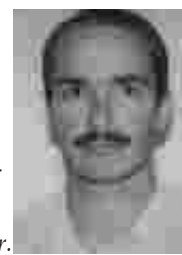


Measuring Oxidized DNA Lesions as Biomarkers of Oxidative Stress: An Analytical Challenge

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Born in 1964 in La Mure, France, Jean-Luc Ravanat received his Master's degree in biochemistry in 1986 and his Ph.D. in organic chemistry in 1992 from the University of Grenoble. From 1993-1994, he was a postdoctoral fellow in the "metabolism of xenobiotics" team at the Nestlé Research Centre in Switzerland. Since 1995, he has been a staff member of the "Lésions des Acides Nucléiques" laboratory at the CEA-Grenoble belonging to the French Atomic Energy Commission. His fields of research include: Identification and characterization of DNA lesions generated by ionizing radiations, photosensitization, reactive oxygen species and chemicals; measurement of DNA lesions as potential biomarkers of oxidative stress; and DNA repair.



Measuring Oxidized DNA Lesions as Biomarkers of Oxidative Stress: An Analytical Challenge

Summary: During the last three decades, considerable efforts have been devoted to the development of accurate assays aimed at measuring oxidative base damage to cellular DNA as a potential biomarker of oxidative stress. Much attention has been focused on the determination of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), the main studied DNA lesion. An overview of the literature indicates that the determined cellular background levels of 8-oxodGuo vary significantly depending on the method used. In this review article, the different approaches aimed at measuring oxidative DNA lesions, including mainly 8-oxodGuo, will be described and compared according to their feasibility, specificity and accuracy. Emphasis will be placed on recent developments that have significantly contributed to reducing the discrepancies between the different available approaches by identifying and overcoming the major drawbacks. In this respect, recommendations will be given to avoid artifactual formation of oxidized DNA lesions during the work-up, and finally recent developments will be discussed.

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Reactive oxygen species (ROS), which are involved in several biological functions and alterations, may damage cellular constituents. Among them, much interest has been focused on DNA, the macromolecule containing the genetic information. Damage to DNA may induce cell death or mutation that could give rise, several cellular divisions later, to cancer^{1,2}. Therefore, there is an increasing interest in using

oxidative DNA lesions as in vivo biomarkers of oxidative stress³⁻⁶. In that respect, efforts have been made during the last thirty years to develop sensitive and accurate assays aimed at measuring oxidative DNA lesions in cells. The developed analytical tools must have a very good sensitivity and specificity to detect levels of damage as low as one lesion per million nucleosides using a few μg of DNA. However, recent

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works, mainly performed through the European network ESCODD (European Standard Committee on Oxidative DNA Damage), have highlighted that the limitation for an accurate measurement of oxidative DNA lesion is not the sensitivity of the assay but rather the possibility that artifactual DNA oxidation occurs during the work-up, giving rise to significantly overestimated levels⁷⁻¹⁰.

In the present review article, the different methods aimed at measuring oxidative DNA lesions, mainly 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), are detailed and their advantages and limitations are discussed. Major attention will be focused on the origin(s) of potential problems that could give rise to over- or under-estimated levels of the measured oxidative DNA lesions.

The methods developed for measuring oxidative DNA lesions can be classified into two categories (Figure 1). The so-called "indirect approaches", also named "enzymic methods", combine the use of a specific DNA glycosylase and a method to measure DNA strand breaks. Specific DNA repair glycosylases are utilized to convert an oxidized DNA base into a strand break that could be measured by reliable sensitive methods including the well known comet, alkaline elution (AE) or DNA unwinding assays. The methods involving the use of specific antibodies or PCR-based methods to detect DNA lesions also could be considered as indirect approaches. The "direct approaches" consist first in the isolation of cellular DNA that is then hydrolyzed either enzymatically or chemically to either the nucleotide, nucleoside or nucleobase level. Then, the mixture of undamaged and damaged DNA constituents is resolved by a chromatographic system and the lesions of interest are detected, either by the use of sensitive physico-chemical techniques or subsequent to post-labeling with radioactive ³²P.

