Artifacts Associated with the Measurement of Oxidized DNA Bases

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In this paper we review recent aspects of the measurement of oxidized DNA bases, currently a matter of debate. There has long been an interest in the determination of the level of oxidized bases in cellular DNA under both normal and oxidative stress conditions. In this respect, the situation is confusing because variations that may be as large as two orders of magnitude have been reported for the yield of the formation of 8-oxo-7,8-dihydroguanine (8-oxoGua) in similar DNA samples. However, recent findings clearly show that application of several assays like gas chromatography-mass spectrometry (GC-MS) and [32P]-postlabeling may lead to a significant overestimation of the level of oxidized bases in cellular DNA. In particular, the silylation step, which is required to make the samples volatile for the GC-MS analysis, has been shown to induce oxidation of normal bases at the level of about one oxidized base per 10^9 normal bases. This has been found to be a general process that applies in particular to 8-oxoGua, 8-oxo-7,8-dihydrodine, 5-hydroxycytosine, 5-(hydroxymethyl)uracil, and 5-formyluracil. Interestingly, purification of the oxidized bases from DNA hydrosylate prior to the derivatization reaction prevents artefactual oxidation. Under these conditions, the level of oxidized bases measured by GC-MS is similar to that obtained by HPLC associated with electrochemical detection (HPLC-EC). It should be added that the level of 8-oxo-7,8-dihydro-2'-deoxyguanosine in control cellular DNA has been found to be about fivefold lower than in earlier HPLC-EC measurements by using different conditions of extraction and enzymatic digestion of DNA. Similar conclusions were reached by measuring formamidopyrimidine-DNA glycosylase sensitive sites as revealed by the single cell gel electrophoresis (comet) assay. Key words: DNA base damage, DNA repair enzymes, oxidative lesions.

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Oxidative damage to DNA is widely recognized to be at least partly involved in cancer and aging processes (1–3). Oxidation of DNA components can be induced by a variety of factors including endogenous cell metabolism, chemicals and drugs, ionizing radiation, and solar light. Oxidation processes that may involve hydroxyl radical, ferryl or perfferyl ion, singlet oxygen, hydrogen peroxide, peroxynitrite, and one-electron oxidation lead to several types of DNA modifications. These include chain breaks, DNA-protein cross-links, abasic sites, purine-reactive aldehyde adducts, and oxidized DNA bases (for recent reviews see (4,5)). Some of the latter classes of lesions have been unambiguously shown either to be mutagenic or to block DNA replication (6–9). In this respect, 8-oxo-7,8-dihydroguanine (8-oxoGua) was found to induce G to T transition, whereas 2,6-diamino-4-hydroxy-5-formamidoprimidine (FapyGua), the related imidazol ring open compound, was found to be lethal (10). Interestingly, various repair activities, mostly of the N-glycosylase type (11–16) that take care of oxidative base damage, have been characterized in both prokaryotic and eukaryotic cells (17–19). It should be noted that the gene of several DNA repair enzymes including those of Escherichia coli and yeast OGG1 have been cloned (20,21). Altogether, this provides a strong impetus to the development of accurate and sensitive methods for monitoring oxidized bases within cellular DNA (22). In particular, the assays should be able to detect at least one modification per 10^9–10^10 normal bases in a few micrograms of DNA. Another major limitation is the possible generation of oxidation processes during the DNA workup and the assay itself. Various chemical and biochemical approaches have been proposed. Indirect measurements based on the use of antibodies, purified repair enzymes, single-cell gel electrophoresis, or ligation-dependent chain reaction (PCR) techniques are usually highly sensitive, but their specificity is still open to debate. A more direct approach involves the extraction of the cellular DNA, followed by its conversion through hydrolysis to either nucleosides or nucleobases. Then, the complex mixture of DNA fragments is usually resolved using appropriate liquid or gas chromatographic methods. The measurement of the analytes is achieved on line by sensitive techniques whose limits of detection are within the femtomole range. High performance liquid chromatography associated with electrochemical detection (HPLC-EC) and gas chromatography coupled to mass spectrometry (GC-MS) have been the most widely applied assays aimed at monitoring oxidative base damage within DNA during the last decade.

