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Analysis of 8-oxo-7,8-dihydro-2′-deoxyguanosine by ultra high pressure liquid chromatography–heat assisted electrospray ionization–tandem mass spectrometry

Gunnar Boysen[a,b,c,*], **Leonard B. Collins**[b,c], **Shengkai Liao**[b], **April M. Luke**[d], **Brian F. Pachkowski**[b], **Joanne L. Watters**[e], and **James A. Swenberg**[b,c,d,e,**]

Gunnar Boysen: gboysen@uams.edu; James A. Swenberg: swenberg@unc.edu

[a]Department of Environmental and Occupational Health, University of Arkansas for Medical Sciences, Little Rock, AR 72205, United States

[b]Department of Environmental Sciences and Engineering, The University of North Carolina at Chapel Hill, Gillings School of Global Public Health, Chapel Hill, NC 27599, United States

[c]Center for Environmental Health and Susceptibility, The University of North Carolina at Chapel Hill, Gillings School of Global Public Health, Chapel Hill, NC 27599, United States

[d]Curriculum in Toxicology, The University of North Carolina at Chapel Hill, Gillings School of Global Public Health, Chapel Hill, NC 27599, United States

[e]Department of Nutrition, The University of North Carolina at Chapel Hill, Gillings School of Global Public Health, Chapel Hill, NC 27599, United States

Abstract

Increased amounts of reactive oxygen species (ROS), generally termed oxidative stress, are frequently hypothesized to be causally associated with many diseases. Analyses of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) in DNA and urine are widely used biomarkers for oxidative stress. Over the years it became clear that analysis of 8-oxo-dG in DNA is challenging due to artifactual formation during sample work up. The present study demonstrates that 8-oxo-dG can be measured reliably and accurately when appropriate precautions are taken. First, the presence of an antioxidant, metal chelator, or free radical trapping agent during sample preparation improves reproducibility. Second, sample enrichment by HPLC fraction collection was used to optimize sensitivity. Third, heat assisted electrospray ionization (HESI) eliminated potential interferences and improved assay performance and sensitivity. Subsequently, the UPLC–HESI–MS/MS method was applied to show the biphasic dose response of 8-oxo-dG in H_2O_2-treated HeLa cells. Application of this method to human lymphocyte DNA ($n = 156$) gave a mean±SD endogenous amount of 1.57±0.88 adducts per 10^6 dG, a value that is in agreement with the suggested amount previously estimated by European Standard Committee on Oxidative DNA Damage (ESCODD) and others. These results suggest that the present method is well suited for

*Corresponding author at: University of Arkansas for Medical Sciences, Department of Environmental and Occupational Health, 4301 West Markham St., Slot 820, Little Rock, AR 72205, United States. Tel.: +1 501 526 4956. **Corresponding author at: University of North Carolina at Chapel Hill, Department of Environmental Sciences and Engineering, 253 Rosenau Hall, Chapel Hill, NC 27599, United States.

application to molecular toxicology and epidemiology studies investigating the role of oxidative stress.

Keywords

8-Oxo-7, 8-dihydro-2′-deoxyguanosine; Oxidative stress; LC–MS

1. Introduction

During the last decades, immense efforts have been directed to identify the molecular mechanisms of human diseases like cancer, obesity and others [1,2]. Numerous endogenous processes form reactive oxygen species (ROS) that are known to damage DNA and proteins. The state where the number of ROS formed exceeds those being detoxified is generally termed oxidative stress and has been identified to significantly increase adverse health effects. The extent of oxidative stress has been assessed by analysis of several endpoints including DNA damage and products of lipid oxidation. Of these endpoints, the most widely determined are 8-oxo-7,8-dihydroguanosine and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) in urine and 8-oxo-dG in DNA.

Despite wide application, analytical methods have been inconsistent in inter-laboratory and intra-laboratory comparisons, and determination of the actual endogenous amount of 8-oxo-dG is still heavily debated. The European Standard Committee on Oxidative DNA Damage (ESCODD) is a consortium of 27 laboratories established to examine the critical aspects involved in the measurement of 8-oxo-dG in DNA. Standard oligonucleotides containing defined amounts of 8-oxo-dG, calf thymus DNA (ctDNA), pig liver and HeLa cells were all sent to the participating laboratories for analysis. Several rounds of comparisons demonstrated significant differences of 8-oxo-dG background in DNA, which varied by several orders of magnitude [2,3]. Techniques for 8-oxo-dG measurement included chromatographic approaches utilizing GC–MS or GC–MS/MS, LC–MS/MS or HPLC with electrochemical detection (HPLC–ECD) [4–10]. In addition, an alternative enzymatic approach was examined utilizing the bacterial DNA repair endonuclease, formamidopyrimidine DNA *N*-glycosylase (FPG). FPG creates strand breaks at 8-oxo-dG sites that subsequently can be quantified via the single cell electrophoresis assay (comet assay) or alkaline elution techniques [10,11].

