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# Comparative analysis of baseline 8-oxo-7,8-dihydroguanine in mammalian cell DNA, by different methods in different laboratories: an approach to consensus

**ESCODD (European Standards Committee on Oxidative DNA Damage)***

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**The European Standards Committee on Oxidative DNA Damage (ESCODD) was set up to resolve the problems associated with the measurement of background levels of oxidative DNA damage (in particular 8-oxo-7,8-dihydroguanine, or 8-oxoGua) in human cells. A tendency for DNA oxidation to occur during sample preparation prior to chromatography has been recognized as the source of a very substantial artefact. To assess the success of attempts to eliminate the artefact, ESCODD has distributed to its members standard samples of pig liver and HeLa cells for analysis. Estimates of 8-oxoGua in pig liver, using chromatographic techniques, ranged from 2.23 to 441 per $10^6$ guanines, with a median of 10.47 per $10^6$ guanines. Chromatographic analysis of HeLa cell DNA gave a range of 1.84 to 214 per $10^6$ guanines with a median of 5.23 per $10^6$ guanines. HeLa cell DNA was also analysed by an enzymic approach, in which whole cell DNA was treated with formamidopyrimidine DNA glycosylase, which nicks DNA at sites of 8-oxoGua, and the breaks measured with the comet assay, alkaline unwinding or alkaline elution. Values with these methods ranged from 0.06 to 4.98 8-oxoGua per $10^6$ guanines, with a median of 0.79 per $10^6$ guanines. Although there are clearly still serious discrepancies between methods and laboratories, the lowest estimates by chromatography (arguably those in which the artefact was best controlled) are only 2.5 times higher than the median value obtained with the enzymic approach.**

**Abbreviations:** FPG, formamidopyrimidine DNA glycosylase; GC, gas chromatography; HPLC, high performance liquid chromatography; MS, mass spectroscopy; 8-oxoGua, 8-oxo-7,8-dihydroguanine.

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## Introduction

In recent years it has become increasingly clear that, when the background level of 8-oxo-7,8-dihydroguanine (8-oxoGua) is estimated in normal human cells, much of what is measured may in fact be artefact, since guanine is easily prone to oxidation if stringent precautions are not taken during preparation of samples for analysis (1). This undoubtedly contributes to the variation seen in published estimates of oxidative damage to DNA in human cells, which amounts to three orders of magnitude (2). Efforts have been made to decrease the extent of the oxidation artefact, for example by decreasing the time and temperature of the derivatization step required for GC–MS (after acid hydrolysis of DNA), and by including antioxidants and iron chelators in solutions used in the procedures for assay by GC–MS, HPLC with electrochemical detection, and HPLC–MS/MS.

The European Standards Committee on Oxidative DNA Damage (ESCODD) was set up following a meeting that explored the reasons for discrepancies in the measurement of oxidative DNA damage (2). Its aims are to optimize detection methods and devise standard protocols, and ultimately to reach consensus over the basal level of DNA damage in human cells. Identical samples have been sent to 27 member laboratories for analysis. So far, ESCODD has published results of comparative measurements of 8-oxo-7,8-dihydroguanine (or the related 2′-deoxyribonucleoside, namely 8-oxo-7,8-dihydro-2′-deoxyguanosine or 8-oxodGuo) in standard 8-oxodGuo solutions, commercial calf thymus DNA, calf thymus DNA with additional experimentally induced base oxidation, and oligonucleotides containing defined proportions of 8-oxoGua (3–5). In the analysis of both 8-oxodGuo and calf thymus DNA (4), about half the results returned were within 20% of the target or median value respectively, and a CV of $<10\%$ was recorded by half the laboratories when measuring identical samples on three different occasions. However, some laboratories returned values several-fold higher or lower than the target or median. Accuracy did not depend on method; GC–MS, HPLC–MS/MS, and HPLC with amperometric as well as coulometric detection were represented among those scoring within 20% of target or median.

