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Determination of 8-Oxoguanine in DNA by Gas Chromatography–Mass Spectrometry and HPLC–Electrochemical Detection: Overestimation of the Background Level of the Oxidized Base by the Gas Chromatography–Mass Spectrometry Assay

Jean-Luc Ravanat,[†] Robert J. Turesky,*,[†] Eric Gremaud,[†] Laura J. Trudel,[‡] and Richard H. Stadler[†]

Nestec Ltd., Nestlé Research Centre, Vers-Chez-Les-Blanc, 1000 Lausanne 26, Switzerland, and Division of Toxicology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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Two analytical methods, one involving the combined use of reverse-phase HPLC and electrochemical detection (HPLC-EC) and one involving a mass spectrometric detection after gas chromatography separation (GC/MS), were developed for the detection of 8-oxoguanine in DNA. In order to obtain quantitative results, 2,6-diamino-8-oxopurine, whose chemical structure and electrochemical response are very similar to 8-oxoguanine, has been employed as an internal standard in the HPLC-EC assay. In the case of the GC/MS method, an isotopically stable (M + 4) 8-oxoguanine has been employed as an internal standard. Both methods are able to detect approximately 1 modification per 10^6 DNA bases. The background level of 8-oxoguanine in DNA as determined by GC/MS is approximately 50-fold higher than that determined by the HPLC-EC assay. The discrepancy between the two methods is due to an artifactual oxidation of guanine during the derivatization reaction as demonstrated by using pure guanine. The amount of 8-oxoguanine in guanine, determined by GC/MS, increases linearly with the time of derivatization, indicating that an oxidation occurs during the silylation reaction. Derivatization under nitrogen atmosphere reduces but does not suppress the artifactual oxidation. The amount of 8-oxoguanine in DNA, quantified by GC/MS, is comparable to that obtained by HPLC-EC when 8-oxoguanine is prepurified by HPLC or by immunoaffinity chromatography, prior to the silylation reaction. The artifactual formation of 8-oxoguanine during the derivatization reaction may explain, at least in part, why the values reported for 8-oxoguanine determination by GC/MS are generally about 1 order of magnitude higher than that determined by HPLC-EC. Prepurification of 8-oxoguanine from guanine is recommended in order to obtain reliable results by GC/MS which may be compared to HPLC-EC.

Introduction

Reactive oxygen species which are formed in vivo after exposure to oxidizing agents or ionizing radiation, or during natural cellular oxidative events, could react with cellular components such as proteins and DNA (*1*, *2*). It is now well established that free radical mediated oxidation of DNA leads to chemical modifications of the macromolecule, including single or double DNA strand breaks, base, and also sugar modifications (*3*). These modifications contribute to aging and age-related diseases such as cancer and atherosclerosis (*4*, *5*).

Among the DNA base modifications induced by such oxidation, 8-oxoguanine (8-oxoGua)[1] is of particular relevance and has been proposed as a biomarker of DNA oxidation (*6*–*8*). Several analytical methods (*9*, *10*), including ^{32}P-postlabeling (*11*, *12*), HPLC in combination with electrochemical detection (*13*, *14*), and a GC/MS assay (*15*, *16*), have been developed for the detection of 8-oxoGua both in vitro and in vivo. There have been continuous improvements in both methods. For example, the limit of 8-oxoGua detection of the HPLC-EC method has been increased (*17*), and isotopically labeled 8-oxoGua (*16*, *18*) or labeled DNA (*19*) has been employed as internal standard for accurate quantification of the modified base by mass spectrometry. In addition, polyclonal antibodies (*20*) and also monoclonal antibodies (*21*) have been raised against 8-oxoGua to purify this lesion from biological fluids by immunoaffinity chromatography. The results indicate that 8-oxoGua is present at cellular "background" levels in both nuclear (*14*) and mitochondrial DNA (*22*). Unfortunately, the different methods used for the detection and quantification of 8-oxoguanine in DNA do not always provide similar background levels of oxidation. The values of 8-oxoGua obtained by GC/MS are generally about 1 order of magnitude higher than those obtained by HPLC-EC (for review see ref 23).

* Address correspondence to this author. Telephone (41)-21-785-8833; Fax (41)-21-785-8553.
[†] Nestec Ltd.
[‡] Massachusetts Institute of Technology.
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[1] Abbreviations: 8-oxoGua, 7,8-dihydro-8-oxoguanine; $(NH_2)_2$-OH-Pur, 2,6-diamino-8-oxopurine; 8-oxodGuo, 7,8-dihydro-8-oxo-2′-deoxyguanosine; dGuo, 2′-deoxyguanosine; [^{3}H]dGTP, deoxy[1′,2′-^{3}H]-guanosine-5′-triphosphate; HPLC-EC, HPLC with electrochemical detection; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; TMS, trimethylsilyl; Mab, monoclonal antibodies; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; SIM, selected ion monitoring; PBS, 10 mM KH_2PO_4/0.9% saline, pH 7.4.