It was quickly recognized that guanine in DNA is readily oxidized to 8-oxo-dG during sample preparation and analysis [3], particularly for chromatographic methods. Subsequently, protocols have been revised to include antioxidants, metal chelators, or free radical trapping agents during sample preparation to prevent artifactual formation of 8-oxo-dG [3]. After standardization and additional inter- and intra-laboratory comparisons, it was suggested that the actual background amount of 8-oxo-dG in human lymphocytes is between 0.3 and 4.2 adducts/10^6 guanines [12]. These values were determined by HPLC–ECD and the enzyme-coupled Comet assay. It was concluded that HPLC-based methods usually overestimate the actual amount of 8-oxo-dG, while the enzymatic approaches provide an

underestimation [4]. Apart from the problem with accuracy, none of the above methods are chemically specific enough to precisely measure 8-oxo-dG [12].

Our laboratory has been working for several years on establishing an easy, robust and reproducible method for the analysis of 8-oxo-dG. Our most current procedure (Fig. 1) includes measures to prevent artifactual formation of 8-oxo-dG at each sample workup step and quantitation by ultra high pressure liquid chromatography–heat assisted electro spray ionization–tandem mass spectrometry (UPLC–HESI–MS/MS) to provide chemically specific quantitation.

2. Materials and methods

2.1. Materials

[$^{15}N_5$]8-Oxo-dG was from Cambridge Isotope Laboratories (Andover, MA). 8-Oxo-dG, DNase I, Type II, 40 kU/bottle, Phosphodiesterase I, 0.74 U/bottle, Alkaline phosphatase, 10,000 U/bottle were from Sigma–Aldrich (St. Louis, MO). 2,2,6,6-Tetramethylpiperidine 1-oxyl (TEMPO) was obtained from Acros (Morris Plains, NJ). All other reagents and solvents were from Fisher Scientific at ACS grade or higher.

2.2. Cell lines

HeLa S3 cells were obtained in suspension from the Lineberger Comprehensive Cancer Center at the University of North Carolina at Chapel Hill. After centrifugation, cells were resuspended in 1× F-12 (Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated fetal bovine serum (Sigma–Aldrich, St. Louis, MO) and 1% (v/v) penicillin and streptomycin (Invitrogen) prior to seeding in 10 cm dishes at a density of ~4×10^5 cells/mL. After an overnight incubation at 37 °C (5% CO_2), cells were washed with warm 1× PBS (Invitrogen), and fresh 1× F-12 without supplements was added to each dish for a 15 min incubation. Cells were then exposed to H_2O_2 (Sigma–Aldrich) for 15 min at 37 °C. After exposure, culture dishes were placed on ice, cells were washed three times with cold PBS, and the harvested cell pellets were stored at −80 °C.

2.3. Rat Liver

Whole, snap frozen rat livers were purchased from Taconics (Germantown, NY).

2.4. Collection of human peripheral blood lymphocytes

DNA from human peripheral blood lymphocytes was collected as part of the Diet, Supplements, and Health (DISH) Study, a cross-sectional study that examined dietary antioxidants and oxidative stress in healthy African American and White adults in North Carolina. Details on the DISH study design, methods and results have been published elsewhere [13]. Briefly, participants were recruited between March and December 2005 via flyers displayed in public venues throughout the Research Triangle area in North Carolina. Eligible persons were 20–45 years of age, generally healthy, free of diseases related to oxidative stress (i.e., cancer, diabetes, heart disease, etc.), and fluent in written and spoken English. Subjects likely to have high levels of oxidative stress, such as current smokers and those with a self-reported body mass index (BMI) of 30 or greater, were ineligible.

Peripheral blood lymphocytes were isolated from 10mL whole blood using Ficoll-PaquePLUS (GE Healthcare, Piscataway, NJ) and stored at −80 °C.