The Assays

HPLC-EC assay aimed at monitoring the formation of oxidized bases with a low oxidation potential. The HPLC-EC assay was initially developed for the measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) (23). The HPLC-EC assay is currently the most widespread method and has received hundreds of applications for determining the level of 8-oxodGuo within the DNA of either isolated cells or animal tissues. The application of the HPLC-EC technique has been extended first to the measurement of 8-oxo-7,8-dihydro-2'-deoxyadenosine (24) and then to 5-hydroxy-2'-deoxycytidine and 5-hydroxy-2'-deoxyuridine. It should be added that the levels of 5-hydroxy-2'-deoxycytidine and 5-hydroxy-2'-deoxyuridine have been determined in the DNA of rat organs and human leukocytes (25). The limit of the sensitivity is on the order of 50 fmol of oxidized base, which corresponds to one lesion per 10^6 normal base, in a DNA sample size of 10 µg. It may be added that the coulometric detection is more sensitive than the amperometric technique. However, one limitation of the assay deals with the fact that only bases with a low oxidation potential can be measured by electrochemical detection.

GC-MS assay for measuring oxidative DNA base damage. GC-MS is a more versatile technique because it is less dependent on the chemical properties of the measured oxidized base (26). The mass spectrometer may be set in the selective ion monitoring mode so that it detects only the ions corresponding to the major peaks of the mass spectrum of the compound of interest. This provides a specific and sensitive detection, which is, on the average, in the same range as that of the HPLC-EC assay. It should be noted that the bases have to be converted into volatile derivatives prior to their injection into the GC-MS apparatus. In an improved version of the method, an isotopically enriched internal standard, which differs by at least three units of mass, is added. An internal standard is used to compensate for any loss of the sample during the derivatization step and also to

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compensate for the possible lack of reproducibility of the injection. The GC-MS assay was first applied to the detection of 8-oxoGua in isolated DNA that was exposed to γ-radiation in aqueous solution (27). Subsequently, the method has been applied numerous times to the measurement of many types of oxidative base damage in isolated DNA (28–30) and within isolated cells (31–37). It should also be mentioned that the GC-MS assay has been widely used to study the specificity of various DNA repair enzymes including endonuclease III and formamidopyrimidine-DNA glycosylase (FPG) protein from E. coli (12,13,38–41). In addition, recent data on the kinetics of the repair of several oxidative base lesions in cellular DNA were inferred from GC-MS measurements (42).

Discrepancies in the results obtained by GC-MS and HPLC-EC measurements. Comparison of the results concerning the level of 8-oxoGua (or 8-oxodGuo) as determined by applying either the GC-MS or the HPLC-EC method revealed large discrepancies (43,44). As a general trend, the amount of 8-oxoGua measured by GC-MS is about 10-fold higher than that inferred from HPLC-EC. The level of 8-oxodGuo determined in DNA by applying the HPLC-EC assay is in the range of 1–20 lesions/10⁸ bases, depending on cells and organs. However, the values are significantly higher, varying from 40 to 140 8-oxoGua residues per 10⁸ normal bases in the samples that were analyzed by GC-MS. Studies using commercial calf thymus DNA are less easily comparable, mostly due to high variability between batches. However, a similar trend, which shows a higher amount of 8-oxoGua as measured by GC-MS with respect to HPLC-EC determinations, can be observed. Conflicting data are also observed in the literature for other oxidative nucleoside lesions such as 5-hydroxy-2'-deoxyctydine and 5-hydroxy-2'-deoxyuridine (25). These inconsistencies may lead to misleading biological observations. For instance, GC-MS analysis showed a relatively high level of 8-oxoGua in breast cancer tissues (32,45), whereas the amount of 8-oxodGuo detected by HPLC-EC in similar samples is close to those of normal tissues (46). In addition, the level of 8-oxoGua in normal breast tissue is much higher when measured by GC-MS than the level of 8-oxodGuo as estimated by HPLC-EC (46). Several hypotheses have been proposed to account for the observed low yield of 8-oxoGua when measured by HPLC-EC. This could be due either to a lack of complete enzymatic digestion of DNA or to the occurrence of a dynamic equilibrium between two tautomeric forms of 8-oxodGuo that would prevent a quantitative detection of 8-oxodGuo (43). However, it may be concluded that at this stage, the proposals, particularly the latter one, are not relevant. In fact, no serious attempts were made to solve the problem until recently, and controversial data were still accumulating.

