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Inter-laboratory Validation of Procedures for Measuring 8-oxo-7,8-dihydroguanine/8-oxo-7,8-dihydro-2′-deoxyguanosine in DNA

ESCODD (European Standards Committee on Oxidative DNA Damage)

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The aim of ESCODD, a European Commission funded Concerted Action, is to improve the precision and accuracy of methods for measuring 8-oxo-7,8-dihydroguanine (8-oxoGua) or the nucleoside (8-oxodG). On two occasions, participating laboratories received samples of different concentrations of 8-oxodG for analysis. About half the results returned (for 8-oxodG) were within 20% of the median values. Coefficients of variation (for three identical samples) were commonly around 10%. A sample of calf thymus DNA was sent, dry, to all laboratories. Analysis of 8-oxoGua/8-oxodG in this sample was a test of hydrolysis methods. Almost half the reported results were within 20% of the median value, and half obtained a CV of less than 10%. In order to test sensitivity, as well as precision, DNA was treated with photosensitiser and light to introduce increasing amounts of 8-oxoGua and samples were sent to members. Median values calculated from all returned results were 45.6 (untreated), 53.9, 60.4 and 65.6 8-oxoGua/10^6 Gua; only seven laboratories detected the increase over the whole range, while all but one detected a dose response over two concentration intervals. Results in this trial reflect a continuing improvement in precision and accuracy. The next challenge will be the analysis of 8-oxodG in DNA isolated from cells or tissue, where the concentration is much lower than in calf thymus DNA.

Keywords: Oxidative DNA damage; 8-Oxodexyguanosine (8-oxo-dG); Methods validation; Photosensitiser

INTRODUCTION

It is now generally accepted that oxidation of guanine in DNA can readily occur during sample preparation and that, as a result, estimates of the base 8-oxo-7,8-dihydroguanine (8-oxoGua) or the

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nucleoside 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in human cellular DNA have generally exaggerated the background level—sometimes by orders of magnitude.[1] Methodological differences between laboratories have resulted in a lack of consistency in the analysis of identical samples of 8-oxodG.[2] The European Standards Committee on Oxidative DNA Damage (ESCODD), set up in 1997 to resolve these issues, has—since February 2000—been supported by the European Commission as a Concerted Action, with 27 laboratories as members. Here we report on the results obtained during the first year of this Action.

The initial requirement was that participating laboratories should be able to measure 8-oxodG, as well as dG itself, in standard solutions distributed from the laboratory of the ESCODD co-ordinator. The next task was to measure 8-oxoGua/8-oxodG in samples of calf thymus DNA, which required hydrolysis either to nucleosides using DNA-degrading enzymes (prior to analysis by HPLC) or to bases with formic acid (the procedure normally used for GC–MS). A more critical test was presented in the form of DNA that had been treated to introduce varying amounts of additional 8-oxoGua. In addition, oligonucleotides containing different proportions of 8-oxodG were analysed; results of this trial will be presented separately.

METHODS

Samples Distributed for Analysis

Samples were sent (by ordinary mail) in three batches. Batch one, in April 2000, consisted of 8-oxodG samples A, B, C and D, plus one sample of dG. Batch two (June 2000) contained a further four 8-oxodG samples—E, F, G and H—together with a sample of calf thymus DNA. Also provided in batch two were standard dG (3.97 mM) and 8-oxodG (68.93 nM) solutions, to be used to prepare standard curves for calibration of the assays for analysis of 8-oxodG in DNA. Batch three was sent in October, and comprised freeze-dried samples of calf thymus DNA solutions (J, K, L and M) that had been treated to induce varying amounts of additional 8-oxoGua.

8-OxodG Samples A, B, C, D, and dG

Solutions of 8-oxodG and dG (Sigma, Poole, UK) were prepared in HPLC-grade water (Rathburn Chemicals, Walkerburn, UK) and sterilised by passage through 0.22 μm filters (Millipore, Molsheim, France). The concentration of the 8-oxodG stock solution was estimated spectrophotometrically ($\epsilon_{245} = 12.3\,\mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$ in water) and diluted to 3.66 nM (solution D), 1.37 nM (B, C) and 0.46 nM (A). The dG solution was approximately 150 μM, estimated by weighing. Solutions were dispensed as 1.5 ml aliquots in sterile low-retention microcentrifuge tubes (AxyGen Scientific, Union City, California, USA); three aliquots of each were sent to each participant.