2.5. DNA isolation

DNA isolation was performed with modification to the Puregene® DNA extraction kit (Qiagen, Valencia, CA) as described previously [14]. To minimize formation of oxidative artifacts during isolation, 2,2,6,6-tetramethyl-piperidinoxyl (TEMPO, 20mM final concentration) was added to all solutions and all procedures were performed on ice. Briefly, frozen solid tissues were thawed at 4 °C and homogenized in 3mL Lysis Solution® with a Tehran homogenizer (Wheaton Instruments, Millville, NJ). Cell culture or peripheral blood lymphocyte pellets were dissolved in 2mL Lysis Solution®. The nuclear pellets form solid tissues, cell culture or peripheral blood lymphocytes were incubated with a mixture of RNase T1 and RNase A for 30 min at 37 °C, then overnight at 4 °C with proteinase K (50 U; Applied Biosystems, Foster City, CA). Protein was precipitated with Protein Precipitation Solution® containg 20mM TEMPO and centrifugation at 2000×*g* for 10 min. The supernatant containing the DNA was transferred to a clean 15 mL tube and precipitated by mixing with 6 mL propanol and subsequent centrifugation at 2000×*g* for 5 min at 4 °C. The DNA pellet was washed with 6 mL 70% ethanol, centrifuged at 2000×*g* for 1 min at 4 °C, air dried, and resuspended in sterilized double distilled water. The DNA solution was stored at −80 °C until assayed. Re-extraction and determination of background amounts of 8-oxo-dG in control ctDNA ($n = 4$) were not different form original ctDNA (1.21±0.08 versus 1.22±0.2, respectively). Conclusively, the DNA extraction method used in this study does not alter the amounts 8-oxo-dG.

2.6. Enzyme hydrolysis

For enzyme hydrolysis, DNA concentrations were determined by UV spectrometry, and concentrations were adjusted to approximately 1μg/μL. Fifty microliters of 80 mM Tris–HCl, 20 mM $MgCl_2$ buffer (pH 7) and 3 μL TEMPO (1.5M) were added to 50 μg DNA solution, and the volume was adjusted with water to 213 μL. For accurate quantitation of 8-oxo-dG, 500 fmol of [$^{15}N_5$]8-oxo-dG were added. The hydrolysis was started by addition of 32U DNase I and incubation at 37 °C for 10 min. This was followed by the addition of 2.7 mU phosphodiesterase I and 2U alkaline phosphatase with continued incubation at 37 °C for 60 min. The final volume of sample containing all reagents and internal standard was 300 μL. After hydrolysis, enzymes were removed by ultra filtration using pre-washed YM-10 microcentrifuge filters (Millipore, Bedford, MA).

2.7. Enrichment of 8-oxo-dG by HPLC

DNA filtrates were transferred to high-recovery, glass autosampler vials, and 8-oxo-dG was purified by reverse phase HPLC using an Ultrasphere ODS C18 4.6 mm×250 mm 5 μm column (Beckman, Fullerton, CA). The gradient program provided by an Agilent 1200 HPLC system (Agilent, Santa Clara, CA) was as follows: 7% methanol, 10 mM ammonium formate (pH 4.3) for 22 min, then increasing methanol linearly to 80% in 1 min and holding for 6 min. The flow rate was 1 mL/min, and the column oven, autosampler tray and fraction collector chamber temperatures were maintained at 20, 4 and 4 °C, respectively. A 275 μL aliquot of the filtered DNA hydrolysylate was injected. The retention time of 8-oxo-dG was

determined by using 2′-deoxyguanosine as a retention time marker and multiplying its retention time by 1.5 to obtain the expected retention time of 8-oxo-dG. Fractions containing 8-oxo-dG were collected from 1.5 min before until 1.5 min after the predicted RT in collection tubes containing 300 μL TEMPO solution (75 mM). Solvents were removed with a SpeedVac concentrator and samples were stored at −20 °C until analysis by UPLC–HESI–MS/MS.

2.8. Quantitation by UPLC–HESI–MS/MS

The quantitative analysis of 8-oxo-dG was performed with an UPLC (Waters, Milford, MA) coupled to a TSQ-Quantum Ultra triple-quadrupole mass analyzer (Thermo Finnigan, San Jose, CA) using a HESI source. A 2.1 mm×100 mm HSS T3 C18, 1.8 μm column (Waters, Milford, MA) was operated with a linear gradient of 1% methanol and 0.1% acetic acid in water to 5% methanol in 2 min and then held for 10 min, followed by an increase to 50% methanol in 2 min, at a flow rate of 200 μl/min. The retention time of 8-oxo-dG was determined with authentic standards. The analyte and internal standard were detected in selected reaction monitoring (SRM) mode, monitoring the transitions of m/z 284.1 → 168.0 and m/z 289.1 → 173.0 for 8-oxo-dG and [$^{15}N_5$]8-oxo-dG, respectively. Samples were dissolved in 20 μL water and 15 μL was injected. The electrospray conditions were as follows: spray voltage of 3000 V, vaporizer (HESI) temperature of 250 °C, sheath gas pressure 35, auxiliary gas pressure 30, capillary temperature of 285 °C and collision energy of 12 eV.

