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# Analysis of a Malondialdehyde−Deoxyguanosine Adduct in Human Leukocyte DNA by Liquid Chromatography Nanoelectrospray−High-Resolution Tandem Mass Spectrometry

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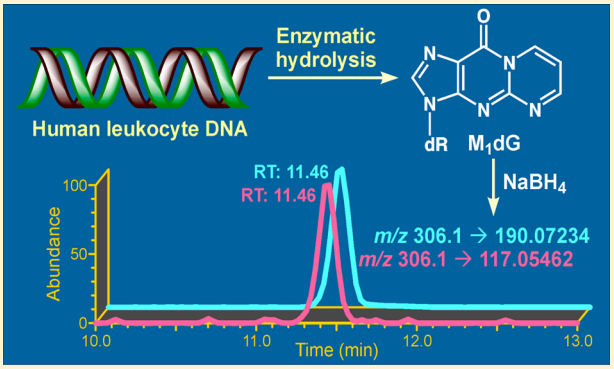
Supporting Information

**ABSTRACT:** Malondialdehyde (MDA), an endogenous genotoxic product formed upon lipid peroxidation and prostaglandin biosynthesis, can react with DNA to form stable adducts. These adducts may contribute to the development of such inflammation-mediated diseases as cancer and cardiovascular and neurodegenerative diseases. The predominant MDA-derived DNA adduct formed under physiological conditions is 3-(2-deoxy-β-D-erythro-pentafuranosyl)pyrimido[1,2-α]purin-10(3*H*)-one deoxyguanosine ($M_1$dG). In this study, we developed a novel liquid chromatography (LC)−nanoelectrospray ionization (NSI)−high-resolution tandem mass spectrometry (HRMS/MS) method for the analysis of $M_1$dG in human leukocyte DNA. After enzymatic hydrolysis of DNA, $M_1$dG and the added internal standard [$^{13}C_3$]$M_1$dG were reduced to their 5,6-dihydro derivatives by addition of sodium borohydride to the hydrolysate and purified by solid-phase extraction and column chromatography. The 5,6-dihydro derivatives in the purified samples were analyzed by LC−NSI−HRMS/MS using higher-energy collisional dissociation (HCD) fragmentation, isolation widths of 1 Da for both the analyte and internal standard, and a resolution of 50 000. The detection limit of the developed method is 5 amol on-column, and the limit of quantitation is 0.125 fmol/mg DNA starting with 200 μg of DNA. Method accuracy and precision were characterized. The developed method was further applied to the analysis of leukocyte DNA from 50 human subjects. $M_1$dG was detected in all samples and ranged from 0.132 to 275 fmol/mg DNA, or 0.004 to 9.15 adducts per $10^8$ bases. This unique and highly sensitive HRMS/MS-based method can be used in future studies investigating the pathophysiological role of $M_1$dG in human diseases.




## INTRODUCTION

Chronic inflammation, a key mechanism in the pathogenesis of cancer and cardiovascular disease,[1−4] induces lipid peroxidation, which in turn generates a spectrum of reactive electrophiles capable of causing extensive damage to DNA and proteins, resulting in toxic and mutagenic events.[5,6] Malondialdehyde is the principal and most studied product of lipid peroxidation (Figure 1).[7] Under physiological conditions, malondialdehyde can react with DNA to form adducts mainly to deoxyguanosine and deoxyadenosine, with the predominant one being 3-(2-deoxy-β-D-erythro-pentafuranosyl)pyrimido-[1,2-α]purin-10(3*H*)-one deoxyguanosine ($M_1$dG, Figure 1).[7] $M_1$dG is a premutagenic lesion and has been shown to induce G → T and G → A mutations in DNA;[8] these mutations are believed to be important steps in carcinogenesis, contributing to the etiology of human cancer.

While $M_1$dG may serve as a valuable biomarker in studies of the role of inflammation in human disease, its sensitivity to changes in inflammation-inducing exposures is not clear. For instance, cigarette smoke contains high levels of pro-oxidants, such as reactive oxygen and nitrogen species,[9] and it is plausible to expect that levels of $M_1$dG would be elevated in DNA from smokers as compared to nonsmokers. Indeed, many studies demonstrated that levels of urinary $F_2$-isoprostane 8-*epi*-$PGF_{2\alpha}$, a reliable urinary biomarker of oxidative stress, are elevated in smokers,[12−15] and chronic inflammation is a major contributing factor in the pathogenesis of cigarette smoke-associated diseases, including lung cancer.[10,11] However, existing reports on $M_1$dG levels in smokers and nonsmokers are inconsistent: some studies demonstrate that levels of $M_1$dG are modestly elevated in smokers,[16−18] whereas other studies show no differences.[19,20] These inconsistencies may be, at least in part, due to differences in the methodologies used by different research groups.

