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Use of LC-MS/MS and Stable Isotopes to Differentiate Hydroxymethyl and Methyl DNA Adducts from Formaldehyde and Nitrosodimethylamine

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Abstract

Formaldehyde is a known human and animal carcinogen that forms DNA adducts, and causes mutations. While there is widespread exposure to formaldehyde in the environment, formaldehyde is also an essential biochemical in all living cells. The presence of both endogenous and exogenous sources of formaldehyde makes it difficult to develop exposure-specific DNA biomarkers. Furthermore, chemicals such as nitrosodimethylamine form one mole of formaldehyde for every mole of methylating agent, raising questions about potential co-carcinogenesis. Formaldehyde-induced hydroxymethyl DNA adducts are not stable and need to be reduced to stable methyl adducts for detection, which adds another layer of complexity to identifying the origins of these adducts. In this study, highly sensitive mass spectrometry methods and isotope labeled compounds were used to differentiate between endogenous and exogenous hydroxymethyl and methyl DNA adducts. We demonstrate that N^2-hydroxymethyl-dG is the primary DNA adduct formed in cells following formaldehyde exposure. In addition, we show that alkylating agents induce methyl adducts at N^2-dG and N^6-dA positions, which are identical to the reduced forms of hydroxymethyl adducts arising from formaldehyde. The use of highly sensitive LC-MS/MS and isotope labeled compounds for exposure solves these challenges and provides mechanistic insights on the formation and role of these DNA adducts.

Introduction

Formaldehyde is classified as a human and animal carcinogen according to the International Agency for Research on Cancer (IARC) [1]. Formaldehyde can react with proteins and DNA to form corresponding protein adducts [2;3], DNA adducts [4-7] and DNA-protein cross-links [8-15]. The sources of formaldehyde exposure in the body can be classified into several categories. Inhaled formaldehyde can enter into the body through environmental exposures such as vehicle emissions, off gassing of building materials and tobacco smoke. At the same time, formaldehyde is endogenously produced from serine, glycine, methionine, and choline as well as being generated from metabolism of foods, drugs, chemicals and proteins by demethylation. The endogenous concentration of formaldehyde in the blood of human subjects is about 0.1 mM/L [16].

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Previous research has demonstrated that formaldehyde is genotoxic in a variety of test systems, causing mutations in multiple genes [17-20]. DNA adducts play an important role in mutagenesis and carcinogenesis. The structures of formaldehyde-induced DNA adducts *in vitro* have been known for decades [4-7]. Formaldehyde can typically result in N^6-hydroxymethyl-dA, N^2-hydroxymethyl-dG and N^4-hydroxymethyl-dC *in vitro* [21-23]. Hydroxymethyl DNA adducts arising from formaldehyde are not stable and need to be reduced to their methyl forms for robust quantitation. However, alkylation is another type of DNA damage in cells, with methyl adducts being induced by a variety of alkylating agents. Such methyl adducts could be mistakenly identified as reduced hydroxymethyl adducts, as their structures are identical. Therefore, in this study, we have applied sensitive LC-ESI-MS/MS-SRM methods to detect and differentiate hydroxymethyl and methyl DNA adducts. In particular, this study was designed to address several critical questions: Which adduct, N^2-hydroxymethyl-dG or N^6-hydroxymethyl-dA, is the primary DNA damage following direct exposure of cells to formaldehyde? Does metabolically formed formaldehyde also induce hydroxymethyl DNA adducts? If so, which adduct is the major DNA lesion formed? Do formaldehyde-generating compounds also result in DNA alkylation at N^2-dG and N^6-dA? If so, how can one differentiate alkylation products from reduced hydroxymethyl DNA adducts originating from formaldehyde? To answer these questions, we largely rely on the use of stable isotope labeled reagents for exposure and our highly sensitive mass spectrometry methods. This allows us to determine the sources of DNA adducts, their chemical characterization and provide mechanistic insights on the formation of these DNA lesions.

In this study, we demonstrate that formaldehyde-DNA adducts arising from endogenous and exogenous sources can be clearly differentiated using [$^{13}CD_2$]-formaldehyde and mass spectrometry. Our results also demonstrate that N^2-hydroxymethyl-dG is the primary DNA adduct formed following formaldehyde exposure in cells. No detectable amounts of exogenous formaldehyde-induced N^6-hydroxymethyl-dA were found in any exposed cells. In addition, we have demonstrated that DNA alkylating agents induced methylation at N^2-dG and N^6-dA positions, which could be confused with hydroxymethyl-dG and hydroxymethyl-dA adducts after their reduction. This further defines the development of formaldehyde-specific DNA biomarkers and clarifies important issues for differentiating between methyl and reduced hydroxymethyl adducts in future research.

Materials and methods

Chemicals and Materials

2-deoxyguanosine, 2-deoxyadenosine, potassium phosphate, Tris-HCl, magnesium chloride ($MgCl_2$), formic acid, sodium cyanoborohydride ($NaCNBH_3$), methanol, acetonitrile, HPLC grade water and 10× PBS were all purchased from Sigma (St. Louis, MO). Formaldehyde (20% in water) came from Tousimis (Rockville, MD). [$^{13}CD_2$]-Formaldehyde (20% in heavy water) was bought from Cambridge Isotopes (Cambridge, MA). D_6-Nitrosodimethylamine and methyl-D_3-methanesulfonate were purchased from CDN Isotopes (Quebec, Canada). DNase I, alkaline phosphatase and phosphodiesterases were purchased from Sigma (St. Louis, MO). [$^{15}N_5$]-2-deoxyadenosine and [$^{13}C_{10}$ $^{15}N_5$]-2-deoxyguanosine were ordered from Cambridge Isotope Lab (Cambridge, MA). N^6-Methyl-dA and N^2-methyl-dG were obtained from Sigma (St. Louis, MO) and Berry & Associates (Dexter, MI), respectively. All chemicals were used as received unless otherwise stated.

Preparation of Internal Standards of N^2-methyl-dG and N^6-methyl-dA

Ten mM [$^{13}C_{10}$$^{15}N_5$]- dG or [$^{15}N_5$]-dA solution was treated with 100 mM formaldehyde in phosphate buffer (pH=7.2) overnight at 37 °C. The reaction mixture was separated by HPLC

using a 150 mm × 2.5 mm C18 T3 analytical column. N^2-hydroxymethyl-dG and N^6-hydroxymethyl-dA eluted at 20.5 and 24.3 min, respectively. N^2-hydroxymethyl-dG and N^6-hydroxymethyl-dA were collected and incubated with 50 mM $NaCNBH_3$ (pH=7.1) overnight at 37 °C, followed by further separation using HPLC. [$^{13}C_{10}$ $^{15}N_5$]-N^2-Methyl-dG and [$^{15}N_5$]-N^6-methyl-dA eluted at 27.2 and 33.5 min on a 150 mm × 2.5 mm T3 column, separately. The concentration of [$^{13}C_{10}$ $^{15}N_5$]-N^2-methyl-dG and [$^{15}N_5$]-N^6-methyl-dA was determined by HPLC with corresponding unlabelled N^6-methyl-dA and N^2-methyl-dG as references. The conversion rate from hydroxymethyl to methyl groups was ~65% to 85%.

Exposure Experiments

Hela S3 cells were obtained from the Tissue Culture Facility of the University of North Carolina in a cell culture flask containing α-MEM culture medium supplemented with 5% fetal bovine serum and 5% horse serum. The exposure experiment was performed when the cell density was about 5.5×10^5 cells/ml. The calculated concentrations of added [$^{13}CD_2$]-formaldehyde in the plates was 0.0625, 0.125, 0.25, 0.50, 1.0 mM. The exposure time varied from 1 h to 24 h. In addition, Hela S3 cells were exposed to [$^{13}CD_2$]-formaldehyde in warm PBS buffer for 1 h to examine the influence of protein and amino acid binding on the formation of DNA adducts. VL17A cells were cultured in DMEM high glucose medium containing 10% fetal bovine serum with 400 μg/ml of zeocin and 400 μg /ml of G418. Cells were exposed to 10 mM *N*-nitrosodimethyl-D_6-amine for 6, 12 or 18 h [24;25], followed by harvest and storage for later use. HepG2 cells were grown in DMEM high glucose medium containing 10% fetal bovine serum. HepG2 cells were exposed to methyl-D_3-methanesulfonate for 4 h. In the methylnitrosourea experiments, HepG2 cells were treated with 0.1, 0.3 and 1 mM MNU for 15 min. After exposure, all the cells were harvested by a scraper and centrifuged at 3500 rpm for 5 min. Then, the cells were washed in 5 ml of PBS and centrifuged again. The wash step was repeated an additional 2 times. The resultant cell pellets were frozen at -80 °C for later use.

