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ESCODD: European Standards Committee on Oxidative DNA Damage

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"There can be no more important goal in a clinical laboratory than that of ensuring that the results produced have the precision and accuracy necessary to aid diagnosis and treatment."

T.P. Whitehead 1976

INTRODUCTION

7,8 dihydro-8-oxo-2′-deoxyguanosine (8-oxodG) was first reported in 1983 as a predominant form of oxidative DNA damage.[1] Since its discovery this lesion has been measured world-wide in peripheral blood cells, solid tissues and urine (in the latter as a putative repair product of oxidative DNA damage). There has been an increasing desire to measure the baseline, steady-state level of 8-oxodG *in vivo* ever since the lesion was discovered. However, the lesion can be measured by at least three different techniques and the lack of a consensus between these methods has inspired a controversy in the literature and at international meetings which is almost unrivalled in medical research.[2–5] All analysts in the field search for the most accurate level of 8-oxodG in the DNA of cells, which represents the steady-state level achieved between damage and "repair" of the lesion. The level detected is an indicator of the overall burden of oxidative damage to the cell.

'The Controversy'

The measurement of 8-oxodG can be performed by three major procedures: (a) gas chromatography mass spectrometry (GCMS); (b) high performance liquid chromatography and (c) enzymically, using the Fapy glycosylase repair enzyme. Discrepancies in measurement of 8-oxodG occur over a range of at least two orders of magnitude, with GCMS measuring the highest levels, ranging two orders of magnitude in the same tissue, and procedures using repair enzymes

the lowest. The discrepancies are even larger between tissues.[2,6] This is a situation which appears untenable, especially since there is a clinical need to establish normal and abnormal ranges in order to test the hypothesis that measurement of this biomarker may be useful in diagnosis, as an indicator for predisposition to disease, or for therapeutic monitoring of patients.

In January 1997 a meeting of interested scientific groups was arranged at the Rowett Institute in order to investigate this problem with a view to discussing a way forward. It was decided that a quality assurance programme was needed to establish precision, accuracy and a quality control material, which would harmonise results, i.e. a material, or number of materials, which would be exchanged between laboratories and the results compared. This meeting established ESCODD, the European Standards Committee on Oxidative DNA Damage.[2]

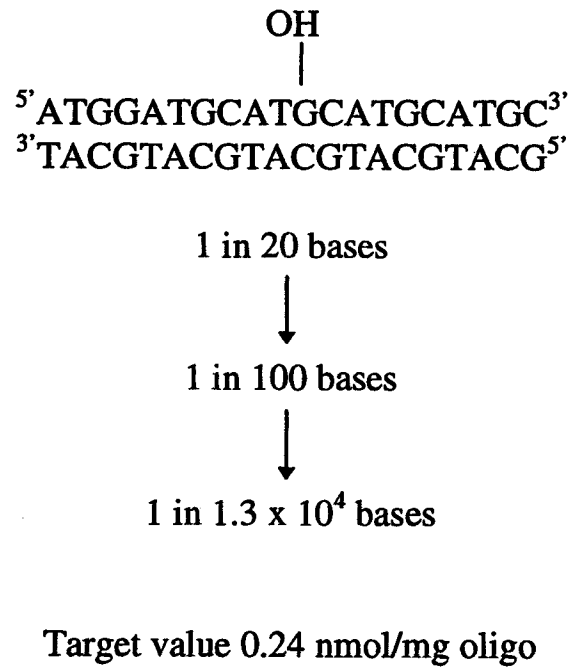

FIGURE 1 Protocol for production of oligomeric (20 mer) standard containing 8-oxodG in a central position.