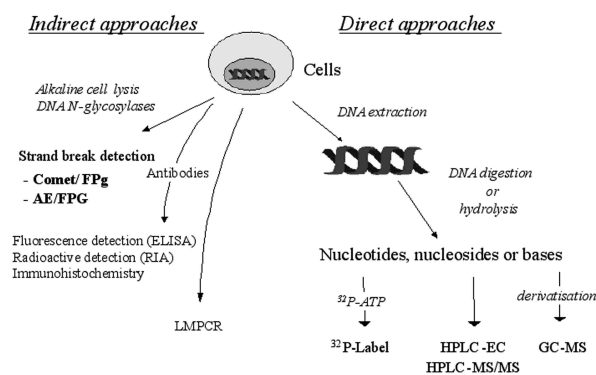


Figure 1. Classification of the different methods developed for the measurement of 8-oxodGuo as a biomarker of oxidative stress.

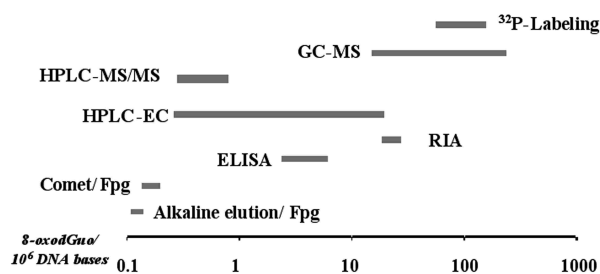


Figure 2. Overview of the reported cellular background levels of 8-oxodGuo according to the method used for the measurement. For indirect methods, variation observed for one method, such as HPLC-EC, could be explained at least in part by the difference in the DNA extraction protocols.

Indirect approaches

The comet assay¹¹ and the alkaline elution (AE) technique¹² were initially developed for the measurement of DNA strand breaks. In fact, both directly induced strand breaks and alkali-labile sites that are converted to strand breaks under the alkaline experimental conditions used are simultaneously measured with such an approach. For the AE technique, cells are lysed onto the top of a filter and then DNA is eluted through the filter using an alkaline solution¹³. The rapidity of DNA elution, determined by quantifying the amount of DNA in the collected fractions using a fluorescent intercalating dye, is directly correlated to the length of the DNA fragments and therefore to the number of DNA strands breaks¹⁴. For the comet assay^{15,16}, cells embedded in an agarose gel

onto a microscope slide are lysed under alkaline conditions and thereafter an electrophoresis is performed. DNA migration toward the anode could occur only if DNA contains strand breaks. DNA is then revealed under a fluorescent microscope using an intercalating dye, and migrated DNA loops form the tail of a comet. The percentage of DNA in the tail is related to the oligonucleotide break frequency¹⁷. Alkaline unwinding has been also used to quantify DNA strand breaks¹⁸. For such a purpose, DNA is allowed to unwind at alkaline pH. Then, following neutralization and sonication, single- and double-stranded DNA are separated and their respective amounts quantified. The proportion of double-stranded DNA decreases with an increase in the number of DNA strand breaks. To measure oxidized DNA bases, the different assays use the same strategy, consisting in the conversion of a DNA lesion into a strand break using specific DNA N-glycosylases. Then, the number of DNA lesions recognized by the DNA repair enzyme can be estimated by subtracting the number of strand breaks measured following incubation with the glycosylase to the number determined in the absence of any enzyme. The incubation with the DNA repair enzyme is performed onto the top of the filter or directly into the agarose gel, subsequent to cell lysis. Such a strategy has been mostly applied using two *E. coli* DNA N-glycosylases, the Fpg protein that is specific for oxidized purines, including 8-oxodGuo, and its corresponding formamidopyrimidine derivative 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), and Endonuclease III, which predominantly excises oxidized pyrimidines¹⁹⁻²¹. As a general trend, since 8-oxodGuo is known to be predominantly produced in DNA and since Fpg protein could excise that lesion efficiently, it is usually estimated that the level of Fpg-sensitive sites corresponds to the number of 8-oxodGuo. The background level of 8-oxodGuo estimated using such indirect approaches has been determined to be around 0.1-0.2 lesion per million nucleosides (Figure 2). This value constitutes one of the lowest estimated levels of the oxidized purine in cellular DNA. In particular, the level is significantly lower to that determined using direct approaches (*vide infra*)⁹. The pending question concerns the quantitative aspect of

