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Original Contribution

IMPORTANCE OF COMPLETE DNA DIGESTION IN MINIMIZING VARIABILITY OF 8-oxo-dG ANALYSES

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Abstract—Estimates of 8-oxo-2′-deoxyguanosine (8-oxo-dG) in DNA vary at least one order of magnitude using different quantitative methods or even the same method. Our hypothesis is that an incomplete DNA hydrolysis to nucleosides by the conventional nuclease P1 (NP1) and alkaline phosphatase (AP) digestion system plays an important role in contributing to the variability of measurements using HPLC coupled with UV and electrochemical (EC) detection. We show here that factors, such as the amount of DNA, choice of enzymes, their activities, and incubation time, can affect DNA digestion and, thus, cause variability in 8-oxo-dG levels. The addition of DNase I and phosphodiesterases I and II to the NP1 + AP system improves the DNA digestion by completely releasing normal nucleosides and 8-oxo-dG, thereby reducing the interday variations of 8-oxo-dG levels. Diethylenetriamine pentaacetic acid (DTPA), an iron chelator, prevented background increases of 8-oxo-dG during DNA digestion, as well as during the waiting period in the autosampler when a batch of DNA samples is analyzed by HPLC. After optimization of the DNA digestion conditions, the interday variability of 8-oxo-dG measurements using commercially available salmon testes DNA (ST DNA) were 26% over a period of 2 years. Under these optimal conditions, our laboratory variability may contribute as little as 13% to the overall variability as shown by assessment of oxidative DNA damage in a population of smokers. Based on our results, we believe that the modified DNA digestion conditions will provide much more accurate 8-oxo-dG determinations and, thus, more reliable estimates of cancer risk. © 2001 Elsevier Science Inc.

Keywords—8-oxo-dG, Oxidative DNA damage, Cancer risk, Biomarker, Enzymatic DNA digestion, Free radicals

INTRODUCTION

Damage to DNA by oxygen radicals occurs in vivo despite the presence of multiple antioxidant defenses and DNA repair systems. Increasing evidence indicates that such damage contributes to the age-related development of cancer [1–3]. Among many oxidatively damaged DNA bases, 8-oxo-2′-deoxyguanosine (8-oxo-dG) is the most investigated because of its mutagenicity and the high sensitivity of its measurement [4]. Indeed, 8-oxo-dG levels in target tissues are correlated with the incidence of some cancers in human and animal models [5,6]. Therefore, 8-oxo-dG in various bio-specimens is considered as an appropriate biomarker of cancer risk.

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Various techniques exist for the detection of 8-oxo-dG. The three most commonly used methods are: (i) high-performance liquid chromatography (HPLC) coupled with electrochemical (EC) detection, (ii) gas chromatography (GC) coupled with mass spectrometry (MS), and (iii) ^{32}P- or ^{3}H-postlabeling [7–9]. Most laboratories studying DNA oxidation use HPLC-EC analysis of enzymatically hydrolyzed DNA to examine levels of 8-oxo-dG, but steady state levels reported from different laboratories, ostensibly using the same method, vary over almost an order of magnitude [4,10,11].

The goal of the present study was to optimize enzymatic DNA digestion conditions using two parameters as criteria: (i) maximal release of normal nucleosides and 8-oxo-dG per μg DNA, and (ii) minimize oxidation of dG and DNA during sample preparation and handling. After complete DNA digestion, we set out to quantitate and minimize the intra- and interday variations in the levels of 8-oxo-dG per 10^6 dG or per 10^6 total nucleo-

sides (N), so that the ratio of 8-oxo-dG/10^6 dG or N can be used as a reliable biomarker for molecular epidemiology studies.

In the present study, we have shown that incomplete DNA digestion significantly contributes to the variability in the 8-oxo-dG levels, particularly under the conventional method using nuclease P1 (NP1) and alkaline phosphatase (AP) digestion conditions. NP1 + AP are probably not efficient in releasing 8-oxo-dG from small oligonucleotides having a pyrimidine residue next to it. We find that using DNase I at the beginning and phosphodiesterases (PDE) I and II at the end of the DNA digestion processes, 8-oxo-dG can be much more efficiently released from DNA than when only NP1 + AP are used. Our results suggest that the modified DNA digestion conditions can significantly improve accuracy and reproducibility of these analyses.

MATERIALS AND METHODS

Materials

Deoxyribonucleic acid from Salmon testes (ST DNA) in a form of sodium salt was purchased from Sigma Chemical Co. (St. Louis, MO, USA) at two different times. One DNA sample obtained approximately 5 years ago was dissolved in HPLC water (Milli Q, 18 MΩ), aliquoted, and stored at −20°C. This DNA was used for most of the experiments in this study. The same type of DNA was purchased from the same vendor towards the end of the study period and used to compare the background levels of 8-oxo-dG with that of the 5 year old ST DNA. DNA isolated from various tissues was used for comparison. DNase I, NP1, AP, PDE I and II, DNase-free RNase, and proteinase K were purchased from Roche Molecular Bio-Chemicals (Indianapolis, IN, USA). Chloroform, isoamyl alcohol, isopropanol, $MgCl_2$, $ZnSO_4$, NaCl, dithiothreitol (DTT), diethylenetriamine pentaacetic acid (DTPA), ethylenediamine tetraacetic acid (EDTA), and deferoxamine (DFO), each of the highest purity available, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade Tris · HCl, sodium acetate, acetonitrile, and methanol were from Fisher Scientific (Pittsburgh, PA, USA).

Preparation of enzyme solutions: DNase I from bovine pancreas (20,000 units) was dissolved in 1 ml containing 20 mM Tris · HCl, pH 7.4, 1 mM $MgCl_2$, and 50% (v/v) glycerol, aliquoted (50 μl) in Ependorff tubes, and stored at 4°C (20 units/μl). NP1 from *Penicillium citrinum* (1 mg with approximately 1000 units of 3′-phosphomonoesterase activity) was dissolved in 100 μl 20 mM sodium acetate buffer (pH 5.2), aliquoted in 10 μl, and stored at −20°C. Upon use, NP1 was further diluted 10 times to a final concentration of 1 U/μl in the above acetate buffer. AP from calf intestine (1 U/μl) was stored in 25 mM Tris · HCl (pH 7.6), 1 mM $MgCl_2$, and 50% glycerol (w/v). Upon receipt, AP was aliquoted and stored at 4°C. PDE I from snake venom (*Crotalus durissis*) and PDE II from calf spleen were dissolved in 10 mM Tris · HCl, pH 8.0, at a concentration of 0.01 U/μl and stored at −20°C. PDE I from other vendors or sources contains adenosine deaminase, causing deamination of dA to deoxyinosine. All enzymes were replaced after 6 months storage or sooner if incomplete DNA digestion occurred using 100 μg ST DNA, as shown by HPLC profiles.