Analytical methods that have been used to detect and quantify $M_1$dG in human biological samples include gas chromatography−mass spectrometry,[19] immunoslot blot,[21] and $^{32}$P-postlabeling technique[22] as well as liquid chromatography−tandem mass spectrometry (LC−MS/MS).[23,24] By

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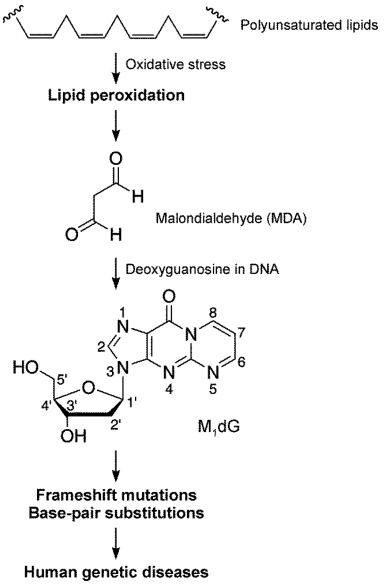



**Figure 1.** Formation of 3-(2-deoxy-β-D-erythro-pentafuranosyl)-pyrimido[1,2-α]purin-10(3*H*)-one deoxyguanosine ($M_1dG$) via lipid peroxidation.

using these methods, $M_1dG$ has been detected in liver, pancreas, breast, leukocytes, and lymphocytes from human subjects, with the levels of this adduct ranging from 1 to 120 adducts/$10^8$ nucleotides, depending on the sample type, subject characteristics or exposures, and applied methodologies for $M_1dG$ analysis.[5,7] One of the potential issues with some methodologies could be overestimation of $M_1dG$ levels, due to either lack of specificity or artifactual formation.[5,7] Thus, to better understand the role of $M_1dG$ in human diseases, there is a need for a robust, specific, and sensitive method that can be applied in large population studies investigating relationships between relevant exposures and health outcomes.

It should be noted that the latest LC−MS/MS-based methods for $M_1dG$ measurement provide high sensitivity and selectivity; however, the published assays involve the use of custom-made immunohistochemistry columns to purify samples prior to analysis.[23,24] Therefore, robust transfer of this methodology to other laboratories is not practical. Our goal in this study was to develop a new robust and sensitive mass spectrometry-based method for the analysis of $M_1dG$ in human leukocyte DNA, with the initial primary focus on modifying the sample preparation procedure to exclude the immunohistochemistry-based purification step.

## EXPERIMENTAL PROCEDURES

**Chemicals and Enzymes.** $M_1dG$ and [$^{13}C_3$]$M_1dG$ were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Reagents and enzymes for DNA isolation were obtained from Qiagen Sciences (Germantown, MD). Calf thymus DNA was purchased from Worthington Biochemical Corporation (Lakewood, NJ). All other chemicals and solvents were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI).

**Synthesis of 5,6-Dihydro-$M_1dG$ and 5,6-Dihydro-[$^{13}C_3$]$M_1dG$.** The synthesis of 5,6-dihydro-$M_1dG$ was performed by reacting $M_1dG$ with $NaBH_4$, employing a previously described protocol.[23] Briefly, $M_1dG$ (5 mg) was dissolved in 2 mL of a MeOH/$H_2O$ (2:8, v/v) solution, 1.3 mg of $NaBH_4$ was added, and the mixture was stirred for 30 min at room temperature. The final product was isolated and purified on a Phenomenex Bondclone $C_{18}$ column (3.9 × 300 mm, 10 μm). $^1$H NMR ($d^6$-DMSO, 500 MHz) of 5,6-dihydro-$M_1dG$ was consistent with that previously reported:[16] δ 7.93 (s, 1H, $H_2$), 7.29 (dt, *J* = 8.4, 1.9 Hz, 1H, $H_8$), 6.08 (dd, *J* = 7.8, 6.1 Hz, 1H, $H_1'$), 5.48 (m, 1H, $H_7$), 4.34 (m, 2H, $H_6$), 4.05 (br s, 1H, $H_3'$), 3.81 (m, 1H, $H_4'$), 3.50 (m, 2H, $H_5'$), 2.48−2.17 (m, 2H, $H_2'$). ESI−MS: $MH^+$, *m*/*z* 306. A similar procedure was employed to synthesize 5,6-dihydro-[$^{13}C_3$]$M_1dG$ from [$^{13}C_3$]$M_1dG$, and $^1$H NMR similar to that of 5,6-dihydro-$M_1dG$ was obtained for the product; $MH^+$ *m*/*z* 309. The yields for 5,6-dihydro-$M_1dG$ and 5,6-dihydro-[$^{13}C_3$]$M_1dG$ were 65% and 55%, respectively.

**Subjects and Blood Collection.** Blood samples from 25 smokers and 25 nonsmokers were obtained from the "Methodology and Development of Tobacco Related Biomarkers" biorepository at the Masonic Cancer Center and Tobacco Research Programs. Collection of these samples was approved by the University of Minnesota Human Research Protection Programs Institutional Review Board (IRB Study no. 0908M70881). All subjects were at least 18 years old, not pregnant or breastfeeding, and were in good physical and mental health. Additional criteria for smokers included smoking at least 10 cigarettes per day (CPD), having been a smoker for at least 5 years with no change greater than 50% in CPD or brand in the last year, and not using any other tobacco products in the last 6 months. Nonsmokers were required to have smoked less than 100 cigarettes in their lifetime and were not using any tobacco products regularly. Smoking status was confirmed by expired carbon monoxide (CO) levels.