that measurement and the calibration of the assays. Could the enzyme be efficient enough to recognize and excise all the oxidized purines when DNA is loaded onto a filter or embedded in an agarose gel? Kinetic studies performed using the AE assay strongly suggest that the efficiency is almost complete and thus that the approach does not underestimate the level of Fpg-sensitive sites¹⁴. However, it is obvious that two closely located DNA lesions will be determined as a single Fpg-sensitive site and this could contribute, though certainly not significantly, to an underestimation of the level of the oxidized purine. The other limitation of the assay concerns the calibration of the measurement. Calibration is usually indirect, using a defined number of strand breaks generated per cells upon X-ray irradiation⁹. All things considered, it seems today that the approaches using the Fpg protein, in combination with a sensitive method for measuring strand breaks, are accurate, very sensitive and give background values lower than those reported using direct approaches (*vide infra*). Another advantage of the assay concerns the reduced number of cells required for the measurement. In addition, DNA extraction, which could potentially induce DNA oxidation, is not required. However, the exact nature of the measured lesions is not completely known, and strongly depends on the specificity of the DNA repair enzyme used. For instance, considering Fpg-sensitive sites as 8-oxodGuo is restrictive since it is known that oxidative stress induces the formation of other DNA lesions recognized by the Fpg protein, including for example FapyGua. In addition, it has been shown that the Fpg protein is also able to excise alkylated DNA damage²². The use of more specific DNA glycosylases, such as hOGG1, the human 8-oxoguanine glycosylase, might overcome this limitation²³. It is also important to mention that the level of DNA damage that could be accurately measured by such assays is rather limited compared to indirect approaches. When levels of damage are too high, the quantification is not more linear, and it is generally considered that the method is quantitative over a range of about two orders of magnitude (from 0.1 up to 10 lesions per million nucleosides). This could explain at least partly the homogeneity of the levels of damage determined by such indirect ap-

proaches (Figure 2).

Concerning the methods involving the use of antibodies (mostly ELISA), only a few attempts have been made to develop specific antibodies raised again oxidative DNA lesions, including 8-oxodGuo²⁴. As a general observation, the specificity of the antibodies is not good enough to allow an accurate determination of the level of the lesion. This is due to a cross-reaction of the antibodies with parent unmodified guanine base which is structurally similar to 8-oxodGua. Therefore, quantitative measurement of the level of 8-oxodGuo using such an approach gives rise to overestimated determinations^{25,26}. However, immunohistochemical determination of 8-oxodGuo in tissues using the specific N45.1 antibodies²⁷ has received interesting applications indicating that these antibodies seem to have an excellent specificity for 8-oxodGuo (or another lesion?) in double-stranded DNA^{28,29}, but quantification is semi-quantitative. Attempts were also made using polymerase chain reaction technology (LMPCR) to identify where the oxidative DNA damage is located, in an inactive or active gene or even in telomeres^{30,31}. The sensitivity of the assay is still a limitation for such a determination and the specificity also depends on the enzyme used to cleave DNA at the site of the oxidized DNA lesion.

Direct approaches

The so-called direct methods developed to measure DNA lesions require, in a first step, isolation of cellular DNA. Thereafter, the macromolecule is hydrolyzed, either enzymatically or chemically, to related nucleosides, nucleobases or nucleotides (Figure 1). Then, the strategy to measure the lesion consists in the separation of the mixture of DNA constituents followed by a more or less specific detection of the lesions. DNA extraction and hydrolysis steps are of particular importance to obtain reliable results. DNA extraction. The major limitation of the indirect approach seems to be due to potential artifactual DNA oxidation during its extraction. This probably explains the origins of the discrepancies between the reported levels of 8-oxodGuo in the literature even when 8-oxodGuo was detected with similar methods of detection such as, for example, high performance