Preparation and storage of standards

Normal nucleosides (dC, dG, dT, and dA) were weighed and dissolved in HPLC-grade water at a concentration of 5 mM except for dA, which was used at 2.5 mM. One ml each of dC, dG, dT, and 2 ml dA were mixed to have a final concentration of 1 mM of nucleosides. The mixture was aliquoted in 200 μl, dried under vacuum (SpeedVac, Savant, Holbrook, NY, USA), and stored at −20°C. 8-oxo-dG obtained from Sigma Chemical Co. (approximately 1 mg) was dissolved in 1 ml HPLC water. The 8-oxo-dG concentration was determined by measuring the absorbance at 248 nm and divided by the extinction coefficient of 12,300. After a series of dilutions, 100 μl aliquots (final concentration 1 μM) were dried and stored at −20°C. Upon use of the standards for a standard curve, one vial of 8-oxodG was dissolved in 1 ml HPLC grade water and nucleosides were dissolved in 200 μl HPLC grade water. Equal volumes of 8-oxo-dG and nucleosides were mixed, which gave final concentrations of nucleosides at 0.5 mM and 8-oxo-dG at 0.05 μM. Five, 10, 20, and 40 μl standards (2.5–20 nmoles nucleosides and 0.25–2 pmoles 8-oxo-dG, respectively) were used in a batch and injected in between samples to serve as a standard curve. If 8-oxo-dG background is expected to be low or high in the DNA samples to be analyzed (e.g., DNA from cultured cells or DNA from smokers, respectively), 0.025 or 0.1 μM 8-oxodG may be used as standards.

Various enzymatic DNA digestion conditions

Three DNA digestion protocols were used in the present study, as follows: (i) NP1 + AP, (ii) DNase I + NP1 + AP, and (iii) DNase I + NP1 + AP + PDE I + PDE II. Completeness of DNA digestion was evaluated using the amounts of normal nucleosides (nmoles) and 8-oxo-dG (pmoles) released per μg DNA and the extent of DNA damage as the molar ratio of 8-oxo-dG/10^6 dG or N.

Most of the DNA digestion protocols were carried out with 100 μg DNA in a total volume of 113 μl during 2.5 h incubation at 37°C. One exception was when various concentrations of DNA were used to test for NP1 and AP (1 U each) digestion conditions, different volumes were used for the given 100 μg DNA. After digestion, the reaction mixtures were filtered through Ultrafree-MC membrane (nominal molecular weight limit 5,000, Millipore, Bedford, MA, USA) by centrifugation (9,000 rpm, Marathon MicroA Centrifuge, Fisher Scientific) to remove enzymes before injection to the HPLC column. The following digestion conditions were used for NP1 + AP: 20 μl 5 μg/μl of ST DNA (100 μg total) were diluted to 100 μl with HPLC grade water. After acidification with 1 μl 3 M acetate buffer (pH 5.2), DNA was first digested with 1 μl 1 U/μl NP1. After 1.5 h incubation, pH of the reaction mixture was increased to pH 7.4 by adding 10 μl 1 M Tris · HCl buffer (pH 8), followed by 1 μl 1U/μl AP for an additional 1 h. Factors such as single- and double-strand DNA, pH of the medium (pH 5.2; water; pH 7.4 or 8.0), Zn^{2+} (as a cofactor of NP1), various amounts of enzymes (NP1 or AP), and DNA incubation time from 2.5 h to 24 h were tested under these conditions. DNA hydrolysates were stored at −20°C for various times in the presence or absence of DTT to check the stability of 8-oxo-dG during storage.

DNase I + NP1 + AP digestion conditions: 20 μl 5 μg/μl of ST DNA (100 μg total) were diluted to 87 μl HPLC water. After addition of 10 μl 100 mM $MgCl_2$ and 1 μl 1 M Tris · HCl (pH 7.4) with or without 100 mM NaCl (final concentration), 2 μl 20 U/μl DNase I were added for 0.5 h incubation at 37°C. After adjusting the pH to 5.2 with 1 μl of 3 M sodium acetate (pH 5.2), the fragmented DNA was digested with 1 μl of NP1 (1 unit/μl) for 1 h. After bringing the acidic pH back to neutral with 10 μl of 1 M Tris · HCl (pH 8.0), 1 μl of AP (1 unit/μl) was added, followed by 1 h incubation.

DNase I + NP1 + AP + PDE I + PDE II digestion conditions: the protocol is almost the same as for DNase I + NP1 + AP except shortening AP incubation time from 1 h to 0.5 h. Finally, 1 μl each of PDE I and II (0.01 U/μl each) were added to the reaction mixture for an additional 0.5 h to ensure the completeness of DNA digestion.

HPLC analysis

Twenty μl DNA hydrolysates (corresponding to 17.7 μg DNA) were analyzed by HPLC coupled with UV and EC detectors. The HPLC system (Beckman Coulter Inc., Fullerton, CA, USA) consists of an autosampler (Model 507e), 2 pumps with 4-way solvent programming (Model 126), and a programmable UV/VIS detector (Model 166). A Coulochem II EC detector (ESA Inc., Chelmsford, MA, USA) is linked to the system. Data from both UV and EC detectors were acquired by Gold Nouveau Software (Beckman Coulter Inc., version 1.6), which was installed on an IBM computer. The HPLC column used was a Rainin ODS C_{18} protected by an Ultrasphere ODS guard column. The mobile phase consisted of 6% aqueous methanol containing 50 mM sodium acetate buffer (pH 5.2) and used at 1 ml/min flow rate. Normal nucleosides (dC, dG, dT, dA) were detected by the UV absorption at 254 nm and 8-oxo-dG was monitored with the Coulochem II EC detector (ESA Inc.) using the analytical cell 1 (at 400 mV and 20 nA). The Coulochem II EC detector (ESA Inc.) consists of a guard cell, a conditioning cell, and two analytical cells. The guard cell (set at 850 mV) is placed between the pump and the injector and is used to oxidize electroactive materials in the mobile phase. The conditioning cell (set at 100 mV) is placed immediately before the analytical cells to provide additional selectivity. Levels of normal nucleosides and oxidized nucleosides were quantified using the standard curves of each corresponding compound. The degree of DNA damage was expressed as 8-oxo-dG per 10^6 of dG or 10^6 total normal nucleosides (N).