The samples distributed in this round of ESCODD were (a) pig liver; and (b) transformed human (HeLa) cells. Both were sent frozen. The level of oxidative damage in this fresh material was expected to be far less than in calf thymus DNA and therefore to present a more demanding test of the various methods.

Because the HeLa cells were frozen in a viable state, they were suitable for measurement of oxidative DNA damage

by a group of methods using the bacterial repair enzyme formamidopyrimidine DNA glycosylase (FPG) to remove 8-oxoGua and nick the DNA via its associated lyase activity against baseless sugars. Measurement of the ensuing strand breaks requires intact, high molecular weight cellular DNA, and these methods could not be used to measure 8-oxoGua in calf thymus DNA in the previous round. The methods are alkaline elution (6), alkaline unwinding (7) and the comet assay (8). 'Alkaline elution' refers to the movement of single-stranded DNA (at high pH) through a filter; elution is accelerated if breaks are present. In the alkaline unwinding technique, lysis of cells at high pH for a fixed period, followed by neutralization, leaves the DNA partially single-stranded, to an extent that depends on break frequency; the fractions of single- and double-stranded DNA are measured by hydroxyapatite chromatography. The comet assay detects the break-induced relaxation of supercoiled loops of DNA in cells embedded in agarose and lysed to form nucleoids; the relaxed loops extend under electrophoresis to form a comet image, the relative intensity of 'comet tail' compared with 'head' reflecting break frequency. All three of these enzymic techniques are calibrated indirectly by comparison with cells irradiated with ionizing radiation to induce breaks at a known dose rate. When applied to the measurement of 8-oxoGua in human cell DNA, they have produced estimates substantially lower than those from other techniques (6,9,10,11).

## Materials and methods

*Pig liver*

A Danish specific pathogen-free female pig, weighing ~60 kg, was killed by a bolt pistol brain shot, followed by exsanguination. The liver was removed and placed on ice. After ~2 h on ice, the liver was cut into pieces (~0.5 $cm^2$) and placed in cryotubes (Nunc, Denmark), which were filled with nitrogen gas and stored at –80°C. Three samples were sent out on dry ice to each laboratory for each method used. Laboratories were requested to store the liver at –80°C until use. The outside of the piece of liver was likely to be oxidized through exposure to air, and therefore removal using surgical knives was recommended, before dividing the sample into three for separate DNA extractions. Each of the DNA preparations was to be analysed in triplicate.

*HeLa cells*

HeLa (human transformed epithelial) cells were grown to confluence in thirty 50 ml flasks (Nunc) in Glasgow-modified Eagle's Minimal Essential Medium (GMEM) with 5% fetal calf serum, 5% calf serum, supplemented with glutamine and non-essential amino acids. The cells were harvested by trypsinization and the cells from each flask were transferred to a polystyrene roller bottle (850 $cm^2$: Corning Incorporated, US) in 75–100 ml medium in an ~6% $CO_2$ atmosphere.

The bottles were placed on a rolling machine and after 6 days the medium was changed; after a further 6 days, the cells were harvested from two bottles at a time as above. Medium was added and the cells were transferred to sterile glass bottles and kept at 37°C until all the cells had been harvested. The cells were combined in one roller bottle and gently mixed by inversion, the volume measured and the cell density determined using a haemocytometer. The cells were collected by centrifugation of 50 ml aliquots at 700 *g* for 7 min at 20°C. The cell pellet was gently dispersed in 1 ml freezing mix (GMEM containing 20% fetal calf serum, 10% DMSO) and further freezing mix was added to a cell density of $5 \times 10^6$/ml. The cell suspension was divided into aliquots of: (a) $55 \times 10^6$ cells for HPLC, HPLC–MS/MS and GC/MS; (b) $1.5 \times 10^6$ cells for the comet assay; (c) $10 \times 10^6$ cells for alkaline elution; and (d) $6 \times 10^6$ cells for alkaline unwinding.

