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COMMENTARY

THE MEASUREMENT OF OXIDATIVE DAMAGE TO DNA BY HPLC AND GC/MS TECHNIQUES

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Oxidative damage to DNA has been measured by quantitating 8-hydroxy-2′-deoxyguanosine (8-OHdGuo) after enzymic digestion of DNA, followed by HPLC separation and electrochemical detection. Alternatively, 8-hydroxyguanine (and a wide range of other base-derived products of free radical attack) may be measured after acidic hydrolysis of DNA or chromatin, followed by derivatization and gas-chromatography/mass spectrometry. Both techniques have comparable sensitivity, but GC/MS enables determination of a wide variety of chemical changes to all four DNA bases and it can be applied to DNA–protein complexes. However, the two techniques do not always give similar results. Potential reasons for this are discussed. Greater attention to methodological questions is required before using measurement of 8-OHdGuo as a "routine" marker of oxidative DNA damage *in vivo*.

KEY WORDS: DNA, hydroxyl radical, singlet oxygen, 8-hydroxyguanine, mass spectrometry, electrochemical detection.

INTRODUCTION

Subjecting cells to oxidative stress can result in severe metabolic dysfunctions (reviewed[1–3]). Indeed, DNA damage (measured as strand breakage or chromosomal aberrations) has been almost invariably observed in a wide range of mammalian cell types exposed to oxidative stress in different ways,[2] some of which are summarized in Table I. This topic is of the greatest importance because of the growing realization that oxidative stress can initiate and promote carcinogenesis.[2]

Oxygen-derived species affect DNA in different ways. Neither $O_2^{\cdot -}$ nor H_2O_2 causes any strand breakage or chemical modification of the purines or pyrimidines in the absence of transition metal ions.[3–6] Their toxicity is thought to result from their metal ion-dependent conversion to hydroxyl radical ($^{\cdot}OH$), which is very reactive towards organic compounds.[3] Hydroxyl radical reacts with the DNA bases at diffusion-controlled rates by addition reactions: abstraction of H from the methyl group of thymine also occurs.[7,8] For example, $^{\cdot}OH$ can add on to guanine at C-4, C-5, and C-8 positions to give OH-adduct radicals that can have different fates.[7] Addition of $^{\cdot}OH$ to C-8 of guanine produces a C-8 OH-adduct radical that can be reduced to 8-hydroxy-7,8-dihydroguanine, oxidized to 8-hydroxyguanine (8-OHGua), or undergo opening of the imidazole ring, followed by one-electron reduction and

TABLE I
Methods used to subject cells to oxidative stress that have produced increased intracellular DNA damage (reviewed in [3])

Exposure to ionizing radiation
Elevated O_2 concentrations
Exposure to activated phagocytic cells
Exposure to "redox cycling" chemicals
Exposure to cigarette smoke
Exposure to ozone
Direct addition of hydrogen peroxide or organic hydroperoxides
Exposure to "autoxidizing" chemicals (e.g., dihydroxyfumarate; adrenalin)
Exposure to xanthine oxidase* plus its substrates (xanthine, hypoxanthine)
Addition of TNFα

*Care must be taken in the use of commercial xanthine oxidase, which is often heavily contaminated with proteases and other material directly injurious to cells.

protonation, to give 2,6-diamino-4-hydroxy-5-formamidopyrimidine, usually abbreviated as FapyGua.[7] Figure 1 shows the structures of these products. Similarly, $^{\cdot}OH$ can add on to C-4, C-5, or C-8 of adenine. Pyrimidines are also attacked by $^{\cdot}OH$ to give multiple products (reviewed in [8]). Thus, thymine can form *cis* and *trans* thymine glycols (5,6-dihydroxy-6-hydrothymines), and 5-hydroxy-5-methylhydantoin, 5-hydroxy-6-hydrothymine, 6-hydroxy-5-hydrothymine, and 5-(hydroxymethyl)uracil. Cytosine can form several products, including cytosine glycol and 5,6-dihydroxycytosine (Figure 1).