**DNA Isolation from Human Leukocytes.** DNA isolation from human blood samples was performed using the commercial protocol for DNA purification from buffy coat (Qiagen, Valencia, CA) with several modifications. Briefly, 3 mL of red blood cell lysis solution was added to 1 mL of buffy coat prepared from 10 mL of blood. The leukocyte pellet was collected by centrifugation (3000 × *g*, 10 min) and mixed with 5 mL of cell lysis solution. Proteinase K (2 μL of 20 mg/mL solution) was added, and the mixture was incubated at room temperature overnight with gentle shaking. The following day, 50 μL of RNase A solution (4 mg/mL) was added, and the sample was incubated at room temperature for 2 h. Protein precipitation solution (1.5 mL) was added to the cell lysate, and the sample was vortex-mixed for 30 s and centrifuged (3000 × *g*, 10 min) to remove proteins. DNA was precipitated from the supernatant by the addition of 5 mL of isopropanol. The DNA pellet was washed with 2 mL of 70% ethanol in $H_2O$ and then 2 mL of 100% ethanol. DNA was dried under a stream of nitrogen and stored at −20 °C until use. Potential RNA contamination was assessed by HPLC analysis of enzymatically hydrolyzed DNA samples for uridine. No uridine was detected. In a previously published study, addition of antioxidants during DNA isolation did not affect the measured $M_1dG$ levels, suggesting that artifactual formation of $M_1dG$ is minimal during this step.[19]

**DNA Hydrolysis and Adduct Enrichment.** The DNA samples were dissolved in 1 mL of 25 μM Tris-HCl (pH 7.4) buffer containing 5 μM $CaCl_2$ and 5 μM $MgCl_2$. The resulting solution was mixed with 25 fmol [$^{13}C_3$]$M_1dG$ (internal standard), followed by the addition of micrococcal nuclease (1 unit), phosphodiesterase I (0.003 units), and alkaline phosphatase (0.25 units). The mixed solution was then incubated overnight at 37 °C. The next day, 25 μL of hydrolysate was taken for the analysis of deoxyguanosine by HPLC,[25] which was used to calculate the amount of DNA as described previously.[26] The remaining volume of hydrolysate was incubated with 50 μL of $NaBH_4$ (2 mg/mL) at room temperature for 30 min to reduce $M_1dG$ and [$^{13}C_3$]$M_1dG$ to their corresponding 5,6-dihydro derivatives. After the incubation, samples were loaded on Bond Elut PBA cartridges (100 mg, Agilent Technologies, Lake Forest, CA) activated with 1 mL of MeOH and 1 mL of $H_2O$. The cartridges were washed with 1 mL of $H_2O$ and 1 mL of 3% MeOH sequentially and finally eluted with 2 mL of 25% MeOH. The 25% MeOH fraction containing analytes was collected and concentrated to dryness in a centrifugal evaporator. The residue was redissolved in 20 μL of deionized $H_2O$ and subjected to column purification on an Agilent 1100 HPLC system equipped with a Zorbax SB $C_{18}$ column (5 μm, 150 × 0.5 mm, Agilent Technologies, Wilmington, DE). The mobile phase consisted of 15 mM $NH_4OAc$ and $CH_3CN$, with a gradient from 4 to 27% $CH_3CN$ within 15 min, increased to 33% $CH_3CN$ over 2 min, then returned to 4% $CH_3CN$ in

1 min and held for 15 min at this composition, at a flow rate of 12 μL/min. The detection wavelength was set at 254 nm, and the column temperature was maintained at 25 °C. Benzamide (2 μg/mL), which has similar retention time (~15.5 min) to that of 5,6-dihydro $M_1$dG under the described HPLC conditions, was used as a UV marker. The fraction eluting at 15−17 min was collected (Supporting Information, Figure S1), evaporated to dryness, and redissolved in $H_2O$ prior to analysis by LC−NSI−HRMS/MS.

To assess possible artifactual $M_1$dG formation during the sample preparation, 5 fmol of $^{15}N_5$-deoxyguanosine was added to calf thymus DNA, and the sample was enzymatically hydrolyzed and purified as described above. No 5,6-dihydro-[$^{15}N_5$]$M_1$dG was detected (Supporting Information, Figure S2).

**LC−Electrospray Ionization (ESI)−(MS/MS).** The LC−ESI−MS/MS analysis was carried out on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, Waltham, MA) interfaced with an Agilent 1100 capillary HPLC system (Agilent, Palo Alto, CA). Analysis was performed on an Agilent Zorbax SB $C_{18}$ column at a flow rate of 12 μL/min with the temperature maintained at 30 °C. Sample injection volume was 8 μL. The mobile phase consisted of 15 mM $NH_4OAc$ and $CH_3CN$ with a linear gradient from 4 to 35% $CH_3CN$ over a period of 18 min and then returned to 4% $CH_3CN$ followed by 15 min re-equilibration. The ESI source was operated in positive ion mode, monitoring $m/z$ 306.2 $[M + H]^+ \rightarrow$ 190.2 $[C_8H_8N_5O]^+$ for 5,6-dihydro-$M_1$dG and corresponding ions at $m/z$ 309.2 → 193.2 for 5,6-dihydro-[$^{13}C_3$]$M_1$dG. The collision gas was Ar at 1 mTorr with collision energy of 12 eV. The quadrupoles were operated at a resolution of 0.2 (Q1) and 0.7 (Q3) Da.