8-OxodG Samples E, F, G, H

Samples were prepared as for batch one and aliquoted into sterile polypropylene cryovials (Nunc, Roskilde). Three aliquots of each were sent to each participant. 8-OxodG concentrations were 0.4 nM (F), 0.9 nM (E, H) and 2.5 nM (G).

Calf Thymus DNA

DNA was obtained from Sigma (type 1, sodium salt, highly polymerised). For batch two, it was cut into pieces using scissors and three pieces of approximately 2 mg were sent in separate microcentrifuge tubes (Eppendorf, Hamburg, Germany). Tubes were left open to the air until all the DNA had been dispensed.

Samples J, K, L, M

For batch three, calf thymus DNA was dissolved at 0.2 mg/ml in 10 mM Tris–HCl, 2 mM Na_2EDTA, pH 8.5. The solution was divided into four portions of 300 ml, and the photosensitiser Ro 19-8022 (Hoffmann-La Roche, Basel, Switzerland) was added from a 1 mM solution to final concentrations of 7.5 μM (K), 20 μM (J), 30 μM (M) or 0 (L). The solutions (as 50 ml aliquots in 150 mm diameter dishes) were irradiated for 5 min on ice at 33 cm from a 1000 W halogen lamp. When not being irradiated, the solutions were stored on ice in the dark. NaCl was added to a concentration of 1.4 M and the DNA was precipitated by addition of ethanol at −20°C. After 15 min on ice, the supernatant was removed and the DNA precipitate washed with 3 × 150 ml of ice-cold 70% ethanol, and then dried under nitrogen. HPLC-grade water was added to redissolve the DNA. Concentrations of DNA in each solution were measured by absorbance at 260 mm. Aliquots were pipetted into microcentrifuge tubes (approximately 250 μg per tube). The tubes were covered with Parafilm (perforated), snap-frozen in liquid nitrogen, and freeze-dried overnight. Lids were closed and samples stored at room temperature in the dark.

Analysis

Samples were analysed on three occasions, at least 2 days apart, using a different tube of sample on each occasion and performing the analysis in triplicate. Thus we were able to calculate the coefficient of

variation (CV) for the triplicate determinations (intra-tube variability) and for the three different samples (inter-tube variability).

Analytical Procedures

Brief details only are given, as methods vary in detail between laboratories.

HPLC: DNA was hydrolysed enzymically with P1 nuclease and alkaline phosphatase (most laboratories), or with P1 nuclease, alkaline phosphatase and T1 ribonuclease (one laboratory), or deoxyribonuclease I, phosphodiesterase I, phosphodiesterase II and alkaline phosphatase (two laboratories). Separation of DNA hydrolysate, 8-oxodG and dG samples on a C18 column was followed by electrochemical detection (coulometric or, occasionally, amperometric) of 8-oxodG, and UV detection of dG.

GC–MS: Samples were hydrolysed to bases with 60% formic acid at 130/140°C for 30–45 min. Bases were derivatised with bis(trimethylsilyl)trifluoroacetamide at room temperature for up to 2 h, under argon or nitrogen and usually with ethanethiol present to prevent oxidation.

LC–MS–MS: Two laboratories used liquid chromatography followed by mass spectroscopy. Nucleosides separated on a C18 column were injected into a triple quadrupole mass spectrometer for identification and quantitation of products. Heavy isotope-labelled standards were used for mass spectroscopy.

RESULTS AND DISCUSSION

Measurement of Standard DG and 8-oxodG Solutions

Three samples of a solution of dG were analysed by all laboratories (in some cases by more than one method), in triplicate, by HPLC, GC–MS and LC–MS–MS against their own standard. The mean value of all the laboratories was 161.8 μM, compared to an expected value of 154.5 μM. Virtually all laboratories achieved a CV of $<5\%$ for intra-tube variability (i.e. CV of triplicate determinations on the same sample) and two-thirds showed a CV of $<5\%$ for inter-tube variability (i.e. CV of the mean values obtained on the three different analysis days).

Two sets of standard 8-oxodG solutions were prepared and distributed, in spring and in summer. Each set of 8-oxodG solutions comprised four samples, coded A, B, C, D (batch one) and E, F, G, H (batch two). B and C were identical, as were E and H. Quality control was carried out in the distributing laboratory. Samples from batch one were repeatedly analysed over a period of 42 days (Fig. 1). Determinations of 8-oxodG were consistent, apart from a few tubes, which gave rather low concentrations. Adsorption onto the storage tubes may have accounted for this loss, and a different brand of tubes was used for subsequent sets of samples.