DNA Isolation

DNA was isolated from cells using a NucleoBond DNA Isolation Kit (Bethlehem, PA). One ml of buffer G1 (ice-cold) and 3 ml of H_2O (ice-cold) were added into 1 ml of cell suspension (approximate 1×10^7 cells in 1 ml of PBS buffer). Then, the suspension was mixed by inverting the tube 6 – 8 times and incubated for 10 min on ice, followed by the centrifugation at 4 °C for 15 min at 3500 rpm. The supernatant was discarded and 5 ml of buffer G2 was added, followed by vortexing for 15 sec. One hundred μl of proteinase K stock solution (20 mg/ml) was added and the mixture was incubated for 60 min at 50 °C. After equilibrating the column with 2 ml of buffer N2, 5 ml of buffer N2 was added to the sample. The mixture was vortexed for 15 sec, followed by loading the sample onto the column. After all the mixture passed through the column, the column was washed 3 times with 4 ml of buffer N3. DNA was eluted with 5 ml of buffer N5, followed by adding 3.5 ml of isopropanol, mixing, and centrifuging at 4 °C for 15 min. The isolated DNA was dissolved in 1X TE buffer or water and frozen for later analysis.

DNA Treatment and Digestion

About 50 μg of DNA was incubated with 50 mM $NaCNBH_3$ at 37°C for 6 hours in phosphate buffer (pH=7.2). Then, the internal standards were added and the DNA was treated with DNase I (200 U) for 10 min in the digestion buffer (80 mM Tris-HCl, 20 mM $MgCl_2$, pH=7.2), followed by the addition of 25 μl of alkaline phosphatase and 25 μl of phosphodiesterases for an additional hour. Enzymes and undigested DNA were removed by a Millipore Microcon YM-10 spin column and the resultant filtrate was separated by HPLC to collect the fractions containing the corresponding DNA adducts.

High Performance Liquid Chromatography (HPLC)

The purification of formaldehyde-DNA adducts was carried out on an Agilent 1200 series HPLC system equipped with a diode-array detector (Santa Clara, CA). Analytes were separated by reverse phase chromatography using a 150 mm × 2.5 mm C18 T3 analytical column from Waters (Milford, MA). The mobile phases were 0.1% formic acid (A) and methanol (B). A linear gradient was run from 2% methanol to 30% methanol over 15 min, at a flow rate of 200 μL/min and monitored at 254 nm. N^6-Methyl-dA and N^2-methyl-dG eluted at 21.2 and 24.5 min on the column in this system, respectively.

Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS analyses were performed on a triple quadrupole mass spectrometer TSQ-Quantum Ultra (Thermo Electron, Waltham, MA) operating in selected reaction monitoring (SRM) mode to detect and quantify the reduced hydroxymethyl-DNA adducts. The mass spectrometer was interfaced with a nano-Ultra Performance Liquid Chromatography (nano-UPLC) system from Waters (Milford, MA). Mobile phases were comprised of water with 0.1% acetic acid (A) or acetonitrile with 0.1% acetic acid (B). Both capillary and nano-flow rates were used to quantify formaldehyde-DNA adducts. For the capillary method, a linear gradient was run from 2% to 60% B over 10 min, at 40 μL/min. The electrospray ionization (ESI) source was set as follows: spray voltage, 4.0 kV; capillary temperature, 300°C; sheath gas pressure, 40 au; aux gas pressure, 10 au. For the nano method, analytes were first retained on a trap column with a flow rate of 5 μL/min of 2% mobile phase B, followed by transfer to the analytical column with an initial starting condition of 2% B at 0.6 μL/min for 1 minute followed by a linear gradient to 60% B over 14 min. The column was then cleaned at 80% B for 1.5 min followed by re-equilibration for an additional 7.5 min. The analytes were introduced to the MS using positive mode electrospray ionization with a source voltage of 2200 V and no additional gasses. The ion transfer tube was held at 325°C and skimmer offset set to zero. Scan speed was set at 0.1 sec, scan width at 1.0 m/z, and peak widths for Q1 and Q3 at 0.3 and 0.5 m/z, respectively. Collision energy was set at 17 eV with Argon as the collision gas set at 1.5 arbitrary units.

Quantitation of formaldehyde-DNA adducts

The adducts were quantified by a triple quadrupole mass spectrometer TSQ-Quantum Ultra (Thermo Electron, Waltham, MA) using SRM mode. N^2-hydroxymethyl-dG was quantified as N^2-methyl-dG after reduction using the transition of m/z 282.2→m/z 166.1 and [$^{13}CD_2$]-N^2-hydroxymethyl-dG was quantified as [$^{13}CD_2$]-N^2-methyl-dG with the transition of m/z 285.2→m/z 169.1. N^6-hydroxymethyl-dA was detected as N^6-methyl-dA after treatment by $NaCNBH_3$ with the transition of m/z 266.2→m/z 150.1. [$^{13}CD_2$]-N^6-hydroxymethyl-dA was monitored as [$^{13}CD_2$]-N^6-methyl-dA with the transition of m/z 269.2→m/z 153.1 after reduction. Four additional transitions including m/z 284.2→m/z 168.1, m/z 283.2→m/z 167.1, m/z 268.2→m/z 152.1, and m/z 267.2→m/z 151.1 were also monitored in case H-D exchange occurred. *N*7-methyl-dG and O^6-methyl-dG were detected using the transition of m/z 282.2→m/z 166.1. The collision energy was set at 17 eV after optimization. The calibration curve for quantitation was obtained using the integrated peak area and amount of injected analytical standard and internal standard.

Derivatization of formaldehyde by acetylacetone

Formaldehyde from freshly prepared solutions and culture media after 24 h exposure was derivatized with acetylacetone. Briefly, 100 μl of formaldehyde solution or culture medium was incubated with 8.8 μl of 200 mM acetylacetone, 10 μl of acetic acid, 100 μl of 4 M ammonium acetate for 30 min at 60 °C. The resultant formaldehyde-acetylacetone was immediately analyzed by an Agilent 6500 Series Quadrupole Time-of-Flight (Q-TOF) LC/

MS (Santa Clara, CA) with an ESI source. A linear gradient was run from 2% acetonitrile in 0.1% formic acid to 60% acetonitrile in 15 min at 200 μL/min. The ESI source was set as follows: gas temperature, 350 °C; drying gas, 10 L/min; Vcap, 4000 V; Nebulizer, 35 psig; fragmentor, 100 V; and skimmer, 65 V. A 150 mm × 2.0 mm T3 C18 column (3 μm particle size) was used.

Results

Methods and adducts measured in this study

Both capillary and nano-LC methods were used in this study, which have been published previously [26;27]. As the hydroxymethyl DNA adducts are not stable, they are usually measured as corresponding methyl adducts after reduction by $NaCNBH_3$. Therefore, hydroxymethyl and methyl DNA adducts cannot be differentiated after the reduction treatment. Fortunately, application of stable-isotope labeled compounds for exposure offers a compelling approach to address this issue. In this study, we have used $^{13}CD_2$-formaldehyde, D_6-nitrosodimethylamine (D_6-NDMA) and methyl-D_3-methanesulfonate (D_3-MMS) to treat cells, which could unambiguously distinguish the sources of diverse DNA adducts listed in Figure 1.

N^2-hydroxymethyl-dG from [$^{13}CD_2$]-formaldehyde exposed cells

Figure 2A shows the LC-ESI-MS/MS-SRM chromatogram from control cells without any exposure to exogenous [$^{13}CD_2$]-formaldehyde. The peak corresponding to the specific transition of m/z 282.2→m/z 166.1 exhibits several-fold increased intensity after reduction and has the same retention time as [$^{13}C_{10}{}^{15}N_5$]-N^2-methyl-dG internal standard, which identified the formation of N^2-hydroxymethyl-dG from endogenous formaldehyde, as shown by the peak at 6.7 min in the top panel of Figure 2A. The endogenous amount of N^2-hydroxymethyl-dG adducts in Hela cells was 3.3±1.9 adducts/10^7dG (n=4). Figure 2B was obtained from the cells after 1 h exposure to 1 mM [$^{13}CD_2$]-formaldehyde. In addition to the peak of endogenous adducts, a new peak corresponding to the transition of m/z 285.2→m/z 169.1 co-eluted with the internal standard, which is attributed to [$^{13}CD_2$]-N^2-hydroxymethyl-dG arising from exogenous [$^{13}CD_2$]-formaldehyde.