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To our knowledge, these two analytical methods have up to now never been employed in the same laboratory; thus no direct comparison between the HPLC-EC and GC/MS assays have been made using the same DNA sample. Therefore, in order to establish the origin of the discrepancy in the results obtained from these methods, we have measured the background level of 8-oxoGua in DNA both by HPLC-EC and by GC/MS. 2,6-Diamino-8-oxopurine (*24*) and isotopically labeled 8-oxoguanine (M + 4) (*18*) have been used as internal standards for the HPLC-EC and GC/MS assays, respectively. The two methods are sufficiently sensitive to detect approximately 1 modification per 10^6 DNA bases. The values obtained using the GC/MS assay are higher than those obtained by HPLC-EC detection, due to an overestimation of the level of 8-oxoguanine in DNA by GC/MS. We have demonstrated, by using pure guanine, that the overestimation is due to the artifactual oxidation of guanine during the derivatization reaction used prior to GC/MS analysis. Furthermore, the amount of 8-oxoguanine in DNA determined by GC/MS is comparable to that obtained by HPLC-EC when 8-oxoguanine is prepurified by HPLC or by immunoaffinity chromatography prior to the derivatization reaction.

Materials and Methods

Chemicals. 2,6-Diamino-8-oxopurine ($(NH_2)_2$-OH-Pur) and 2-amino-6,8-dihydroxypurine (8-oxoGua) were purchased from Janssen Chemica (Geel, Belgium). *N,O*-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA), pyridine, and acetonitrile used for the derivatization reaction were obtained from Pierce (Pierce Chemical Co., Rockford, IL). Guanine, cyanogen bromide-activated Sepharose 4B, calf thymus DNA, lipid-free type IV bovine serum albumin (BSA), and PBS were purchased from Sigma (St. Louis, MO). Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem-Novabiochem (Lucerne, Switzerland). Radiolabeled deoxy[1′,2′-^{3}H]guanosine 5′-triphosphate ([^{3}H]dGTP), specific activity 34 Ci/mmol, and [8-^{14}C]guanine, specific activity 60 mCi/mmol, were obtained from the Amersham (Zurich, Switzerland) and Dupont de Nemour (Dreieck, Germany), respectively. Isotopically labeled 8-oxoGua (M + 4) was synthesized according to Stadler *et al.* (*18*). All the other reagents were of analytical grade and were used without further purification.

Chemical Syntheses. Radiolabeled 8-oxoGua and 8-oxodGuo were synthesized using a Fenton type reaction as previously described by Shigenaga *et al.* (*6*) with the following modifications.

Synthesis of [^{3}H]dGuo from [^{3}H]dGTP. An aliquot (100 μL = 100 μCi) of [^{3}H]dGTP was concentrated under a stream of nitrogen at 30 °C (heater block). The residual solution was taken up in a minimal amount of water and again concentrated to ensure removal of residual ethanol. Into the same Eppendorf tube was added the following: 50 μL of 0.1 M Tris-HCl buffer (pH 8.5); 20 μL of 0.2 M glycine/NaOH (pH 8.8); 20 μL of 50 mM $MgCl_2$; 1 unit of alkaline phosphatase (desalted with Centricons MW cutoff 30 000 and taken up in a 10 mM Tris-HCl buffer, pH 8.5). Incubation was for 1.5 h at 37 °C with slight agitation in a tightly closed Eppendorf "Safe-lock" tube. Termination of the reaction and workup were by direct injection of the incubation mixture onto the HPLC. Chromatography was performed with a Supelcosil LC-18 DB column (25 cm × 4.6 mm i.d.) with peak monitoring at 290 and 254 nm, and collecting [^{3}H]dGuo manually (t_R 8.2 min) from the column during isocratic elution with 10% MeOH in water. The yield was 94.1 μCi (94.1%) of chromatographically pure [^{3}H]dGuo with a specific activity of 34 μCi/nmol.