ESCODD I: METHODOLOGY

The first role of ESCODD was to attract interested scientists who would co-operate in a scheme where four samples would be sent to each of the contributing laboratories. Analysis would be performed in triplicate on each sample on three separate occasions. The first sample was a lyophilised sample of the pure standard deoxynucleoside (8-oxodG). Its purpose was to test the calibration curve used by the contributing laboratory. The sample was freeze dried and laboratories were asked to reconstitute in 0.5 ml aqueous solvent. The second sample was a synthesised oligomer (20 mer) which had 8-oxodG positioned centrally, in order to minimise "end effects". Sequence identity of the 20 mer is shown in Figure 1. The oligomer was made double-stranded with a complimentary-sequence oligomer and diluted five times, in excess, with this complimentary sequence. An appropriate dilution of this standard was then made with polydA.polydT. The oligomeric standard was included as an appropriate quality control material because it was artificially manufactured and unlikely to undergo appreciable oxidation *in vitro*. The absorbance of the oligomer at 260 nm was used for quantification (1.0OD = 20 μg/ml). The target value for the oligomer was set at 0.24 nmol/mg or 1 in 1.3×10^4 bases. The third sample sent to all contributors was a sample of lyophilised calf thymus DNA. Calf thymus DNA was chosen because it was a sample many laboratories had analysed previously. Laboratories had already established an international consensus for the level of 8-oxodG present in calf thymus DNA (see Table II). In addition it was considered a legitimate and natural sample of DNA unlike the oligomer. The further advantage of the ESCODD sample of calf thymus DNA was its origin; it was distributed from one single batch isolated by one single procedure, thus eliminating possible variation in extraction procedures. The fourth and last sample was a sample of pig liver. This relatively homogeneous tissue was processed into small pieces of

approximately 300–400 mg, and sent on dry ice to each participating laboratory. The measurement of 8-oxodG in pig liver allowed the procedures for DNA extraction to be compared between laboratories. Much of the controversy surrounding discrepancies between levels of 8-oxodG revolves around the DNA extraction procedures, which have been criticised because they may induce artefact (i.e. increased 8-oxodG levels due to oxidation occurring during extraction with organic reagents).[7–12] Therefore, it was particularly important to achieve consensus in the measurement of 8-oxodG in whole tissue.

In addition to sending samples to each laboratory a questionnaire was also provided, the purpose being to establish the variations in analytical methodology used by contributors. Each participant in the trial was asked to return the first page of the questionnaire to an independent assessor who would hold the codes for each laboratory. The co-ordinating laboratory would, in this way, be unaware of the identity of any of the contributors.

RESULTS AND DISCUSSION

Eight laboratories took part in ESCODD 1. All contributors were from the European Community and all were well established in the field of free radicals and DNA damage. Seven of the contributing laboratories submitted results on the popular HPLC procedure, which utilises an enzymic degradation of DNA to corresponding deoxynucleoside fragments. Two laboratories submitted results on the less popular GCMS method used for determination of the base following derivatisation.[13] One of the laboratories also submitted HPLC data and results using a 'hybrid' HPLC method i.e. a method of analysis of the base using acid hydrolysis followed by guanase treatment to remove guanine interference.[14] Table I shows the distribution of data for all methods and defines the mean and coefficient of variation for the inter-batch analysis for each of the four test samples. Intra-batch CV's varied widely ranging from 1.1% to 52.8%. Inter-batch variation ranged from 0.7% to 63.3%. These variations were largest for both calf thymus DNA and pig liver analyses. However, the variation with consecutive analyses indicates that there had been a time-dependent, oxidation of samples *in vitro* which could have accounted for the variation in at least a few of the results (see solid lines, Figures 4 and 5). Three laboratories came within 10% of the target value for the calibration standard as shown in Table I. Nevertheless, it is clear that calibration is an important issue since all HPLC analyses underestimated the value of the 8-oxodG standard, while GCMS methods overestimated this deoxynucleoside standard value (Figure 2). All contributors overestimated the value of the oligomeric standard except for the HPLC-base procedure. HPLC overestimated, overall, by a mean of approximately 280% (Figure 3). The GCMS methods, on average, overestimated by approximately 400%. It is extremely difficult to explain these results on the basis of differences between the analytical procedures, although the discrepancy between HPLC and GCMS was very clear. No other patterns emerged within each major analytical group. The oligomer value was calculated from its extinction coefficient. Although this was valid for the aqueous deoxynucleoside the value may not be valid for 8-oxodG within an oligomeric structure. The essential differences between the two types of procedures are that the HPLC procedure is calibrated using deoxynucleoside standard and DNA is degraded via enzymic hydrolysis, while the GCMS measurement relies on use of the base product (8-oxoG) for calibration, together with formic acid hydrolysis for digestion and release of bases from DNA.[13] In all calibrations performed it is essential that standards are put through the same procedure as test samples. However, because the standard deoxynucleoside may be decomposed differentially in formic acid compared to the base, or undergo a different rate of reaction with derivatising agent in the presence of DNA,