**LC−Nanoelectrospray Ionization (NSI)−High-Resolution (HR) MS/MS.** The LC−NSI−HRMS/MS was performed on an LTQ Orbitrap Velos instrument (Thermo Scientific, Waltham, MA) interfaced with a Nano2D−LC HPLC (Eksigent, Dublin, CA) system using nanoelectrospray ionization. The analysis was performed using a capillary column (75 μm i.d., 10 cm length, 15 μm orifice) created by hand packing a commercially available fused-silica emitter (New Objective, Woburn, MA) with Luna $C_{18}$ bonded separation media (Phenomenex, Torrance, CA). The mobile phase consisted of 5 mM $NH_4OAc$ and $CH_3CN$. A 5 μL injection loop was used, and the sample (3 μL) was loaded onto the capillary column with a 1000 nL/min flow under the initial conditions for 5.5 min. Separation on the capillary column was performed using a linear gradient at a flow rate of 300 nL/min with increasing $CH_3CN$ from 2 to 33% over 12 min, followed by ramping to 98% $CH_3CN$ within 1 min and holding at this composition for additional 2 min. The gradient was then returned to 2% $CH_3CN$ (initial condition) in 1 min, and the system was re-equilibrated at this mobile phase composition for 6 min at 1000 nL/min before next injection. The nanoelectrospray source voltage was set at 1.6 kV. The capillary temperature was 350 °C, and the S-Lens RF Level was set at 40%. The analysis was performed using accurate mass extracted ion chromatograms of $m/z$ 190.0723 $[C_8H_8N_5O]^+$ and 117.0546 $[C_5H_9O_3]^+$ (parent ion $m/z$ 306.1) for 5,6-dihydro-$M_1$dG and corresponding fragments ($m/z$ 193.0824 and 117.0546) for 5,6-dihydro-[$^{13}C_3$]$M_1$dG with a mass tolerance of 2 ppm. The scan events were performed using higher-energy collisional dissociation (HCD) fragmentation with a normalized collision energy of 20 units, isolation widths of 1 Da for both the analyte and internal standard, and product ion spectra acquisition at a resolution of 50 000. The quantitation of $M_1$dG was based on the peak area ratio of 5,6-dihydro-$M_1$dG ($m/z$ 306.1 → 190.0723) to 5,6-dihydro-[$^{13}C_3$]$M_1$dG ($m/z$ 309.1 → 193.0824), the constructed calibration curves, and the amount of internal standard added.

A calibration curve was constructed before each analysis using a series of standard solutions of 5,6-dihydro-$M_1$dG and 5,6-dihydro-[$^{13}C_3$]$M_1$dG. The calibration standard solutions contained a constant amount of 5,6-dihydro-[$^{13}C_3$]$M_1$dG (2.5 fmol on column) and varying amounts of 5,6-dihydro-$M_1$dG (0.005, 0.025, 0.1, 0.5, 2.5, and 5 fmol on column).

**Method Characterization and Sample Analysis.** Accuracy was determined by adding different amounts of $M_1$dG (0.025, 0.1, 0.5, 2.5 fmol) and 2.5 fmol of internal standard to 200 μg of calf thymus DNA in 1 mL of 25 μM Tris-HCl (pH 7.4) buffer containing 5 μM $CaCl_2$ and 5 μM $MgCl_2$, followed by hydrolysis and purification as described above. Samples at each level of added $M_1$dG were analyzed in triplicate.[27] To characterize method precision, 0.5 fmol of $M_1$dG and 2.5 fmol of internal standard were added to 200 μg of calf thymus DNA, followed by the described hydrolysis and purification protocol. The precision was determined as intraday and interday coefficients of variation (% CV), which were calculated based on the analyses of three aliquots of the samples on three separate days. Trace levels of $M_1$dG were present in calf thymus DNA; these levels were quantified and subtracted from the levels of $M_1$dG measured in the samples during the method characterization.

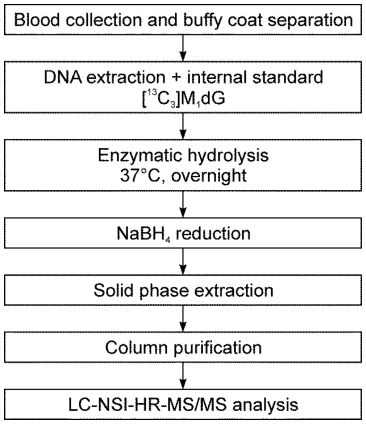
The limit of detection (LOD) was determined using standard solutions of 5,6-dihydro-$M_1$dG. The limit of quantitation (LOQ) was established in calf thymus DNA samples by adding $M_1$dG (0.005, 0.01, 0.025, and 0.05 fmol) and internal standard (2.5 fmol) to calf thymus DNA samples, followed by hydrolysis and purification, and analyzing each sample in triplicate. The LOQ was defined by identification of the lowest $M_1$dG level that produced a coefficient of variation (CV) lower than 5%.[28]

Recovery was determined by comparing the results of samples to which [$^{13}C_3$]$M_1$dG (2.5 fmol) was added to 200 μg of calf thymus DNA at the beginning and at the end of sample preparation procedure.[29] All data are presented as mean ± standard deviation (SD). Two-tailed unpaired Student's $t$-test was used for two group comparison. A $p$ value less than 0.05 was considered significant.