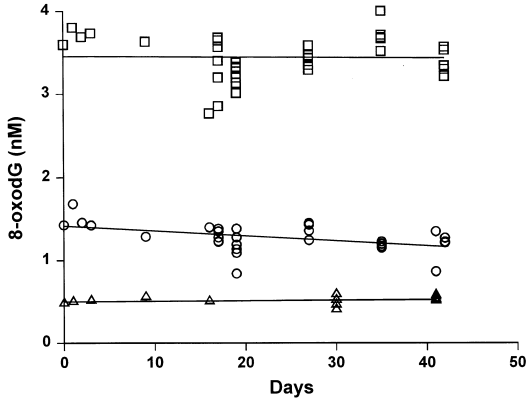

FIGURE 1 Quality control; samples of 8-oxodG solutions A (△), B/C (○) and D (□) were analysed by HPLC with coulometric electrochemical detection over a period of 42 days which included the interval allowed to collaborators for analysis. Lines indicate linear regression for each sample.

Results from the partner laboratories were compared with the concentrations as determined in the distributing laboratory. Figure 2 displays the results for the first set, ordered according to increasing values obtained for the solutions of intermediate concentration, B/C. (Four laboratories failed to detect one or more of the samples; their zero values are not included in the figure, nor in the following analysis). The pattern of increasing values is replicated, though not precisely, for samples D and A. Overall, about half the results received were within 20% of the expected value. Intra-tube variability was reasonable: for sample D (highest concentration), two-thirds of responses had a CV of $<5\%$, while for sample A (lowest concentration) one-third had a CV of $<5\%$. Inter-tube CVs of $<10\%$ were achieved by about half the laboratories/methods.

The second set of samples (E, F, G, H) gave a similar pattern of results in terms of accordance with the expected value for the intermediate and highest

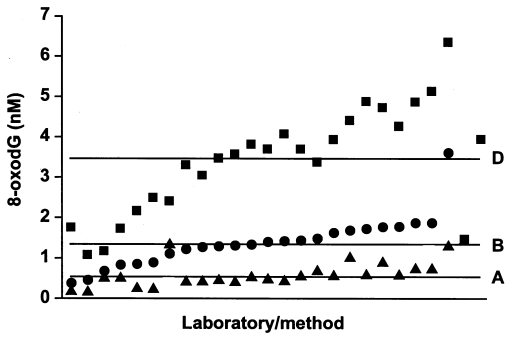

FIGURE 2 Inter-laboratory comparison of 8-oxodG analyses, samples A (▲), B/C (•), D (■). The order of laboratories/methods on the *x*-axis is determined by the order of values reported for sample B/C. Horizontal lines for A, B/C and D indicate the mean of concentrations as determined by the distributing laboratory.

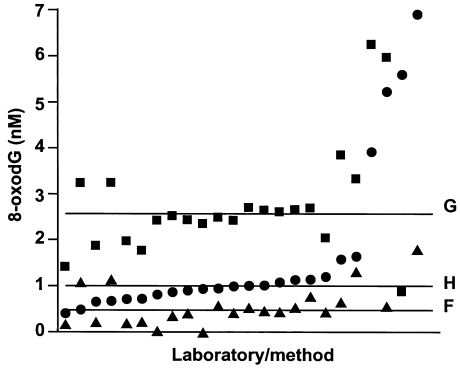

FIGURE 3 Inter-laboratory comparison of 8-oxodG analyses, samples F (▲), E/H (•), G (■). The order of laboratories/methods on the *x*-axis is determined by the order of values reported for sample E/H. Horizontal lines for F, E/H and G indicate the mean of concentrations as determined in the distributing laboratory.

concentrations, as shown in Fig. 3. However, results outwith this band of consensus were apparently more erratic than in the first set. CVs were also poorer (Fig. 4). It was possible that high laboratory temperatures contributed to the variability (a temperature of over 30°C was reported by one member); but in control experiments (not shown), storage at 30°C or 37°C for 3 weeks had no effect on 8-oxodG concentration. The increased variability in this batch of results remains unexplained.