TABLE I Summary chart of mean and inter-batch coefficients of variation (%) generated by all laboratories contributing to ESCODD 1

					8-oxodG	CV	Oligo	CV	C-T	CV	P-L	CV
Lab no.	*Technique*	*Enz.*	*Extract*	*Coul/Amp*								
28500	HPLC	2	Pronase	Amp	8.4	5.8	0.45	5.8	10.5	51.8	10.7	60.9
80181	HPLC	4	Prot K	Coul	9.8	5.1	0.45	1.9	2.0	9.0	2.2	9.9
70138	HPLC	2	Protease + desferal	Coul	11.2	8.3	0.79	9.5	3.0	11.5	0.7	19.3
75319	HPLC	2		Coul	11.1	6.6	NR		3.1		0.9	20.7
9561	HPLC	2	Protease	Amp	8.0	43.3	NR		21.3	9.8	18.1	42.4
991	HPLC	2	Phenol	Coul	11.2	26.5	0.61	6.3	2.3	8.3	0.3	5.3
64650	HPLC	?	?	?	9.4	0.7	0.60	11.6	1.9	9.4	3.0	18.0
Lab no.	*Technique*	*Derivn.*	*Int. Std.*	*Extract*								
26665	GC-MS	RT BSTFA/ACN ethanethiol	DAP	Phenol	18.1	30.5	0.93	23.3	3.6	22.2	3.1	19.4
28500	GC-MS	RT/ACN BSTFA	M+4	Pronase	22.9	12.6	0.76	61.7	8.5	29.9	14.6	14.3
28500	HPLC (base)	—	—	Pronase	14.5	9.5	0.22	7.7	15.8	63.3	41.3	30.5

NR indicates no return of results. This was usually due to reasons of "insufficient sample". DAP – Diaminopurine internal standard. RT = Room temperature; BSTFA = N,O-bis(trimethylsilyl) trifluoro acetamide; ACN = acetonitrile; M + 4 = isotopically labelled 8-oxoG. Target value for 8-oxodG aqueous standard = 12.6 nmol/l. Target value for 8-oxodG oligomer standard = 0.24 nmol/mg oligomer.