## RESULTS

**Development of the Analytical Procedure.** The purpose of this study was to develop a robust and sensitive analytical procedure for the analysis of $M_1$dG by LC−MS/MS. The developed protocol is outlined in Scheme 1. The addition of $NaBH_4$ after enzymatic hydrolysis was used to increase the detection sensitivity by reducing $M_1$dG to 5,6-dihydro-$M_1$dG.[23] In agreement with the previous report,[23] the detection sensitivity of 5,6-dihydro-$M_1$dG increased 12-fold as compared to that of $M_1$dG. Because it has been previously reported that $M_1$dG can form a conjugate with Tris buffer,[30] we tested the stability of $M_1$dG upon overnight incubation in Tris-HCl and in other two buffers commonly used in DNA hydrolysis: MOPS and sodium succinate buffer. The subsequent reduction with $NaBH_4$ and quantification of the formed 5,6-dihydro-$M_1$dG showed no difference among the three tested buffers, suggesting no effect of Tris-HCl on the sensitivity of our assay. This could be due to the reported instability of Tris−$M_1$dG conjugate in aqueous solutions at room temperature[30] or

**Scheme 1. Analytical Scheme for the Analysis of $M_1$dG in Human Leukocyte DNA**





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due to deconjugation of this product during the $NaBH_4$ reduction step.

We initially used low-resolution triple quadrupole mass spectrometry (LC−ESI−MS/MS), monitoring $m/z$ 306.2 → 190.2 for 5,6-dihydro-$M_1$dG and $m/z$ 309.2 → 193.2 for 5,6-dihydro-[$^{13}C_3$]$M_1$dG. By using this method, an LOQ of 3.3 fmol (on-column) was achieved in calf thymus DNA (data not shown). However, when the method was applied to human leukocyte DNA, high noise levels and co-eluting peaks were observed for both 5,6-dihydro-$M_1$dG and its internal standard, with no obvious peak being detected at the retention time of 5,6-dihydro-$M_1$dG (Figure 2A). Analysis of the same human

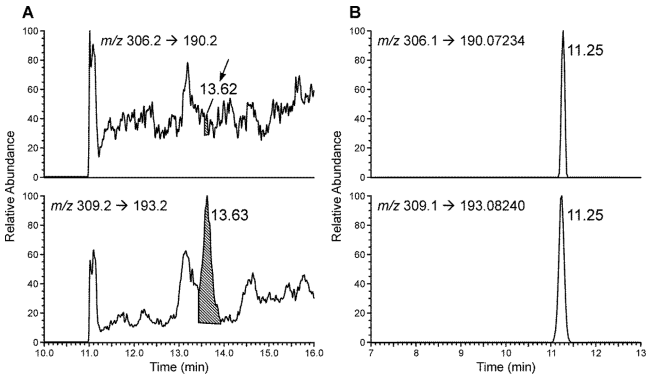


**Figure 2.** Chromatograms obtained upon analysis of $M_1$dG in the same human leukocyte DNA sample by using (A) LC−ESI−MS/MS and (B) LC−NSI−HRMS/MS.

leukocyte DNA sample using the high resolving and accurate mass capabilities of the Orbitrap detector produced clear peaks for both 5,6-dihydro-$M_1$dG and its internal standard without any baseline noise or coeluting peaks (Figure 2B). The product scans of the analytes using HCD fragmentation generated two major fragments for 5,6-dihydro-$M_1$dG at $m/z$ 190.0723 and $m/z$ 117.0546 and two major fragments for 5,6-dihydro-[$^{13}C_3$]$M_1$dG at $m/z$ 193.0824 and $m/z$ 117.0546 (Supporting Information, Figure S3). Because of the higher signal intensities, the transitions $m/z$ 306.1 → 190.0723 and $m/z$ 309.1 → 193.0824 were selected for quantitation of 5,6-dihydro-$M_1$dG and 5,6-dihydro-[$^{13}C_3$]$M_1$dG, respectively; the peak area ratios between the two major fragments of 5,6-dihydro-$M_1$dG were used to confirm its identity, and the corresponding ratio was used to confirm the identity of the internal standard (Supporting Information, Figure S3).