Synthesis of [^{3}H]8-OxodGuo from [^{3}H]dGuo. The radiolabeled C-8 oxopurine was synthesized by addition in the following order at ambient temperature: [^{3}H]dGuo (94.1 μCi in 30 μL of H_2O); 50 μL of 1.7 M ascorbic acid; 2 μL of 0.2 M $CuSO_4$; and finally 50 μL of H_2O_2 (30% w/v). Immediately after addition of H_2O_2, the incubation mix was subjected to HPLC purification employing a Supelcosil LC-18 DB column (25 cm × 4.6 mm i.d.) and monitoring at 290 and 254 nm. Elution of [^{3}H]8-oxodGuo was with a gradient going from 100% solvent A (50 mM ammonium acetate, pH 5.0) after 20 min to 6% solvent B (MeOH) over 20 min and then to 100% B over 5 min, collecting [^{3}H]8-oxodGuo manually at t_R 38 min. These chromatographic conditions enabled separation of contaminant material eluting ca. 2 min before 8-oxodGuo, giving 22 μCi of chromatographically pure [^{3}H]8-oxodGuo (23% yield).

Synthesis of [^{14}C]8-OxoGua from [^{14}C]Gua. To 50 μg of [^{14}C]Gua (59.8 mCi/mmol) dried under reduced pressure (0.05 mbar) were added the following at room temperature in the following order: 250 μL of H_2O, 100 μL of 1.7 M ascorbic acid; 10 μL of 0.2 M $CuSO_4$; and finally 200 μL of H_2O_2 (30% w/v). The incubation mixture was then injected onto a Supelcosil LC-18 DB column (25 cm × 4.6 mm i.d.) eluted under isocratic conditions with 50 mM ammonium acetate (pH 5.5) as the mobile phase, monitoring at 295 nm. Lyophilization of the collected fraction (t_R = 19 min) gave 11.5 μg (59.8 mCi/mmol) of chromatographically pure [^{14}C]8-oxoGua (23% yield). The radiolabeled compounds were diluted with authentic unlabeled material to a final specific activity of 30 mCi/mmol.

Standard Solutions. Lyophilized calf thymus DNA was dissolved in deionized distilled water to a 0.5 mg/mL final concentration as determined by UV (20 OD corresponding to 1 mg/mL). Commercial 8-oxoGua, and $(NH_2)_2$-OH-Pur were dissolved in deionized distilled water, and the concentration was determined by UV (*25*); then the solution was adjusted to a 10 pg/μL final concentration. The nucleosidic solutions are stable for several months when stored at −30 °C.

Apparatus. The HPLC system consisted of a Waters (Waters Millipores, Milford, MA) Model 625 LC HPLC pump, a Waters Model 994 programmable photodiode array detector, and a Waters Model 464 pulsed electrochemical detector. The Hewlett-Packard GC/MS system consisted of a Model 5890 Series II gas chromatograph equipped with a 7673 automatic injector and connected to a Model 5972 mass selective detector.

8-OxoGua Analysis by HPLC-EC. The separation of the nucleobases was achieved using a Supelco LC18-DB 5 μm column (25 cm × 4.6 mm i.d.) under isocratic conditions. The flow rate was set at 1 mL/min with 2% methanol in 50 mM ammonium acetate buffer, pH 5.5, as the mobile phase. Unmodified nucleobases were detected by UV at 260 nm, detection set at 1.5 OD, and 8-oxoGua and $(NH_2)_2$-OH-Pur were detected by amperometry using a glassy carbon electrode setup at a potential of +550 mV versus the AgCl/KCl reference electrode, detection set at 5 nA. For purification of 8-oxoguanine by HPLC, the electrochemical detector was disconnected and a second UV channel was set up at 295 nm, for the UV detection of 8-oxoGua.

GC/MS Analysis of 8-OxoGua. Separations were performed using a Rtx5 (Restek Corp., Bellefonte, PA) column (20 m × 0.18 mm, film thickness 0.2 μm) with helium as the carrier gas at a constant flow rate of 30 cm/s. The oven program was set at 120 °C for 1 min to 300 °C at 10 °C/min and resting 5 min. Injections were performed in the splitless mode with an injector port temperature of 240 °C. The selected ion monitoring (SIM) mode was used in order to detect the ions 440 and 455 for 8-oxoGua, and 444 and 459 for the isotopically labeled internal standard (*18*). The derivatization reactions were performed in a tightly closed screw cap vials (Reacti-vials, Pierce Chemical Co., Rockford, IL) using a mixture of 25 μL of BSTFA, 15 μL of acetonitrile, and 10 μL of pyridine at a reaction temperature of 130 °C for 30 min. For silylation under reduced oxygen concentration, the vials were purged with nitrogen for 1 min, then the derivatization mixture was added under nitrogen atmosphere, and the reaction vial screw caps were closed prior to derivatization at 130 °C for 30 min. After adjustment to room temperature, 1 μL of the derivatized solution was injected in the splitless mode without further treatment.