Measurement of 8-oxodG in Calf Thymus DNA

A dry sample of calf thymus DNA, taken from the same purchased stock, was sent to all participating laboratories, together with standard solutions of 8-oxodG and dG for calibration. Storage at room temperature in the dark was specified. From the results returned, the median value for 8-oxoGua/8-oxodG concentration was 51.9 per 10^6 Gua or dG. Almost half the reported results were within 20% of the median value; half obtained a CV of $<5\%$ for intra-tube variability, and half obtained a CV of $<10\%$ for inter-tube variability. However, one GC–MS and one HPLC method gave consistently very high values. The GC–MS method also gave high results for the 8-oxodG samples (E, F, G, H) analysed at this time. One HPLC method gave consistently low figures for 8-oxodG/10^6 dG and also for the 8-oxodG samples. An obvious explanation would be an error in preparing standards; but in this case the standards were supplied with the DNA sample.

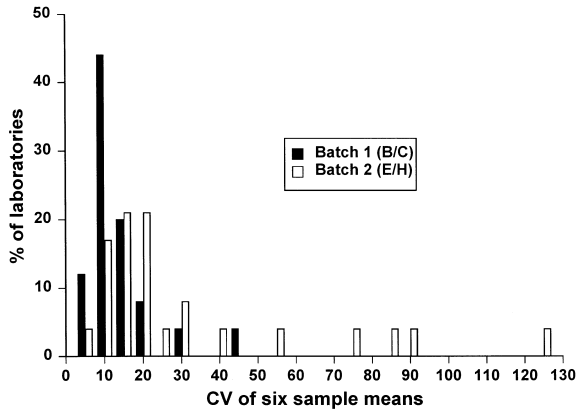

FIGURE 4 Reproducibility assessed for samples B/C and samples E/H; distribution of values of CVs.

For quality control, samples of DNA stored at different temperatures were analysed by the distributing laboratory every few days for 3 weeks. In this time, the 8-oxodG content of dry DNA increased by 8% at 30°C and by 16% at 37°C. There was no increase at room temperature (approximately 20°C). The standard dG solution was also tested; at 37°C the concentration decreased unpredictably, though by at least 20%. At 30°C there was no change. A decrease in the standard dG solution would lead to an apparent increase in 8-oxodG concentration in DNA.

As a test of sensitivity and precision, samples of calf thymus DNA were prepared with additional 8-oxoGua residues by irradiating a solution of DNA with visible light in the presence of the photosensitiser Ro 19-8022. The concentration of photosensitiser was varied (0, 7.5, 20 and 30 μM) and the

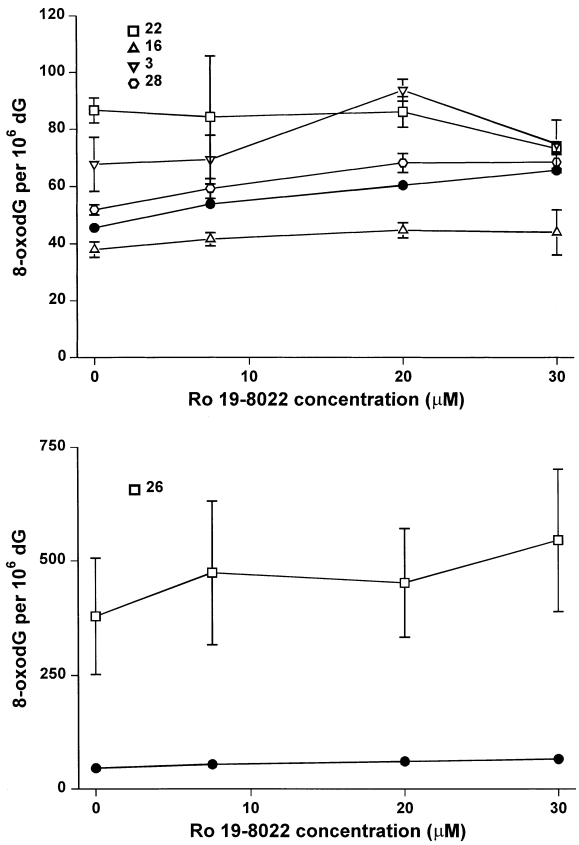

FIGURE 5 Values reported for calf thymus DNA samples by laboratories using GC–MS or LC–MS–MS (method/laboratory details are given in Table I). Median values are shown as filled circles. Bars represent SD. Note different scale on *y*-axis of second panel.

irradiation time was constant. Samples were coded (L, K, J, M in order of increasing Ro 19-8022 concentration). They were analysed against the laboratories' own standards. Median values calculated from all returned results were 45.6, 53.9, 60.4 and 65.6 8-oxodG/10^6 dG, respectively.