The first harvest of cells gave a total of $24.4 \times 10^8$ cells; the aliquots were labelled '1'. The second harvest gave $42.6 \times 10^8$ cells; half the aliquots from this second harvest were labelled '2' and the other half were labelled '3', so that the cells in tubes 2 and 3 were identical. The cells were cooled slowly in a –80°C freezer. For distribution to participating laboratories, the tubes of cells were sent out in expanded polystyrene boxes containing dry ice. On receipt, the laboratories were requested to store the cells at –80°C until use. For chromatographic analysis, two DNA preparations were to be carried out on each tube, each preparation to be analysed in triplicate. In the case of the enzymic methods, triplicate measurements were requested from each sample of cells.

Tubes retained for quality control were placed in polystyrene boxes with dry ice for 24 h, except for two tubes of harvest 1 and one tube of harvest 2/3, which were kept at –80°C throughout to test for any effect of transfer to, and transport in, dry ice.

*DNA preparation, hydrolysis and analysis*

*Isolation of DNA.* Methods for isolating DNA varied too widely to be described in detail. They included different homogenization buffers, with and without the antioxidant Desferal, at different temperatures; different times and temperatures of incubation with SDS, RNase and protease; use of a commercial DNA extraction kit; use of chloroform/3-methyl-1-butanol to partition DNA, and NaCl or NaI during DNA precipitation with ethanol or 2-propanol; and different methods for drying and subsequently dissolving DNA.

As analytical methods also vary in particulars between laboratories, only brief details are given here.

*HPLC.* DNA was hydrolysed enzymically with P1 nuclease and alkaline phosphatase, or with P1 nuclease and acid phosphatase (one laboratory), or deoxyribonuclease, P1 nuclease and alkaline phosphatase (one laboratory). Separation of DNA hydrolysate, 8-oxodGuo and dGuo samples on a C18 column was followed by electrochemical detection (coulometric or, in two laboratories, amperometric) of 8-oxodGuo, and UV detection of dGuo.

*GC–MS.* Samples were hydrolysed to bases with 60% formic acid at 120/130°C for 30–45 min. One laboratory carried out hydrolysis under argon. Bases were derivatized with bis(trimethylsilyl)trifluoroacetamide in acetonitrile at room temperature for up to 2 h, under argon or nitrogen; one laboratory added ethanethiol to prevent oxidation.

*HPLC–MS/MS.* Three laboratories used liquid chromatography followed by mass spectroscopy. Nucleosides were separated on a C18 column and injected into a triple quadrupole mass spectrometer for identification and quantitation of products.

*Enzymic methods.* The three methods used to measure DNA breaks have been described in the Introduction. Eight laboratories used the comet assay, one alkaline elution and one alkaline unwinding. The enzyme FPG, used to convert 8-oxoGua in DNA to single strand breaks, was from various sources. Of those laboratories employing the comet assay, two used purified preparations from commercial sources (BD Biosciences Pharmingen, Heidelberg, Germany, and Trevigen, Gaithersburg, USA); the others used crude extract prepared in the Rowett Research Institute. FPG for the alkaline unwinding and alkaline elution methods was obtained from Serge Boiteaux (CNRS–CEA/Fontenay aux Roses, France).

## Results

### *Chromatographic analysis*

*Pig liver.* Twenty-one sets of results were returned (19 complete). Three were analysed by GC–MS, three by HPLC–MS/MS and the rest by HPLC (13 using coulometric and two amperometric detection). The mean values for each laboratory (or for each method where one laboratory used two methods) are presented in Figure 1 in ascending order on a logarithmic scale. The median value, 10.47 8-oxoGua lesions per $10^6$ guanines, is indicated by a line. Individual mean values show a range of 200-fold, with no sign of a consensus. The highest value reported was obtained by a laboratory using GC–MS and taking the most rigorous precautions to prevent oxidation; hydrolysis and derivatization were performed under argon and derivatization in the presence of ethanethiol. The lowest estimates (from three laboratories using HPLC with coulometric detection, HPLC with amperometric detection and HPLC–MS/MS) are below three 8-oxoGua per $10^6$ guanines. Inter-tube CVs were calculated; only two of 20 laboratories achieved a CV of $<10\%$, and 12 of $<20\%$.