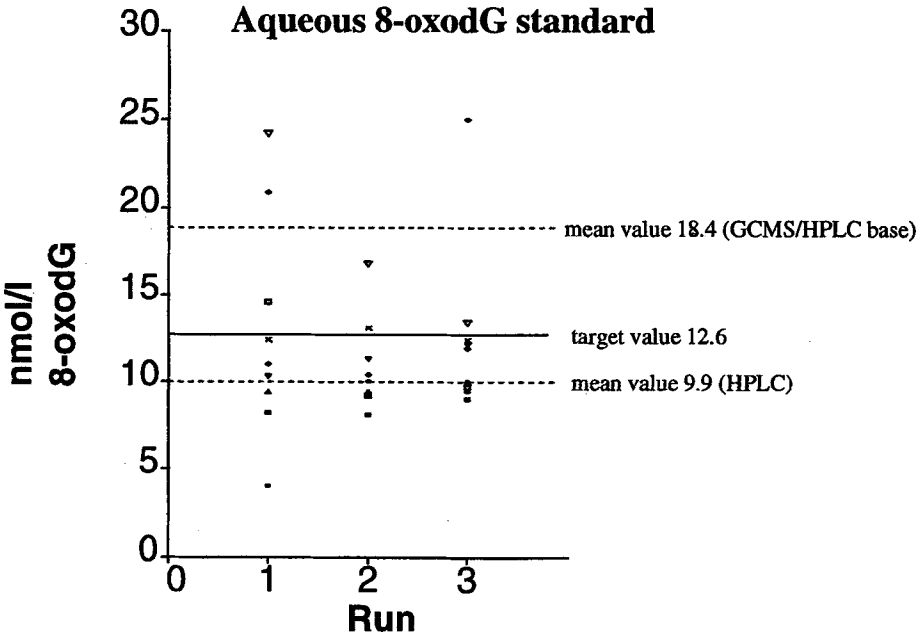

FIGURE 2 Distribution of 8-oxodG results for standard 8-oxodeoxyguanosine. The symbols represent the mean of triplicate analyses from each individual laboratory; 1, 2 and 3 represent consecutive batch results. Average time interval 1–2 weeks.

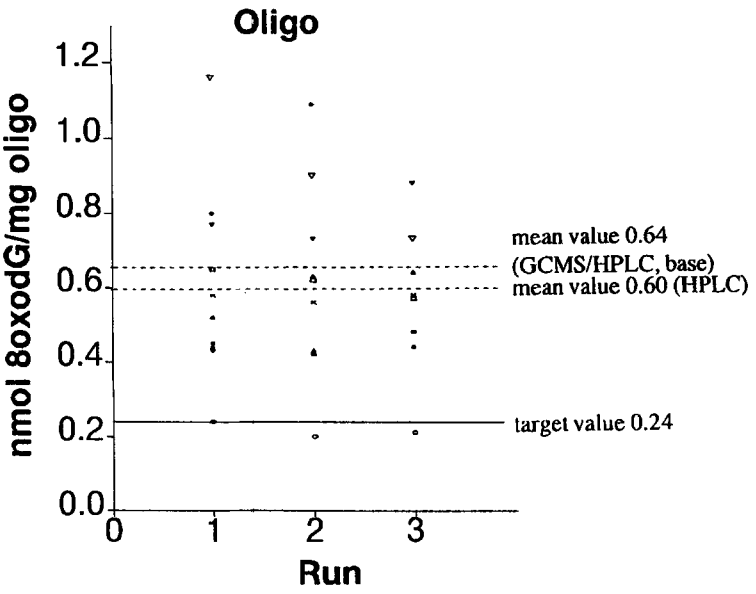

FIGURE 3 Distribution of 8-oxodG results for oligomer. The symbols represent the mean of triplicate analyses from each individual laboratory; 1, 2 and 3 represent consecutive batch results. Average time interval 1–2 weeks.

