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Comparison of Different Methods of Measuring 8-oxoguanine as a Marker of Oxidative DNA Damage

ESCODD (European Standards Committee on Oxidative DNA Damage)*

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We are attempting to resolve some of the problems encountered in measuring 8-hydroxy-2′-deoxyguanosine (8-oxodG) in human cellular DNA as a marker of oxidative stress. Samples of authentic 8-oxodG were distributed, and participating laboratories undertook to analyse this material within a specified period. Most HPLC procedures gave values for 8-oxodG within ±40% of the target, as did two of four GC-MS procedures, and both LC-MS-MS methods. Calf thymus DNA samples containing increasing amounts of 8-oxodG were also distributed for analysis. Fewer than half the procedures tested were able to detect the dose response; those that were successful tended to be procedures with low coefficients of variation. For the analysis of 8-oxodG in human cells, where it is likely to be present at much lower concentrations than in the calf thymus DNA, it is crucial to reduce analytical variation to a minimum; a coefficient of variation of less than 10% should be the aim, to give reasonable precision. HPLC with amperometric electrochemical detection is not recommended, as it is less sensitive than coulometric detection. Immunological detection, ^{32}P-postlabelling and LC-MS-MS are alternative approaches to measurement of 8-oxodG in DNA that, on the grounds of precision and detection of dose response, cannot at present be recommended.

Keywords: Oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine, HPLC, GC-MS, methods validation

INTRODUCTION

Measurement of oxidative DNA damage in human cells is crucial to an understanding of the consequences of oxidative stress in health and disease, and of the influence of dietary antioxidants. 8-Hydroxy-2′-deoxyguanosine (8-oxodG), or the corresponding base 8-oxoguanine (8-oxogua), are most commonly measured as a marker of oxidative damage, but estimates of the levels of damage in normal human cells by GC-MS and HPLC range over several orders of magnitude.[1] The European Standards Committee for Oxidative DNA Damage (ESCODD) was set up in 1997 to resolve methodological problems and to reach agreement on the basal level of oxidative damage in human cells. In phase 1, laboratories participating in ESCODD received samples of calf thymus DNA and liver tissue, as well as standard 8-oxodG and 8-oxodG-containing oligonucleotides for analysis. Results confirmed the existence of wide variations between methods and indicated that even the determination of standard 8-oxodG

*Address for Correspondence: Dr. Andrew Collins, DNA Instability Group, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, Scotland, UK. Tel.: +44(0)1224 716634. Fax: +44(0)1224 716629. E-mail: a.collins@rri.sari.ac.uk.

showed considerable inter- and intra-laboratory variations.[2] Therefore, in phase 2, reported here, the analytical tasks were simpler. First, aliquots of a standard solution of 8-oxodG (freeze-dried) were distributed to participants for analysis (triplicate injections). Second, four coded samples of calf thymus DNA were sent out, including one untreated sample, two with a relatively low level of 8-oxodG introduced by visible light plus photosensitiser, and one with a higher level of damage. The aim of this part of the study was to assess the sensitivity of each procedure by its ability to detect relatively small increases in DNA oxidation. The analysis of a standard 8-oxodG solution was repeated in phase 3, and is also reported here.

MATERIALS AND METHODS

Preparation of Standard (Phase 2)

8-oxodG from Calbiochem was dissolved in ultrapure water (pH 7.4) at a concentration of 143.3 μM (determined by absorbance at 245 nm). It was diluted 10,000-fold and dispensed as 0.5 ml aliquots into siliconised 1.5 ml Eppendorf tubes. The tube openings were covered with Parafilm pierced with a needle, the tubes frozen at −80°C and water removed by freeze-drying overnight. The Parafilm was removed, the tubes capped and (after storage at 4–6°C) distributed to participating laboratories.