DNA Hydrolysis. Calf thymus DNA (50 μg) was dried under reduced pressure (0.05 mbar) in a screw cap vial after addition of the appropriate internal standard, i.e., 500 pg of $(NH_2)_2$-OH-Pur for HPLC-EC or 2.5 ng of 8-oxoGua (M + 4) for GC/MS analysis. Thereafter, 500 μL of 60% formic acid was added and hydrolysis was performed for 30 min at 130 °C. After cooling to room temperature, formic acid was removed by centrifugation under vacuum (0.05 mbar). The residual nucleobases were then dissolved in 50 μL of the HPLC mobile phase prior to injection onto the HPLC system or subsequently derivatized prior to GC/MS analysis.

Preparation of Haptens. 8-Oxoguanosine was synthesized according to Lin *et al.* (*26*) with a purity of at least 98% as shown by HPLC and ^{1}H NMR analysis. Treatment of 8-oxoguanosine (167 μmol) with sodium periodate (1.5-fold molar excess) formed the dialdehyde (*20*), which was then reacted separately with 2 μmol of bovine serum albumin (BSA) and 23.3 nmol of keyhole limpet hemocyanin (KLH). After reduction of the Schiff base with sodium borohydride (*27*), the modified proteins were dialyzed for 36 h against cold distilled water. The extent of protein modification was determined by UV measurement at 292 nm for BSA (176 nmol of 8-oxoGua/mg of BSA) and at 283 nm for KLH (163 nmol of 8-oxoGua/mg of KLH).

Immunization of Mice. Four BALB/cby mice (Jackson Laboratories), 4–6 weeks of age, were immunized by ip injection with 14 ng of 8-oxoGua–KLH in RIBI adjuvant (RIBI Immunochem Research Inc., Hamilton, MT) and boosted as above 14 days later. Twelve days post boost, serum was obtained from the tail vein. The antibody titer was determined by ELISA using 8-oxoGua–BSA as the test antigen as previously described (*28*). All 4 mice had titers >1:18 000 above normal mouse sera control.

Cell Fusion. The mice were boosted with 100 μg of 8-oxoGua–KLH 3 days before fusion to the Sp2 myeloma cell line as previously described (*28, 29*). Resulting clones were screened by ELISA for recognition to 8-oxoGua–BSA. Control plates coated with BSA only were used to eliminate clones with BSA crossreactivity.

Immunoassays. ELISA, used to determine antibody titer in mouse sera and hybridoma supernatants, was performed as described previously (*29*) using haptenized BSA. Competitive radioimmunoassay (RIA) was used to determine affinity constants (*30*). The affinity constant for 8-oxodG was 4.3×10^7 M^{-1}.

Monoclonal Antibodies Immobilization for Affinity Chromatography. The immunoaffinity matrix was prepared from monoclonal antibodies (Mab) obtained from ascites fluid and partially purified by ammonium sulfate precipitation (40% v/v), followed by dialysis against coupling buffer pH 8.3 (*29*). The protein was bound to CNBr-activated Sepharose 4B (2 mg off protein/mL of gel) which had been swelled in 1 mM HCl as previously described (*30*). The binding of the antibody was 80–95%. The immobilized gel was stored at 4 °C in phosphate-buffered saline containing 0.02% sodium azide.

Immunoaffinity columns were prepared by packing Bio-Rad Poly-prep columns with 0.5 mL of the immobilized gel. Purification of 8-oxoGua by immunoaffinity was performed under the conditions previously described by Park *et al.* (*21*) with minor modifications. Typically, 8-oxoGua or other chemicals of interest were applied to the immunoaffinity column at room temperature in 0.5 mL of PBS. Then the column was washed under gravity-induced flow with 2.5 mL of PBS buffer followed by 2.5 mL of H_2O. The bound chemicals were then eluted with 2.5 mL of MeOH. The binding capacity of the column was determined using 500 ng (30 mCi/mmol) of the appropriate radiolabeled tracer.

Results

Reliable and quantitative methods of 8-oxoguanine detection in DNA require internal standards with similar chemical properties to 8-oxoGua. In the case of the GC/MS method, we have synthesized a stable isotopically labeled derivative (*18*) which has been employed for isotope dilution mass spectrometry. In the case of the HPLC-EC method, we have used $(NH_2)_2$-OH-Pur which is chemically and electrochemically similar to 8-oxoGua as an internal standard (Figure 1). Both standards were added to DNA prior to acid hydrolysis.