**GC-MS overestimation rather than HPLC-EC underestimation.** The observation of such discrepancies in the level of measured oxidative base damage to DNA, and particularly of 8-oxoGua, is likely to have its origin in at least one critical parameter, which has to be specific for the experimental protocol of either the GC-MS or the HPLC-EC assay. Let us first consider the method used to hydrolyze DNA prior to the chromatographic analytical step. An enzymatic digestion into nucleosides by incubation of DNA with nuclease P1 and alkaline phosphatase is applied mostly in the HPLC-EC method. On the other hand, GC-MS analysis is usually achieved subsequent to acidic hydrolysis of DNA by either 60 or 88% formic acid at 140°C. As already mentioned, the enzymatic digestion could be incomplete; if this occurred, there would be an underestimation of the level of 8-oxoGua (43). As evidence against this, it was observed that an increase in either the amount of nuclease P1 or the period of digestion does not affect the level of released 8-oxodGuo (Douki and Cadet, unpublished data). It was also argued that 8-oxodGuo may depurinate during the nuclease P1 treatment carried out at pH 5.5 (44). However, no experimental evidence was provided to support such an assumption. In contrast, it was shown that the N-glycosidic bond of 8-oxodGuo is much more stable than that of normal purine nucleosides (47,48). Other results seem to show that both explanations are unlikely. When the same oxidized calf thymus DNA was hydrolyzed by using either enzymatic digestion or hydrogen fluoride/pyridine treatment and subsequently analyzed for its 8-oxoGua (or 8-oxodGuo) content by HPLC-EC, similar results were obtained (49). Interestingly, similar levels of 8-oxoGua background within cellular DNA were inferred from HPLC-EC measurements involving the use of an internal standard, irrespective of either enzymatic digestion or formic acid treatment (Ravanat and Turesky, unpublished data).

Another major difference between the HPLC-EC and GC-MS assays is the requirement of the derivatization of the DNA samples prior to their GC-MS analysis (26). The oxidized bases or nucleosides are usually made volatile by silylation, using either N-bis-(trimethylsilyl)-trifluoroacetamide or N-trimethylsilyl-N-methyltrifluoroacetamide. Another alternative is pentfluorobenzylolation, which constitutes a convenient approach for chemical ionization detection of oxidized nucleosides in the negative mode as a GC/electron capture negative ion chemical ionization-mass spectrometry assay (50,51). The silylation is usually carried out at a high temperature (120–140°C) for at least 40 min. In fact, this appears to be the critical factor in the discrepancies between the results of the GC-MS and the HPLC-EC methods. Unambiguous support for the artifactual generation of 8-oxoGua in the derivatization step (52) was provided by a careful comparative study involving analysis of the same DNA sample by both HPLC-EC and GC-MS (Fig. 1). Formic acid was used to hydrolyze DNA in both cases. In addition, an aliquot of the hydrolyzed DNA sample was purified by immunoaffinity chromatography. With the exclusion of guanine, the fraction containing 8-oxoGua was collected and then analyzed by GC-MS. In agreement with previous observations, the values for 8-oxoGua obtained by applying the crude GC-MS assay were higher than those inferred from the HPLC-EC method. In contrast, the levels of 8-oxoGua measured by GC-MS after the prepurification step were similar to those given by HPLC-EC. The latter observation strongly suggested that unmodified guanine present in the nonpurified DNA hydrolysate was partly

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**Figure 1.** Comparative measurement of the background level of 8-oxo-7,8-dihydroguanine (8-oxoGua), 8-oxo-7,8-dihydroadenine (8-oxoAde), and 5-hydroxycytosine (5-OHCyt) within calf thymus DNA using three analytical approaches including the usual GC-MS assay, its improved version (HPLC/GC-MS), and the HPLC associated with electrophotometric detection (HPLC-EC) method. The results, expressed in number of modifications per 10⁸ bases, represent the average of three independent measurements.
oxidized into 8-oxoGua during the silylation step. This received further confirmation from the fact that the level of oxidized bases increased with the time of derivatization (52,53). On the other hand, there was a decrease in the measured level of 8-oxoGua in DNA when the temperature of the silylation was lowered under conditions in which the derivatization of the sample was still achieved (54). The artificial oxidation of the overwhelming normal bases during derivatization appears to be a general process. This is inferred from the observation of increasing yields of 5-hydroxyuracil (5-OHdCyt), 8-oxo-7,8-dihydrodoadenine (8-oxoAdo), 5-formyluracil (5-FoUra), and 5-(hydroxymethyl)uracil with time during the silylation of purified cytosine, adenine, and thymine, respectively (55). It should be noted that the levels of isotopically labeled derivatives of the three oxidized bases used as internal standards were not affected during the silylation reaction. This rules out any kinetic effect of the reaction. In addition, comparative measurement of 5-hydroxyuracil and 8-oxo-7,8-dihydroadenine within isolated DNA was achieved by both GC-MS and HPLC-EC; thus, we concluded that the detection methods by themselves are not involved in the former observed discrepancies. It may be concluded that cytosine, thymine, adenine, and guanine are subject to oxidation reactions during silylation. In this respect, it is worth mentioning that the oxidizing ability of silylating agents was already recognized more than 15 years ago. An interesting example dealt with the preparation of silylated derivatives of unsaturated pyrimidines from dihydropyrimidines (56).