8-Hydroxyguanine is one of the major products of base damage when DNA is exposed to physiologically-relevant systems producing $^{\cdot}OH$. For example, in the ferric ion/hypoxanthine/xanthine oxidase system and ferric chelate/H_2O_2 systems (± ascorbate), 8-OHGua represents over 30% of the total base modification products measured.[4,5] In copper ion-dependent systems producing $^{\cdot}OH$, 8-OHGua is more than half the total base products measured.[6,9,10] Singlet O_2 has much less effect on the DNA bases than does $^{\cdot}OH$, but 8-OHGua is again produced.[11,12] Measurement of 8-OHGua is now frequently used as a method of assessing the extent of free radical-mediated DNA damage in cells subjected to oxidative stress, in animal tissues, and (as a putative index of whole body oxidative DNA damage) in urine.[13–18]

METHODS OF MEASURING 8-HYDROXYGUANINE AND 8-HYDROXYDEOXYGUANOSINE

We should first clarify a point of nomenclature. 8-Hydroxyguanine (8-OHGua) describes the purine base guanine in which the H atom at position 8 is replaced by an OH group (Figure 1). It can be released from DNA by acidic hydrolysis. If enzymic hydrolysis is used instead, 8-OHGua may be released still attached to the 2-deoxyribose sugar. This product is called 8-hydroxy-2′-deoxyguanosine (8-OHdGuo). Guanine is the base, guanosine the nucleoside (base-deoxyribose).