**Method Characteristics.** The calf thymus DNA that was used for the method development contained 13 fmol of $M_1$dG/mg DNA. This value was subtracted during the analysis of data obtained for the samples used for method characterization. By using the developed LC−NSI−HRMS/MS method, an LOD of 5 amol (on-column) was obtained. The instrument response and the 5,6-dihydro-$M_1$dG/5,6-dihydro-[$^{13}C_3$]$M_1$dG ratio were linear in the 0.005−5 fmol (on-column) range of $M_1$dG ($R^2$ = 0.9997, Figure 3A). The LOQ was 25 amol on-column based on a CV of 3.21%. The accuracy of measured levels of 5,6-dihydro-$M_1$dG (expressed as % of added $M_1$dG) at 0.025, 0.1, 0.5, and 2.5 fmol was 99.6, 103, 100, and 100%, respectively, exhibiting excellent linearity ($R^2$ = 0.9981, Figure 3B). The interday CV was 6.0%. The recovery of the assay was 41.3 ± 3.47% ($n$ = 5).

**Quantitation of $M_1$dG Adducts in Human Leucocyte DNA.** The method was applied to the analysis of leukocyte

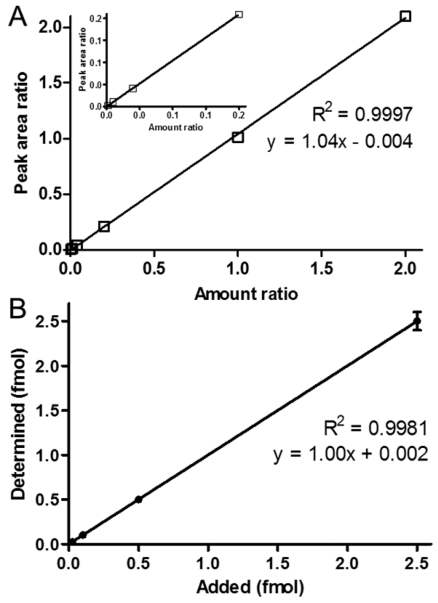


**Figure 3.** Method characteristics. (A) Linearity of 5,6-dihydro-$M_1$dG/5,6-dihydro-[$^{13}C_3$]$M_1$dG peak area ratio at constant level of 5,6-dihydro-[$^{13}C_3$]$M_1$dG (2.5 fmol on-column) and varying levels of 5,6-dihydro-$M_1$dG (from 5 to 5000 amol on-column). (B) Relationship between added $M_1$dG and measured 5,6-dihydro-$M_1$dG in calf thymus DNA ($R^2$ = 0.998) in the range from 0.025 to 2.5 fmol of $M_1$dG per 200 $\mu$g of DNA; $M_1$dG originally present in the calf thymus DNA was determined and subtracted from each value.

DNA samples from 50 human subjects. To investigate the potential effect of cigarette smoking on levels of $M_1$dG in human leukocyte DNA, samples from 25 smokers and 25 nonsmokers were selected for this analysis. Typical chromatograms upon analysis of $M_1$dG in human leukocytes from a smoker and a nonsmoker are presented in Figure 4. The results of $M_1$dG levels from the 50 subjects are summarized in Table 1. The yield of DNA in this study averaged 136 ± 55 $\mu$g, which was sufficient to detect and quantify the $M_1$dG adduct in all samples. The levels of $M_1$dG in the analyzed samples ranged from 0.004 to 9.15 adducts/$10^8$ nucleotides, averaging (±SD) 2.02 ± 2.17 adducts/$10^8$ nucleotides. Levels of $M_1$dG in leukocyte DNA from smokers averaged 64.9 ± 71.9 fmol/mg DNA and in nonsmokers, 56.5 ± 58.8 fmol/mg DNA, or 2.16 ± 2.40 and 1.89 ± 1.96 adducts/$10^8$ nucleotides, respectively. Although the average $M_1$dG level in smokers was slightly higher than that in nonsmokers, this difference was not significant.

## ■ DISCUSSION

Measurement of lipid peroxidation-induced DNA damage in populations with varying environmental and occupational exposures and lifestyle and dietary habits could greatly advance our understanding of the role of these factors in the induction of chronic inflammation and the associated diseases. In this study, we developed a novel LC−NSI−HRMS/MS method for the analysis of $M_1$dG, the major DNA adduct derived from the lipid peroxidation product malondialdehyde, in human leukocyte DNA. This robust and sensitive method was successfully applied to the analysis of leukocyte DNA from 25 smokers and 25 nonsmokers. Studies are underway to investigate how the levels of this highly mutagenic adduct in humans are related to various exposures and disease risk.