Figure 5 shows results from laboratories using GC–MS and LC–MS–MS. Median values are also indicated. Most results are relatively close to the median, but one method gave extremely high values, shown in the separate chart. HPLC results are displayed in Fig. 6, with the median values as reference. These results allowed us to assess the sensitivity as well as the precision of each laboratory/method. Table I indicates the ability or otherwise to detect increases in 8-oxoGua/8-oxodG content over the range of samples, and also gives CVs. Generally, the ability to detect the dose response in these samples corresponded with low CVs. The requirements in the table are stringent, excluding laboratories/methods that failed to detect the dose response over specified concentration ranges. In fact, all laboratories/methods apart from one (GC–MS) detected the positive dose response over at least two of the three concentration intervals. These results are a distinct improvement over the similar trial reported from an earlier round of ESCODD,[2] in which only about half the laboratories/methods successfully detected a dose response over two concentration intervals.

CONCLUSIONS

This is the third report on the activities of ESCODD, and represents clear improvements compared with our earlier endeavours.[2,3] Analysis of 8-oxodG in standard solutions has clearly become both more precise and more accurate (as shown in Figs. 2 and 3 by the clustering of results around the mean value obtained from the distributing laboratory). The degree of agreement over the level of 8-oxoGua/8-oxodG in calf thymus DNA is in marked contrast to previous trials.[2,3] LC–MS–MS–although used by only two laboratories—is demonstrably as reliable, sensitive and precise as the best HPLC procedures, and has the added advantage of giving unambiguous information on the identity of analytes. The amperometric version of electrochemical detection with HPLC is less sensitive than the coulometric version, but the one laboratory returning results with this method for batch three performed reasonably well in terms of CV and sensitivity in detecting the gradient of 8-oxodG in calf thymus DNA.

Of the large number of laboratories taking part in ESCODD, several were novices at the measurement

TABLE I Measurement of 8-oxoGua/8-oxodG in calf thymus DNA treated with Ro 19-8022 and light; estimates of sensitivity and precision for different laboratories and methods

Method	Laboratory number	Ability to detect increase in 8-oxodG over range of Ro 19-8022 concentrations (μM)			Lowest CV (inter-tube, %)
		0–7.5	0–7.5–20	0–7.5–20–30	
HPLC (amperometric)	4	+	+	−	4
HPLC (coulometric)	1	+	+	+	3
	6	+	+	+	1
	13	+	+	+	4
	16	+	+	+	3
	21	+	+	+	0
	28	+	+	+	8
	3	+	+	−	1
	5	+	+	−	11
	8	+	+	−	4
	12	+	+	−	8
	14	+	+	−	1
	15	+	+	−	0
	20	+	+	−	4
	7	+	−	−	1
	17	+	−	−	38
	2	−	−	−	19
	23	−	−	−	91
	25	−	−	−	3
GC–MS	16	+	+	−	5
	26	+	−	−	26
	22	−	−	−	2
LC–MS–MS	28	+	+	+	3
	3	+	+	−	4

Numbers identifying laboratories in the figures and table do not bear any relation to the order of authors.