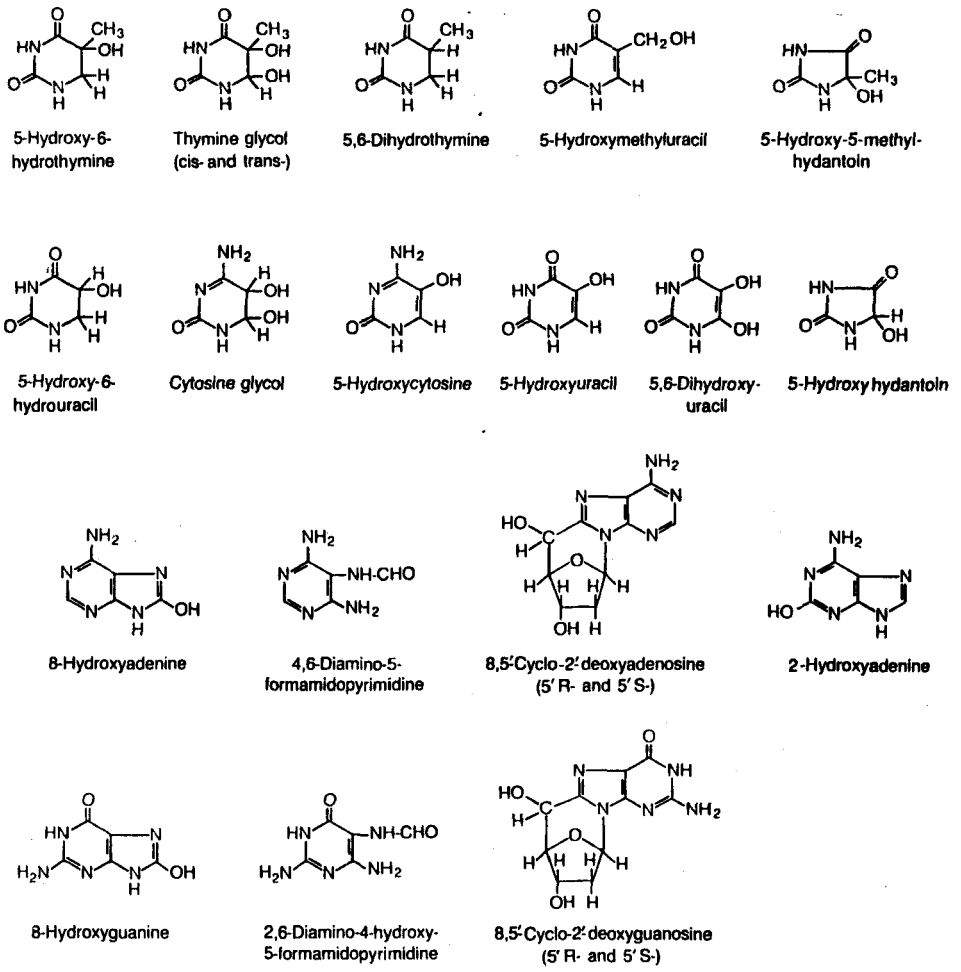


FIGURE 1 Chemical structures of modified bases. These modified bases (except for 5,6-dihydrothymine) are formed in DNA by attack of hydroxyl radical upon the DNA bases. 5,6-Dihydrothymine is a product of attack of H atoms or hydrated electrons upon thymine (H atoms and hydrated electrons result from water radiolysis).[8]

High Performance Liquid Chromatography (HPLC)

The development of an HPLC-based technique, coupled with highly-sensitive electrochemical detection, for the measurement of 8-OHdGuo released from DNA by enzymic digestion was the main impetus that led to the choice of this product for measurement as a putative index of oxidative DNA damage.[13–18,20] In retrospect this was a good choice since 8-OHGua is a major product of free radical damage to DNA by biologically-relevant oxygen-derived species and ionizing radiation,[3–6,19–26] i.e., if one had to pick a single product to measure, this would be the one to choose.

HPLC-based measurement of 8-OHdGuo has rapidly gained popularity as a means of getting some information about free radical damage to DNA in intact cells and

whole organisms. For example, the amount of 8-OHdGuo in the DNA of certain sub-populations of rat liver mitochondria was found to be considerably higher than in rat liver nuclear DNA, leading to proposals about the role of mitochondria in aging and carcinogenesis (discussed in [16]). Exposure of numerous cell-types to oxidative stress has been reported to increase the 8-OHdGuo content of their DNA (reviewed in [13, 17]).

These pioneering measurements of 8-OHdGuo have produced evidence that oxidative damage to DNA does occur in isolated cells and in whole organisms, although care must be used in interpreting the data (discussed in [3]). One must be especially cautious in attempting to use levels of 8-OHdGuo (or of any other single product) as a *quantitative* measure of DNA base damage by oxygen-derived species such as $^{\cdot}OH$. When $^{\cdot}OH$ attacks DNA bases, radicals are formed that have different fates, depending on experimental conditions (reviewed in [7, 8, 19]). Thus, attack of $^{\cdot}OH$ upon guanine can lead to formation of 8-OHGua by oxidation of the C8 OH-adduct radical, but this radical can lead to other products as well (such as FapyGua).[7,19] Hence, *different amounts* of 8-OHGua can result from attack of *the same amount* of $^{\cdot}OH$ upon guanine in DNA. This is one reason why *changes in 8-OHdGua levels do not necessarily reflect changes in the amount of free radical attack upon DNA*.[3] To take some examples, iron ion-dependent systems generating $^{\cdot}OH$ cause substantial formation of FapyGua as well as 8-OHGua in DNA,[4,5,10] whereas systems containing copper ions and H_2O_2 greatly favor 8-OHGua over FapyGua.[6,9,10] When mammalian chromatin is isolated, suspended in aqueous buffer solution and exposed to radiation-generated $^{\cdot}OH$, the relative amounts of 8-hydroxypurines and formamidopyrimidines generated depend upon the environment provided by gases used to saturate the aqueous solution.[24] For example, anoxic conditions favor formamidopyrimidines over 8-hydroxypurines.

Hence, HPLC-based analysis of 8-OHdGuo as a method of measuring oxidative DNA damage, despite its undoubted value, has intrinsic limitations. Another problem is the frequency with which co-eluting peaks occur when HPLC is applied to complex biological fluids: a peak must *never* be assumed to represent 8-OHdGuo merely on the basis of its retention time. Electrochemical behavior and (if concentrations permit) absorbance spectrum or fluorescence spectrum should be checked for identity with those of authentic 8-OHdGuo. Mass spectrometry can be employed for unequivocal identification.