3. Results

3.1. Method validation

Before analysis of unknown biological DNA samples, the method was validated by repetitive analysis of rat liver DNA spiked with various amounts of authentic 8-oxo-dG standards as described in material and methods (Fig. 2). Spiked rat liver DNA was analyzed in triplicate on a single day or at different days and the intraday and interday reproducibility were determined. In addition, analysis of commercially available calf thymus DNA showed much higher high endogenous amounts of 8-oxo-dG and were not suitable as control DNA (data not shown). In rat liver DNA the mean±SD amount of 8-oxo-dG was 1.24±0.17 adducts/10^6 dG (n = 6; CV = 14%). The interday result for the same rat liver DNA was 1.03±0.24 adducts/10^6 dG(n = 9,CV = 20%).A second analyst tested a separate batch of calf thymus DNA freshly isolated in our laboratory over multiple days and obtained 1.68±0.33 adducts/10^6 dG (n = 10, CV = 20%).

3.2. H_2O_2 induced 8-oxo-dG in HeLA cell cultures

Subsequently, DNA samples from HeLa cells treated with various concentrations (0.1–15 mM) of H_2O_2 were analyzed for 8-oxo-dG. The dose response of 8-oxo-dG, as determined by the UPLC–HESI–MS/MS method is shown in Fig. 3. The formation of 8-oxo-dG followed a biphasic dose response similar to those reported for single strand breaks and apurinic sites in H_2O_2-treated HeLa cells [14]. These data demonstrate the suitability of the UPLC–HESI–MS/MS method for analysis of 8-oxo-dG.

3.3. Establishment of 8-oxo-dG amounts in human peripheral blood lymphocyte DNA

To establish the endogenous amounts of 8-oxo-dG in humans, peripheral blood lymphocyte DNA from 156 healthy subjects were analyzed for the presence of 8-oxo-dG. 8-oxo-dG was detected in all samples with a mean±SD amount of $1.57\pm0.88/10^6$ dG. The values ranged from 0.46 to 5.98 8-oxo-dG/10^6 dG and >90% were within one SD of the mean and >97% were within two×SD.

4. Discussion

Initially, a method was developed in our lab and successfully applied to study the involvement of oxidative stress in acetaminophen-induced liver toxicity [15]. This method was based on enzyme digestion, HPLC clean up and capillary LC–MS/MS. While this method appeared to be suitable for small studies, it became quickly apparent that it was unsuitable for larger studies. A main disadvantage was that the published method depended on optimum column performance for the separation of 8-oxo-dG from several interfering peaks (Fig. 4A). Therefore, important steps for improvement were identified and are discussed below.

4.1. Enrichment of 8-oxo-dG by automated HPLC

During subsequent analysis of larger sample sets, the 8-oxo-dG enrichment by HPLC was automated. While convenient, the recovery of 8-oxo-dG was reduced when samples remained on the fraction collector for a long period of time. This decrease in recovery appeared to increase with exposure of 8-oxo-dG to laboratory air and light leading to analyte degradation. To stabilize 8-oxo-dG during automated fraction collection, 300 μL of TEMPO (75 mM) were added to the collection tubes. This stabilized the overall recoveries and also increased precision, as shown by a comparison of analyses of rat liver DNA with or without TEMPO in the fraction collection vial (data not shown). In the previously published study [15], this step was performed manually, and the fraction was either immediately capped and stored at −80 °C or the solvents were removed in the SpeedVac.

To determine whether TEMPO prevents further oxidation of 8-oxo-dG, 100 nM 8-oxo-dG in aqueous solution were incubated with 15 mM TEMPO for 24 h at room temperature. Solutions of 100 nM dG were also incubated with 15 mM TEMPO to show prevention of artifactual formation of 8-oxo-dG. Samples were analyzed by UPLC–HESI–MS/MS monitoring for both dG and 8-oxo-dG. The amounts of 8-oxo-dG remained constant in presence of TEMPO indicating that TEMPO prevents further reactions of 8-oxo-dG such as oxidation (data not shown). Further, the dG solutions also did not show any detectable 8-oxo-dG, which indicates that the presence of TEMPO inhibits artifactual formation of 8-oxo-dG. These results are in agreement with observations made by Hofer and Möller [16].

4.2. Liquid chromatography mass spectrometry

Our initial approach for measuring 8-oxo-dG was DNA digestion, HPLC enrichment and analysis by capillary LC–MS/MS. This approach was successful; however, the HPLC column lifetime was drastically shortened due to the consistent use of high pressure. Several other solvent compositions and columns were evaluated, but any changes resulted in

merging of the interfering peaks with 8-oxo-dG (Fig. 4A). For example changing the pH from 4.3 to 3.4 moved deoxyadenosine (dA) from the right of 8-oxo-dG to on top of 8-oxo-dG, which resulted in a strong interfering peak in the 8-oxo-dG signal (data not shown). It was previously reported that 2′-deoxyadenosine forms solvent clusters which mimicked 8-oxo-dG during LC–MS/MS analysis [17]. To guarantee separation of 8-oxo-dG from interfering peaks, the previously described capillary LC–MS/MS needed to operate a 3.5 μm particle capillary column at pressures up to 350 bar at 30 °C (Fig. 4A). Alternatively, sufficient separation of 8-oxo-dG from interfering peaks can be obtained by using a 5 μm particle capillary column that is operated at 10 °C [18].