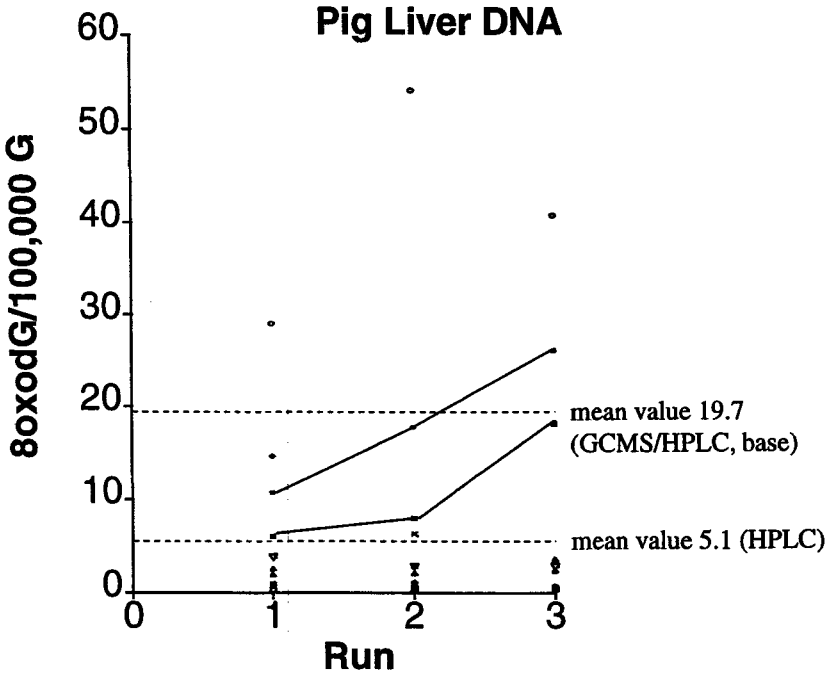

FIGURE 4 Distribution of 8-oxodG results for pig liver DNA. The symbols represent the mean of triplicate analyses from each individual laboratory; 1, 2 and 3 represent consecutive batch results. Average time interval 1–2 weeks.

over-estimation of 8-oxodG in either pure standard or oligomer may occur due to a downward shift in the calibration curve. The underestimate of 8-oxodG may be explained by the observation made by Frenkel *et al.*, 1991, that acid pH, frequently used for nuclease P_1 digestion, can promote hydrolysis of 8-oxodG to 8-oxoG.[15] The overestimate of 8-oxodG by HPLC in the oligomer is less easily explained. The level of 8-oxodG obtained for the oligomeric standard could not be due to incomplete enzymic digestion, as this would have led to an underestimate. Obviously no DNA extraction is involved with analysis of either deoxynucleoside standard or oligomeric standard which means it cannot be implicated in what appears to be a consistent discrepancy between HPLC and GCMS. The results on calf thymus DNA shed some light on the results of the standards. The range obtained for all HPLC results and the GCMS/HPLC base results are shown in Figure 5. Levels for all HPLC results show a mean of 6.3 per 10^5 G and 9.3 per 10^5 G for the GCMS/HPLC base procedures. These results compare very well with literature values for calf thymus DNA, although the range is much larger for HPLC than GCMS (see Table II). It is noteworthy that the mean for the HPLC results is elevated mainly because of two data points, both produced by laboratories using amperometric detection. The amperometric procedures had the highest coefficient of variation for 8-oxodG, probably because the detection system is not as sensitive as the coulometric procedure. For calf thymus DNA the discrepancy between mean level for HPLC versus mean level for GCMS is of the order of 50–100%. This lack of consensus is independent of any extraction procedure and can only be associated with either standardisation or digestion. Most of the discrepancy in measuring 8-oxodG in calf thymus DNA may be accounted for by calibration differences (Table II).

The final test related to incorporating a DNA extraction procedure. The results on the time-dependent analysis of pig liver indicated, as did analysis of calf-thymus DNA, that shipment delays or storage of some specimens gave rise to artefactual oxidation when means of first analysis were compared to last analysis means (see Figures 4 and 5). It is significant that, again as in the calf thymus analysis, laboratories using GCMS techniques did not report the highest levels, although overall the means of GCMS results were of the order of twice the means of HPLC results. The two laboratories using amperometric detection of deoxynucleoside reported a higher mean level of 8-oxodG than those using coulometric analysis. Both means were entirely consistent with literature values, with GCMS values averaging approximately four times the HPLC values, a level of discrepancy consistent with two previous reports.[5, 16] Laboratory 28500 reported similar discrepancies between three different procedures, despite having a common extraction procedure. The question of artefact production

TABLE II Summary comparison of mean and ranges generated by laboratories contributing to ESCODD 1 with target values and literature values