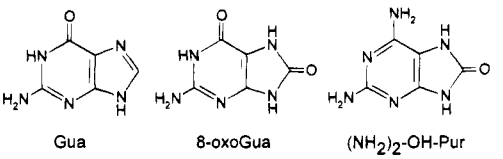

Figure 1. Chemical structures of Gua, 8-oxoGua, and $(NH_2)_2$-OH-Pur.

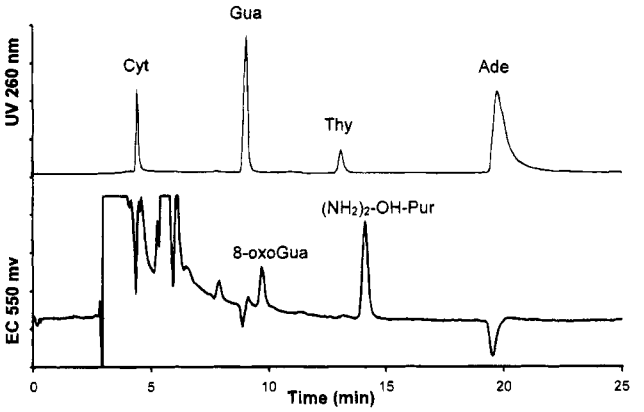

Figure 2. HPLC-EC/UV chromatogram of 50 μg of hydrolyzed calf thymus DNA, with the UV profile (260 nm) showing hydrolyzed DNA bases and the EC profile showing 8-oxoGua and the internal standard $(NH_2)_2$-OH-Pur.

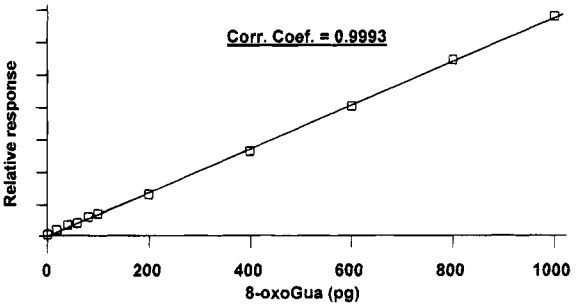

Figure 3. HPLC-EC calibration curve of 8-oxoGua determination using 500 pg of 2,6-diamino-8-oxopurine ($(NH_2)_2$-OH-Pur) as the internal standard.

8-OxoGua Determination by HPLC-EC. For the calibration curve, 8-oxoGua and $(NH_2)_2$-OH-Pur were dried under vacuum and then treated with formic acid under conditions that mimic DNA hydrolysis. There was no evidence of decomposition of either product when subjected to such acid hydrolysis. Typically, after formic acid hydrolysis, the pure standards or the nucleobases resulting from the formic acid hydrolysis of DNA were dried under vacuum and dissolved in 50 μL of the HPLC buffer prior to injection onto a C-18 reverse-phase column connected to the amperometric detector. 8-OxoGua and $(NH_2)_2$-OH-Pur elute at 10 and 14 min, respectively (Figure 2), under the conditions used. The calibration curve (Figure 3), obtained using varying amounts of 8-oxoGua and 500 pg of $(NH_2)_2$-OH-Pur, was found to be linear from 10 pg to 1 ng of 8-oxoguanine injected (correlation coefficient = 0.9993).

8-OxoGua Determination by GC/MS. The detection of 8-oxoGua and its isotopically labeled 8-oxoGua (M +

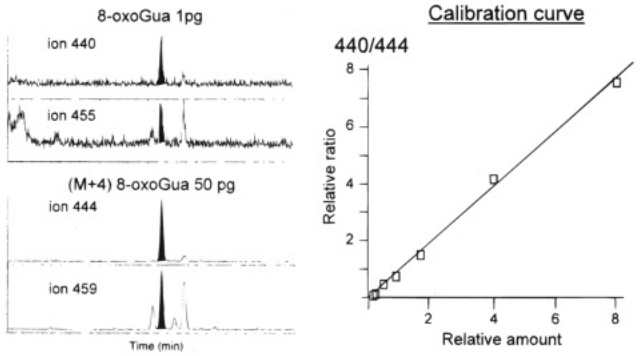

Figure 4. GC/MS (SIM) chromatograms and 8-oxoGua calibration curve using (M + 4) 8-oxoGua (50 pg injected) as the internal standard.

Table 1. Background Levels of 8-OxoGua[a] in Calf Thymus DNA

8-oxoGua (pg/μg of DNA)	HPLC-EC[b]	GC/MS[c]	HPLC GC/MS[d]
DNA	8.9 (±0.9)	405.7 (±22.1)	7.6 (±0.6)
spiked DNA[e]	99.5 (±4.8)	490.2 (±25.9)	109.7 (±7.9)

[a] Values, given in pg of 8-oxoGua/μg of DNA, represent the average ± the standard deviation of three independent determinations. [b] HPLC-EC determination. [c] GC/MS determination. [d] GC/MS determination after HPLC purification of 8-oxoGua prior to the derivatization reaction. [e] The DNA samples were spiked with 100 pg of 8-oxoGua/μg of DNA prior to hydrolysis.