Gas Chromatography/Mass Spectrometry (GC/MS)

Characterization of various types of damage to DNA by oxygen-derived species can be achieved by the technique of gas chromatography/mass spectrometry, which may be applied to DNA itself or to DNA-protein complexes such as chromatin (reviewed in [19]). For GC/MS, the DNA or chromatin are hydrolyzed (usually by formic acid) and the products converted to volatile derivatives, which are separated by gas chromatography and *conclusively* identified by the structural evidence provided by a mass spectrometer. Higher sensitivity and selectivity of detection can be achieved by operating the mass spectrometer in the selected ion monitoring (SIM) mode. In SIM, the mass spectrometer is set to monitor several ions derived by fragmentation of a particular product during the time at which this product is expected to emerge from the GC column. The GC/MS-SIM technique can be used to study the precise *mechanism*

by which DNA is damaged in cells subjected to oxidative stress. Thus, if damage is due to $^{\cdot}OH$ generation, then *a variety of base products* characteristic of $^{\cdot}OH$ attack should be detected (Figure 1), as has been observed in chromatin isolated from γ-irradiated human cells in culture (Nackerdien, Olinski and Dizdaroglu, submitted for publication), from murine hybridoma cells after treatment with H_2O_2[25] and in DNA isolated from primate tracheal epithelial cells exposed to ozone or to cigarette smoke (Aruoma, Wu and Halliwell, in preparation). If, for example, singlet O_2 were responsible for the DNA damage, then a much more limited range of products should be measurable, such as 8-OHGua[11,12,26] and FapyGua.[26]

The results of GC/MS analysis of modified bases in DNA have usually been expressed as nanomoles of modified bases per mg of DNA[4-6,9,10] (equivalent to pmol/μg DNA). However, it is easy to convert this data into the actual *number* of bases modified. Thus, dividing the figure of nmol bases/mg DNA by 3.14 (or multiplying by 0.318) gives the number of modified bases per 10^3 bases in DNA, i.e. 1 nmol/mg DNA corresponds to about 318 modified bases per 10^6 DNA bases.

QUESTIONS OF SENSITIVITY

The HPLC-based measurement of 8-OHdGuo is a highly-sensitive method, largely because of the use of electrochemical detection, introduced by Floyd *et al.*[15] Floyd[27,28] quotes a detection limit of 20 femtomoles, or one 8-OHdGuo per 10^6 nucleosides. Shigenaga *et al.*[29] quote 5–50 fmol on 40–100 μg samples of DNA.

How much 8-OHdGuo does the technique measure in cellular DNA? Floyd[17] quotes background levels in cells or tissues (not deliberately subjected to oxidative stress) as 0.5–2.0 8-OHdGuo per 10^5 guanosines, or between 1 and 5 8-OHdGuo per 10^6 DNA bases (assuming that DNA has, on average, 25% guanine bases). Ames[13] gives similar figures (one 8-OHdGuo per 130 000 bases in rat liver nuclear DNA, or about 8 per 10^6 bases, but 1/8000 in mitochondrial DNA, or 125 per 10^6 bases). Other scientists obtain broadly-comparable results (some published data are summarized in Table II). The range is between 3 and 20 8-OHdGuo per 10^6 DNA bases. Part of the variation might be accounted for by an effect of age[18] in some rat tissues. By contrast, commercial calf-thymus DNA (frequently used in studies *in vitro*) contains much more 8-OHdGuo than freshly-isolated DNA (Table II). Floyd[12] quotes a range of 8–320 8-OHdGuo per 10^6 bases, depending on the batch of commercial DNA used.