The main breakthrough towards a robust and reproducible method, however, was the availability of a heat assisted electrospray ionization source (HESI). In addition, the availability of UPLC promised higher sample loading capacities and increased sensitivity due to sharper peaks. Unfortunately, direct injection of the DNA hydrolysate was unsuccessful and produced larger coefficient of variation (CV) and background amounts (data not shown). Therefore it was concluded that sample purification was still necessary for accurate and artifact free analysis of 8-oxo-dG. The application of UPLC in combination with HESI provided sufficient separation and sensitivity for the accurate detection of background amounts of 8-oxo-dG in 50 μg DNA. More importantly, interfering peaks were essentially removed using HESI (Fig. 4B), thereby eliminating previous challenges of maintaining sufficient separation and sensitivity.

4.3. Method validation

Repetitive analysis of rat liver DNA spiked with various amounts of authentic 8-oxo-dG standards demonstrate the reproducibility and precision of the current method for analysis of 8-oxo-dG in DNA (Fig. 2). The LOQ of the present method was determined to be 10 fmol per injection, which corresponds to 0.2 fmol/μg DNA, or ~0.4 adducts 8-oxo-dG/10^6 dG. Many of the rat liver and calf thymus DNA control samples analyzed contained 8-oxo-dG amounts close to the LOQ when starting with 50 μg DNA. To be confident the endogenous level can be quantitated, a 50 μg sample of DNA was determined to be the minimum necessary for analysis. Subsequently, DNA samples from H_2O_2-treated HeLa cells were analyzed for 8-oxo-dG. The treatment concentrations were based on a well-established model, which produces a characteristic biphasic dose response for H_2O_2 induced DNA lesions [19]. The dose response of 8-oxo-dG, as determined by the UPLC–HESI–MS/MS method is shown in Fig. 3. As expected, the formation of 8-oxo-dG followed the same biphasic dose response as previously reported for single strand breaks measured by the comet assay and formation of apurinic sites [19]. This “proof of principle” experiment clearly demonstrates the suitability of the UPLC–HESI–MS/MS method for accurate quantitation of 8-oxo-dG in cell DNA in response to chemical treatment.

4.4. Establishment of 8-oxo-dG amounts in human peripheral blood lymphocyte DNA

A previous criticism of 8-oxo-dG as a viable biomarker for oxidative stress was related to disagreements concerning endogenous background amounts. To investigate this issue, 8-oxo-dG was measured in peripheral blood lymphocyte DNA from 156 healthy subjects. The DNA specimens were part of a study comparing multiple biomarkers for oxidative stress. 8-

Oxo-dG was detected in all samples with a mean±SD amount of 1.57±0.88 adducts per 10^6 dG and 98% of the values were within 0.3 and 4.2 adducts/10^6 guanines, which was the range ESCODD estimated as the 8-oxo-dG background (Fig. 5) [3]. Bianchini at al. [20] reported 1.19–2.17 8-oxo-dG/10^6 dG in lymphocytes of 115 premenopausal non-smoking women. Lenton et al. [21] reported somewhat higher amounts of 4.5±1.8 8-oxo-dG/10^6 dG in lymphocytes of 105 healthy volunteers. Together, these data provide confidence that the presented UPLC–HESI–MS/MS method measures 8-oxo-dG accurately and free of artifact. The ability of the UPLC–HESI–MS/MS to detect endogenous amounts of 8-oxo-dG in 50 μg of human DNA is a significant improvement, when considering the limited amount of DNA obtained in molecular epidemiology studies.