Technique		8-oxo(d)G (aq) (nmol/l)	Oligo (nmol/mg)	Calf thymus* (8-oxo(d)G/10^5 G)	Pig liver* (8-oxo(d)G/10^5 G)
8-oxoG					
GCMS	ESCODD mean	18.4	0.64	9.3	19.7
($n=3$)	ESCODD range	(14.5 → 22.9)	(0.22 → 0.93)	(3.6 → 15.8)	(3.1 → 41)
(inc GUANASE)	Target/lit value	(12.6)	(0.24)	(0.3 → 22)	(5 → 20)
8-oxodG	ESCODD mean	9.9	0.60	6.3	5.1
HPLC	ESCODD range	(8.0 → 11.2)	(0.45 → 0.79)	(1.9 → 21.3)	(0.3 → 18.1)
($n=7$)	Target/lit value	(12.6)	(0.24)	(3 → 128)	(0.6 → 20)‡

*Results for GCMS have been converted from nmol/mg DNA by using the following conversion factor: 1 nmol of 8-oxoguanine per mg DNA is equivalent to approximately 124 8-oxoguanines per 10^5 G. ‡ Range includes literature levels for rat liver also.

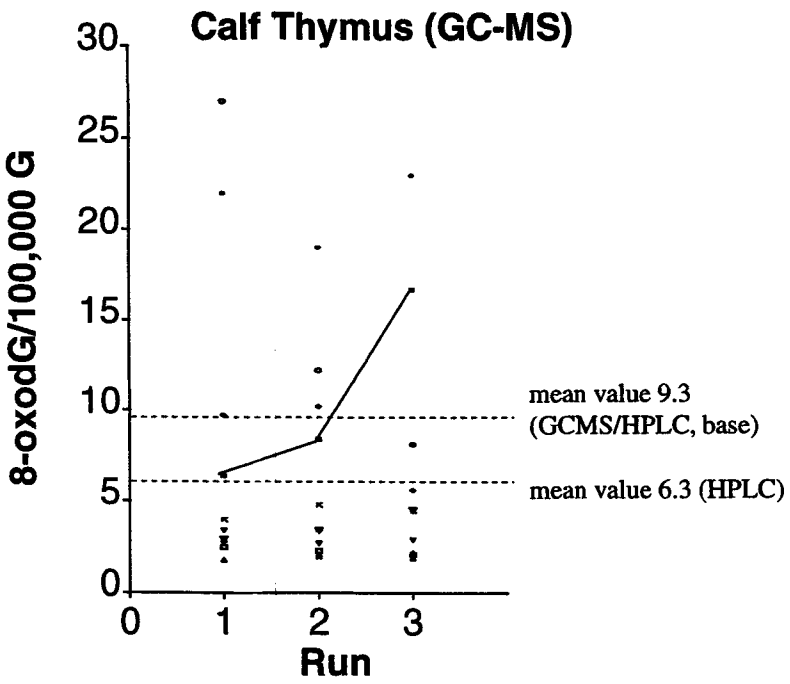

FIGURE 5 Distribution of 8-oxodG results for calf thymus. The symbols represent the mean of triplicate analyses from each individual laboratory; 1, 2 and 3 represent consecutive batch results. Average time interval 1–2 weeks.

during derivatisation is an important one,[17] and it is clear from differences between laboratories 26665 and 28500 for GCMS that the use of ethanethiol, to prevent *in vitro* oxidation during derivatisation, produces mean levels more comparable with HPLC techniques. This development is a major step forward in reaching consensus between the two techniques.[18] A similar procedure has been used by other workers to prevent adventitious formation of 8-oxoG during derivatisation.[19]

Herein we have reported the results of the first ESCODD trial. We have confirmed that there are discrepancies between GCMS techniques and HPLC ECD techniques, but they appear to be less than often reported and could be explained on the basis of calibration and relatively small changes occurring during derivatisation. It seems clear that because the base (8-oxoG) used for GCMS calibration is known to be insoluble in water, unless the pH is altered with sodium hydroxide, that it would be advisable to use a labelled deoxynucleoside standard for the GCMS procedure. In addition there appears to be discrepant results between HPLC methods using coulometric versus amperometric procedures, the latter having mean values significantly higher than the former and more comparable with the GCMS techniques. The question of extraction procedure remains open, but clearly is an important issue, common to both HPLC and GCMS techniques, which needs to be standardised in a future ESCODD trial.

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