Formation of *N*^{6}-hydroxymethyl-dA

Several previous *in vitro* studies have shown that N^6-hydroxymethyl-dA is a primary formaldehyde-DNA monoadduct [21-23]. Here, we examined the possible formation of N^6-hydroxymethyl-dA in cells exposed to different doses of formaldehyde, as shown in Figure 3. Figure 3 A and B give the LC-ESI-MS/MS-SRM chromatograms of N^2-methyl-dG in cells exposed to 0.5 mM or 1 mM [$^{13}CD_2$]-formaldehyde for 3 h, while Figures 3C and 3D show the typical chromatograms of N^6-methyl-dA in cells. N^2-hydroxymethyl-dG originating from endogenous and exogenous sources was detected as its corresponding N^2-methyl-dG adduct in both 0.5 mM and 1 mM exposed cells, clearly demonstrating that [$^{13}CD_2$]-formaldehyde entered cells and induced exogenous DNA adducts. However, the peak of [$^{13}CD_2$]-N^6-hydroxymethyl-dA in cells exposed to [$^{13}CD_2$]-formaldehyde was not detected, while the peak of reduced N^6-hydroxymethyl-dA from endogenous formaldehyde could be clearly observed, as shown in Figures 3C and 3D. The endogenous amount of N^6-hydroxymethyl-dA adducts in Hela cells was 4.2±0.6 adducts/10^7 dA (n=4). These results suggest that N^2-hydroxymethyl-dG is a more sensitive DNA biomarker of formaldehyde exposure, compared to N^6-hydroxymethyl-dA.

Hydrogen-Deuterium exchange in cell culture

It is well known that deuterium is an isotope that can exchange with neighboring hydrogen atoms, especially in basic buffer. Thus, the application of formaldehyde labeled with ^{13}C and deuterium raises the issue of possible H-D exchange. If significant H-D exchange does occur, this may affect the identity of adducts since the assignment of each peak is based on the specific transitions of masses of different ions. To evaluate this effect, the mass spectrometer was setup to monitor all possible SRM transitions after losing either 1 or 2 deuterium atoms, as shown in Figure 4.

Figure 4A shows the peaks acquired after scanning all the transitions for 15 fmol of N^2-methyl-dG analytical standard loaded on the column. The $peak_{283\rightarrow167}$ is the consequence of natural isotope abundance of the $peak_{282\rightarrow166}$ and the area ratio of these two peaks is ~0.07. The area ratio of $peak_{284\rightarrow168}$ to the $peak_{282\rightarrow166}$ is ~0.01. Figure 4B is the LC-ESI-MS/MS-SRM chromatogram obtained from cells treated with 0.5 mM [$^{13}CD_2$]-formaldehyde for 3 h. The $peak_{282\rightarrow166}$ and $peak_{285\rightarrow169}$ are assigned to endogenous and exogenous dG adducts, respectively. The area ratio of $peak_{283\rightarrow167}$ to the $peak_{282\rightarrow166}$ is ~0.06, which corresponds to the natural abundance of isotopes. Figure 4C shows the results from cells treated by 0.5 mM [$^{13}CD_2$]-formaldehyde for 24 h. The $peak_{282\rightarrow166}$ is attributed to the endogenous formaldehyde-induced adduct. However, there is no peak corresponding to the transition of m/z 285.2→ m/z 169.1, which would form from [$^{13}CD_2$]-formaldehyde, as shown in Figure 4B. However, the area ratio of the $peak_{283\rightarrow167}$ to the $peak_{282\rightarrow166}$ is 1.26, which is 18-fold higher than the case in Figure 4A. This demonstrates that [$^{13}CD_2$]-formaldehyde is contributing to this peak in addition to the natural abundance of isotope (the ratio is around ~0.07). Additionally, the ratio of the $peak_{284\rightarrow168}$ to the $peak_{282\rightarrow166}$ is also significantly higher than that in Figure 4A (0.13 versus 0.01). These data clearly demonstrate that H-D exchange takes place under this experimental condition (24 h exposure in cell culture media).

H-D exchange occurs more readily under higher pH conditions. The pH value of normal cell culture medium is slightly basic (pH is about 7~8). Therefore, H-D exchange may happen in culture medium instead of cells during a 24 hour exposure. To examine this effect, we analyzed formaldehyde in culture medium after derivatizing it with acetylacetone, followed by measurement using a high resolution Q-TOF mass spectrometer. As shown in Figure 5, the primary ions observed were m/z=197.1331 and m/z=197.1335, corresponding to acetylacetone derivatives of [$^{13}CD_2$]-formaldehyde (theoretical m/z=197.1335). If H-D exchange occurs in the culture medium, the theoretical isotopic mass of new peaks should be m/z=196.1272 after losing 1 deuterium atom. Figure 5 shows that two tiny peaks at m/z=196.1282 and m/z=196.1277 were detected from freshly prepared [$^{13}CD_2$]-formaldehyde solution and 24 h culture medium, respectively. In addition, the area ratios of $peak_{196}$/$peak_{197}$ gave very similar small values for fresh formaldehyde solution and 24 h culture medium (0.016 versus 0.017). Therefore, there is no significant H-D exchange under either condition and no difference between freshly prepared formaldehyde solution and formaldehyde extracted from culture medium after a 24 h exposure. However, 100% of [$^{13}CD_2$]-formaldehyde lost at least 1 deuterium atom in formaldehyde-DNA adduct molecules formed during a 24 h exposure, as shown in Figure 4C. Taken together, these results suggest that H-D exchange primarily occurred inside the cells rather than in culture medium.

Now, we reconsider our inability of detecting [$^{13}CD_2$]-N^6-hydroxymethyl-dA in exposed cells. Is this the consequence of significant H-D exchange? As we have seen in Figure 4, significant H-D exchange occurred in 24 h treated cells. Therefore, we compared the area ratios of various peaks for N^6-methyl-dA analytical standard and 24 h [$^{13}CD_2$]-formaldehyde treated samples to examine whether H-D exchange interfered with the

identification of this adduct. Figure 6A gives the peaks obtained after scanning multiple transitions for 15 fmol of N^6-methyl-dA analytical standard, while the LC-ESI-MS/MS-SRM chromatogram of the 24 h 1mM [$^{13}CD_2$]-formaldehyde treated cells is shown in Figure 6B. No peak corresponding to the transition of m/z 269.2→m/z 153.1 was observed. Moreover, the area ratio of the peak$_{267\rightarrow151}$ to the peak$_{266\rightarrow150}$ in the 24 hour treated sample is not significantly higher than the analytical standard (0.09 versus 0.08). These data show no accumulation of the peak$_{267\rightarrow151}$ and only the normal isotope distribution of the peak$_{266\rightarrow150}$. Therefore, no detectable amount of [$^{13}CD_2$]-N^6-hydroxymethyl-dA was found in the samples, even under a 24 h high concentration formaldehyde exposure.

Differentiating methyl-DNA adducts originating from alkylation and reduction of hydroxymethyl-DNA adducts

A previous study demonstrated that metabolically formed formaldehyde lead to increased amounts of N^6-methyl-dA in multiple tissues from rats exposed to *N*-nitrosodimethylamine (NDMA) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [28;29], which was attributed to formaldehyde-induced N^6-hydroxymethyl-dA. We performed an *in vitro* experiment by exposing HepG2 (2E1+) to *N*-nitrosodimethyl-D_6-amine to examine if metabolically formed formaldehyde could induce hydroxymethyl dA and dG adducts. *N*-nitrosodimethyl-D_6-amine is metabolized by P450 2E1 to generate D_2-labeled formaldehyde (CD_2O), which could potentially lead to the formation of hydroxymethyl DNA adducts. Simultaneously, a diazonium ion and ultimately carbonium ion CD_3^+ are also formed during the metabolism of *N*-nitrosodimethyl-D_6-amine, resulting in the formation of DNA alkylation adducts at multiple positions of DNA bases. As shown in Figure 7, the peak at m/z=284.2 was attributed to exogenous N^2-D_2-methyl-dG adducts, indicating nitrosamine-derived formaldehyde caused N^2-hydroxymethyl-dG adducts, however, there was no signal corresponding to exogenous N^6-D_2-methyl-dA (m/z=268.2) after reduction. Two additional peaks at m/z=285.2 and 269.2 were clearly detected, corresponding to N^2-D_3-methyl-dG and N^6-D_3-methyl-dA, respectively, which were not formaldehyde-induced adducts due to the presence of an additional 1 Da mass shift. In addition, removal of the reduction step did not decrease the intensity of the peaks at m/z=285.2 and 269.2, further supporting that formaldehyde-induced N^2-hydroxymethyl-dG and N^6-hydroxymethyl-dA were not the precursors of these adducts. They were attributed to the alkylation products at N^2-dG and N^6-dA positions resulting from carbonium ion CD_3^+, generated during the metabolism of *N*-nitrosodimethyl-D_6-amine. These data indicate that nitrosamine-derived formaldehyde induces N^2-hydroxymethyl-dG as the primary formaldehyde-DNA adducts in cultured cells.