Measurement of 8-oxo-dG has been conducted by many different means utilizing different chromatography technology including HPLC–ECD, GC–MS, GC–MS/MS, HPLC–MS/MS and HPLC–MS/MS. Essentially all mass spectrometry-based methods rquire sample work up that has been known to introduce artifactual formation of 8-oxo-dG. Since evaluation of several chromatography methods by ESCODD, attention has been focused to eliminate such artifactual formation during sample handling and work up. The reported UPLC–MS/MS method described herein uses TEMPO to prevent artifactual formation or degradation of 8-oxo-dG and is comparable to two other recently published and many other published LC–MS/MS methods. Singh el al [22] recently reported an online column switching LC–MS/MS method for simultaneous quantitation of 8-oxo-dG and 8-oxo-2′-deoxyadenosine in DNA. Endogenous amounts of 8-oxo-dG were different in rat liver DNA isolated with different commercially available DNA isolation kits. Values were 2–3-fold higher when DNA was isolated from 600 to 800 mg or from 300 to 400 mg tissue, using the Wako or Qiagen kit, respectively. Further, it was shown that treatment with carbon tetrachloride did not increase amounts of 8-oxo-dG in rat liver, suggesting that it does not induce oxidative stress. However, the reported endogenous amounts, of 1.03 8-oxo-dG/10^6 dG (reported as ~20 8-oxo-dG/10^8 normal nucleotides) are similar to the amounts observed in our control rat livers, suggesting that endogenous amounts of 8-oxo-dG are similar, independent of the method used for quantitation. Also, whether amounts of the tissue used for extraction or amounts of DNA obtained from tissue may have caused the different background amounts are not known. Mangal et al. [23] recently reported analysis of 8-oxo-dG in cell lines using immunoaffinity purification prior to quantitation by LC–MS/MS based on an approach previously published by Singh et al. [17]. They also compared different commercial DNA isolation (DNAzol and NaI) procedures. Additions of desferal or TEMPO significantly reduced artifactual formation of endogenous amounts of 8-oxo-dG, suggesting artifactual formation during DNA isolation [23]. The amounts of endogenous 8-oxo-dG reported in untreated H358 cells were 2-fold lower compared to amounts of 8-oxo-dG in HeLa cells (2.8 8-oxo-dG/10^6 dGua) reported herein. This difference is most likely due to a real difference between the cell lines and culture conditions.

Altogether, these and other reported methods demonstrate that 8-oxo-dG can be reliably measured in biological specimens if precautions are taken during DNA isolation and hydrolysis. Further, to obtain sufficient sensitivity and to increase reproducibility, adduct enrichment is needed. This can be achieved by immunoaffinity chromatography, HPLC clean up or online column switching prior to quantitation by tandem mass spectrometry. The

advantage of our procedure is that sample work up, including HPLC purification, can be automated and performed without utilizing expensive mass spectrometer time. Further, samples can be processed in most contemporary laboratories and sent to a mass spectrometry facility for analysis were subsequent sample analysis is performed in <20 min per sample, making it suitable for application to multi laboratory molecular epidemiology studies. In direct comparison to the other two recent publications, our method does not require column switching technology [22] or custom made antibodies that to our knowledge are not commercially available [17,23].

The assay requires 5 days to complete starting from DNA extraction and ending with LC–MS/MS data workup. Extraction of DNA from tissue typically requires 2 full workdays to process about 16 samples. One complete set of 22 isolated DNA samples, which includes duplicates of reagent blanks, negative controls, and positive controls, takes 1 day for DNA digestion and fraction collector setup. Automated HPLC fraction collection can be performed overnight, and the fractions usually require 1 full day for evaporation. The second evaporation step in HPLC autosampler vials is completed in less than 4 h, and LC–MS/MS can be performed overnight. Data workup can be automated and typically takes less than 1 h.

In conclusion, we have demonstrated that UPLC–HESI–MS/MS can be routinely employed to measure 8-oxo-dG with high accuracy, if certain precautions are taken. First, the presence of the antioxidant TEMPO improves reproducibility by preventing conversion of dG or 8-oxo-dG. Second, sample enrichment by HPLC fraction collection, immunoaffinity chromatography [17,23] or online column switching [22] is essential to achieve sufficient sensitivity and to remove potential interferences. Third, utilization of HESI eliminates important interfering ions, thereby drastically improving assay performance. Fourth, the UPLC–HESI–MS/MS method has been successfully applied to show the biphasic dose response of 8-oxo-dG in H_2O_2-treated HeLa cells. Lastly, the mean±SD background amount of 8-oxo-dG in peripheral blood lymphocytes (n = 156) from healthy humans was 1.57±0.88 adducts per 10^6 dG, a value that is in agreement with the background amount previously estimated by ESCODD and others [2,20,21].

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Abbreviations

8-oxo-dG	8-oxo-7,8-dihydro-2′-deoxyguanosine
AP	apurinic sites
ctDNA	calf thymus DNA
ESCODD	European Standard Committee on Oxidative DNA Damage
ECD	electrochemical detection
FPG	formamidopyrimidine DNA *N*-glycosylase

HESI	heat assisted electrospray ionization
ROS	reactive oxygen species
TEMPO	2,2,6,6-tetramethylpiperidine 1-oxyl
UPLC–HESI–MS/MS	ultra high pressure liquid chromatography–heat assisted electro spray ionization–tandem mass spectrometry