*HeLa cells.* Nineteen sets of results were returned (17 complete); 15 analysed by HPLC (two amperometric), two by GC–MS and two by HPLC–MS/MS. The mean values are presented in Figure 2 in ascending order with the median value of 5.23 8-oxoGua lesions per $10^6$ guanines indicated. A factor of over 100 separates the highest and lowest values.

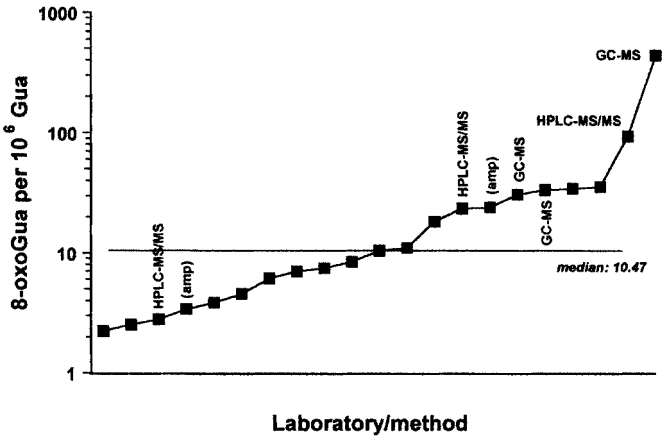


**Fig. 1.** Results of chromatographic analysis of 8-oxoGua content of pig liver DNA, arranged in order of increasing values found; note log scale. HPLC with coulometric detection was employed except where otherwise stated; two laboratories used HPLC with amperometric detection (amp).

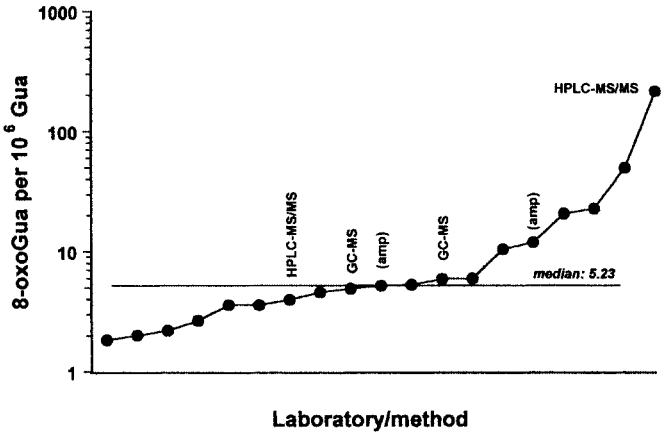


**Fig. 2.** Results of chromatographic analysis of 8-oxoGua content of HeLa cell DNA, arranged in order of increasing values found; note log scale. HPLC with coulometric detection was employed except where otherwise stated; two laboratories used HPLC with amperometric detection (amp).

Three of 18 achieved inter-tube CVs of <10%, and nine of 18 of <20%.

*HeLa cells; analysis by enzymic methods*

Samples of HeLa cells were analysed with the comet assay (in the coordinating laboratory) over a period of 65 days including the period allocated for analysis by participating laboratories (Figure 3). There was no significant change in 8-oxoGua concentration with time, and no difference between samples of the two harvests: mean values were 0.98 ± 0.20 (SD) per $10^6$ guanines for harvest 1 ($n$ = 11), and 0.95 ± 0.11 (SD) per $10^6$ guanines for harvest 2/3 ($n$ = 9). Samples 1, 2 and 3 were therefore regarded as identical in the analysis of results. Cells transferred from –80°C to dry ice for 24 h and then returned to –80°C gave the same value for background breaks and 8-oxoGua concentration as did cells kept at –80°C throughout (results not shown).