Table 1 lists the adduct quantitation results in HepG2(+2E1) cells treated by D_6-NDMA for 6 h and 18 h, which were measured in non-reduced and reduced DNA samples. In the non-reduced samples, both N^6-methyl-dA and N^2-methyl-dG were present in small amounts, indicating the formation of these two types of endogenous DNA alkylation adducts. N^2-D_2-Methyl-dG was detected in all D_6-NDMA treated cells, with much higher amounts in reduced DNA samples. However, N^6-D_2-methyl-dA was below the detection limit in either reduced or non-reduced samples. Both N^2-D_3-methyl-dG and N^6-D_3-methyl-dA were readily detected in either non-reduced or reduced samples, with 10-fold higher amounts of N^2-D_3-methyl-dG adducts, compared to N^2-D_2-methyl-dG adducts. A marked increase of N^2-methyl-dG and N^6-methyl-dA adducts after reduction indicates endogenous hydroxymethyl-DNA adducts are predominant, compared to endogenous methyl adducts.

Alkylation at N^2-dG and N^6-dA positions in cells treated by D_3-MMS

To further confirm the formation of alkylation products at N^2-dG and N^6-dA positions, D_3-MMS was used for exposure, which does not generate formaldehyde, removing this confounding factor for the identification of adducts. As shown in Figure 8A and 8B, the

peaks corresponding to transitions at m/z 285.2→ m/z 169.1 and m/z 269.2→ m/z 153.1 co-elute with internal standards, unequivocally supporting the formation of N^2-D_3-methyl-dG and N^6-D_3-methyl-dA in D_3-MMS treated cells.

Relative abundance of the alkylation at N^2-dG and N^6-dA positions

Since methylation at N^2-dG and N^6-dA had not previously been reported in mammalian DNA, the relative abundance of alkylation at N^2-dG and N^6-dA were established by analyzing methylnitrosourea (MNU)-treated hepG2 cells. Cells were exposed to different concentrations of MNU for 15 min to induce DNA alkylation, under conditions that minimize the influence of DNA repair due to the short period of exposure. As shown in Figure 8C and 8D, similar to the well-studied *N*7-methyl-dG and O^6-methyl-dG adducts, the formation of methylation DNA adducts at N^2-dG and N^6-dA positions shows an approximately linear dose response. Moreover, N^2-methyl-dG and N^6-methyl-dA are minor alkylation products, with N^2-methyl-dG having several-fold higher amounts than N^6-methyl-dA. The amounts of *N*7-methyl-dG and O^6-methyl-dG are approximately one hundred and one thousand times higher than those of N^2-methyl-dG, and five hundred and five thousand fold greater than N^6-methyl-dA.

Influence of protein binding on the formation of formaldehyde-DNA adducts

We hypothesize that formaldehyde's high reactivity with proteins plays an important role in the formation of formaldehyde-specific DNA adducts. Formaldehyde is a very reactive compound, so it could target serum proteins or other components in culture medium, reducing formaldehyde DNA adduct formation. To evaluate this effect, a parallel exposure experiment was performed in either normal culture medium or PBS buffer.

Figure 9 shows the amount of exogenous N^2-hydroxymethyl-dG adducts in cells exposed to 125-500 μM formaldehyde in either culture medium or PBS. At the 125 μM concentration, exogenous dG adducts were not detected in cells exposed in culture medium by the capillary LC-MS/MS method. However, we could detect this adduct in cells exposed to formaldehyde in PBS buffer. At 250 and 500μM concentration, the numbers of exogenous dG adducts in cells exposed to formaldehyde in culture medium was roughly 30%-50% of those in cells exposed to formaldehyde in PBS. These data show that formaldehyde has high reactivity with serum proteins or amino acids present in culture medium, with considerable amounts of formaldehyde actually being consumed by protein binding during exposure.

Discussion

In this study, we have applied highly sensitive LC-ESI-MS/MS-SRM methods to detect and quantify hydroxymethyl and methyl DNA adducts in cells treated by different isotope labeled compounds. Both endogenous formaldehyde-induced N^2-hydroxymethyl-dG and exogenous [$^{13}CD_2$]-N^2-hydroxymethyl-dG arising from [$^{13}CD_2$]-formaldehyde were detected. However, we did not observe [$^{13}CD_2$]-N^6-hydroxymethyl-dA from exogenous formaldehyde in any exposed cells. In addition, we have demonstrated that metabolically generated formaldehyde also induces hydroxymethyl-dG as the primary DNA adduct in cells. The results support N^2-hydroxymethyl-dG as a sensitive DNA biomarker to evaluate formaldehyde exposure. We have also confirmed the formation of two types of minor DNA alkylation adducts at N^2-dG and N^6-dA positions in treated cells, which were not previously reported in the literature due to their low abundance.

The utilization of [$^{13}CD_2$]-formaldehyde allowed us to unambiguously differentiate formaldehyde-DNA adducts originating from endogenous and exogenous sources. This study offers a unique approach to investigate potential effects of exogenous formaldehyde

exposure. However, it is well documented that deuterium can exchange with hydrogen, which is especially evident under alkaline conditions. Therefore, the unambiguous identification and accurate quantification of specific DNA adducts may be influenced if H-D exchange occurs in the analyzed samples. Our results show that short-term exposures in cell culture do not lead to significant H-D exchange, which is consistent with our previous results using 6 hour-exposed rats. Therefore, peak assignment and accurate quantitation are reliable for these samples, based on transition scanning in the mass spectrometer. However, when analyzing the exposed samples for a longer period, or studying the DNA adducts induced by formaldehyde from metabolic formation, special caution is needed in interpreting the peaks due to potential H-D exchange, as we have seen in the cell samples after 24 h exposure. In addition, we have concluded that H-D exchange occurred inside of the cells instead of in the culture medium after analyzing [$^{13}CD_2$]-formaldehyde extracted from culture medium after 24 h exposure.

Previous studies show that N^6-hydroxymethyl-dA is a primary formaldehyde-induced DNA monoadduct [21-23]. Surprisingly, our data did not support the formation of detectable amounts of [$^{13}CD_2$]-N^6-hydroxymethyl-dA from exogenous formaldehyde in cell culture. The detection limit of N^6-methyl-dA was more sensitive than that of N^2-methyl-dG, so a lack of sensitivity does not account for our inability to detect exogenous [$^{13}CD_2$]-N^6-hydroxymethyl-dA. Moreover, possible H-D exchange could hinder the identification of this adduct, however, this possibility was also ruled out after calculating the area ratios of various peaks. In addition, endogenous adducts and internal standards were clearly resolved at the expected retention times, supporting the conversion of N^6-hydroxymethyl-dA to N^6-methyl-dA and that recovery of the adduct was not an issue. All current evidence supports that exogenous formaldehyde does not result in detectable amounts of [$^{13}CD_2$]-N^6-hydroxymethyl-dA in either cells exposed to [$^{13}CD_2$]-formaldehyde or rats exposed to 10 ppm inhalation exposures to [$^{13}CD_2$]-formaldehyde for 6 hours or 5 days [30]. In this study, the endogenous amounts of formaldehyde-DNA adducts in Hela cells were determined to be ~3-4 adducts/10^7 nucleosides. It should be noticed that formaldehyde-induced hydroxymethyl DNA adducts were measured as corresponding methyl adducts after reduction, which may underestimate the adduct amounts due to the loss of adducts during sample processing and reduction.

In addition, we have clearly demonstrated the formation of endogenous and exogenous methylation DNA adducts at N^2-dG and N^6-dA positions. The endogenous amount of N^2-methyl-dG and N^6-methyl-dA is about 0.5~0.8 adducts/10^7dG or dA. Alkylating agents induce exogenous N^2-methyl-dG and N^6-methyl-dA in a linear manner, with several-fold higher amounts of N^2-methyl-dG than N^6-methyl-dA. Moreover, these two adducts are minor alkylation products, being ~ one hundred and one thousand times lower than those of O^6-methyl-dG and *N*7-methyl-dG. These observations are consistent with previous conclusion that a violation of the Swain-Scott principle, and not S_N1 versus S_N2 reaction mechanism, governs DNA alkylation spectra [31]. The formation and biological significance of N^2-methyl-dG and N^6-methyl-dA in mammalian cells have not been reported [32]. Likewise, the sources of endogenous N^2-methyl-dG and N^6-methyl-dA adducts are unknown. They could be generated by endogenous DNA alkylating species, or they could result from formaldehyde-induced hydroxymethyl DNA adducts that were reduced by cellular reducing compounds such as ascorbic acid.