4) was performed in the electron ionization (EI) mode. The mass spectra of tetra-trimethylsilylated 8-oxoGua and of the isotopically labeled internal standard were previously described (*18*). Except for the fragment corresponding to the TMS group (ion 73), two predominant ions, corresponding to the molecular ion (M^+) and the characteristic $(M - 15)^+$ fragment, were detected for both compounds that elute together at 11.2 min (*18*). The ions 440, 455 for 8-oxoGua and 444, 459 for 8-oxoGua (M + 4) were used to detect the modified bases in the SIM mode (Figure 4). The calibration curve of the isotopic dilution mass spectrometric assay, represented in Figure 4, was obtained using an internal standard concentration of 50 pg/μL. The limit of the detection of the assay approaches 1 pg of 8-oxoguanine injected, and the calibration curve is linear up to 1 ng (correlation coefficient = 0.999).

Comparative Analysis of 8-OxoGua by HPLC-EC and GC/MS. The two methods were used in order to determine if the background levels of 8-oxoGua measured in DNA were the same. The amount of 8-oxoGua determined by using either the HPLC-EC or GC/MS assay is represented in Table 1. In order to check the accuracy of the method, the determination of 8-oxoGua was also done after spiking of DNA with 100 pg of 8-oxoGua/μg of DNA, the results are also presented in Table 1. In the last column of the table are also presented the results obtained by GC/MS after HPLC purification of 8-oxoGua prior to the derivatization reaction. The results show that the background level of 8-oxoGua in calf thymus DNA determined by GC/MS is approximately 50 times higher than that recorded by HPLC-EC. However, the estimated background levels are the same for both methods if, for GC/MS analysis, 8-oxoGua is purified by HPLC prior to the derivatization reaction.

Artifact Formation during the Derivatization Reaction. In order to determine if the discrepancy in results between the two analytical methods was attributed to 8-oxoGua formation during the high temperature derivatization reaction, the amount of 8-oxoGua in Gua was determined by GC/MS after increasing time periods of derivatization. The amount of 8-oxoGua in Gua was found to increase almost linearly with the time of derivatization (Figure 5), suggesting an artifactual formation of 8-oxoGua during the silylation reaction. In addition, purging the solution with nitrogen reduced, but did not completely suppress, the artifactual formation of 8-oxoGua (Figure 5), confirming the oxidation of Gua during the derivatization.

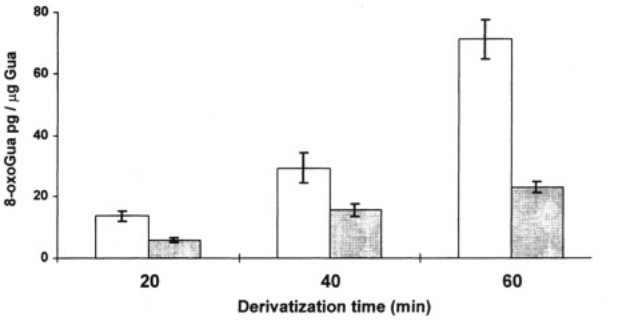

Figure 5. Influence of the derivatization reaction time on the level of 8-oxoGua (in pg/μg of Gua) detected in Gua by GC/MS. The silylation mixture was purged (shaded columns) or nonpurged (open columns) with nitrogen prior to heating at 130 °C for 30 min.

Table 2. Specificity of the Monoclonal Antibody Clone 3F10[a]

Mab 3F10	PBS[b] (%)	H_2O (%)	MeOH 1 (%)	MeOH 2 (%)
dGuo	99	0	0	0
8-oxodGuo	29	0	69	2
Gua	89	5	1	0
8-oxoGua	15	1	82	1

[a] The specificity of the Mab was determined using 500 ng (30 mCi/mmol) of the appropriate radiolabeled tracer in PBS buffer. The results are given in percentage of the radioactivity collected into the different fractions. [b] The column was washed with 2.5 mL of PBS, followed by water and methanol, respectively. The column was then washed a second time with 2.5 mL of MeOH (MeOH 2) in order to ensure that no compound remained bound to the Mab.