The GC/MS-SIM technique also provides high sensitivity and selectivity. The highest sensitivity for a compound under analysis (generally about 5 fmol per compound for products of DNA base modification) is achieved by monitoring the most abundant characteristic ion in its mass spectrum. For initial processing of DNA samples, 50–100 μg of DNA has usually been used[4-6,9,10,24] but smaller amounts are feasible since usually only 0.1–0.4 μg of hydrolyzed and derivatized DNA is injected onto the GC column for actual analysis.

The lowest background level of a base modification in DNA, or in chromatin, that is measurable by currently-used GC/MS-SIM techniques corresponds to 1 modified base in approximately 10^6 bases.[19] This makes the technique broadly comparable in sensitivity to measurement of 8-OHdGuo by HPLC with electrochemical detection. The exact sensitivity achieved is affected by, among other factors, the GC/MS instrument used and the type of column. In most cases, it is not the absolute sensitivity

TABLE II
"Baseline" levels of 8-hydroxydeoxyguanosine in DNA as determined by HPLC. Calculations assume that guanine is, on average, 25% of the DNA bases. Data abstracted from the references quoted.

Source of DNA	Content of 8-OHdGuo		Ref.
	per 10^5 guanines	per 10^6 DNA bases	
Rat liver (Total DNA or nuclear DNA)	1.21 ± 0.45	~3	30
	4.0 ± 1.3)		
	3.1 ± 0.4)	~8	31
	7.4 ± 0.9	~19	32
	1.3–3.3	3–8	18
	–	~20	33
Mouse liver (Total DNA)	0.6–1.4	1.5–3.5	14
Hamster liver	~7	~17	53
Rat liver (mitochondrial DNA)	~13	~33	34
Human phagocytes	0.3 ± 0.008*	~1	35
	–	<10	28
Human sperm	–	13–25	21
Commercial calf-thymus DNA	3–128†	8–320	12
	–	26.8 ± 12.6	27
	22 ± 2	~55	35
	–	25	36
	–	70	54
Rat kidney DNA	2.4–4.7	~6–12	18
	~1.7	~4	37
	~1	~3	38
Hamster kidney DNA	~3	~7	53
HeLa cell DNA	0.6–1.4	1.5–3.5	14
S. typhimurium DNA	0.6–1.4	1.5–3.5	14

*From non-smokers.
†Depending on batch of commercial DNA used.

of the technique that matters, but the "background" levels of base modification in untreated DNA, or DNA from "unstressed" cells or tissues. Examination of Tables II and III shows that GC/MS-SIM is sensitive enough to detect the levels of 8-OHdGuo that have been recorded by HPLC analysis.

Commercial calf thymus DNA usually contains 0.5–1.0 nmol of 8-OHGuo per mg, as measured by GC/MS after formic acid hydrolysis and trimethylsilylation. This corresponds to 159–318 bases/10^6 bases, towards the upper end of the range of HPLC-determined values quoted by Floyd[12] (Table II). To date, fewer studies upon DNA freshly-isolated from cells and tissues have been done with GC/MS-SIM than with HPLC, but the figures available show around 40 8-OHGua per 10^6 DNA bases (Table III), about 2- to 11-fold greater than the figures recorded by HPLC (Table II).

TABLE III
GC/MS-based measurement of 8-hydroxyguanine in DNA. After acidic hydrolysis of DNA, unless otherwise stated

Source of DNA	8-OHGua		Total modified bases* (per 10^6 DNA bases)	Ref.
	nmol/mg DNA	bases/10^6 DNA bases		
Commercial calf-thymus DNA	0.5–1.0	159–318	~640	4–6
Mouse liver† mitochondrial DNA	–	~3500	–	39
Fish liver	0.13 ± 0.06	~41	–	40
Chromatin isolated from murine hybridoma cells‡	–	35–40	~138	25
Human breast tissue	0.13 ± 0.03		~41	52
Human neutrophils	0.20–0.23		64–73	52

*Total of all the modified bases measured in the DNA.
†HPLC/MS analysis after enzymic hydrolysis of DNA.
‡Freshly-isolated chromatin. Chromatin stored after extensive dialysis gave higher figures (e.g., about 182 8-OHGua[10] per 10^6 bases).