liquid chromatography-electrochemical (HPLC-EC) detection. As reported in Figure 2, values for 8-oxodGuo determined by HPLC-EC detection range from around 0.2 up to 40 lesions per million nucleosides in untreated cells. Efforts have been made recently, mostly through the European network ESCODD, to overcome that limitation. It could be first abusively estimated that any improvement in the DNA extraction method should result in a decrease in the measured level of 8-oxodGuo. However, the lowest value is not necessarily the best. For instance, with 8-oxodGuo being more easily oxidizable than parent 2'-deoxyguanosine, a lower level of 8-oxodGuo could be explained by a specific oxidation of 8-oxodGuo. With the help of the ESCODD network, an elegant approach was designed to allow an unambiguous determination of any improvement in the extraction protocol. For that purpose, a chemical precursor of labeled singlet oxygen³² is used to generate 18O-labeled 8-oxodGuo in cellular DNA³³ that is used as an internal standard. Then, different methods are used to extract DNA, and both unlabeled and labeled 8-oxodGuo are individually measured by HPLC-mass spectrometry (MS)/MS (vide infra). The method giving the highest level of 18O-8-oxodGuo and the lowest levels of 8-oxodGuo is the one that minimizes occurrence of DNA oxidation during the work-up. If the extraction method induces decomposition of the oxidized purine, then lower levels of 18O-labeled 8-oxodGuo are measured. Using such an approach, an optimized protocol³⁴ was set-up. Results indicate that to minimize DNA oxidation during extraction, in agreement with other works^{35,36}, DNA isolation requires first the transient isolation of nuclei followed by DNA precipitation using sodium iodide subsequent to RNA and protein hydrolysis, using desferoxamine-containing buffers. The protective role of desferoxamine indicates that DNA oxidation that can take place during extraction is due to Fenton type reactions initiated by transition metals. Using the optimized protocol, background levels of 8-oxodGuo in cellular DNA were determined around 0.5 lesion per million normal nucleosides, using either HPLC-EC or HPLC-MS/MS detection, a level only about 3 to 5 times higher than those reported by indirect approaches. Furthermore, it was noted that DNA precipitation

using phenol/chloroform³⁷⁻³⁹ is not recommended, nor are anion exchange columns³⁴, whereas guanidine thiocyanate seems more appropriate⁴⁰. It is also important to mention that extraction of about 50 µg of DNA is required to minimize artifactual DNA oxidation. When lower amounts are extracted, side oxidation becomes more important.

DNA hydrolysis. The accuracy of determination of the level of the lesion depends on the quantitative aspect of hydrolysis⁴¹. DNA could be hydrolyzed either enzymatically to release nucleosides or nucleotides, or chemically, generating the free bases. As a general trend, enzymatic digestion is used prior to liquid chromatography analysis including HPLC-EC and HPLC-MS/MS, whereas for gas chromatography (GC)-MS analysis, acid hydrolysis is preferred. Enzymatic digestion is usually performed in two steps: first DNA is digested to nucleotides that are subsequently hydrolyzed to nucleosides. To digest DNA into nucleotides, nuclease P1 has been mostly used. Such an enzyme hydrolyzes 8-oxodGuo with a similar efficiency to normal nucleosides. However, a few DNA lesions, including thymidine glycols⁴² and 5'-8-cyclo-2'-deoxyadenosine, are not good substrates for the enzyme⁴³. Therefore, the combined use of nuclease P1 together with exonucleases is recommended for a quantitative digestion⁴². Alkaline phosphatase, which has been mostly used to hydrolyze nucleotides into nucleosides, shows a broad specificity and is able to hydrolyze almost any nucleotide. It is important to mention that for the detection of a new DNA lesion, the efficiency of the enzymes to quantitatively liberate the nucleoside from DNA should be determined. If digestion is not complete compared to normal nucleosides, then the determined level of the lesion could be underestimated. The other potential problem concerns the possibility of artifactual generation of oxidative DNA lesions during DNA digestion⁴¹. However, it has been shown recently that if oxidation occurs during enzymatic digestion this is to a non-significant level³⁴.

Concerning acid hydrolysis, it has been shown that 8-oxoGua is quantitatively released upon DNA hydrolysis using formic acid treatment, as confirmed by comparison with enzymatic digestion⁴⁴. This is not the case with its corresponding ring-opened de-

rivative FapyGua, which has been shown to be unstable under these conditions^{45,46}. In conclusion, for an accurate determination of a defined DNA lesion, the quantitative aspect of the hydrolysis, together with the stability of the released compound under the conditions used, has to be checked.

