amount of DNA. While similar levels of O^6Me-dG were observed in untreated mouse liver reported here (<LOD to 5.5 ± 6.7 adducts/10^8 nucleotides) and previously in untreated rat liver (4 adducts/10^8 nucleotides [18]), the high degree of variability suggests that caution may be indicated in the interpretation of background adduct levels in rodent liver. Similar caution may also be warranted for O^6Et-dG given the quite low and variable results from different strains of untreated mice reported here.

When the method was applied to liver DNA from mice treated with equimolar amounts (342 μmol/kg body weight) of either MNU or ENU, O^6Me-dG and O^6Et-dG, respectively, were readily detected. The level of O^6Me-dG (5.0 O^6Me-dG/10^8 nucleotides/μmol MNU/kg body weight) was 6.5-fold higher than that of O^6Et-dG (0.76 O^6Et-dG/10^8 nucleotides/μmol ENU/kg body weight). In an earlier study, Frei et al. [9] assessed the levels of O^6Me-dG and O^6Et-dG in DNA from various organs of C57BL mice administered a variety of doses of radiolabeled MNU and ENU. As in our experiment, O^6Me-dG (1.8 O^6Me-dG/10^8 nucleotides/μmol MNU/kg body weight) was found at higher levels than O^6Et-dG (0.44 O^6Et-dG/10^8 nucleotides/μmol ENU/kg body weight), with the difference being 4.1-fold. When reactions were conducted with DNA in vitro (Tables 1 and 2), MNU gave adduct levels (8700 O^6Me-dG/10^8 nucleotides/μmol MNU) that were approximately 20-fold higher than ENU (430 O^6Et-dG/10^8 nucleotides/μmol ENU). The differences in the ratios observed in vitro and in vivo may reflect the more efficient removal of O^6Me-dG compared to O^6Et-dG by O^6-alkylguanine-DNA alkyltransferase [20].

The methods described here demonstrate that LC with tandem mass spectrometric detection can replace ^{32}P-postlabeling as a means to detect DNA adducts from alkylating agents with much higher analytical specificity and precision without loss in sensitivity. This methodology was shown to be useful for analyzing both background adduct levels in untreated rodents and adduct levels in rodents treated with chemical carcinogens. When the analytical sensitivity reported here, which is below 1 adduct/10^9nucleotides, is considered in light of the 4.6×10^9 nucleotide content of haploid human genome [21], it is striking that modern LC/MS/MS methodology can provide data near the level of one modified base per genome copy. Future studies will use these techniques to explore cellular mechanisms that affect steady state DNA adduct levels and modify toxicological and carcinogenic responses in experimental animals and the relationship between such adduct determinations and human cancers.

References

[1] D.T. Beranek, Mutat. Res. 231 (1990) 11.
[2] D.E.G. Shuker, H. Bartsch, in: K. Hemminki, A. Dipple, D.E.G. Shuker, F.F. Kadlubar, Segerbäck, H. Bartsch (Eds.), DNA Adducts: Identification and Biological Significance, IARC Scientific Publications No. 125, International Agency for Research on Cancer, Lyon, 1994, p. 73.
[3] M.C. Poirier, F.A. Beland, Chem. Res. Toxicol. 5 (1992) 749.
[4] D.H. Phillips, P.B. Farmer, F.A. Beland, R.G. Nath, M.C. Poirier, M.V. Reddy, K.W. Turteltaub, Environ. Mol. Mutagen. 35 (2000) 222.
[5] M.C. Poirier, R.M. Santella, A. Weston, Carcinogenesis 21 (2000) 353.
[6] F.A. Beland, D.R. Doerge, M.I. Churchwell, M.C. Poirier, M.C. Poirier, M.M. Marques, Chem. Res. Toxicol. 12 (1999) 68.
[7] F.A. Beland, M.I. Churchwell, D.R. Doerge, D.R. Parkin, D. Malejka-Giganti, D.A. Hewer, D.H. Phillips, P.L. Carmichael, G. Gamboa da Costa, M.M. Marques, J. Nat. Cancer Inst. 96 (2004) 1099.
[8] F.A. Beland, M.I. Churchwell, L.S. Von Tungeln, S. Chen, P.P. Fu, S.J. Culp, B. Schoket, E. Gyorffy, J. Minarovits, M.C. Poirier, E.D. Bowman, A. Weston, D.R. Doerge, Chem. Res. Toxicol. 18 (2005) 1306.
[9] J.V. Frei, D.H. Swenson, W. Warren, P.D. Lawley, Biochem. J. 174 (1978) 1031.
[10] D.R. Doerge, M.I. Churchwell, F.A. Beland, Environ. Carcin. Ecotoxicol. Rev. C20 (2002) 1.
[11] A.L. Wilds, A.L. Meader, J. Chem. Soc. (1948) 763.
[12] P.B. Farmer, A.B. Foster, M. Jarman, M.J. Tisdale, Biochem. J. 135 (1973) 203.
[13] J. Basler, N.D. Hastie, D. Pietras, S.I. Matsui, A.A. Sandberg, R. Berezney, Biochemistry 20 (1981) 6921.
[14] F.A. Beland, N.F. Fullerton, R.H. Heflich, J. Chromatogr. 308 (1984) 121.
[15] B. Blömeke, M.J. Greenblatt, V.D. Doan, E.D. Bowman, S.E. Murphy, C.C. Chen, S. Kato, P.G. Shields, Carcinogenesis 17 (1996) 741.
[16] Y. Yang, D. Nikolic, S.M. Swanson, R.B. van Breeman, Anal. Chem. 74 (2002) 5376.
[17] G.P. Margison, J.M. Margison, R. Montesano, Biochem. J. 157 (1976) 627.
[18] A. Brink, U. Lutz, W. Völkel, W.K. Lutz, J. Chromatogr. B 830 (2005) 255.
[19] F. Zhang, M.J. Bartels, L.H. Pottenger, B.B. Gollapudi, M.R. Schisler, J. Chromatogr. B 833 (2006) 141.
[20] A.E. Pegg, D. Scicchitano, M.E. Dolan, Cancer Res. 44 (1984) 3806.
[21] L.H. Pottenger, M. Penman, N.P. Moore, R.A.J. Priston, M. Thomas, Regul. Pharmacol. Toxicol. 39 (2004) 403.