EXPLANATIONS OF THE DISCREPANCY BETWEEN GC/MS AND HPLC-ECD

Modifications of DNA bases affect cell metabolism and may be related to carcinogenesis,[2,13,16,17,41,42] so it is important to understand this apparent discrepancy. What are the possible explanations? First, HPLC may underestimate the real amount of 8-OHdGuo in DNA. Second, GC/MS may overestimate it, e.g., if the derivatization and hydrolysis procedures somehow artifactually *cause* formation of 8-OHGua. Third, both errors could occur. Before discussing these potential explanations in detail, it is worth pointing out that isolation of DNA from cells may introduce some oxidative modification, particularly if phenol-based methods are used, since oxidizing phenols produce a wide range of reactive radicals (reviewed in [43]). This is one of the reasons why extraction of chromatin for analysis may be preferable.[19,24,25] This technique is milder than those used for DNA extraction and may minimize the loss of extensively fragmented DNA and of DNA that has become cross-linked to protein as a result of oxidative damage.

How could HPLC underestimate 8-OHdGuo?

HPLC analysis relies upon extraction of DNA from cells and complete enzymic hydrolysis before quantitative measurement of 8-OHdGuo can be achieved. First, extraction of DNA that has undergone extensive modification and fragmentation (Table I) may be impaired, because of the easy loss of small DNA fragments[19,28] and cross-linking of the DNA bases to amino acid residues in nuclear proteins.[19] However,

TABLE IV

Effect of formic acid on 8-hydroxyguanine. The stability of 8-OHGua at different concentrations of formic acid was evaluated. The relative molar response factor [RMRF = (amount of the analyte)/(amount of the standard) × (peak area of the ion of the standard)/(peak area of the ion of the analyte)] of the trimethylsilyl derivative of 8-OHGua was determined without the use of formic acid and after treatment with formic acid at various concentrations. The table shows the RMRF values obtained [data from Z. Nackerdien, R. Olinski, and M. Dizdaroglu, *Biochemistry* [(submitted)]. These results show that 8-OHGua is stable under all acidic conditions used.

No acid	60%	70%	88%
0.415 ± 0.030	0.398 ± 0.046	0.396 ± 0.049	0.452 ± 0.008

although this artifact could lead to an underestimate of DNA damage in heavily-stressed cells, it would not be expected to affect measurement of the low levels of DNA base damage in cells not deliberately subjected to oxidative stress (the word "deliberately" is used in this review because cell culture conditions may often themselves impose a mild oxidative stress).

Second, the efficiency of exonucleases and endonucleases in hydrolysing DNA is greatly affected by modification of the bases.[42,50] For example, a recent paper[45] showed that 8-OHGua severely inhibits digestion of dinucleotides by phosphodiesterases. Thus it is not always certain that modified bases are completely hydrolyzed from DNA, especially when published hydrolysis techniques are transplanted from one laboratory to another and not re-validated.

Third, the HPLC technique as usually used measures 8-OHdGuo, and not 8-OHGua which is separated on the columns used (e.g. reference [11]). Frenkel *et al.*[46] showed that acidic pH (frequently used for nuclease P_1 digestion) can promote hydrolysis of 8-OHdGuo to 8-OHGua, causing a loss of HPLC-detectable material. Extensive free radical damage might also lead to release of modified guanine from the DNA backbone to leave an abasic site. Frenkel *et al.*[46] considered these artifacts and, using a more-complex HPLC technique than is commonly employed, they found (page 134 of [46]) that DNA extracted from murine epidermal cells contains baseline 8-OHdGuo levels of at least 30 per 10^6 DNA bases, closer to the values measured by GC/MS (Table III) than to those measured by HPLC (Table II).