Following DNA extraction and hydrolysis, different methods were developed for the quantitative determination of the level of 8-oxodGuo.

³²P-post-labeling

Few attempts have been made to use the ³²P-postlabeling approach to measure oxidative DNA lesions⁴⁷⁻⁴⁹. The strategy consists in the enzymatic incorporation of a radioactive phosphorous atom into a DNA lesion. The obtained radioactive nucleotide could then be sensitively detected and quantified after separation mostly using thin layer chromatography or HPLC. Initially, such an assay has been successfully developed for the detection of bulky DNA adducts^{50,51}. The limitation of the assay, which has the theoretical sensitivity to detect one lesion per 100 million nucleosides using few µg of DNA, is due to the required purification of the lesion prior to labeling. This purification is absolutely necessary since the presence of radioactive phosphorus may induce generation of lesions in presence of overwhelming normal nucleosides^{52,53}. For bulky DNA adducts, this could be efficiently performed using solid phase extraction, but for oxidative DNA lesions, HPLC purification has to be used. The difficulty is due to the fact that the product of interest, such as for example 8-oxodGuo, has to be collected in a "blind" manner, before being labeled. Therefore, large fractions are usually collected to provide a quantitative purification and then the presence of a contaminating product that could affect labeling efficiency can not be excluded. The second limitation of the assay concerns the accuracy, which is dependent on the efficiency of the enzymatic radioactive labeling⁵⁴⁻⁵⁶. Unfortunately, such efficiency has never been checked carefully but is often considered (abusively?) quantitative. It would not be surprising that the efficiency strongly depends on the nature of the DNA lesion, and the use of an internal standard would certainly significantly improve the assay. Finally, the specificity

of the method depends on the approach used to isolate the labeled products prior to the measurement of the radioactivity that allows determination of the amount of the lesion. Usually 2D thin layer chromatography is used for such a purpose and, at least in the field of oxidative DNA lesions, it can not be totally excluded that two lesions have similar chromatographic features under the conditions used. A specific post-reaction method to confirm the identity of the detected lesion could overcome that limitation⁵⁷. This also applied to HPLC, which has been used connected to a radioactive detector⁵⁸.

The main advantage of the ³²P-postlabeling is due to its high sensitivity that allows working with limited amounts of DNA. However, for oxidative DNA lesions, extraction of large amounts of DNA seems to be required to obtain reliable results. All things considered, it seems that the post-labeling assay is not suitable for measuring oxidative DNA lesions^{55,59,60}. The possible drawbacks of this approach, including self radiolysis processes, explain the high levels of 8-oxodGuo detected in cellular DNA using such an assay (Figure 2).

HPLC-EC. Actually, the developed HPLC separation coupled to EC detection^{61,62} method has significantly contributed to the popularity of 8-oxodGuo to the detriment of other oxidative DNA lesions. The method uses the fact that the oxidized purine has a redox potential far below that of normal nucleosides. The EC detector, coupled on-line to a HPLC separation, could thus specifically oxidize 8-oxodGuo, and such an oxidation liberates electrons that are sensitively detected. Overwhelming natural nucleosides are not detected since they are not oxidized at the defined potential, giving a specific and sensitive detection. About 0.1 pmole of 8-oxodGuo could then be detected in a hydrolyzed DNA sample. Such an assay has been usually used subsequent to enzymatic digestion of DNA to nucleosides. Interestingly, the combined use of nuclease P1 and alkaline phosphatase has been shown to digest efficiently normal nucleosides and, with a similar efficiency, 8-oxodGuo (vide infra). The HPLC system is usually also equipped with an UV detector set at 260 nm allowing the detection and quantification of normal nucleosides⁶³. Results could

then be expressed in the number of 8-oxodGuo per million nucleosides. It should be noted as well that the HPLC-EC assay could also be applied subsequently to acidic DNA hydrolysis, and then the free base 8-oxodGua is measured⁴⁴. Interestingly, both approaches give similar results. In addition, an internal standard could be used to compensate for any eventual loss of sensitivity during analyses. The HPLC-EC approach has been extended as well to the detection of other DNA lesions, including 5-hydroxycytosine⁶⁴ and 8-oxo-7,8-dihydro-2'-deoxyadenosine⁶⁵. However, since separation has to be performed under isocratic conditions, it is almost impossible to detect the three nucleosides in one injection. It could be mentioned in addition that FapyGua could, in theory, be detected by such an approach⁶⁶, but the limitation concerns the polarity of the product that is eluted in the void volume of the octadecylsilyl silica gel column used.