DNA isolation

To validate our optimal DNA digestion protocol, DNA isolated from human breast epithelial MCF-10A cells grown in cell culture and rat liver was subjected to the enzymatic digestion. Rat liver samples were first ground under liquid nitrogen. After that, cells (25×10^6), or liver (100–200 mg) samples were repeatedly washed in 3 ml ice-cold nuclei isolation solution [10 mM Tris · HCl, pH 8.0, 1% (v/v) Triton X-100, 0.32 M sucrose, 0.2 mM EDTA, 0.1 mM DTPA, and 5 mM $MgCl_2$] to remove cytoplasm and red blood cells (RBC) (at least two times for MCF-10A cells and three to five times for liver samples). Addition of DTPA was intended to chelate iron released from RBC or cytoplasm, thus avoiding possible spurious DNA oxidation. The nuclei were suspended in 1.5 ml RNase solution (10 mM Tris · HCl, pH 8.0, 5 mM EDTA, and 0.1 mM DTPA). One μl DNase-free RNase was then added and the mixtures were incubated at 37°C for 0.5 h. The nuclei suspension was then mixed with 1.5 ml cell lysis solution [10 mM Tris · HCl, pH 8.0, 1% (v/v) SDS, 5 mM EDTA, and 0.1 mM DTPA]. Proteinase K (20 μl of 20 μg/μl) was added and incubated at 55°C for 1 h, followed by an additional 10 μl proteinase K and incubation for 2 h more.

After cooling the solution on ice, 1 ml protein precipitation solution (Puregene, Gentra Systems, Minneapolis, MN, USA) was added and shaken vigorously. Precipitated protein was removed by centrifugation and the

Table 1. Standard Intra- and Interday Variation and Laboratory Coefficient of Variations (COV) at Different Concentrations (4 Injections/Concentration)

	dG			8-oxo-dG		
[C]	Mean	Standard deviation	COV (%)	Mean	Standard deviation	COV (%)
Standard intra-[a]	724737	12786	1.8	281715	4337	1.5
Standard inter-						
1[b]	92348[c]	2619	2.8	96223	2845	3.0
2	176530	6673	3.8	177067	6951	3.9
3	346776	11097	3.2	358194	22870	6.4
4	692512	25969	3.7	707523	40087	5.7

[a] 20 nmoles dC, dG, dT, dA and 2 pmoles 8-oxo-dG with Coulochem II EC detector running at 50 nA.
[b] 2.5 nmoles dC, dG, dT, dA, and 0.25 pmoles 8-oxo-dG were injected all together for concentration [C] 1; 5 nmoles dC, dG, dT, dA, and 0.5 pmoles 8-oxo-dG for [C] 2; 10 nmoles dC, dG, dT, dA, and 1 pmoles 8-oxo-dG for [C] 3; and 20 nmoles dC, dG, dT, dA, and 2 pmoles 8-oxo-dG for [C] 4. EC was set at 20 nA.
[c] Units in the Table as the height of the peak were the actual reading by HPLC using the Gold Nouveau software (Beckman Coulter Inc.).

supernatants were extracted with equal volume of choloform:isoamyl alcohol (24:1 v/v). The upper aqueous phase was treated again with 1 μl DNase-free RNase for 15 min at 37°C. Elimination of RNA is very important in enhancing efficiency of enzymatic digestion and in obtaining clean HPLC profiles detected by both UV and EC. The aqueous phase was extracted once more with equal volume of choloform:isoamyl alcohol. DNA was precipitated in the aqueous phase with equal volume of ice-cold isopropanol and stored at −20°C overnight. After centrifugation, DNA was washed twice with 70% ethanol and dissolved in HPLC grade water for immediate enzymatic digestion after measuring the ratio of 260/280.

RESULTS

Intraday and interday variability of the standards

Various amounts of normal nucleosides and 8-oxo-dG were injected by an autosampler (Beckman Coulter Inc., Model 507e) with a μl pickup mode. Table 1 shows that the intraday coefficients of variation for standard dG and 8-oxo-dG were 1.8% and 1.5% ($n = 4$), respectively. The interday coefficients of variation for four different doses of dG and 8-oxo-dG injected varied from 2.8% to 6.4% over a period of 2 weeks ($n = 4$, 4 injections per dose). The intra- and interday coefficients of variation for dC, dT, and dA were all less than 5% (data not shown). The correlation coefficients for the linearity of the standard curves of each nucleoside including 8-oxo-dG were above 0.99. To eliminate the systematic errors (e.g., decreased sensitivity or shift in the retention time), standards containing dC, dG, dT, dA, and 8-oxo-dG at four different amounts were interspersed with other samples in each batch to construct the standard curve for that batch. This allowed more accurate quantitation of DNA samples being evaluated, and, sometimes, easy identification of 8-oxo-dG peak (through chromatography overlap with standards) in case that multiple peaks appeared in EC chromatogram (e.g., often the case with DNA of smokers, data not shown).

Effects of DNA concentration, amount of DNA, and enzyme activities on DNA digestion

Enzymatic digestion of DNA depends on the enzyme activity, the amount of DNA used for the enzymatic digestion, DNA concentration, and the incubation time. Incomplete enzymatic DNA digestion probably contributes to the interlaboratory variations and inter- and intraday variability within the same laboratory. Figure 1 shows the UV and EC separation profiles of normal nucleosides (UV) and 8-oxo-dG (EC), illustrating the difference in those profiles when ST DNA (100 μg) was used at a concentration of 5 or 0.5 μg/μl for the digestion with NP1 (1 unit) for 1.5 h at pH 5.2, followed by AP (1 unit) for 1 h at pH 7.4. At 5 μg/μl of DNA, NP1 + AP released 10% lower amounts of 8-oxo-dG (peak height comparing to that of 0.5 μg/μl), and 31.0, 2.3, 31.1, and 9.4% lower release of dC, dG, dT, and dA, respectively. It points to the effect of DNA concentration on the nucleoside release, and particularly to the impairment in the release of pyrimidines. Figure 2 shows 8-oxo-dG release when various DNA concentrations (0.2, 0.5, 1.0, 2.0, and 5.0 μg/μl) were tested under NP1 and AP conditions. As judged by the release of normal nucleosides (nmoles/μg DNA), NP1 and AP (1 unit each) produced approximately the same amounts of nucleosides for up to 2 μg/μl of DNA, and then exhibited a 22.9% drop in the total release of normal nucleosides (N) when 5 μg/μl DNA was used for the assay (Fig. 2A). However, if judged by the release of 8-oxo-dG, 0.5