Preparation of Standard (Phase 3)

8-oxodG (Sigma) was dissolved in HPLC-grade water (pH 5.2) at approximately 6 nM and divided into 1 ml aliquots in cryotubes (Nunc) for distribution to participating laboratories. Storage was at room temperature. On four occasions during the period of 25 days allowed for analysis, viz. on days 2, 21, 22 and 23, six aliquots were analysed by HPLC, against a standard curve prepared on the same day using a stock solution of 8-oxodG. The absorbance at 245 nm of this standard solution was measured on the same day. The mean of these determinations (6.9 ± 0.5 nM) is the target value indicated by the broken line in Figure 1. For comparison, some aliquots were also stored at 4°C, and in siliconised microcentrifuge tubes (at room temperature and at 4°C), in the distributing laboratory; no significant differences were seen in 8-oxodG levels as determined by HPLC (data not shown). Thus there was no adsorption of 8-oxodG onto the tubes under any of these conditions.

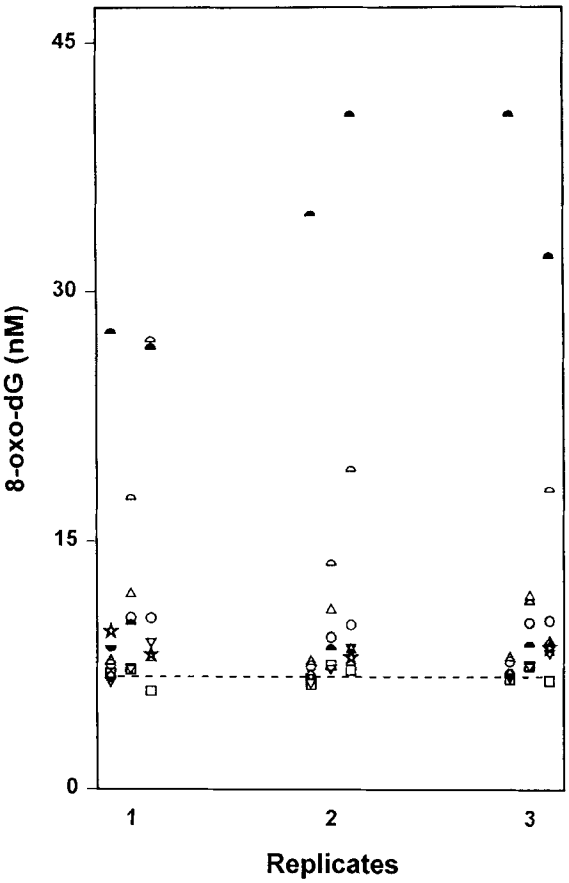

FIGURE 1 Values obtained for three replicate determinations of the 8-oxodG standard (ostensibly 6.9 nM, indicated by the horizontal broken line). Each laboratory/procedure is identified uniquely by a symbol within one of the three (arbitrary) series of symbols for each replicate position on the *x*-axis. Thus, for example, the circle in the third series for replicate 1 represents the same laboratory/procedure as the circle in the third series for replicates 2 and 3. Open symbols represent HPLC; stars represent LC-MS; solid semicircles represent GC-MS.

Preparation of Calf Thymus DNA Samples (Phase 2)

The preparation is described in full in Ref. [3]. Briefly, calf thymus DNA solution was treated with the photosensitiser Ro 19-8022 (Hoffmann-La Roche) at 5 and 20 μM or with no Ro 19-8022. All three solutions were then irradiated for 5 min at 33 cm from a 1000 W tungsten halogen lamp. DNA precipitated with NaCl and ethanol at −20°C was washed with 70% ice-cold ethanol, dried under nitrogen at room temperature and redissolved overnight in HPLC-grade water. Aliquots containing approximately 120 μg of DNA in Eppendorf tubes were snap-frozen in liquid nitrogen and lyophilised overnight. Tubes were checked for visible pellets, sealed and distributed by mail, coded as A (20 μM Ro 19-8022), B and D (5 μM Ro 19-8022) and C (control, without Ro 19-8022).

Analytical Procedures

Brief details are given here, since methods vary in particulars from laboratory to laboratory. For analysis by GC-MS, samples hydrolysed to bases by incubation with formic acid (60–88%, at between 130°C and 150°C) were derivatised with bis(trimethylsilyl) trifluoroacetamide at temperatures indicated in Table I.