Specificity of Monoclonal Antibody toward 8-OxoGua and Its Analogues. The specificity of the monoclonal antibody clone 3F10 toward 8-oxoGua and purine analogues was checked by the ability of the Mab linked to the Sepharose gel to bind radiolabeled tracers of 8-oxoGua and 8-oxodGuo, as well as dGuo and Gua. The results are represented in the Table 2. The C8 oxidized nucleobase (8-oxoGua) and nucleoside (8-oxodGuo), which bind to the Mab, were eluted in the methanolic fraction. It was unexpected that 8-oxoGua bound to the Mab because it was not recognized by this antibody in ELISA.[2] One the other hand, the immobilized Mab does not bind Gua, dGuo (Table 2), and also $(NH_2)_2$-OH-Pur, the internal standard used for the HPLC-EC assay (as determined by HPLC-EC analysis of the different fractions), and thus they elute in the PBS fraction. Therefore, the 6,8-oxo moiety is the critical epitope center for this Mab, and immunoaffinity chromatography could be used to purify both 8-oxoGua and 8-oxodGuo from nonoxidized nucleobases after formic acid hydrolysis of DNA or from biological fluids.

Methodology for the Determination of 8-OxoGua in DNA. The background level of 8-oxoGua in freshly

[2] L. Trudel, unpublished observation.

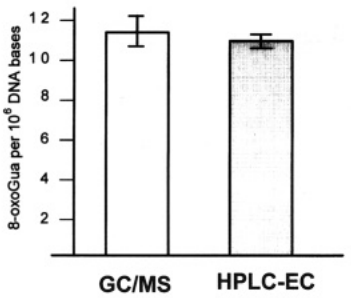

Figure 6. Level of 8-oxoGua in calf thymus DNA determined either by HPLC-EC or by GC/MS assay after immunoaffinity chromatography purification of the modified DNA base. Results, in number of 8-oxoGua residues per 10^6 DNA bases, represent the average and standard deviation of 3 independent determinations.

dissolved calf thymus DNA was determined by HPLC-EC and also by GC/MS. For the GC/MS assay, 8-oxoGua was purified from DNA hydrolysate by immunoaffinity chromatography prior to the silylation reaction in order to avoid the artifactual oxidation of Gua during the derivatization. Using both methods, the background level of 8-oxoGua in DNA was found to be close to 10 residues of 8-oxoGua per 10^6 DNA bases (Figure 6).

Discussion

The growing interest and the need to measure oxidative DNA damage both in vitro and in vivo demand that the analytical methods employed must be accurate and reproducible. The major problem associated with the measurement of oxidative DNA damage is the possibility of artifactual formation of DNA oxidation products during the isolation of DNA and during the analyses of 8-oxoGua. Most of the recent analytical methods on the determination of 8-oxoGua in DNA have employed either the HPLC-EC or the GC/MS techniques. As reported recently (*23*), the two methods do not provide comparable levels of oxidized DNA base formation, particularly for 8-oxoGua. For example, the background level of 8-oxoGua in commercial calf thymus DNA as determined by HPLC-EC has been reported to range between 8 and 70 8-oxodGuo/10^6 DNA bases (*31*–*33*), compared to 159–318 8-oxoGua/10^6 DNA bases measured by the GC/MS techniques (*34*–*36*).

The discrepancies between the two analytical methods may in part be attributed to the choice of the DNA hydrolysis method. An enzymatic digestion procedure usually has been used prior to the detection of the modified DNA bases by HPLC-EC. An advantage of the enzymatic hydrolysis method, over acid hydrolysis of DNA, is that the retention time of the nucleosides is longer than that of the free bases on the reverse-phase column, which facilitates the separation of the modified and unmodified DNA nucleosides. However, enzymatic hydrolysis and recovery of 8-oxoGua in DNA may not be complete (*37*, *38*). On the other hand, acid hydrolysis is preferred when the detection of the modified DNA base is performed using GC/MS because the free base is less polar. In order to bypass this problem of evaluating different analytical methods to measure 8-oxoGua formation with different hydrolysis schemes, identical DNA hydrolysis conditions were employed in this study to detect 8-oxoGua either by HPLC-EC or by GC/MS. Formic acid hydrolysis was chosen, and the HPLC-EC assay was developed for the detection of the free base 8-oxoGua.