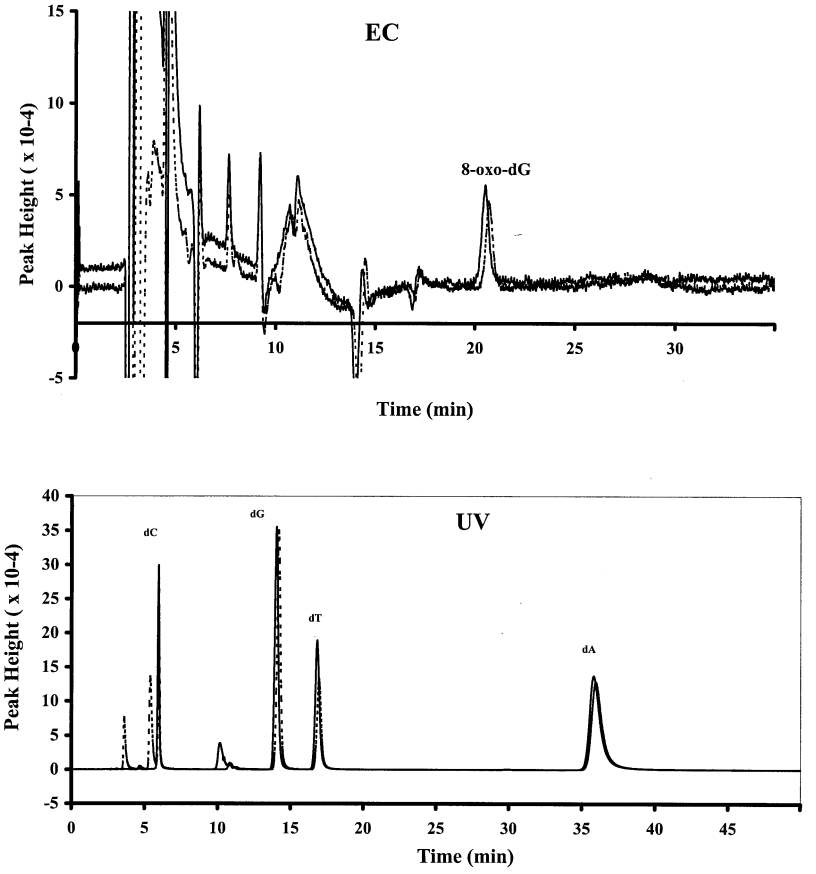

Fig. 1. Separations of enzymatically digested ST DNA at 0.5 and 5 μg/μl by NP1 and AP. The dotted line in the UV spectrum shows incomplete DNA digestion at a concentration of 5 μg/μl, resulting in less amount of 8-oxo-dG released as shown in the EC spectrum. The same amounts of DNA were injected for both analyses.

μg/μl DNA gave the highest yield (79 pmoles/mg DNA) (Fig. 2B).

To test the completeness of DNA digestion as a function of enzyme activity, 100 μg ST DNA at 1 μg/μl were used. Figure 3A shows the results of experiments when NP1 from 0.5 U to 10 U was used while AP was kept constant at 1 U. We found that NP1 from 1, 2.5, 5, to 10 U released the same amounts of normal nucleosides and 8-oxo-dG [average level of 8-oxo-dG/10^6 N: 15.35 $\pm$ 1.18 (SD), $n = 4$]. At 0.5 U NP1, molar ratio of 8-oxo-dG/10^6 N was 9.27, representing only 62% of those at $\geq$ 1 U NP1. By varying the concentrations of AP from 0.2, 0.5, 1, 2, to 4 U and keeping NP1 at 1 U, no significant differences in the release of normal nucleosides and 8-oxo-dG were observed among the five samples (Fig. 3B). Addition of Zn^{2+} (1 mM) to the reaction mixture did not improve the DNA digestion by NP1.

Maintaining the acidic pH (pH 5.2 buffered by sodium acetate) was the key to the effectiveness of NP1 acting on double-stranded DNA. Using single-stranded ST DNA (after boiling 5 min and rapid cooling in ice-water bath), NP1 worked at pH 5.2, pH 6.0 (in water), pH 7.4 or 8.0 (Tris · HCl buffers). Boiling DNA for 5 min increased levels of 8-oxo-dG/10^6 N by 24.6% [20.56 $\pm$ 5.13 (SD), $n = 4$)] (using 2.5 h incubation for NP1 + AP digestion) in comparison to that released from double-stranded ST DNA (16.5 $\pm$ 1.5, $n = 9$). A 24 h incubation of single-stranded ST DNA with NP1 and AP induced a 27.2% increase in the levels of 8-oxo-dG/10^6 N [26.15 $\pm$ 3.53 (SD), $n = 4$] in comparison with 2.5 h incubation. Based on these results, 1 U each of NP1 and AP for a total of 2.5 h incubation were used for digestion of double-stranded DNA up to 100 μg.

Effects of DNase I and phosphodiesterases I and II on DNA digestion

To search for DNA digestion conditions that can release maximal 8-oxo-dG and normal nucleosides with minimal variations among assays and from day to day, DNase I, PDE I, and PDE II were used. Table 2 shows that the addition of DNase I (40 U), an endonuclease that can cut DNA into small oligonucleotides in the presence of Mg^{2+}, increased release of 8-oxo-dG/10^6 dG or N,

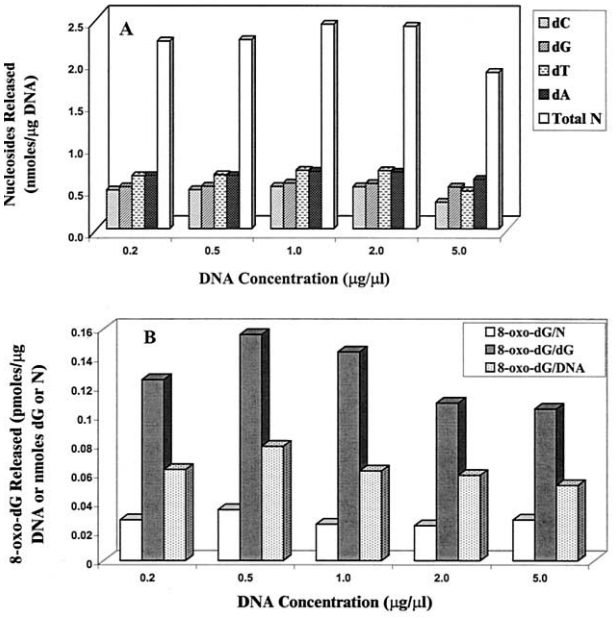

Fig. 2. DNA digestion as a function of DNA concentration. NP1 (1U) + AP (1U) during 2.5 h incubation were used for 100 μg DNA digestion at various concentrations. Data presented are an average of two experiments.