In general, for HPLC analysis, enzymic hydrolysis was employed to break down the DNA to nucleosides with P1 nuclease and alkaline phosphatase; with P1 nuclease and acid phosphatase; or with deoxyribonuclease I, phosphodiesterases I and II, and alkaline phosphatase. Alternatively, hot formic acid was used (in two laboratories) to release the bases. Separation on a C18 column was followed by electrochemical detection (amperometric or coulometric) of 8-oxodG or 8-oxogua, and UV detection of deoxyguanosine (dG) or guanine (gua).

One laboratory used liquid chromatography followed by mass spectroscopy (LC-MS-MS). After enzymic hydrolysis with nuclease P1 and acid phosphatase, and separation on a C18

TABLE I Methods used by laboratories participating in ESCODD 2/3

Laboratory	Method	Features of method
1a	HPLC	P1/alk P'ase; ethanol added. Ampero.
1b	HPLC	P1/alk P'ase. Ampero.
2	HPLC	Acid hydrolysis to bases. Ampero.
3	HPLC	4-enzyme hydrolysis. Coulo.
4	HPLC	P1/alk P'ase. Coulo.
5a	GC-MS	Ethanethiol. Rt derivatisation. M + 4 standards (gua and 8-oxogua).
5b	HPLC	P1/alk P'ase. Ampero.
5c	Immunological	
6	GC-MS	Ethanethiol. Rt derivatisation. M + 4 8-oxogua standard.
7	GC-MS	Ethanethiol. Rt derivatisation. M + 4 8-oxogua standard; unlabelled gua standard.
8a	HPLC	P1/acid P'ase. Coulo.
8b	LC-MS-MS	P1/acid P'ase.
9a	HPLC	P1/alk P'ase. Coulo.
9b	HPLC	4 enzyme hydrolysis. Coulo.
10a	GC-MS	Prepurification. Derivatisation at 130°C. M + 4 8-oxogua standard; unlabelled gua standard.
10b	HPLC	P1/alk P'ase. Coulo.
10c	LC-MS-MS	M + 4 8-oxogua standard.
11a	HPLC	P1/alk P'ase.Coulo.
11b	HPLC	Acid hydrolysis to bases. Coulo.
12	HPLC	P1/acid P'ase. Coulo.
13a	HPLC	Coulo.
13b	GC-MS	Ethanethiol. Derivatisation at 120°C. M + 4 8-oxogua standard.
13c	GC-MS	Ethanethiol. Rt derivatisation. M + 4 standards (gua and 8-oxogua).
14a	HPLC	P1/alk P'ase. Coulo.
14b	^{32}P postlabelling	
15	HPLC	Coulo.
16	HPLC	Ampero.
17	HPLC	Coulo.
18a	HPLC	Coulo.
18b	HPLC	Gradient. Coulo.
19	HPLC	Coulo.
20	HPLC	Coulo.

Abbreviations: P1/alk P'ase, digestion with P1 nuclease and alkaline phosphatase; P1/acid P'ase, digestion with P1 nuclease and acid phosphatase; Ampero., amperometric; Coulo., coulometric; Rt, room temperature. HPLC systems were isocratic except where 'gradient' is indicated. Where method of hydrolysis is not specified, laboratory participated only in the measurement of standard, not of calf thymus DNA.

column, the eluate was injected into a triple quadrupole mass spectrometer for identification and quantitation of products.

For the ^{32}P-postlabelling procedure, DNA was first hydrolysed with micrococcal nuclease and spleen phosphodiesterase. The preparation was enriched for 8-oxodG-3′-phosphate by HPLC, and then postlabelled with [^{32}P] ATP and T4 polynucleotide kinase. 3′-Phosphates were hydrolysed with nuclease P1, and 5′-^{32}P-labelled modified nucleotides separated by HPLC.

A highly specific antibody to 8-oxodG was used in a competitive ELISA to measure 8-oxodG in the DNA hydrolysate as used for HPLC determination; the dG concentration was obtained from the corresponding HPLC analysis. The system was externally calibrated against authentic 8-oxodG standards.

Participation

Phase 2 Twenty-one laboratories received standard 8-oxodG and calf thymus DNA samples. The samples were to be analysed within 55 days from October 10, 1998. Fourteen returned results of analyses by the deadline. Several laboratories carried out more than one procedure; from a total of 33 laboratory procedures, the number of returns was 22. Laboratories were coded and the results remain unattributed. For the purpose of this paper, each laboratory is identified by a number and different methods within the same laboratory by a letter. Table I lists laboratories and methods and notes distinctive features of the methods.