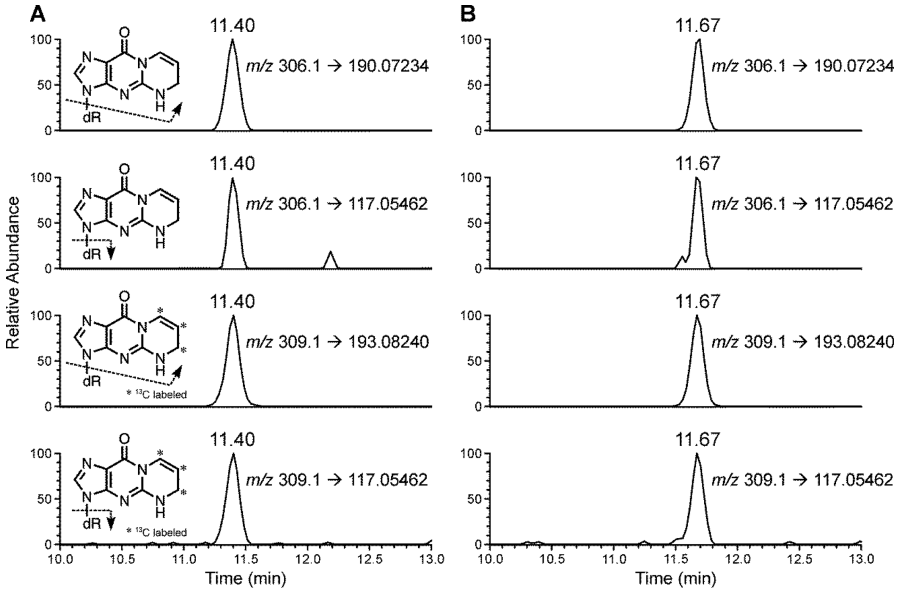


**Figure 4.** Typical LC–NSI–HRMS/MS chromatograms obtained upon analysis of 5,6-dihydro-$M_1$dG in human leukocyte DNA from a (A) smoker and (B) nonsmoker.

**Table 1. Levels of $M_1$dG in Smokers' and Nonsmokers' Leukocyte DNA**

| smokers | | | nonsmokers | | |
|---|---|---|---|---|---|
| subject no. | DNA yield ($\mu$g) | $M_1$dG (fmol/mg DNA) | subject no. | DNA yield ($\mu$g) | $M_1$dG (fmol/mg DNA) |
| 1 | 128 | 2.14 | 1 | 61.1 | 72.1 |
| 2 | 196 | 6.06 | 2 | 49.4 | 47.9 |
| 3 | 90.7 | 18.5 | 3 | 79.4 | 37.0 |
| 4 | 200 | 14.0 | 4 | 46.8 | 75.8 |
| 5 | 164 | 14.8 | 5 | 54.6 | 95.3 |
| 6 | 99.1 | 106 | 6 | 94.9 | 132 |
| 7 | 69.4 | 275 | 7 | 67.3 | 79.9 |
| 8 | 90.2 | 79.4 | 8 | 125 | 135 |
| 9 | 149 | 125 | 9 | 87.8 | 134 |
| 10 | 71.3 | 139 | 10 | 124 | 30.3 |
| 11 | 89.4 | 220 | 11 | 168 | 93.0 |
| 12 | 132 | 57.3 | 12 | 194 | 0.714 |
| 13 | 139 | 48.7 | 13 | 209 | 2.22 |
| 14 | 165 | 102 | 14 | 162 | 55.9 |
| 15 | 122 | 120 | 15 | 236 | 27.9 |
| 16 | 160 | 5.49 | 16 | 244 | 2.82 |
| 17 | 165 | 13.0 | 17 | 69.8 | 9.83 |
| 18 | 93.1 | 0.132 | 18 | 113 | 17.7 |
| 19 | 183 | 16.0 | 19 | 199 | 7.89 |
| 20 | 223 | 1.14 | 20 | 166 | 251 |
| 21 | 213 | 3.19 | 21 | 155 | 28.0 |
| 22 | 39.2 | 73.3 | 22 | 140 | 12.5 |
| 23 | 170 | 43.1 | 23 | 182 | 17.3 |
| 24 | 204 | 117 | 24 | 158 | 21.4 |
| 25 | 166 | 22.9 | 25 | 78.7 | 26.6 |
| mean ± SD | 141 ± 50.2 | 64.9 ± 71.9 | mean ± SD | 130 ± 60.3 | 56.5 ± 58.8 |

In the process of method development, we initially explored the use of low-resolution triple quadrupole mass spectrometry and were able to quantify $M_1$dG in calf thymus DNA at levels as low as 3.3 fmol, which was comparable to the previously developed LC–MS method.[24] However, application of the method to human leukocyte DNA produced high background noise and co-eluting peaks, leading to inaccurate quantitation of the adduct levels in these samples. As an alternative, we employed an accurate mass high-resolution Orbitrap mass spectrometer, which proved to be highly sensitive and selective in a previous study on another DNA adduct, 7-ethylguanine.[29]

After the transition to HRMS/MS, both capillary-ESI at a flow rate of 10 $\mu$L/min and nano-ESI at a flow rate of 300 nL/min were investigated, with nano-ESI showing at least 50-fold