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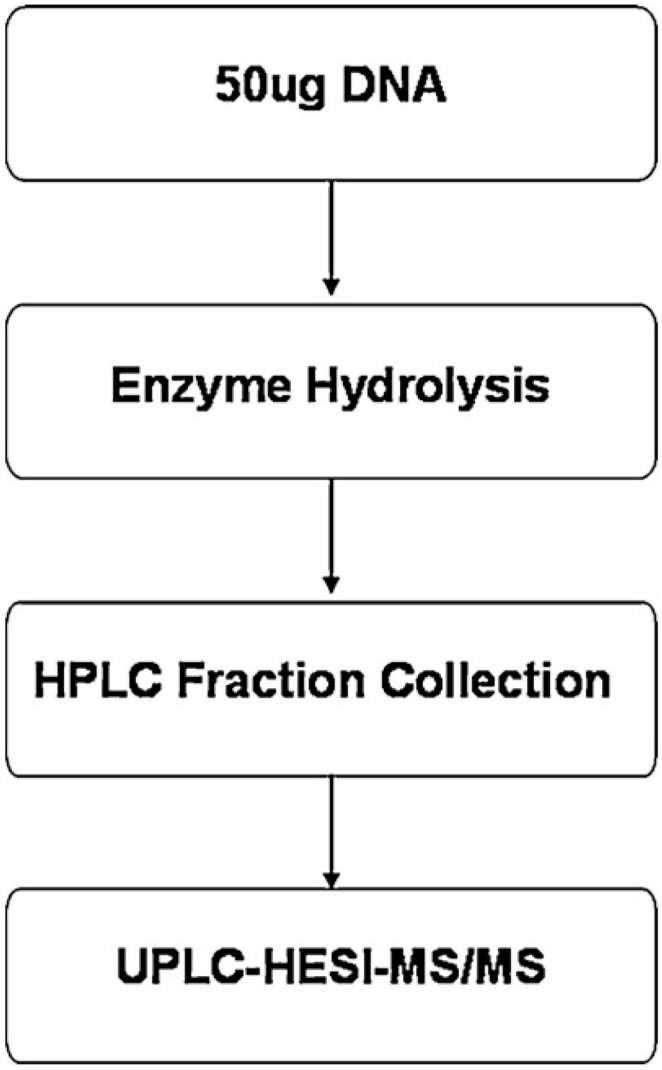

Fig. 1.
Schematic outline of analysis protocol.

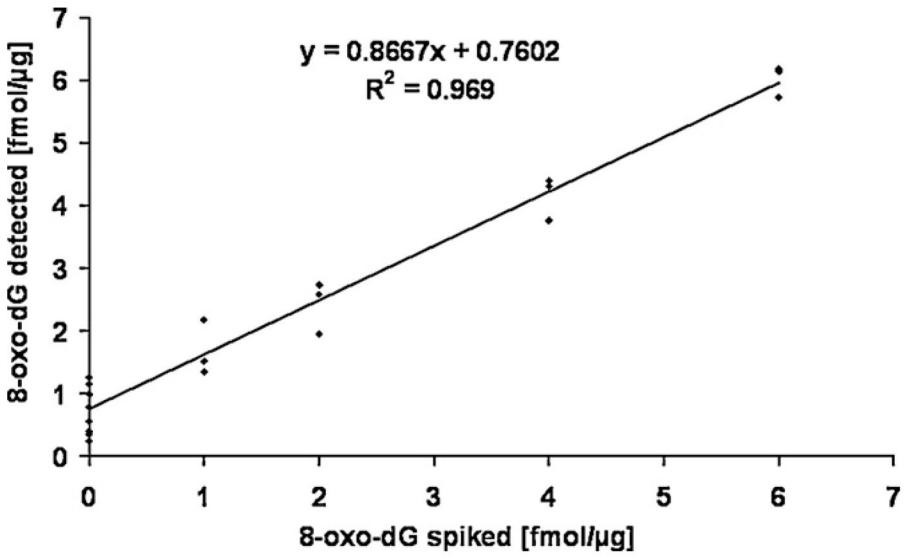

Fig. 2.

Control rat liver DNA spiked with authentic 8-oxo-dG (negative control, $n = 9$; positive control, $n = 3$) was analyzed for 8-oxo-dG by UPLC–HESI–MS/MS as described in materials and methods.