and releases of normal nucleosides per μg DNA. However, those increases were not statistically significant in comparison to those obtained from the same DNA digested by NP1 + AP. Interestingly, adding PDE I and II to the DNase I + NP1 + AP digestion system significantly increased the release of 8-oxo-dG/10^6 dG or total N in comparison to those mediated by DNase I + NP1 + AP ($p < .04$ for 8-oxo-dG/10^6 dG or $p < .002$ for 8-oxo-dG/10^6 N) and NP1 + AP ($p < .02$ for 8-oxo-dG/10^6 dG or $p < .00008$ for 8-oxo-dG/10^6 N). Releases of normal nucleosides were also increased as compared to those mediated by NP1 + AP, approaching statistical significance ($p < .09$).

PDE I and II are two exonucleases that attack the 3′- and 5′-terminal OH-groups, releasing 5′- and 3′-mononucleotides, respectively. These results suggest that DNA digestion with NP1 + AP with or without DNase I may not be complete, and probably yields dinucleotide monophosphates and/or trinucleotide diphosphates, which contain 8-oxo-dG. PDE I and II are likely helping to digest these 8-oxo-dG containing di- or trinucleotides, especially if this oxidized purine is flanked by pyrimidines. Thus, the amount of 8-oxo-dG released was increased about 20% when PDE I and II were included in the DNA digestion mixture. The amount of normal nucleosides was not significantly enhanced because these 8-oxo-dG containing di- or trinucleotides constitute only a very small fraction of normal nucleosides. Most interestingly, due to the completeness of DNA digestion by DNase I + NP1 + AP + PDE I and II, the variability of 8-oxo-dG/10^6 dG or N from day to day over a period of 2 months was the lowest among the three different DNA digestion conditions. A low interday variability would be advantageous in reducing laboratory errors in molecular epidemiological studies when a large number of DNA samples are analyzed over an extended period of time.

Changes of 8-oxo-dG levels during HPLC batch run and DNA hydrolysate storage

A complete HPLC separation required 50 min per sample as shown here or by others [8,12,23]. In epidemiological studies, one batch of samples is often analyzed for 8-oxo-dG in DNA, preferably within the same day. In our experiments, one batch usually contained 20 or more samples including the 4 standards (nucleosides and 8-oxo-dG) and two ST DNA as controls, which would have taken 24 h for HPLC analysis. Figure 4 shows the significant increase in 8-oxo-dG as a function of the waiting time in the autosampler. These results suggest that DNA hydrolysates may contain trace amounts of transition metals, particularly iron, although the highest purity of chemicals and water were used for

Table 2. Comparison of Various DNA Digestions Conditions and Its COV over a Period of Two Months

Enzymes	Nucleosides (nmoles/ μg DNA mean ± SD)	COV	8-oxo-dG/10^6 N (mean ± SD)	COV	8-oxo-dG/10^6 dG (mean ± SD)	COV
NP1 + AP[a]	2.34 ± 0.22 (n = 9)	9.4%	19.30 ± 2.58	13.4%	92.89 ± 19.75	21.3%
DNase I +NP1 + AP[b]	2.42 ± 0.33 (n = 11)	13.6%	20.69 ± 3.00	14.5%	96.68 ± 17.57	18.2%
DNase I + NP1 + AP + PDE I & II[c]	2.47 ± 0.16 (n = 8)	6.47%	24.53 ± 1.53[d]	6.2%	109.35 ± 7.09[e]	6.5%

[a] 100 μg ST DNA were digested with 1 U NP1 for 1.5 h at pH 5.2, followed by 1 U AP for 1 h at pH 8.0 in a total volume of 113 μl. Twenty μl DNA hydrolysates were subjected to HPLC-EC analysis.

[b] 100 μg ST DNA were digested with 40 U DNase I for 0.5 h at pH 7.4, followed by 1 U NP1 for 1 h at pH 5.2, and 1 U AP for 1 h at pH 8.0.

[c] 100 μg ST DNA were digested with 40 U DNase I for 0.5 h at pH 7.4, followed by 1 U NP1 for 1 h at pH 5.2, 1 U AP for 0.5 h at pH 8.0, and 0.01 U each of PDE I and II simultaneously for 0.5 h at pH 8.0.

[d] Significantly different from NP1 + AP ($p < .00008$) or DNase I + NP1 + AP ($p < .002$) by t-test: two samples assuming equal variances.

[e] Significantly different from NP1 + AP ($p < .02$) or DNase I + NP1 + AP ($p < .04$) by t-test: two samples assuming equal variances.

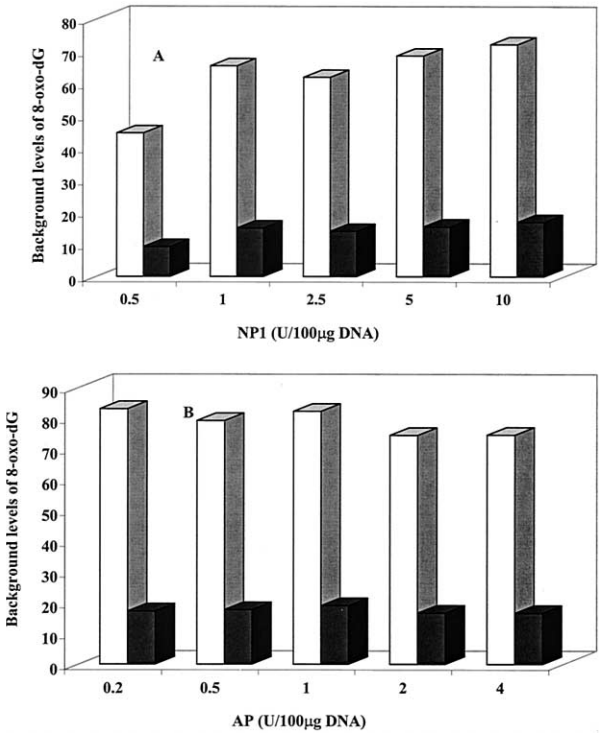

Fig. 3. DNA digestion as a function of enzyme activity. (A) Amount of NP1 was varied while AP was kept constant at 1 unit. (B) Amount of AP was varied while NP1 was kept constant at 1 unit. 100 μg ST DNA were digested with NP1 for 1.5 h at pH 5.2, followed by AP for 1 h at pH 7.4. (□) 8-oxo-dG/10^6 dG; (■)8-oxo-dG/10^6 N. Data presented are an average of two experiments.