We have demonstrated that formaldehyde induces N^2-hydroxymethyl-dG as the primary DNA monoadduct in cells exposed to formaldehyde or other formaldehyde-generating compounds. However, a previous study demonstrated that formaldehyde lead to increased amounts of N^6-hydroxymethyl-dA in multiple tissues from rats exposed to *N*-nitrosodimethylamine (NDMA) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

(NNK) [28;29]. Well characterized pathways support that formaldehyde is released during intracellular metabolism of carcinogenic NDMA and NNK [28]. Thus, increased amounts of N^6-hydroxymethyl-dA could be the consequence of formaldehyde formed via intracellular metabolism of these compounds [28]. However, this is in marked contrast to the current study's findings and to our earlier report [26;30] where there was no detectable [$^{13}CD_2$]-N^6-hydroxymethyl-dA in either formaldehyde-exposed cells or nasal epithelial DNA following inhalation exposure to 10 ppm [$^{13}CD_2$]-formaldehyde. Likewise, exogenous N^2-hydroxymethyl-dG, but not N^6-hydroxymethyl-dA, was found in HepG2 cells exposed to *N*-nitrosodimethyl-D_6-amine. The previous study did not use isotope labeled compound for exposure, which did not allow the differentiation between formaldehyde-induced hydroxymethyl-DNA adducts and alkylation products after $NaCNBH_3$ treatment. Furthermore, N^2-hydroxymethyl-dG was not measured in the previous study [28], but we would predict that it would be increased. Our data do not rule out the possibility of forming exogenous N^6-hydroxymethyl-dA. However, we suggest that exogenous N^6-hydroxymethyl-dA, if formed, can only be induced by formaldehyde generated from metabolic formation. Therefore, the way formaldehyde enters the tissue (from inhalation or intracellular metabolism) may play a critical role in the formation of specific DNA adducts in metabolically active tissues. This is an important issue since the exposure route may determine which formaldehyde-specific DNA biomarker should be used to evaluate the risk of formaldehyde through different exposure routes [33]. Our findings challenge the relevance of dA adducts to current risk assessment of inhaled formaldehyde, other than contributing to the number of endogenous formaldehyde adducts that could form mutations due to enhanced cell proliferation.

We readily detected both endogenous dG and dA hydroxymethyl adducts, but only exogenous dG adducts were observed. What may cause this difference between dG and dA toward exogenous formaldehyde during exposure? Since the *in vitro* reactivity of dG and dA toward formaldehyde is similar, the formation of exogenous DNA adducts should not be different from direct reaction between formaldehyde and the DNA bases, so dA adducts would be expected to occur. We hypothesize that the difference could be a consequence of their different involvement in the formation of DPC or DNA-protein interaction and suggest that exogenous dG adducts are actually formed through DPC intermediates. Our previous study demonstrated that dG actively forms DPC through cross-linking with lysine and cysteine, while dA only cross-linked with cysteine and histidine in much lower amounts [34]. Lysine-dG cross-links are the primary DPC formed, however, they are very labile [34]. The glutathione-dG conjugate induced by formaldehyde that was previously identified by our laboratory [8] is also not stable. Decomposition of these labile DNA-protein cross-links may lead to, or facilitate, the formation of dG monoadducts. However, we suggest that this does not occur with dA since it is much less involved in the formation of DPC. Under our exposure conditions, exogenous formaldehyde may first target neighboring proteins due to the higher reactivity of lysine residues [3], followed by the further condensation with DNA to form DPC. In this study, we have demonstrated that formaldehyde induced fewer DNA adducts in culture medium than in PBS buffer, further highlighting the high reactivity between formaldehyde and proteins. Thus, there is a reduced chance for exogenous formaldehyde to directly react with DNA to form adducts. In contrast, endogenous dA and dG monoaducts may arise from the direct attack of higher concentrations of intracellular formaldehyde, estimated to be present in μM concentrations [35]. Finally, we cannot rule out differences in repair.

In summary, the results of this study clearly demonstrate that endogenous and exogenous formaldehyde-induced hydroxymethyl DNA adducts can be unambiguously differentiated utilizing [$^{13}CD_2$]-formaldehyde and mass spectrometry. Moreover, we have clearly shown that N^2-hydroxymethyl-dG is the primary DNA adduct formed following formaldehyde

exposure, which has important implications on available biomarkers for current risk assessment of inhaled formaldehyde. Hydroxymethyl and methyl DNA adducts cannot be differentiated after reduction, which could cause potentially misleading assignments for sources of these adducts. However, the application of isotope labeled compounds for exposure can successfully solve this problem and provide better mechanistic insights about the formation of these adducts. Taken together, this study clearly shows that integrating highly sensitive mass spectrometry methods and the use of stable isotope labeled compounds for exposure are extremely useful to accurately identify the origins of different DNA adducts when they are formed from endogenous and exogenous sources.

Acknowledgments

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Abbreviations

LC-MS/MS	Liquid chromatography-Mass spectrometry/Mass spectrometry
IARC	International Agency for Research on Cancer
dA	2-deoxyadenosine
dG	2-deoxyguanosine
NDMA	nitrosodimethylamine
MMS	methyl-methanesulfonate
MNU	methylnitrosourea
LC-MS	Liquid chromatography-Mass spectrometry
LC-ESI-MS/MS-SRM	Liquid chromatography-Electrospray ionization-Mass spectrometry/Mass spectrometry-Selected Reaction Monitoring
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
HPLC	High Performance Liquid Chromatography
Q-TOF	Quadrupole Time-of-Flight
nano-UPLC	nano-Ultra Performance Liquid Chromatography

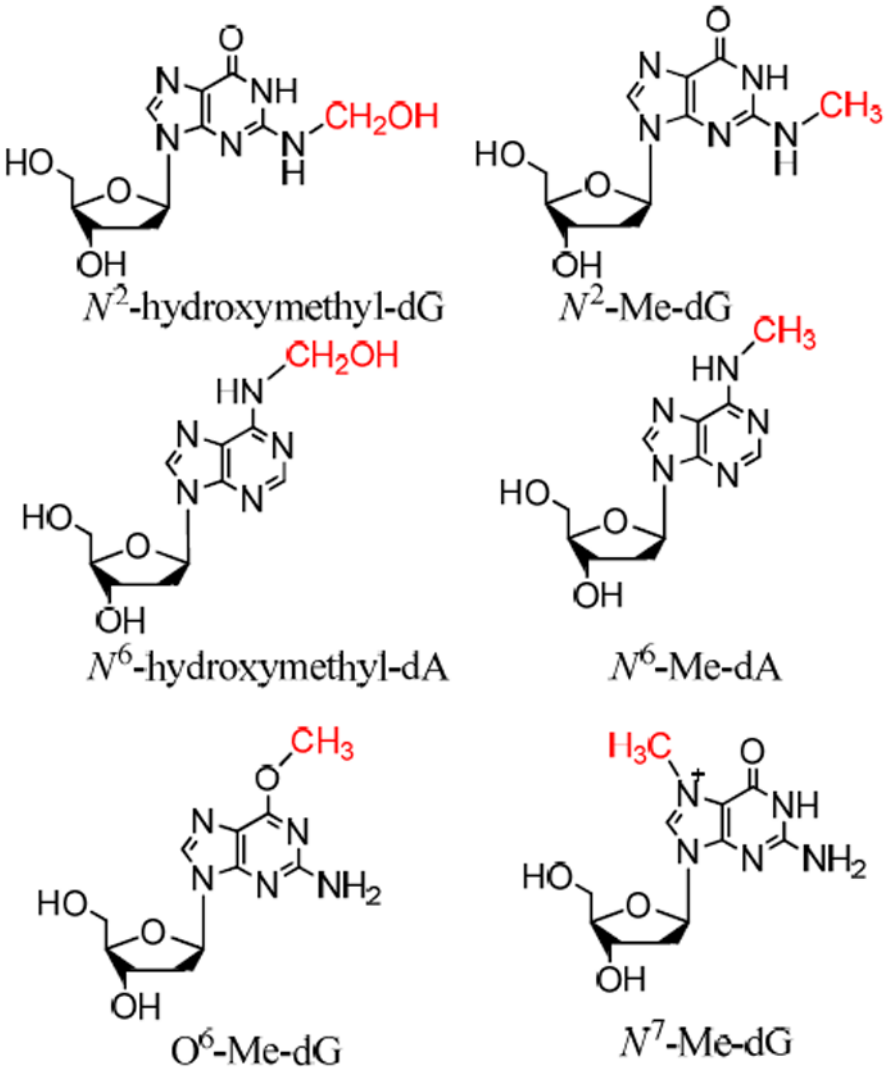

Figure 1.
The structures of hydroxymethyl and methyl DNA adducts measured in this study.