GC-MS. Also in the early 80s, another assay using gas chromatography mass spectrometry (GC-MS) was developed for the detection of several DNA lesions, including 8-oxoguanine⁶⁷. The assay requires in the first step acid hydrolysis of DNA into the corresponding free bases. Then, since GC can be performed only using volatile compounds, the DNA bases have to be chemically converted into volatile derivatives, using either trimethylsilyl (TMS) or tert-butyldimethylsilyl (TBDMS) groups. To obtain a good efficiency, such a derivatization reaction was initially performed at elevated temperature (130°C)⁶⁸. Then, the DNA lesions are detected using a sensitive MS approach, usually using at least two specific ions allowing a good specificity. Compared to the EC detection, the MS detector is more versatile. Therefore, in a DNA sample, in addition to 8-oxodGua, several DNA lesions could be quantified simultaneously^{69,70} with a good sensitivity. In addition, one of the advantages of using a mass spectrometric detection concerns the possibility of using isotopically labeled internal standard to improve the accuracy of determination⁷¹. Therefore, several isotopically labeled DNA lesions were synthesized and used for isotope dilution MS⁷²⁻⁷⁴.

The two methods, HPLC-EC and GC-MS, were de-

veloped almost simultaneously in the early 80s, and as early as 1992⁷⁵, it was observed that the level of 8-oxodGuo determined in DNA strongly depends on the method used for the measurement. In general, values obtained by the GC-MS assay were significantly higher (one or two orders of magnitude) than those reported by the HPLC-EC approach⁷⁶. The difference in DNA hydrolysis method was postulated to be at the origin of the discrepancy between the results of the two approaches, but this hypothesis was ruled-out a couple of years later. In fact, it has been now clearly established that the higher levels reported by the GC-MS approach were due to an artifactual DNA oxidation occurring during the silylation step performed at high temperature^{77,78}. The derivatization reaction that has to be performed in order to increase the volatility of the modified bases has been shown to induce significant oxidation of normal DNA bases. Purification of 8-oxodGua, either by HPLC or immunoaffinity chromatography, prior to the derivatization reaction, prevents the artifactual oxidation from occurring⁷⁷. However, purging the derivatization solution with nitrogen, or reducing the temperature of silylation, was found insufficient to completely suppress the occurrence of artifactual oxidation of DNA. It is interesting to note that 8-oxodGua levels determined by GC-MS after HPLC purification of the lesion are similar to those determined using the HPLC-EC approach. Artifactual oxidation of normal DNA bases was found not to be limited to 8-oxodGua but to also take place in other DNA lesions⁷⁹. Therefore, it appears that the GC-MS method is not suitable for an accurate determination of oxidative DNA lesions, the pre-purification step of the lesion to be measured prior to derivatization being inconvenient for routine analyses of DNA samples. To overcome this limitation, it has been proposed to use specific DNA repair enzymes (such as Fpg and Endo III) to specifically hydrolyze DNA lesions prior to derivatization. Values reported using such an approach are significantly lower than those reported following acidic DNA hydrolysis and similar to those determined by HPLC-EC⁸⁰. However, as already mentioned for the indirect approach, quantitative hydrolysis of the lesions is questionable since *in vitro* efficiency of excision is usually not quantitative^{81,82}. In addition, using such

an approach, the simultaneous measurement of normal DNA bases is not possible and the amount of DNA has to be determined separately.