DNA digestion. Adding DTPA (20 μM, an iron chelator) into the DNA hydrolysates effectively prevented an artifactual increase in 8-oxo-dG level when HPLC analysis was completed within 24 h. After 120 h of seating in the autosampler, 8-oxo-dG levels were significantly increased with or without DTPA. However, that increase was much higher in the absence of DTPA.

DFO, another iron chelator, was also effective in preventing the background increase of 8-oxo-dG. However, DFO caused a baseline shift evident in an EC chromatogram right before the elution of 8-oxo-dG. Bubbling the hydrolysates with argon in the HPLC vials had no effect. When dG was used instead of DNA following exactly the same steps of DNA digestion conditions, the molar ratio of 8-oxo-dG/10^6 dG was increased in comparison to that obtained when dG was incubated in HPLC water only (data not shown), indicating that the contamination of trace metals originated from ST DNA and/or the enzymes and the salts used for DNA digestion. Addition of 20 μM DTPA prevented such an increase in 8-oxo-dG/10^6 dG. Addition of DTT (0–100 μM) to the DNA hydrolysates for storage at −20°C resulted in big variations. As a result of these findings, DTPA (40 μM final) was added to the DNA digestion mixtures, and

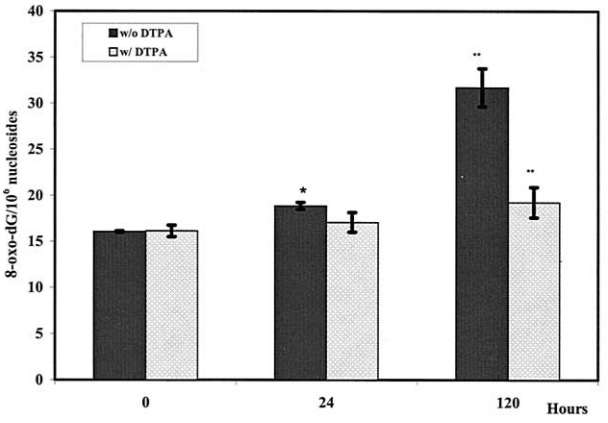

Fig. 4. Increase of 8-oxo-dG levels during the waiting period in the autosampler prior to HPLC analysis and the protective effect of DTPA on the increase. *Significantly different from controls with or without DTPA ($p < .001$) by Student's *t*-test. **Significantly different from controls with or without DTPA ($p < .00001$) by Student's *t*-test.

DNA hydrolysates were analyzed within 24 h, immediately after DNA digestion was completed.

8-oxo-dG levels in DNA from various tissues

To further validate our DNA digestion protocol, DNA was isolated from MCF-10A cells, an immortalized breast epithelial cell line, and rat liver. A new batch of ST DNA was also used expecting that the newly purchased ST DNA may be less oxidatively damaged. Table 3 shows the background levels of 8-oxo-dG in those tissues. Interestingly, oxidative damage in the newly purchased ST DNA was lower [14.0 ± 1.48 (SD), $n = 3$] than in the old batch ST DNA (24.53 ± 1.53), which was used for most of the previous experiments of the present study. The interday variation in the newly purchased ST DNA was within 10.6% ($n = 3$). To check whether there is an artifactual formation of 8-oxo-dG during the DNA isolation procedure, 100 μg ST DNA were added to the RNase solution after nuclei isolation. We found that the average recovery rate of DNA was 86.6 ± 2.6% ($n = 3$), and there was no spurious increase or decrease in 8-oxo-dG levels in the ST DNA comparing to the same DNA without going through the isolation procedure. DNA isolated from MCF-10A was least oxidatively damaged among all samples tested.

The ten independent isolations from the same cell line but different passages revealed an interassay variation of 16.8% and 16.9% as 8-oxo-dG/10^6 N or dG, respectively. Consistent with data published by others [12,13], we confirmed that DNA damage in livers of older rats (22 months old) was higher than in younger rats (3 months old) but the difference was not statistically significant, perhaps due to a small sample size ($n = 3$ for

Table 3. Background Levels of 8-oxo-dG in Various Tissues

Tissue	8-oxo-dG/10^6 dG	8-oxo-dG/10^6 N
OLD ST DNA	109.35 ± 7.09[a] (n = 8)	24.53 ± 1.53 (n = 8)
ST DNA (Newly purchased)	59.72 ± 5.56 (n = 3)	14.00 ± 1.48 (n = 3)
MCF 10A cells	13.76 ± 2.32 (n = 10)	2.97 ± 0.50 (n = 10)
Rat liver DNA (3 months old)	30.81 ± 9.26 (n = 3)	6.77 ± 2.07 (n = 3)
Rat liver DNA (22 months old)	39.04 ± 14.16 (n = 3)	8.53 ± 3.07 (n = 3)

[a] Data are expressed as mean ± SD. DNA was isolated following the protocol described in the Materials and Methods section.

each group). The interindividual variability was higher in old rats (36.0%) than in young rats (30.6%), suggesting that aging contributes to an increased heterogeneity. This variability is high in comparison with the interday variability using ST DNA. These data should be considered as supporting our point of view that minimizing the daily assay variability in the level of 8-oxo-dG/10^6 N due to incomplete DNA digestion is important, if we want to detect the differences in levels of oxidative DNA damage in human populations.

Changes in standards over a period of 3 years and ST DNA over 2 years

A linear relationship between detector response and concentration was demonstrated in the investigated range for 8-oxo-dG (0.125–4 pmoles) and for normal nucleosides (dC, dG, dT, dA, ranging from 2.5 to 20 nmoles). Over a period of three years, over twenty batches of standards were prepared and analyzed by HPLC. In the middle of course, the HPLC column (decreased resolution), the UV lamp in the UV detector (decreased sensitivity), and analytical cells, as well as guard and conditioning cells in the EC detector (noisy background reading) were replaced once. Those changes in analytical capabilities lead to variations in the instrument readings, which were given by the Gold Nouveau software (readings illustrated in Table 1), of the standard nucleosides and 8-oxo-dG.