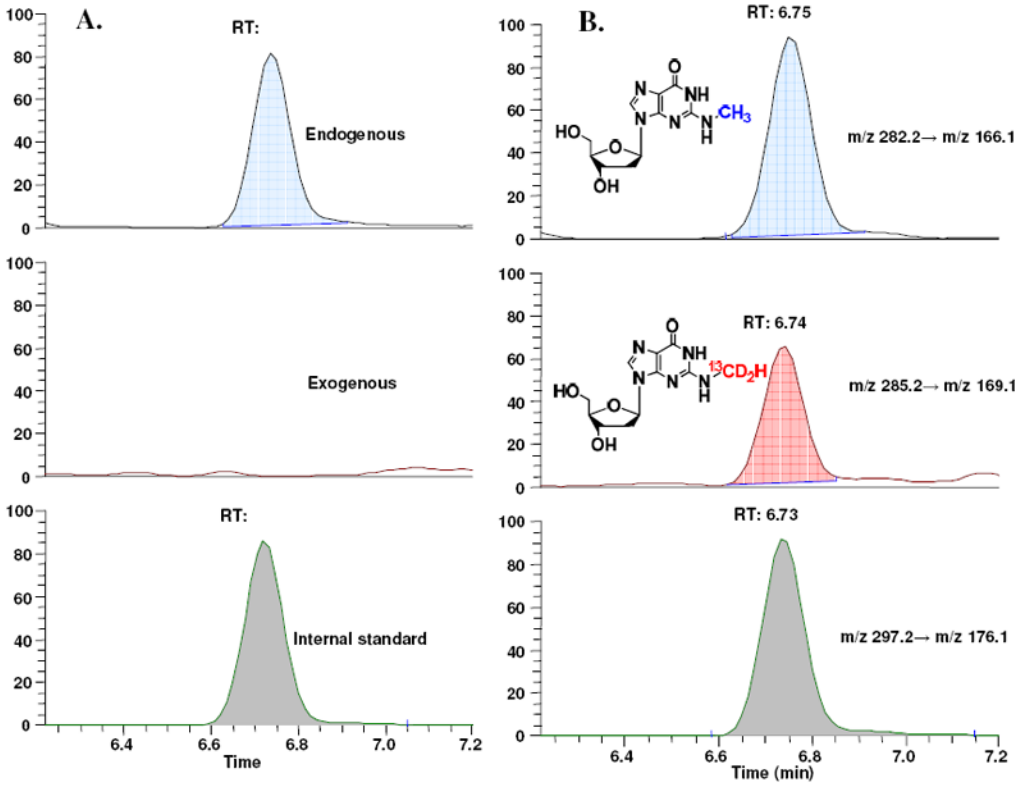

Figure 2.

Capillary-LC-ESI-MS/MS-SRM chromatograms of N^2-Me-dG from control cells (A) and 1 mM [$^{13}CD_2$]-formaldehyde 1 h treated cells (B).

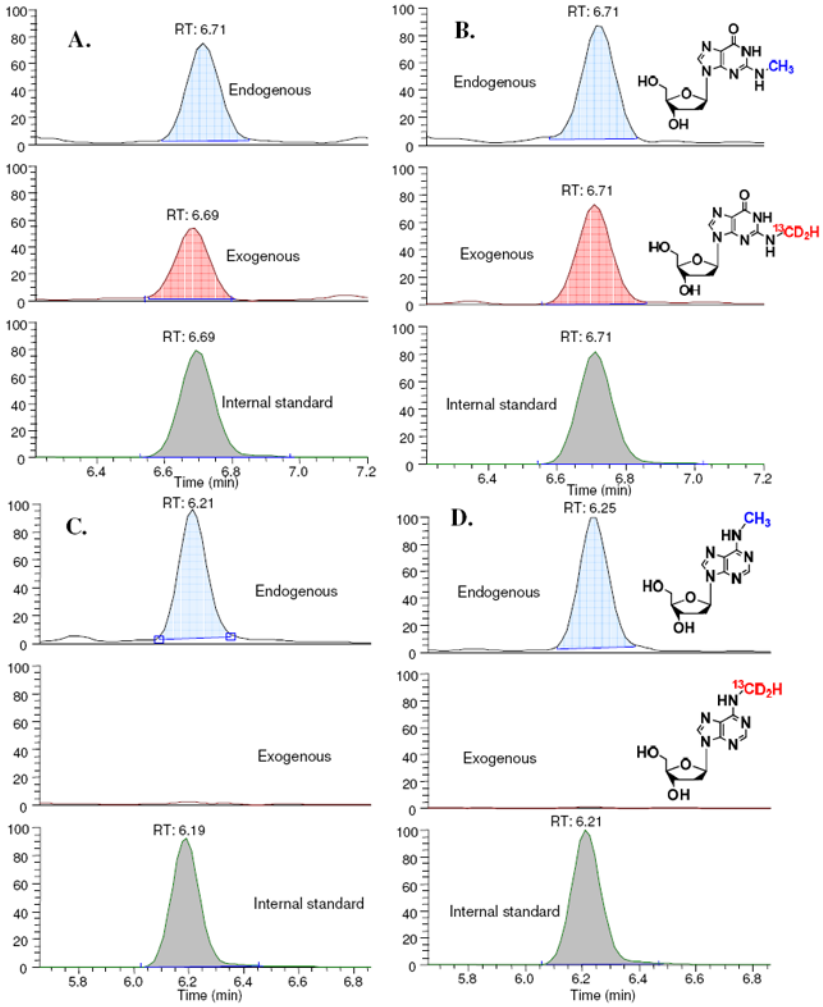

Figure 3.

Capillary-LC-ESI-MS/MS-SRM chromatograms of N^2-Me-dG and N^6-Me-dA from cells: N^2-Me-dG from 0.5 mM [$^{13}CD_2$]-formaldehyde 3 h treated cells (A); N^2-Me-dG from 1mM [$^{13}CD_2$]-formaldehyde 3 h treated cells (B); N^6-Me-dA from 0.5 mM [$^{13}CD_2$]-formaldehyde 3 h treated cells (C); N^6-Me-dA from 1 mM [$^{13}CD_2$]-formaldehyde 3 h treated cells (D).

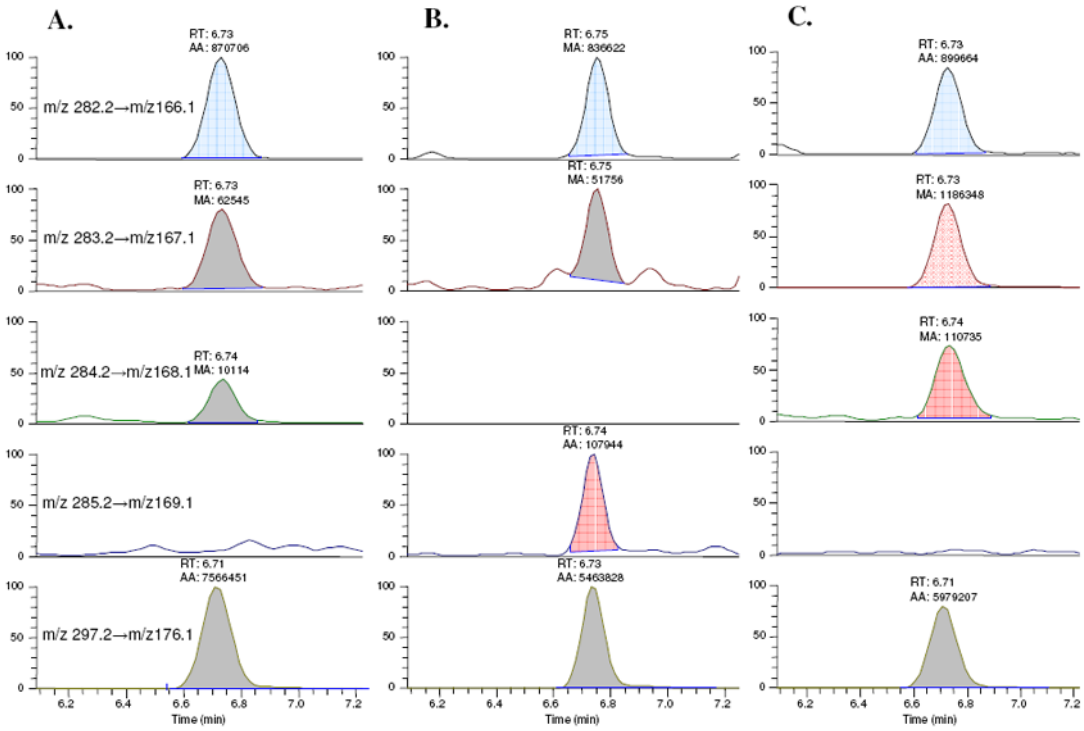

Figure 4.