Phase 3 Most of the laboratories that took part in phase 2 also participated in phase 3, together with some additional laboratories, assigned numbers 15–20 in Table I. The standard solution was to be analysed within 25 days from April 30, 1999.

RESULTS

Determination of 8-oxodG Standard

Standards for phase 2 were prepared and distributed by laboratory number 5. When reconstituted, the standard solution was expected to have a concentration of 14.3 nM. It was found after the aliquots had been distributed that recovery was not always 100%; apparently some material was lost from certain tubes, presumably during or after the freeze-drying stage. In view of this problem, and also the difficulties experienced by some laboratories in reconstituting the freeze-dried material, a new standard solution was prepared (by laboratory number 9) and aliquots distributed without freeze-drying (phase 3). Figure 1 shows the range of values obtained by each laboratory/procedure and for each of the replicate analyses. CVs and means of the standard determinations are given in Table II. Most of the HPLC procedures gave mean values within ±40% of the target. Two of the four GC-MS procedures and both LC-MS-MS methods were also within

TABLE II Determinations of 8-oxodG standard by different methods

Laboratory	8-oxodG (nM)	CV (%)
GC-MS		
5a	34.2	19
6	33.1	21
13b	9.1	8.8
13c	8.7*	
HPLC		
3	6.7	2.4
4	6.7	9.3
5b	7.4	1.2
8a	8.5	5.7
10b	11.4	4.6
11	8.6	3.8
12	9.9	6.1
13a	7.9	1.6
14	10.2	1.9
15	14.1	23
16	21.4	23
17	6.9	2.3
18a	7.5	1.8
18b	7.6	1.7
19	6.7	4.7
20	7.0	1.2
LC-MS-MS		
8b	8.3	3.5
10c	9.6*	

CVs are calculated from the means of triplicate determinations of the three replicates. *Indicates value from one replicate only (mean of triplicate determinations).

this range. CVs for most HPLC methods were reasonably low; for GC-MS, they tended to be higher.

Calf Thymus DNA Determinations

In the laboratory (number 9) responsible for preparing the calf thymus DNA, aliquots of each of the three calf thymus DNA preparations were assayed (in triplicate) for 8-oxodG, using HPLC with 4-enzyme hydrolysis at various times during the testing period of 55 days. In total, there were at least 10 determinations of each. Consistent with the treatment with increasing concentrations of Ro 19-8022, a linear dose response was found. These determinations provide the reference line shown (with SDs) in Figures 2–4. Full details of the preparation and analysis of these samples appear in Ref. [3].

Figure 2 shows results from the five laboratories measuring 8-oxogua by GC-MS. Note that two laboratories reported values higher than the others, and their results are accommodated on a different scale. One of these laboratories (6) based the values for 8-oxoguanine on the approximate amount of DNA in the sample as supplied, rather than on the amount of guanine actually present. Solid lines indicate laboratories finding a positive dose response in this set of samples, defined as an increase (or at least no decrease) in 8-oxogua from sample C (not treated with Ro 19-8022) to the mean of B and D (5 μM Ro 19-8022) and again an increase (or no decrease) from B/D to A (20 μM Ro 19-8022). Only two laboratories met these not very rigorous criteria.

Results from laboratories employing HPLC are depicted in Figure 3(a) and (b). Again, detection of