To eliminate effects of the unforeseen systematic changes, such as decreased sensitivity on the quantitation of 8-oxo-dG in various DNA samples, two ST DNA samples (30 and 100 μg) and four doses of standards were included in the batch with the samples to be analyzed. For example, in a clinical trial study, six to eighteen DNA samples (30 μg DNA/sample) were digested in 113 μl each and 60 μl were injected into HPLC [14]. The 30 μg ST DNA served as a control for DNA digestion conditions. If all enzymes work well, a consistent background level of 8-oxo-dG/10^6 N should be obtained. The 100 μg ST DNA served as a prewarning sample. When the DNA digestion enzymes have decreased activities, this 100-μg DNA sample would first yield additional peaks in the UV profile due to the incompletely digested oligonucleotides.

Table 4 summarizes the variability of background levels of 8-oxo-dG released from ST DNA over a period of 24 months. Thirty-eight independent ST DNA digestions were performed by two investigators. During this period, HPLC column, UV lamp, and analytical cells of EC detector were replaced once. As a result of these changes, the COV was 50% over the 2 year period. For the first 6 months, the same DNA and HPLC system provide a COV of less than 15% with 8-oxo-dG levels

Table 4. Variation of 8-oxo-dG/10^6 dG or N in ST DNA over 24 Months

Experimental conditions	8-oxo-dG/10^6 dG (mean ± SD)	COV (%) (range)	8-oxo-dG/10^6 N (mean ± SD)	COV (%) (range)
Total 24 months period[a] (100 and 30 μg ST DNA)	54.76 ± 27.12 (n = 38)	49.5% (25.06–134.29)	12.64 ± 5.89 (n = 38)	46.6% (5.79–30.31)
First 6 months[b] (100 μg ST DNA)	42.15 ± 6.22 (n = 10)	14.8% (31.84–49.64)	10.05 ± 1.47 (n = 10)	14.6% (7.62–12.00)
Last 12 months[c] (100 μg ST DNA)	57.17 ± 39.40 (n = 14)	68.9% (25.06–134.29)	15.41 ± 8.59 (n = 14)	55.7% (5.79–30.31)
Total 24 months (30 μg ST DNA)	50.88 ± 13.01 (n = 14)	25.6% (35.48–75.32)	11.71 ± 3.01 (n = 14)	25.7% (8.09–17.29)

[a] A total of 38 independent ST DNA digestions by two investigators. HPLC column, UV lamp, and analytical cells of EC detector were replaced once during this period.

[b] For the first 6 months experiments, mostly 100 μg ST DNA samples were enzymatically digested and were run together with other DNA samples as a batch.

[c] During this period, two ST DNA (30 and 100 μg) were included in the each of batches; 100 μg ST DNA served as a pre-warning of the enzyme inefficiency and 30 μg ST DNA was equal to the amount used for real DNA sample analysis.

increasing as a function of time. It is noteworthy that an incomplete DNA digestion using 100 μg DNA during the last 12 months ($n = 14$) produced a comparable ratio of 8-oxo-dG/10^6 dG to that of DNA samples analyzed over 24 months ($n = 38$) but with a higher COV (68.9%). In this 100 μg DNA set, 8-oxo-dG/10^6 N ratio was high because dT and dC were not completely released (see Fig. 1), probably due to the decreased enzyme activity. These results strengthen our point of view that incomplete DNA digestion can lead to a great interday variation. For the 30 μg ST DNA set, the COV over 24 months was 26%, which gives molar ratios of 50.88 $\pm$ 3.48 for 8-oxo-dG/10^6 dG.

Measurements of 8-oxo-dG in smokers

Using this DNA digestion method in our laboratory, levels of 8-oxo-dG/10^6 N in DNA isolated from white blood cells of "healthy smokers" ranged from 1.7 to 586, with a mean of 35 $\pm$ 68.5 (SD), implying a COV for interindividual variation of approximately 200%. Under tightly controlled laboratory conditions, the COV for the assay using ST DNA is 26% (Table 4); hence, laboratory variability may contribute as little as 13% to the overall variability when assessing oxidative damage in a population of smokers. On the other hand, when conditions (amounts of DNA, enzyme activity, waiting time, etc.) are not as tightly controlled, the COV of laboratory variability can easily be as large as 69% (Table 4), and will contribute a relatively greater proportion to the overall variability, thus reducing the ability of the assay to detect differences among individuals.

DISCUSSION

Oxidative DNA lesions are considered to be important events related to aging and the development of cancer. Since the introduction and the development of a simple electrochemical detection method linked to HPLC, the measurement of 8-oxo-dG has become the most widely used technique for assessing oxidative DNA damage [15,16]. Evidence supporting the usefulness of 8-oxo-dG measurements includes its mutagenicity, its easy formation in DNA by a range of reactive oxygen species (ROS), such as hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1O_2$), as well as the existence of DNA repair by 8-oxo-dG DNA glycosylase [17–20].

Discrepancies in quantitation of 8-oxo-dG occur over a range of at least one order of magnitude, with GC-MS providing the highest values [4,21]. This is a situation that appears untenable, since there is a clinical need to establish normal and abnormal ranges in order to test the hypothesis that measurement of this biomarker may be useful in diagnosis, as an indicator of oxidative stress induced by diet and/or environmental and occupational exposures (e.g., smoking), or for monitoring of patients undergoing therapeutic or chemopreventive interventions. A wide variation in the background levels of 8-oxo-dG restrains the application of this biomarker in epidemiological studies.

Procedures such as phenol extraction, dialysis of isolated DNA, storage of tissues prior to DNA extraction, and nuclease digestion of DNA to liberate oxidized nucleosides for HPLC analysis have been shown to result in artifactual oxidation [22–25]. In the present study, we mainly focused on the DNA digestion conditions, which were shown to cause variations by NP1 and AP among the same samples at different time points. We have confirmed our previous study that introduction of DNase I improves DNA digestions [26]. Addition of PDE I and II to the DNase I + NP1 + AP digestion mixture showed a considerable improvement over the traditional NP1 + AP digestion in terms of its completeness of digestion and reproducibility over a long period of 24 months. To the best of our knowledge, no other studies have validated release of 8-oxo-dG over a 2 year period. Our studies suggest that NP1 + AP condition has its limitation in digesting the 8-oxo-dG containing dinucleotide monophosphates and/or trinucleotide diphosphates. As shown in Fig. 3, increasing NP1 from 1 unit to 10 units did not significantly enhance the release of 8-oxo-dG. However, PDE I and II, which attack 3′- or 5′-terminal OH groups, releasing 5′- or 3′-mononucleotides, respectively, are probably more efficient in hydrolyzing these 8-oxo-dG-containing oligonucleotides, especially if 8-oxo-dG is flanked by pyrimidines. Therefore, a significant increase in 8-oxo-dG but not in normal nucleosides was observed (see Table 2).