Capillary-LC-ESI-MS/MS-SRM chromatograms of 15 fmol of N^2-Me-dG analytical standard (A), N^2-Me-dG from 0.5 mM [$^{13}CD_2$]-formaldehyde 3 h treated cells (B) and N^2-Me-dG from 0.5 mM [$^{13}CD_2$]-formaldehyde 24 h treated cells (C).

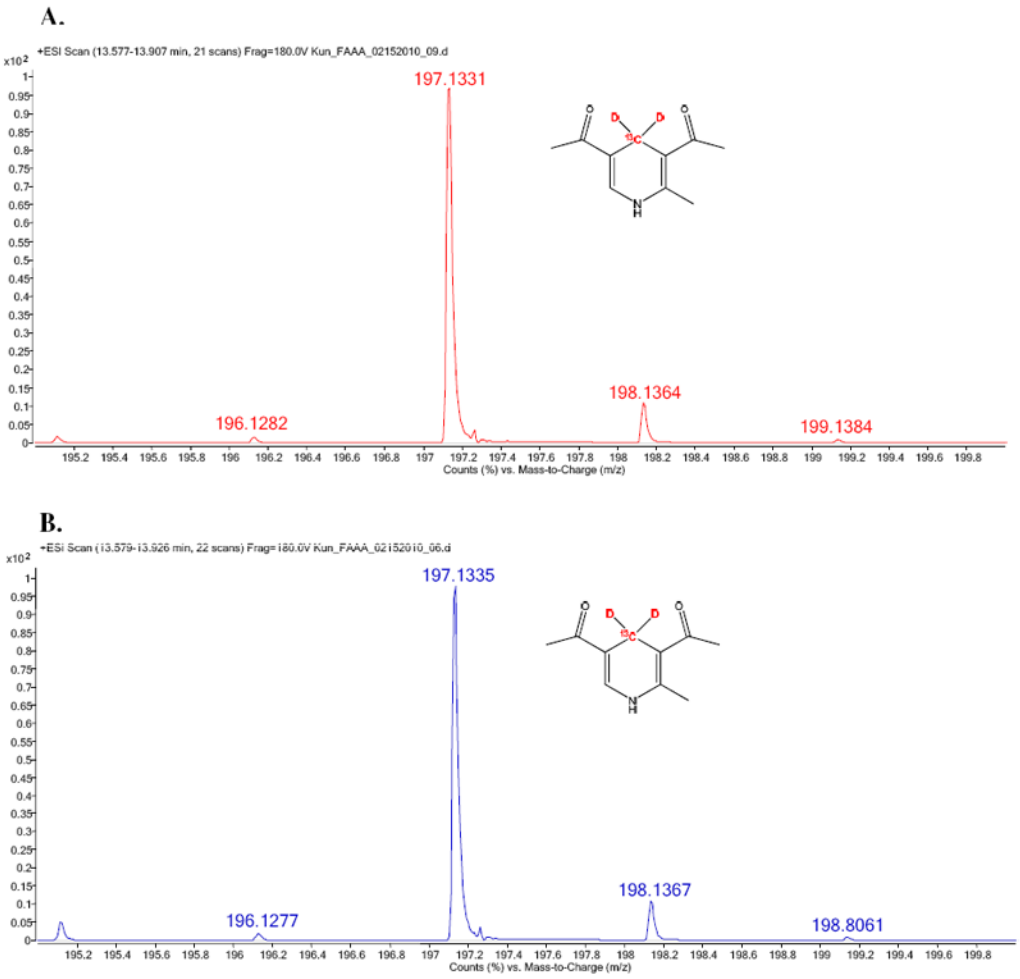

Figure 5.

ESI-Q-TOF mass spectra of formaldehyde-acetylacetone complex prepared from [$^{13}CD_2$]-formaldehyde fresh solution (A) and culture medium after 24 h exposure (B)

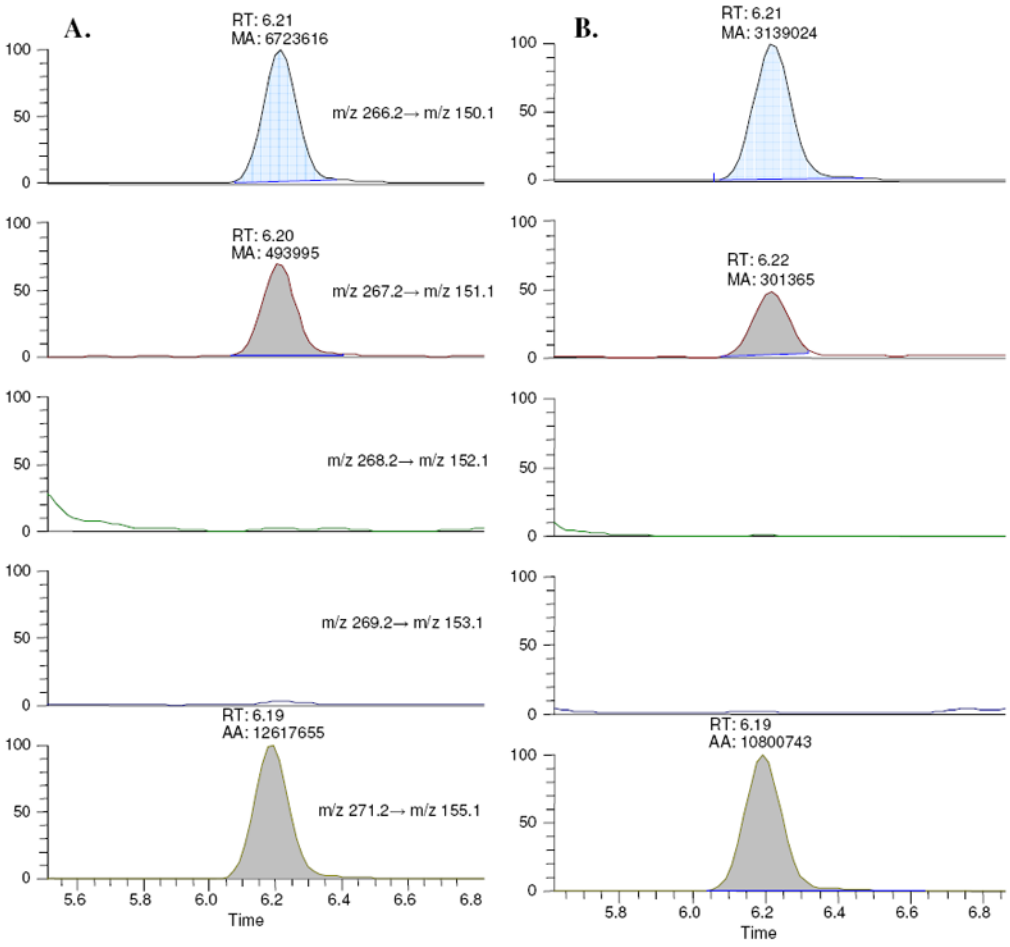

Figure 6.

LC-ESI-MS/MS-SRM chromatograms of N^6-CH_3-dA analytical standard (A) and N^6- Me-dA from 0.5 mM [$^{13}CD_2$]-formaldehyde 24 h treated cells (B).

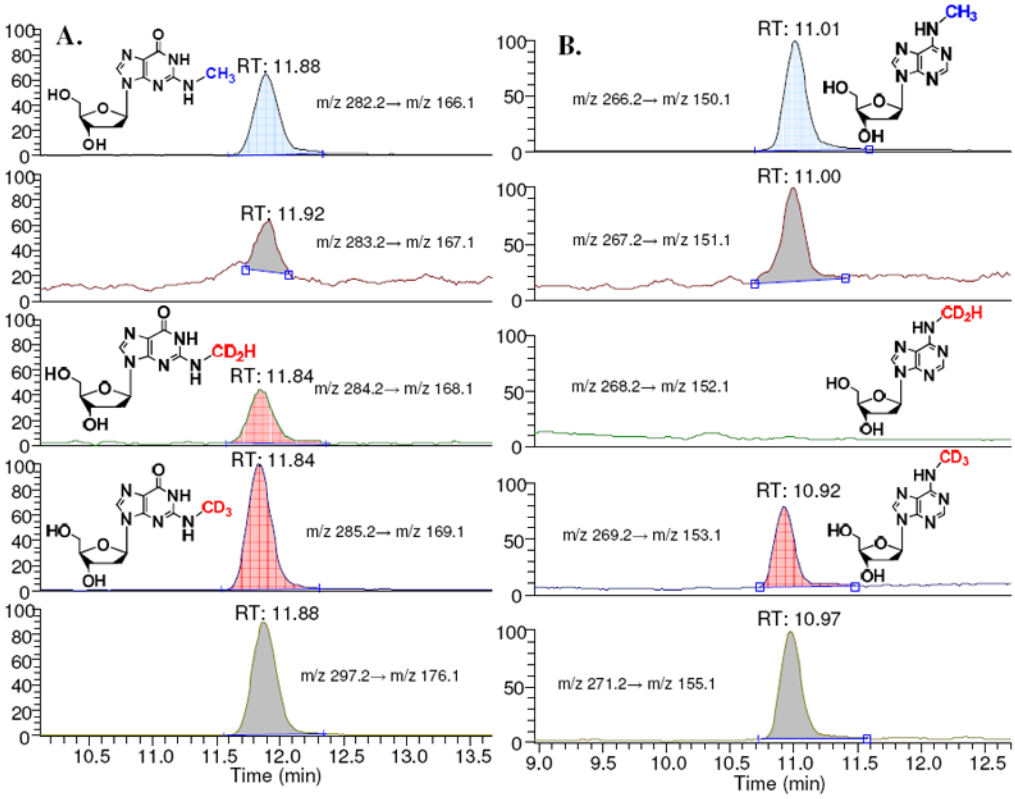

Figure 7.