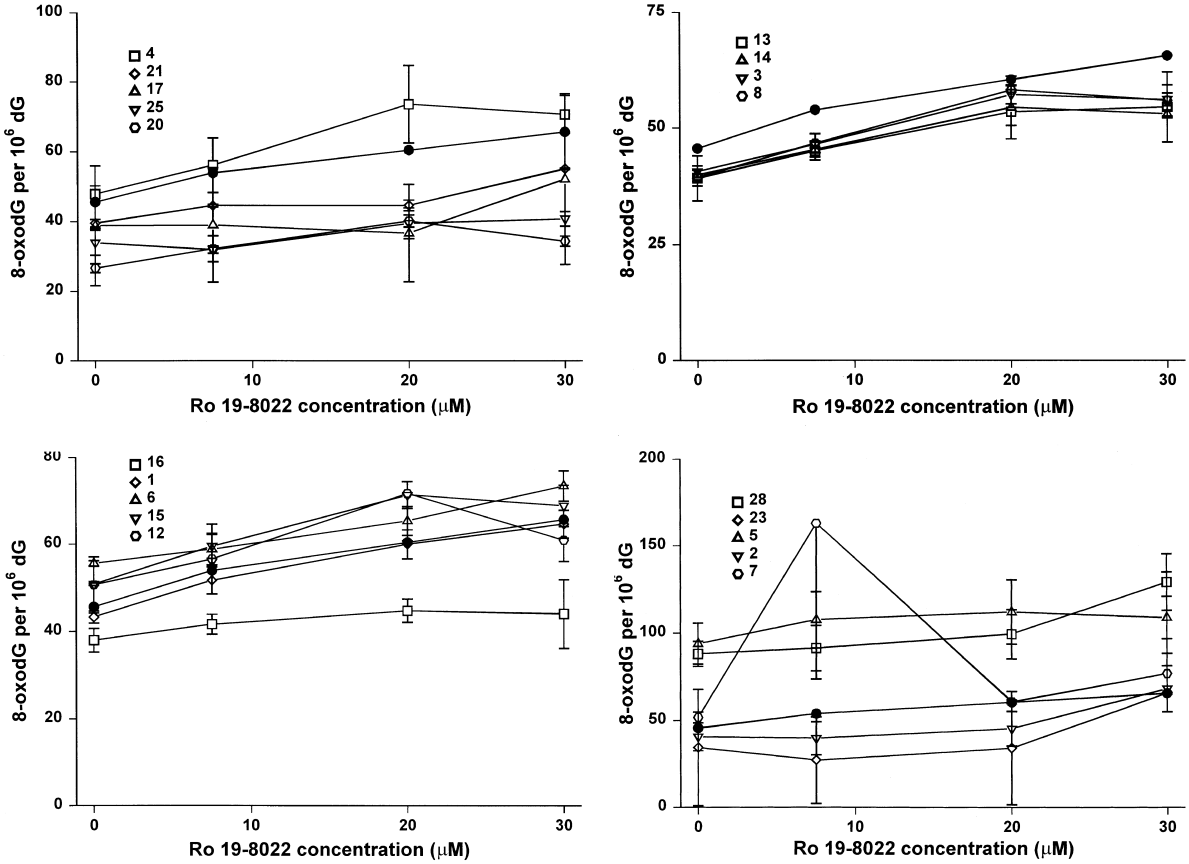

FIGURE 6 Values reported for calf thymus DNA samples by laboratories using HPLC (method/laboratory details are given in Table I). Median values are shown as filled circles. Bars represent SD.

of 8-oxodG, and it is pleasing that their performance was as satisfactory as that of laboratories with several years' experience.

There are still some problems, particularly with GC–MS. High values for 8-oxoGua may be the result of oxidation occurring during sample preparation. Milder conditions are used now for derivatisation than were routine in the past, but acid hydrolysis is still done at high temperature.

ESCODD was founded because the discrepancies between different ways of measuring oxidative DNA damage in normal human cell samples were too glaringly evident to be ignored. It is still rare for non-clinical biomarkers to be subjected to such rigorous validation procedures as we have used, but it seems more than likely that other assays in use in population studies are also prone to inconsistencies, and our approach could be seen as a useful paradigm. Salient aspects include:

- Quality control carried out in the laboratory distributing the samples, monitoring all stages in the production, shipping, storage and analysis of the material;
- distribution of coded samples in aliquots ready for repeat analysis;
- information obtained on details of procedures in use in each laboratory, by means of questionnaires;
- use of median values as reference points, in preference to the values determined by the laboratory responsible for sending samples, and in preference to mean values, which are distorted by anomalous high values.

Lest we appear complacent, it should be remembered that calf thymus DNA contains at least 10 times more 8-oxodG than is reported to be present in the DNA of human white blood cells or human cells in culture. Subsequent phases of ESCODD require the measurement of 8-oxodG in animal tissue, cultured cells and lymphocytes, and far higher sensitivity will be demanded. In addition, with measurement of damage in intact cells, other techniques will be brought into the comparison, namely alkaline elution, alkaline unwinding and the comet assay, all of which make use of the enzyme formamidopyrimidine DNA glycosylase to convert 8-oxoGua lesions to DNA breaks. Reconciling the results from these methods with those from HPLC, LC–MS–MS and GC–MS will be a considerable challenge. We are still far from our ultimate aim of reaching a consensus over the level of background damage in normal cells.

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