To check whether addition of PDE I and II may induce contamination of transition metals to the digestion mixture, thus resulting in an increase of 8-oxo-dG but not nucleosides, we have used 0.5 mM dG and followed the whole digestion procedure. As mentioned earlier, dG is generally present in large excess in DNA samples. Oxidation of a very small fraction of dG could result in a relatively high amount of 8-oxo-dG. We found that during 2.5 h of enzymatic digestion, the enzyme mixtures did not induce any 8-oxo-dG formation as compared to 0.5 mM dG incubated for 2.5 h in HPLC water. At 40 μM, DTPA effectively inhibited background increases in 8-oxo-dG during 24 h incubation at 37°C or during the waiting period in the autosampler (see Fig. 4). Without DTPA, even in the HPLC grade water, levels of 8-oxo-dG were increased in the dG solution after 24 h incubation (data not shown). Contamination with iron or even oxygen may also cause a background increase, which can be prevented by the addition of DTPA. These

results indicate that in the presence of DTPA, an increased level of 8-oxo-dG release is due to the enzymatic effects of PDE I and II.

Our research certainly showed that levels of 8-oxo-dG in the ST DNA samples change in the course of storage. As mentioned in the Materials and Methods section, DNA was dissolved in HPLC grade water at 5 μg/μl and stored at −20°C. At the beginning of this work, an average level of 74 8-oxo-dG/10^6 dG or 16 8-oxo-dG/10^6 N was observed when NP1 + AP were tested (see Figs. 3 and 4). After 9–12 months of storage in HPLC grade water at −20°C, background 8-oxo-dG levels in the same ST DNA were increased to approximately 93 8-oxo-dG/10^6 dG or 19 8-oxo-dG/10^6 N (Table 2). Storage of DNA under dry conditions (after lyophilization) seems to prevent such increases. Dissolving and drying and redissolving DNA in water can result in 8-oxo-dG background level increase (data not shown). For these reasons, control DNA should be aliquoted in small amounts, lyophilized, and reconstituted only once.

DNA isolation is one of the factors that have been considered extremely important in contributing to the wide variation of 8-oxo-dG analysis [10,22,23]. We have taken into account factors known to affect 8-oxo-dG baseline during DNA isolation. For example, all chemicals were of highest purity available (low in transition metals). The incubation and processing times were very limited (DNA can be precipitated within 8 h). Two steps of DNase-free RNase digestion completely removed the contamination by RNA, presence of which can decrease enzyme efficiency for DNA digestion and complicates both UV and EC chromatograms and, thus, 8-oxo-dG quantitation. As an additional precaution, EDTA and DTPA (metal chelators) were present in all solutions during DNA isolation and digestion. DTPA can prevent the redox action of iron, which may be released from tissues such as RBC. Addition of ST DNA to the DNA isolation process (in the absence of another source of DNA) resulted in the recovery rate of 86%, and 8-oxo-dG levels in that ST DNA were comparable to the same ST DNA, which did not go through this process. These results indicate that our DNA isolation did not induce any artifactual oxidation of dG nor decrease 8-oxo-dG present in the ST DNA. The levels of 8-oxo-dG in various tissue samples were comparable to most of the previously published data, except one, in which DNA extracted using the NaI chaotropic method showed 40 times less 8-oxo-dG levels [24].

As shown by our results, molar ratio of 8-oxo-dG/10^6 N showed less variability than oxidative damage expressed as 8-oxo-dG/10^6 dG (Tables 2 and 4). NP1 preferentially releases dG and dA (Fig. 1), so when DNA digestion is not complete, 8-oxo-dG/10^6 dG may reveal a better value closer to the "true" value of the DNA damage, but with a greater variability than the 8-oxo-dG/10^6 N measure (Table 4).

In summary, the optimal DNA digestion conditions are: 30 μg of DNA isolated from tissues should be immediately digested in 100 μl of solution containing 100 mM NaCl, 10 mM Mg^{2+}, 10 mM Tris (pH 7.4), 40 μM DTPA, and 40 units of DNase I for 30 min at 37°C. After adjusting the pH to 5.2 with 1 μl of 3 M sodium acetate (pH 5.2), the fragmented DNA should be digested with 1 μl of NP1 (1 unit/μl) for 1 h. After bringing the acidic pH back to neutral with 10 μl of 1 M Tris · HCl (pH 8.0), 1 μl of AP (1 unit/μl) should be added, followed by 30 min incubation. Finally, 1 μl each of PDE I and II (0.01 unit/μl each) should be added to the reaction mixture for an additional 30 min to ensure the completeness of DNA digestion. After a total of 2.5 h of digestion, the reaction mixtures should be centrifuged through Ultrafree-MC membrane (NMWL 5,000, Millipore) to remove enzymes before injection into an HPLC column.

Two DNA standards should be included in every batch. DNA at 30 μg serves as a control of daily operation to calculate interday variations, and 100 μg DNA serves as a sentinel of a declining efficacy of that digestion. Four standards should also be included in each batch for the quantitation in order to eliminate systematic errors, such as changes of instrument sensitivity. A minimum of 5 nmoles of each of the nucleosides obtained from digested DNA is preferred in order to have a reliable calculation of molar ratio of 8-oxo-dG/10^6 dG or N. If less than 5 nmoles of nucleosides are injected from the digested DNA, it is likely that a high ratio 8-oxo-dG/10^6 dG or N would be obtained. Therefore, the amount of DNA should be 10 μg or more per injection.

In conclusion, DNA digestion with NP1 and AP is not complete, thus causing wide interday variations. By adding DNase I, PDE I, and PDE II, the amount of 8-oxo-dG released was increased up to 30%, although the amount of normal nucleosides is not changed. Based on our results, we believe that the modified DNA digestion conditions will provide much more accurate 8-oxo-dG determinations and, thus, more reliable estimates of cancer risk.

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ABBREVIATIONS

AP—alkaline phosphatase
COV—coefficient of variation
DFO—deferoxamine
DTPA—diethylenetriamine pentaacetic acid
DTT—dithiothreitol
EC—electrochemical
EDTA—ethylenediamine tetraacetic acid
8-oxo-dA—8-oxo-2′-deoxyadenosine
8-oxo-dG—8-oxo-2′-deoxyguanosine
5-OHdC—5-hydroxy-2′-deoxycytidine
GC—gas chromatography
HPLC—high-performance liquid chromatography
MS—mass spectrometry
N—nucleosides
NP1—nuclease P1
PDE I and II—phosphodiesterases I and II
RBC—red blood cells
ROS—reactive oxygen species
ST DNA—Salmon testes DNA