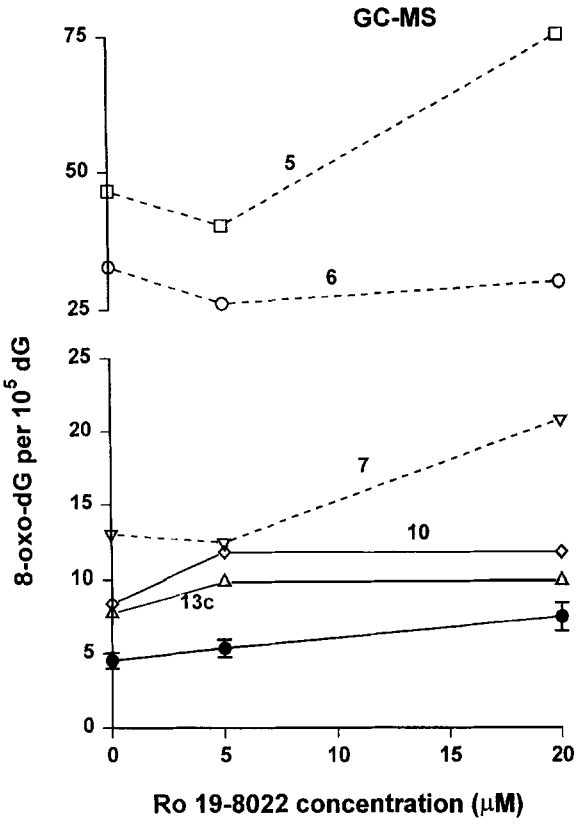

FIGURE 2 Values reported for calf thymus DNA samples by laboratories using GC-MS. The numbers beside lines relate to Table I. The line with solid circles represents the dose response for these samples established in the source laboratory; bars indicate SD. Procedures which show a positive dose response (for definition, see text) are indicated by solid lines.

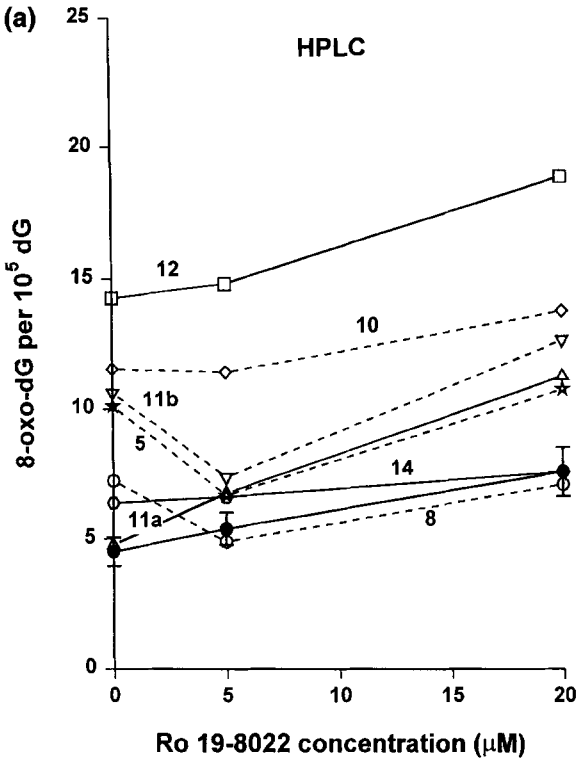

FIGURE 3(a)

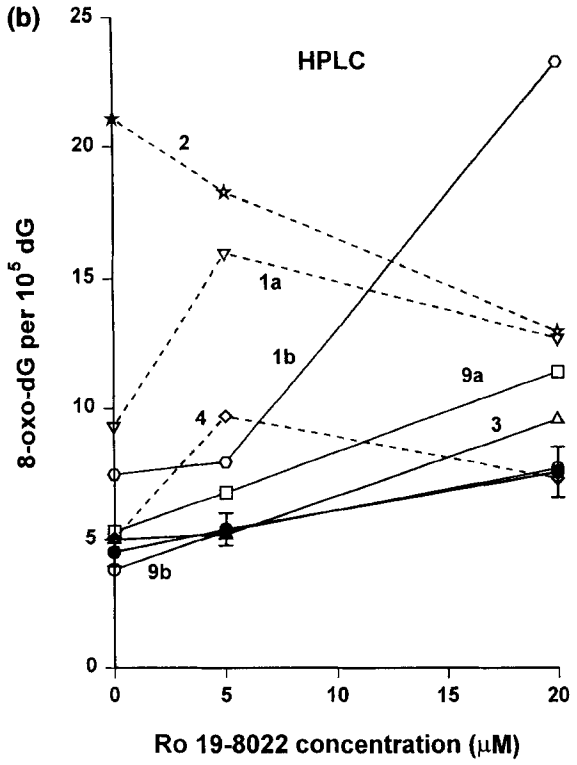

FIGURE 3(b)

FIGURE 3 (a) and (b) Values reported for calf thymus DNA samples by laboratories using HPLC with electrochemical detection. The numbers beside lines relate to Table I. The line with solid circles represents the dose response for these samples established in the source laboratory; bars indicate SD. Procedures which show a positive dose response (for definition, see text) are indicated by solid lines.