The relatively high levels of several oxidized purine and pyrimidine bases, which were determined by using the conventional GC-MS analysis in the control samples of nuclear DNA from rat liver and lung (36), are thus highly questionable. Also questionable are the high levels of 8-oxo-7,8-dihydroadenine and 4,6-diamino-5-formamidopyrimidine that were measured in metastatic and nonmetastatic cells from human breast tumors (57) by applying the earlier GC-MS assay. It should also be mentioned that the level of 5-(hydroxymethyl)-2'-deoxyuridine in the DNA of normal and cancerous breast cells as determined by a similar GC-MS assay was estimated to be around 3.5·10^(-9) thymidine (58).

Optimized GC-MS assay. It is clear from the above observations that the GC-MS analysis of targeted oxidized bases or nucleosides requires their prepurification from the related overwhelming normal DNA components prior to the silylation step. This could be achieved by either HPLC or immunosaffinity chromatography. A second major condition to be fulfilled involves the use of stable isotopically labeled oxidized bases or nucleosides as internal standards for calibration purposes. The use of 8-azaadenine in place of authentic isotopically labeled internal standards may provide flaws in the quantitative measurement. In this respect, it was recently reported that 8-azaadenine was not stable under the acidic conditions used for DNA hydrolysis (59).

In addition, two other important parameters, which are not specific to the GC-MS assay, have to be considered. One parameter, the conditions of acidic hydrolysis used for inducing the quantitative release of the oxidized bases to be measured, has been neglected during the last decade. It is a requisite that the conditions of hydrolysis should be optimized for each new compound to be measured. Emphasis has to be placed on the stability of the compounds under the conditions where the N-glycosidic bond of the related nucleotides is quantitatively cleaved. It was recently shown that treatment of several major DNA base oxidation products by hot 60% formic acid, which is a recent alternative to the use of concentrated 88% formic acid, led to significant degradation processes (59,60). In addition, the formamidopyrimidine derivatives of guanine and adenine were found to undergo recycilation under conditions of formic acid hydrolysis. However, the integrity of the latter imidazole open ring compounds is maintained upon treatment by hydrogen fluoride in pyridine, a mild DNA hydrolyzing reagent (Douki et al., unpublished data). Another major aspect that may be a limiting factor in the sensitivity of the assays is the artificial oxidation of the overwhelming normal nucleobases during DNA workup including extraction and hydrolysis.

Improved conditions of extraction and enzymatic digestion of DNA. During the past 3 years, efforts have been made to minimize the artificial increase in the level of 8-oxoGua during DNA extraction by using suitable solvents and antioxidants (61–66). Particularly relevant are the recent reported results showing at least a fivefold reduction in the level of cellular background of 8-oxo-dGuo in cellular DNA with respect to the lowest values in the literature (65). The improvement in the DNA extraction and the simplification of the enzymic digestion process, which both lead to a significant reduction of the workup period, are likely to expand the low values of cellular 8-oxo-dGuo background. Thus the levels of 8-oxo-dGuo were determined to be 2.6–3.0 and 3.1·10^6 2'-deoxyguanosine residues within the DNA of rat liver (66) and human leukocytes, respectively (67). It should also be noted that 8-oxo-dGuo values as low as 3.07±1.45 and 2.37±1.21·10^6 2'-deoxyguanosine residues were determined in human polymorphonuclear and mononuclear leukocyte DNA using an anaerobic DNA extraction followed by HPLC-EC measurement (68,69). These data, which were obtained using the conventional and accurate HPLC-EC assay, clearly indicate that it should now be possible to search for the formation of 8-oxo-dGuo under mild conditions of oxidative stress by applying the above improved conditions of DNA extraction. In addition, kinetic studies of 8-oxo-dGuo repair within cells should also be facilitated. However, the main message of these interesting findings is that the background level of 8-oxo-dGuo is less than 1 lesion/10^10 normal bases in cellular DNA as inferred from HPLC-EC measurement.