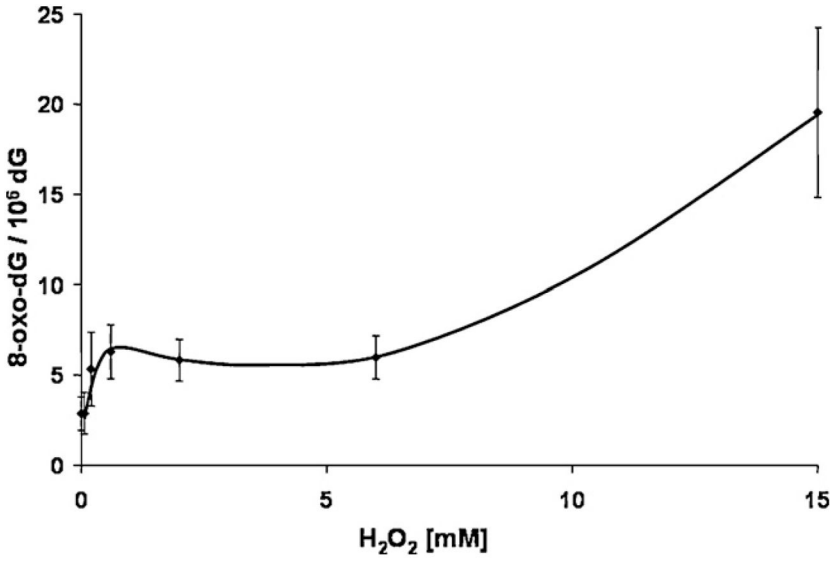

Fig. 3.

Biphasic dose response in H_2O_2-treated HeLa cells ($n = 6$). HeLa cells were treated with different concentrations of H_2O_2 for 15 min and amounts of 8-oxo-dG in DNA were measured by UPLC–HESI–MS/MS as described in materials and method.

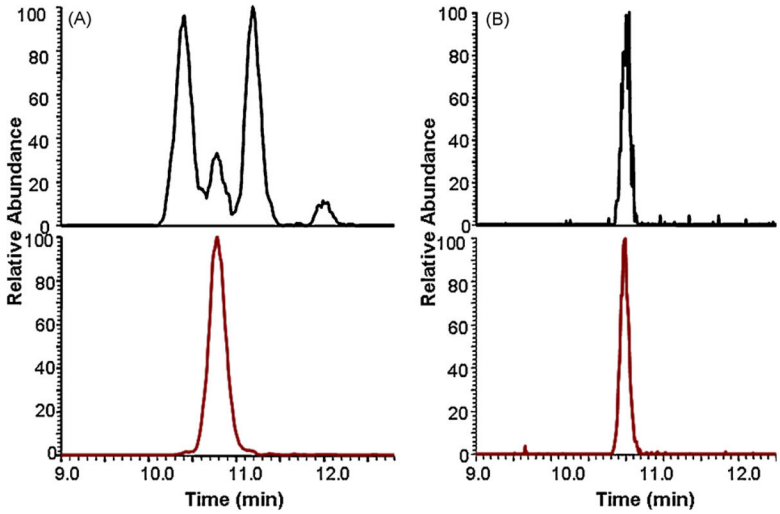

Fig. 4.

Extracted ion chromatogram of 8-oxo-dG and [$^{15}N_5$]8-oxo-dG in rat liver DNA analyzed by (A) capillary LC–MS/MS or (B) UPLC–HESI–MS/MS. Shown are the transitions of *m*/*z* 284.1 → 168.0 and *m*/*z* 289.1 → 173.0 for 8-oxo-dG (upper traces) and [$^{15}N_5$]8-oxo-dG (lower traces), respectively.

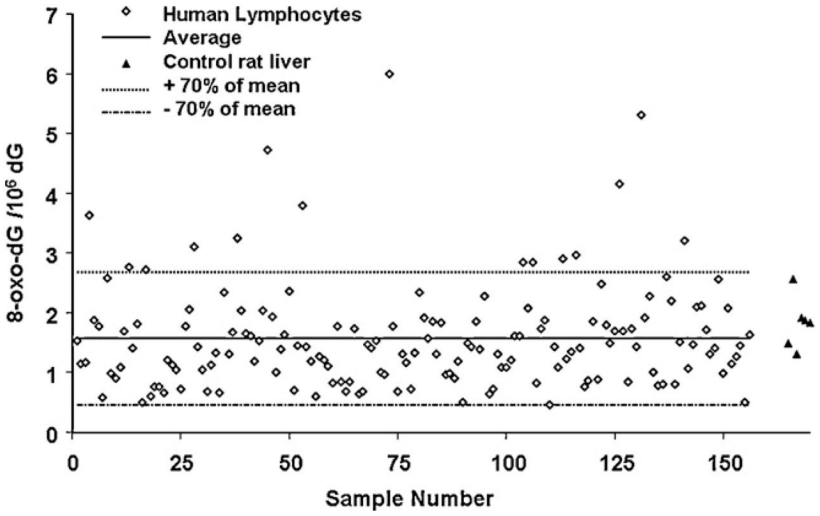

Fig. 5.

Endogenous 8-oxo-dG in human peripheral blood lymphocytes and snap frozen rat liver DNA measured by UPLC–HESI–MS/MS. The mean±SD amount of 8-oxo-dG in human lymphocytes was 1.57±0.88 8-oxo-dG/10^6 dG (n = 156).