HPLC-MS/MS. With the development of electrospray ionization^{83,84} at the end of the last century, compared to non-sensitive thermo-spray ionization⁸⁵, HPLC coupled to MS represents a promising alternative combining the advantages of the two above-mentioned methods (i.e. HPLC-EC and GC-MS)^{86,87}. However, it has been rapidly shown that to achieve the required sensitivity for measuring oxidative DNA lesions in cells, tandem mass spectrometry is necessary using the so-called "multiple reaction monitoring" (MRM) mode^{88,89}. Typically, the either protonated (positive mode) or deprotonated (negative mode) pseudo-molecular ion of the molecule of interest generated during the ionization process⁹⁰ is filtered in the first quadrupole and then fragmented in the collision cell. For 8-oxodGuo, detected with a higher sensitivity in the positive ionization mode, collision-induced dissociation of the $[M+H]^+$ ion at $m/z = 284$ generates as the major fragment an ion at $m/z = 168$ corresponding to the protonated base due to a loss of the 2-deoxyribose moiety. Such a fragmentation has been found to be common for most nucleosides, at least in the positive ionization mode⁹⁰. Then the third quadrupole is used to select the most abundant daughter ion, 168 Da for 8-oxodGuo, that is then sensitively detected. Such a HPLC-MS/MS detection method using the transition 284->168 is very specific and also very sensitive. It should be noted that a few works^{91,92} have reported the measurement of low levels of DNA lesions using a HPLC-MS approach using only one quadrupole in the so-called "selected ion monitoring" (SIM) mode, with a claimed sensitivity close to that obtained by other groups using HPLC-MS/MS. Nevertheless, it should be remembered that tandem mass spectrometry was developed to increase both specificity and sensitivity of HPLC-MS, and this is of major importance to achieve the required sensitivity compatible with the low cellular levels of DNA lesion. Such an assay was first applied to the detection of 8-oxodGuo in DNA or biological fluids using^{88,93} or not⁹⁴ isotopically labeled internal standard. Then the assay was extended to several oxidative DNA lesions^{42,95-98} with almost similar sensitivity for the

different modified nucleosides.

The main advantage of HPLC-MS/MS⁹⁹⁻¹⁰¹ compared to GC-MS is that the derivatization reaction, which could induce artifactual DNA oxidation (vide supra), is not required. In addition, compared to HPLC-EC detection, HPLC-MS/MS is much more versatile and specific. In terms of sensitivity, all three methods have similar limits of detection but recent development of new generation tandem mass spectrometers now allows detection of amounts of DNA lesions at the fmole range. In addition, as already reported for GC-MS, isotopically labeled internal standard could be used to increase the accuracy of the detection. Interestingly, HPLC-MS/MS detection is not limited to oxidative DNA lesions¹⁰² but has been also successfully applied for the detection of several DNA adducts, including adducts arising from lipid peroxidation¹⁰³, UV-induced pyrimidine dimers¹⁰⁴, chlorinated nucleosides¹⁰⁵ and also bulky DNA adducts^{106,107}. Tandem mass spectrometry could have additional advantages as well¹⁰⁸, allowing for example the measurement of isotopically labeled compounds for mechanistic purposes^{33,109,110}. In addition, the assay could be used to search for unknown DNA lesions^{111,112} and also to detect spin trapped DNA radicals¹¹³, nucleotides¹¹⁴ and RNA lesions¹¹⁵. It should be mentioned that such a detection method is particularly well adapted for the measurement of oxidized DNA bases in biological fluids such as urine^{88,116,117}.

CONCLUSION

It is clear today that the choice of using 8-oxodGuo as a marker of DNA oxidation, mostly dictated by the availability of a sensitive method for its detection (i.e. HPLC-EC), was well adapted since 8-oxodGuo appears to be a predominant DNA lesion generated under various conditions of stress. However, the fact that 8-oxodGuo can be "easily" generated in DNA explains the difficulties encountered in its accurate quantitative determination in cellular DNA. This is due to the possible artifactual formation of the lesion during the work-up. The possible origins of such an artifactual formation are now identified and could be minimized using optimized protocols. As a consequence of such an optimization, the method used to measure biologically relevant levels of 8-oxodGuo

should have an excellent sensitivity allowing a determination of less than one lesion per million normal nucleosides. In that respect, the costly HPLC-MS/MS assay represents the most powerful detection method that unfortunately remains inaccessible to most academic laboratories. Less specific, indirect approaches combined with specific DNA repair enzymes seem to represent an interesting alternative for biological studies, which probably explains the extensive use of the comet assay reported in the literature during the last decade.

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