When formic acid-hydrolyzed DNA was derivatized with BSTFA without prior purification of 8-oxoGua, the level of 8-oxoGua detected in commercial calf thymus DNA, as determined by GC/MS, was found to be 50-fold higher than that obtained by HPLC-EC (Table 1). The spiking experiment with 8-oxoGua demonstrated that the two methods are quantitative; the recovery of added 8-oxoGua is close to 100%. Therefore, the discrepancy between 8-oxoGua determination in the two methods could not be due to an underestimation of the level of 8-oxoGua by the HPLC-EC assay. In addition, the level of 8-oxoGua determined by GC/MS, after prepurification of the modified base by HPLC prior to the derivatization reaction, is similar to that obtained using the HPLC-EC assay. Therefore, it appears that the GC/MS assay results in an overestimation of the amount of 8-oxoGua in DNA. To confirm the origin of the artifactual formation of 8-oxoGua, the amount of 8-oxoGua in pure guanine was determined by GC/MS. The level of the oxidized base measured increases as a function of derivatization time (Figure 5). Such an increase is due to an oxidation of Gua under the conditions used for the derivatization. In addition, purging the derivatization solution with nitrogen decreases, but does not fully suppress, the amount of 8-oxoGua formed (Figure 5).

Artifactual formation of 8-oxoGua can be minimized by purification of the modified base prior to the derivatization reaction. Immunoaffinity chromatography is the method of choice for such purification. Monoclonal antibodies raised against 8-oxoGua were developed and bound to a Sepharose gel. The amount of 8-oxoGua in freshly dissolved calf thymus DNA was then determined either by HPLC-EC or by GC/MS after prepurification of the lesion. The results, representing the average and standard deviation of 3 independent determinations, are shown in Figure 6. No significant differences are noted between the results obtained by the two different analytical techniques.

It is important to note that, even if an oxidation occurs during derivatization, the GC/MS method is quantitative, as demonstrated by spiking DNA with a known amount of 8-oxoGua (Table 1). The previous studies, done by GC/MS without prepurification of 8-oxoGua, may have overestimated the true amount of 8-oxoGua in DNA. However, the differences in the levels of 8-oxoGua observed between controls and treated samples should not be due to the artifactual oxidation of guanine during the derivatization, assuming that the samples were treated in an identical fashion. It is also important to emphasize that the silylation reaction performed under nitrogen atmosphere significantly reduces the amount of artifactual 8-oxoGua formed. However, interpretation of the results obtained with the two different analytical methods could give rise to opposing conclusions as inferred from the literature. For example, using HPLC-EC, Nagashima *et al.* (*39*) have determined that the overall levels of 8-oxoGua in breast cancer cells are not different from those of surrounding normal tissues. These results are in contradiction to those obtained by Malins *et al.* (*40*) using GC/MS. As suggested by the first authors, the differences in 8-oxoGua levels reported may be due to the artifact formation of 8-oxoGua during the derivatization (and also 8-hydroxyadenine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine). However, the methods to purify DNA in the two studies were different, and discrepancies in results could also be due to oxidation processes which occur during DNA isolation.

It seems important to determine if artifactual oxidation occurs to other DNA bases during the derivatization process. Preliminary work in our laboratory indicates that an oxidation takes place when adenine is derivatized, generating 8-oxoadenine.[3] This oxidation of purine nucleoside during the derivatization reaction has recently been observed by another group, which has also detected an artifactual oxidation of pyrimidines.[4] Discrepancies have also been reported between the results of the background level of some pyrimidine oxidation products determined either by GC/MS or by HPLC-EC. The steady state level of the modified pyrimidines 5-hydroxycytosine and 5-hydroxyuracil determined by Wagner *et al.* (*41*) using an HPLC-EC method was found to be 8-fold lower then the values recorded by GC/MS (*36*). Further work is necessary in order to establish if an artifactual oxidation of other DNA bases also occurs during the derivatization reaction.

The HPLC-EC method is a suitable method for the detection of 8-oxoGua in DNA; and the internal standard $(NH_2)_2$-OH-Pur increases the accuracy of the method. When available, a GC/MS assay used in combination with immunoaffinity chromatography represents an alternative way to increase the specificity and reliability of 8-oxoGua detection.

For the in vivo detection of 8-oxoGua in DNA, the method which is used to purify DNA from other cell constituents is also very important (*42*). Factors which may result in artifactual formation of 8-oxoGua in DNA, such as extraction using pro-oxidative agents (phenol/chloroform extraction), should be avoided because they can induce, under certain conditions, DNA oxidation (*43*). The different methods to hydrolyze DNA should also be compared in order to determine the most convenient method (acid or enzymatic hydrolysis) which does not produce artifactual oxidation. Work is in progress in order to develop such a methodology for monitoring the formation of other oxidized nucleosides in DNA or in biological fluids. With established analytical methods, it will be possible to determine if compounds such as 8-oxoGua can be used as biomarkers of in vivo oxidation. Such techniques may then be employed to assess the pro- or anti-oxidant properties of foods and beverages, and their impact on human health.

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