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increase in sensitivity compared to that of capillary-ESI. Moreover, nano-ESI has been shown to be more tolerant of salt contamination than conventional ESI.[31] Therefore, nano-ESI was finally adopted. However, it has been reported that substantial ion suppression can occur at flow rates as low as 50 nL/min.[32] In our developed method, a flow rate of 300 nL/min is used and therefore the analysis could be susceptible to signal suppression if sufficient purification of the sample is not performed. Indeed, analysis of $M_1$dG in samples that underwent only one purification step, extraction on Bond Elut PBA cartridges, showed a 4-fold signal decrease due to ion suppression effect (data not shown) compared to samples that were also subjected to a column purification step (Scheme 1). We also investigated mass tolerance, which reflects measurement and calibration errors of the Orbitrap instrument. Changing the mass tolerance setting for ion extraction from 10 to 5 to 2 ppm did not alter the peak area of the analyte (Supporting Information, Figure S4). Consequently, the mass tolerance was set at 2 ppm to achieve better selectivity and accuracy. Overall, the transition to high-resolution MS/MS improved the sensitivity of the method by approximately 130-fold compared with the originally used low-resolution MS/MS. Moreover, the baseline noise and occurrence of co-eluting peaks were completely eliminated, greatly improving selectivity of the method (Figures 2 and 3). The average $M_1$dG level determined in our study was 2.02 ± 2.17 adducts/$10^8$ nucleotides, which is comparable to the previously reported levels.[19] The lowest DNA yield among the analyzed human leukocyte DNA samples was 39 μg, and $M_1$dG was reliably quantified in that sample. The sensitivity and selectivity of the developed method indicates that measurement of $M_1$dG in even lower amounts of DNA is possible in future studies.

Although the average $M_1$dG levels were slightly different between smokers and nonsmokers in this study (Table 1), the observed difference was not statistically significant. This is consistent with the results of several studies that compared $M_1$dG levels in leukocyte DNA from smokers and nonsmokers. For instance, Peluso et al. reported that $M_1$dG levels per $10^8$ nucleotides were 4.8 ± 0.4 in leukocyte DNA of current smokers, 4.2 ± 0.7 in ex-smokers, and 3.7 ± 0.4 in nonsmokers; however, the differences were not statistically significant.[17] Another study investigating the effect of formaldehyde exposure on leukocyte $M_1$dG levels in a group of Italian pathologists found that the levels of $M_1$dG per $10^8$ nucleotides tended to increase in smokers compared to nonsmokers, 4.5 ± 1.3 vs 3.8 ± 0.9, respectively, but without reaching statistical significance.[18] Cigarette smoke is a rich source of exposure to free radicals capable of inducing oxidative damage to DNA and promoting oxidative stress in smokers.[9−11] However, the levels of $M_1$dG in humans may also be affected by such factors as age, gender, diet, environmental or occupational exposures, alcohol consumption, and inflammatory diseases. For instance, $M_1$dG has been reported to be lower in leukocyte DNA of women as compared to men: 5.1 ± 0.4 adducts/$10^8$ nucleotides vs 6.7 ± 1.1 adducts/$10^8$ nucleotides, respectively.[19] The potential contribution of the diet to the measured $M_1$dG is exemplified by the findings of a study in which levels of this adduct in leukocyte DNA of female subjects who consumed a diet rich in polyunsaturated fatty acids were nearly 20-fold higher than in the control group.[33] In addition to the potential contribution of the mentioned demographic and lifestyle factors, a small sample size in the studies that compared $M_1$dG in smokers and nonsmokers, including the present study, may have prevented detection of statistically significant differences between these two groups. Furthermore, $M_1$dG analysis in different cell types that are characterized by different lifespans may potentially reveal differences between smokers and nonsmokers and should be considered in future studies.

Availability of a validated and accurate method for the detection and quantitation of $M_1$dG in humans may be extremely useful not only for investigations of the role of environmental exposures or lifestyle factors in health outcomes but also for the prevention, prognosis, and diagnosis of diseases associated with inflammation and oxidative stress. For instance, Wang et al. reported that $M_1$dG levels in the normal breast tissue of women with breast cancer were increased 2- to 3-fold compared to the normal tissue of women without breast cancer.[34] In another study, $M_1$dG levels in lymphocyte DNA of thalassemia patients were 4-fold higher than in healthy control subjects, which indicates elevated oxidative stress and LPO-induced DNA damage in internal organs.[35] Further applications of the developed methodology to measure $M_1$dG levels in specific cohorts could facilitate our understanding of the importance of malondialdehyde-induced DNA damage in these and other diseases.

In summary, we developed a novel LC−NSI−HRMS/MS method for the quantitation of $M_1$dG in human leukocyte DNA, and successfully applied this method to the analysis of $M_1$dG in leukocyte DNA from 50 human subjects. Our approach features a unique application of high-resolution mass spectrometry to achieve the requisite sensitivity and specificity. The method can be used in future studies aimed at understanding the pathophysiological role of $M_1$dG in humans.

## ■ ASSOCIATED CONTENT

### Supporting Information

Chromatogram of a standard mixture of benzamide and 5,6-dihydro-$M_1$dG during column chromatography purification, chromatograms obtained upon analysis of possible artifactual $M_1$dG production during the sample preparation, product ion spectra of 5,6-dihydro-$M_1$dG and 5,6-dihydro-[$^{13}C_3$]$M_1$dG, and extracted ion chromatograms for 5,6-dihydro-$M_1$dG at mass tolerances of 10, 5, and 2 ppm using the Orbitrap mass analyzer. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

CPD, cigarettes per day; HCD, higher energy collisional dissociation; LOD, limit of detection; LOQ, limit of quantitation; LPO, lipid peroxidation; $M_1$dG, 3-(2-deoxy-$\beta$-D-erythro-pentafuranosyl)pyrimido[1,2-$\alpha$]purin-10(3*H*)-one deoxyguanosine; MDA, malondialdehyde

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