TABLE V

Effect of formic acid on guanine. Commercial guanine (which is contaminated with 8-OHGua as measured by either GC/MS or by HPLC-based methods) was subjected to derivatization and GC/MS after incubation in water or with various concentrations of formic acid. The results show that 8-OHGua is not produced by treatment of guanine with formic acid under the conditions used for DNA hydrolysis. Values represent the number of molecules of 8-OHGua per 10^5 guanines. [Data from Z. Nackerdien, R. Olinski, and M. Dizdaroglu, *Biochemistry* (submitted)]

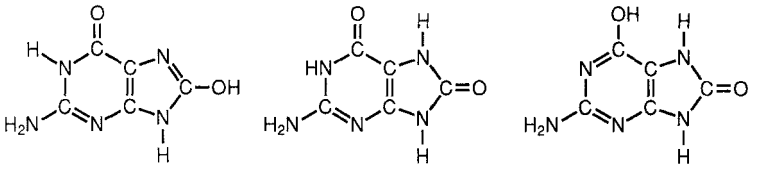
	Water	
	No incubation	With incubation
8-OHGua	76.40 ± 11.28	80.64 ± 14.11

	Formic acid			
	50%	60%	70%	88%
8-OHGua	82.55 ± 5.15	74.85 ± 11.27	68.67 ± 5.81	78.00 ± 25.90

TABLE VI

Effect of different formic acid concentrations on the release of 8-hydroxyguanine from DNA as measured by GC/MS. The release of 8-OHGua by formic acid from γ-irradiated DNA and from control DNA was investigated (values represent the number of molecules of 8-OHGua per 10^4 DNA bases). Data from Z. Nackerdien, R. Olinski, and M. Dizdaroglu, *Biochemistry* (submitted). Except at 50% formic acid (which may be insufficient to hydrolyze DNA completely under the conditions used), concentrations of formic acid of 60% or higher do not cause any more 8-OH Gua to be detected.

50%		60%		70%		88%	
Control	Irradiated	Control	Irradiated	Control	Irradiated	Control	Irradiated
4.89 $\pm$ 0.77	15.26 $\pm$ 2.16	5.09 $\pm$ 0.99	21.44 $\pm$ 1.88	5.25 $\pm$ 0.32	24.19 $\pm$ 2.28	5.50 $\pm$ 1.18	25.12 $\pm$ 0.42



Chemical structure of 8-hydroxyguanine

FIGURE 2 Keto-enol tautomerism of 8-hydroxyguanine.

Another potential artifact may result from keto-enol tautomerism of 8-OHGua[17,47,48] (Figure 2). It has been suggested that the equilibrium favors the 6,8-diketo form. Perhaps 8-OHGua should more correctly be called 8-oxo-7-hydroguanine: the term 8-oxo-7,8-dihydroguanine (frequently used in the literature) is incorrect because C-8 does not bear an H-atom (Figure 2). How tautomerism would affect HPLC analysis is uncertain. It should not affect GC/MS analysis because derivatization occurs at the 8-OH group and the equilibrium will adjust to replace it until all the 8-OHGua has been trimethylsilylated.

Artifacts in GC/MS techniques?

Do derivatization and hydrolysis (especially the vigorous formic acid hydrolysis usually employed) cause artifactual formation of 8-OHGua? This question has been addressed experimentally (Nackerdien, Olinski and Dizdaroglu, submitted for publication). First, 8-OHGua is not destroyed by formic acid, tested from 50% to 88% acid (Table IV). Second, subjecting guanine to acid hydrolysis causes no formation of 8-OHGua (Table V). Third, the apparent release of 8-OHGua from control or γ-irradiated calf-thymus DNA was the same at formic acid concentrations from 60 to 88% (Table VI). These data suggest that 8-OHGua is not formed by the hydrolysis procedures used. Whether it is formed during derivatization is a question currently under investigation.

CONCLUSION

We do not know the "baseline" level of products of DNA damage by oxygen-derived species *in vivo*, since different measurement techniques give different results. Greater attention must be given to resolving these methodological questions before the widespread adoption of such methods as a true index of oxidative DNA damage.

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