Ten laboratories measured 8-oxoGua in HeLa cell DNA using methods based on the induction of DNA breaks by FPG at sites of the oxidized base, and subsequent measurement of breaks using the comet assay (eight laboratories), alkaline elution or alkaline unwinding (one laboratory each). Figure 4 shows the results. The median value of 0.79 8-oxoGua lesions per $10^6$ guanines is 7× less than the median for chromatographic methods. Five of the ten laboratories were within ± 25% of the median. Three of ten results showed inter-tube CVs of <10%, and five of ten <20%.

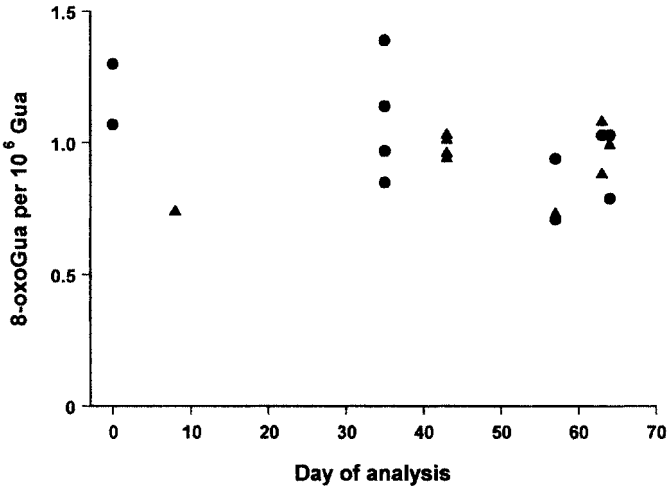


**Fig. 3.** Comet assay analysis of 8-oxoGua content of samples of HeLa cell DNA from cell harvest 1 (circles) and cell harvest 2/3 (triangles) over a period of 65 days; a quality control check.

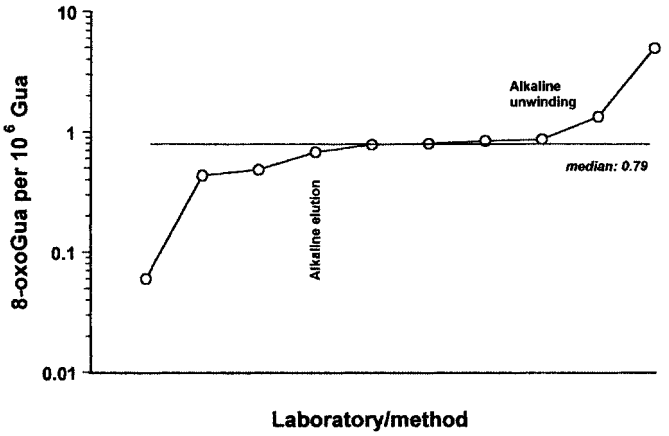


**Fig. 4.** Results of analysis of 8-oxoGua content of HeLa cell DNA by enzymic methods, arranged in order of increasing values found; note log scale. The comet assay was employed except where otherwise stated.

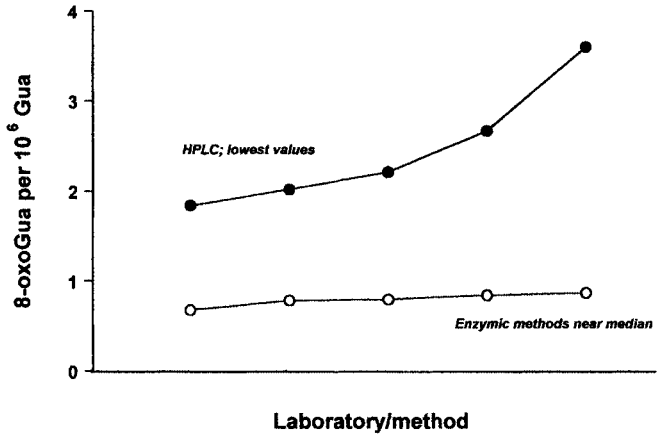


**Fig. 5.** Comparison of determinations of 8-oxoGua content of HeLa cell DNA by HPLC (lowest five values; solid circles) and enzymic methods (results closest to median; open circles).