Single cell gel electrophoresis (comet) assay for visualizing FPG-sensitive sites. Interesting information on the estimate of 8-oxo-dGuo in the DNA of isolated cells was obtained by making the comet assay more specific. This was achieved by digesting the DNA of the embedded cells in agarose gel with E. coli FPG enzyme added (70,71). The latter FPG protein is able to cleave DNA at the sites of 8-oxo-dGuo, and the additional nicks thus generated were subsequently revealed by the analysis of the comet assay. The level of 8-oxo-dGuo measured in single cells is about 10-fold lower than was determined by the HPLC-EC method. This is in agreement with previous measurements of oligonucleotide strand breaks when an alkaline elution assay was performed after extraction of the DNA from mammalian cells and its subsequent incubation with the E. coli FPG protein (72–74). The level of 8-oxo-dGuo thus determined in the DNA of human leukocytes (1.7·10^6 2'-dGuo) is similar to the values obtained in related DNA samples when improved conditions of extraction and digestion of DNA are used (67).

Overestimation of the level of 8-oxo-dGuo by [32P]-postlabeling and immunoblot measurements. Another recent source of confusion in the field of the measurement of oxidative base damage to DNA is provided by a recently introduced [32P]-postlabeling assay aimed at monitoring the formation of 8-oxo-dGuo (75). The [32P]-postlabeling assay of the basal level of 8-oxo-dGuo within the DNA of various rat tissues gave values 10–50-fold higher than those reported by HPLC-EC detection. For example, the amount of 8-oxo-dGuo was found to be 87±29–133±49·10^6 normal bases. These high values may be explained by self-radioysis.
processes associated with the presence of the predominat[32P]-2'-deoxyguanosine 5’-monophosphate in the reaction mixture. The emission of the β particle is likely to induce the formation of 8-oxo-7,8-dihydro-2’-deoxyguanosine 5’-monophosphate through radical reactions. In this respect, the incorporation of [3H]-thymidine within cellular DNA gives rise to significant amounts of 5,6-dihydroxy-5,6-dihydrothymidine as the result of self-radiolysis reactions. The levels of thymidine glycols as estimated by the acetol assay (76) were within the range of 1 lesion/105 thymine residues (77). Also, in this case, the high level of 5,6-dihydroxy-5,6-dihydrothymidine background precludes any application of the assay at the cellular level (78). Work is in progress in our laboratory to develop an accurate and sensitive [32P]-post-labeling assay for measuring 8-oxodGuo.

This involves a HPLC prepurification step to prevent artifactual formation of 8-oxodGuo through radical oxidation. A similar HPLC-[32P]-post-labeling approach was already applied for the determination of 2’-deoxyadenosine N-1 oxide and 5-(hydroxymethyl)-2’-deoxyuridine (79,80). Another example of overestimation of the measurement level of 8-oxodGuo is provided by an immunol dot blot assay (81). Musarrat and Wani (82) reported that the polyclonal antibodies directed against 8-oxodGuo were able to detect one residue of the latter oxidized nucleoside per 15 and 9 normal nucleosides in the DNA of human skin fibroblast cells upon exposure to 10 and 100 μM H2O2, respectively. This is probably due to the lack of specificity of the antibodies because it was shown in other studies that both monoclonal and polyclonal antibodies raised against 8-oxodGuo show significant cross-reactivity with 2’-deoxyguanosine (82,83).

**Conclusion**

It is now well established by at least three independent groups that the conventional GC-MS assay (26) suffers from a major drawback: the overestimation of oxidized DNA bases or nucleosides. This is due to the artifactual oxidation of the related normal DNA components that occurs during the silylation step. However, this artificial oxidation may be easily prevented by, for example, prepurifying the compounds of interest. Therefore, an accurate version of the GC-MS assay that also requires the use of isotopically labeled internal standards is now available. In addition, significant improvements have been achieved in the techniques of DNA extraction and hydrolysis in the GC-MS assay and also in the HPLC-EC assay. Most of the reported GC-MS measurements of oxidative base damage to DNA have been artificially overestimated.

This raises the question of the validity of most of the conclusions concerning the levels of oxidized bases not only in cellular DNA but also in isolated DNA. In addition, the quantitative aspect of GC-MS studies dealing with the specificity of DNA repair endonucleases is also open to debate. Most of the previously reported works should be reassessed using either the more restricted HPLC-EC assay and/or the improved GC-MS method, which has a wider application. Interlaboratory trials should compare, optimize, and validate the various available assays. These include immunological, [32P]-post-labeling, ligation-mediated PCR, and single cell gel electrophoresis methods, for which calibration is also required.

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