Capillary-LC-ESI-MS/MS-SRM chromatograms of N^2-Me-dG (A) and N^6-Me-dA (B) from HepG2 cells (2E1+) exposed to 10mM *N*-nitrosodimethyl-D_6-amine for 12 h.

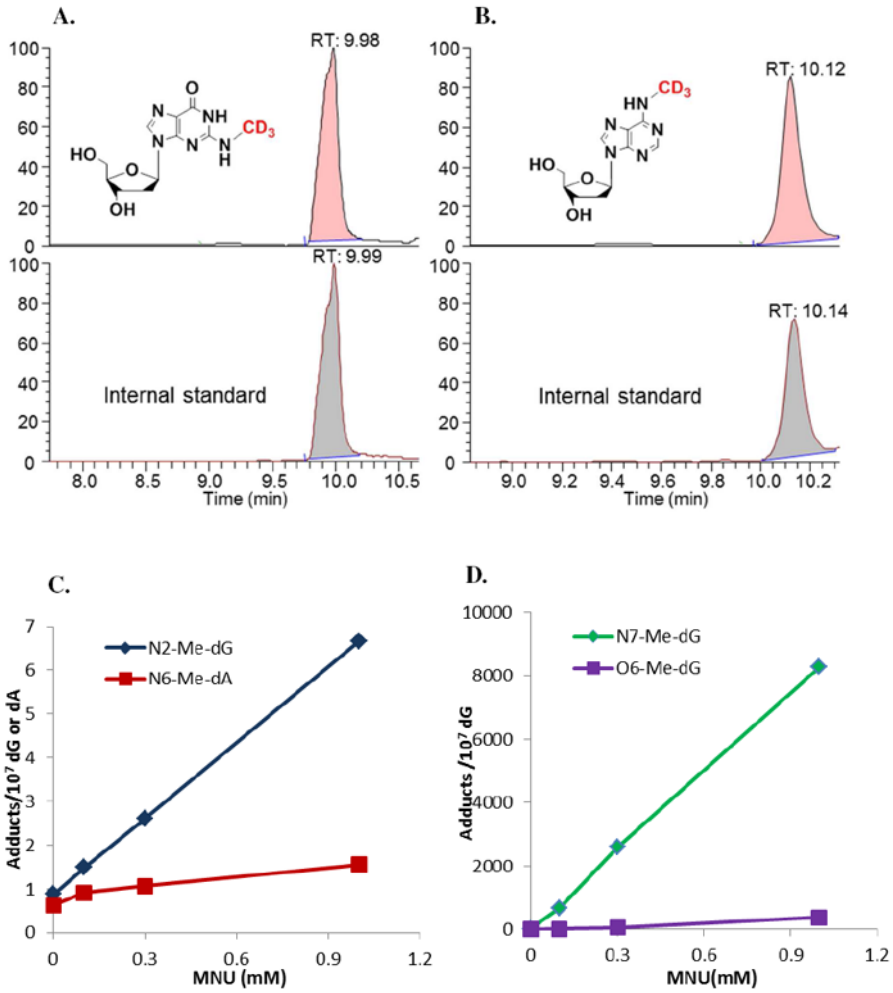

Figure 8.

Nano-LC-ESI-MS/MS-SRM chromatograms of N^2-D_3-Me-dG (A) and N^6-D_3-Me-dA (B) in hepG2 cells treated with 1mM D_3-MMS. The dose responses of DNA adducts in MNU-treated HepG2 cells for 15min; N^2-Me-dG and N^6-Me-dA (C) and $N7$-Me-dG and O^6-Me-dG (D).

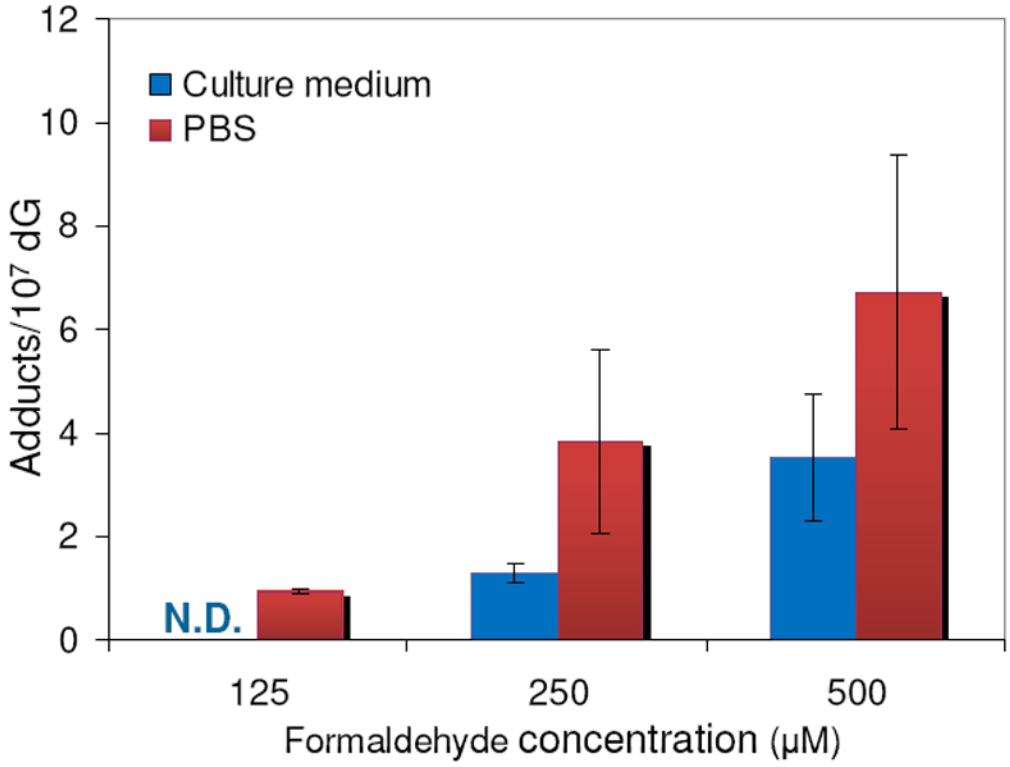

Figure 9.
Exogenous N^2-hydroxymethyl-dG adducts in cells exposed to formaldehyde for 1 hour in either culture medium or PBS (n=3). N.D. stands for Non Detectable.

Table 1

Adduct numbers in HepG2 (+2E1) cells treated by 10mM D_6-NDMA for 6 h or 18 h.

Adducts	Non-Reduced Samples (adducts per 10^7 bases)			Reduced Samples (adducts per 10^7 bases)		
	Control	10mM-6h	10mM-18h	Control	10mM-6h	10mM-18h
N^2-Me-dG	0.3±0.2	0.38±0.03	0.13±0.05	3.3±0.5	3.2±0.49	2.9±0.52
N^2-D_2-Me-dG	N.D.	Detectable	Detectable	N.D.	0.1±0.07	0.57±0.01
N^2-D_3-Me-dG	N.D.	3.5±0.06	18.9±4.5	N.D.	2.0±0.4	14.4±1.1
N^6-Me-dA	0.8±0.4	0.7±0.3	0.9±0.4	3.1±0.13	4.7±1.3	3.5±0.92
N^6-D_2-Me-dA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
N^6-D_3-Me-dA	N.D.	0.2±0.02	1.3±0.04	N.D.	0.23±0.05	1.16±0.03

Limit of detection (LOD) for N^2-methyl-dG and N^6-methyl-dA was ~240 and ~75 amol on the column, respectively.