## Discussion

The range of values of 8-oxoGua reported for liver DNA, in spite of the efforts of ESCODD, is disappointing. The range is distorted by a few very high values, and among the others there is no consensus. GC–MS analyses of liver DNA are high compared with most HPLC results. Although the results with HeLa cell DNA also include some very high values, in contrast to pig liver, GC–MS analyses gave values at the median, and one of two HPLC–MS/MS methods gave a very high value. The DNA extraction procedure which gave rise to this high value was subsequently found to be particularly prone to adventitious oxidation (12). Oxidation of DNA is more likely to occur during isolation from liver than from HeLa cells, since during tissue homogenization and cell lysis, iron released from the cells might act as a redox catalyst.

Clearly some laboratories must address very serious problems, either in their analytical methods, or in handling DNA without introducing spurious oxidation. The improvement in accuracy achieved through the use of common 8-oxodGuo standards (4) implies that the oxidation artefact is now of greater concern than analytical variation. This is borne out by results from a subsequent ESCODD trial (manuscript in preparation). Results from different laboratories are quite consistent between the two trials reported here; six of the seven laboratories which reported values for HeLa above the median also reported pig liver values above the median. It may be significant that the lowest estimates of 8-oxoGua for liver and for HeLa cells are very similar, around 2 per $10^6$ guanines. It is also worth noting that HPLC with amperometric detection – which is in theory less sensitive than coulometric detection – is capable of measuring low levels of damage.

Apart from one result that is very low and one that is several times the median, the results of the enzymic assays agree well. All laboratories employing the comet assay used the same calibration curve, based on X-irradiation of cells to introduce breaks (8), to convert results to the standard units (8-oxoGua per $10^6$ guanines), and this might account for some of the concordance. However, alkaline unwinding and alkaline elution were calibrated completely independently, and the closeness of the results obtained with all three methods is therefore remarkable. FPG came from four different sources, and the results that were close to the median were derived using enzymes from all four sources. The possibility of systemic errors in the enzymic assays has been discussed (13). Some lesions may occur in a particular DNA structure that renders them resistant to FPG. If lesions occur in clusters, very close together in the DNA molecule, they will register as a single break. Both these factors would result in underestimation of damage. But FPG recognizes ring-opened purines (formamidopyrimidines) as well as 8-oxoGua, and if these lesions are also present, the enzymic assays will *over*estimate 8-oxoGua. There is a need for investigation into whether these factors are significant.

A 'hybrid' method has recently been described, in which 8-oxoGua is released from DNA by FPG and measured by HPLC (14). The level of 8-oxoGua in HeLa cell DNA was at the limit of detection, estimated at $<0.4$ per $10^6$ Gua – consistent with the low values obtained here with the enzymic approach.

We can probably regard the median value as the best estimate of the background level of base damage according to the methods using FPG. In the case of the chromatographic methods, it can be argued that spurious oxidation during work-up has been a problem and is still a problem for certain laboratories, and so probably the most trustworthy values from those techniques are the lowest. In Figure 5, therefore, we have plotted (on a linear scale) the five values from enzymic assays closest to the median (all within 10%) and the five lowest chromatographic determinations, all HPLC with coulometric detection. A factor of only 2.5 separates the very lowest HPLC value from the median of the enzymic assays.

The artefact of oxidation during DNA isolation has been widely recognized, and as attempts are made to eliminate it, published values for basal 8-oxoGua concentrations using chromatographic techniques have decreased. A value of between 1 and 5 8-oxoGua residues per $10^6$ guanines for the background level of DNA base oxidation in HeLa cells is consistent with recent reports for human white blood cells measured by HPLC (15–17). It may be necessary to reassess earlier studies in which much higher levels of 8-oxoGua were reported in these cells.

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