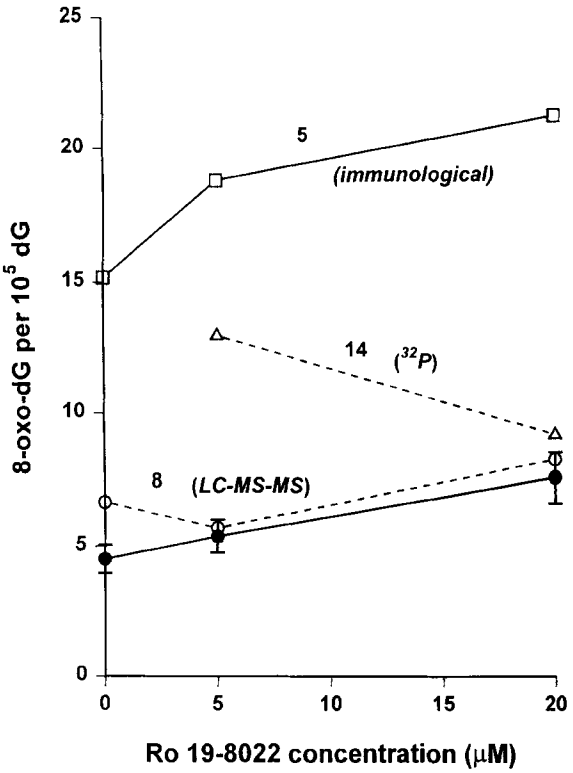

FIGURE 4 Values reported for calf thymus DNA samples by laboratories using procedures other than GC-MS or HPLC. The numbers beside lines relate to Table I. The line with solid circles represents the dose response for these samples established in the source laboratory; bars indicate SD. Procedures which show a positive dose response (for definition, see text) are indicated by solid lines.

a positive dose response is shown by a solid line. Because of the large number of sets of data, the laboratories are arbitrarily split between the two figures, each of which includes the reference line. Seven of 14 laboratories correctly identified the dose response.

Three laboratories used alternative techniques – LC-MS-MS, ^{32}P-postlabelling, and an immunoassay. Their results are shown in Figure 4. One procedure gave a positive dose response.

The performance of a method should also be assessed by examining CVs. Therefore, the values obtained by each laboratory for the duplicate samples B and D were subjected to statistical analysis. The *precision* of a method can be assessed by calculating the CV of repeated measurements of the same sample. As B and D were identical, and as (in most cases) triplicate injections were performed for each, the CV was calculated from the six values. Two GC-MS laboratories, six HPLC procedures but none of the alternative methods show a CV of 10% or less (Table III).

An alternative approach is to estimate the *reproducibility* of a method in terms of the variance associated with determinations of identical samples. In this case, the mean determinations of the two samples B and D were compared (Table III). Slight changes in the ranking of laboratories/methods according to CV are seen, compared with the ranking on the basis of CV of six replicates.

TABLE III Determinations of 8-oxodG in calf thymus DNA by different methods

Laboratory	8-oxodG/10^5 dG mean, B and D	CV of 6 (%)	CV of means of B and D (%)	Detection of dose response
GC-MS				
10	11.8	7.1	6.2	+
13c	10.0	10*	7.0	+
5	40.3	11**	11	
6	26.1	15	18	
7	12.4	23	29	
HPLC				
12	14.8	3.6*	3.6	+
11a	6.7	4.2**	3.8	+
14	6.6	6.2	7.1	+
10	11.5	7.1	8.0	
9b	5.3	7.4	5.2	+
9a	7.7	7.9	9.1	+
3	5.2	14	17	+
5	6.6	16	17	
8	4.9	18	3.8	
11b	7.3	20	24	
2	18.3	24	5.8	
1b	7.9	32	23	+
4	9.7	35	46	
10a	15.9	55	49	
Others				
8	5.7	13	7.1	
14	13.0	22	20	
5	17.3	48**	53	+

Statistical data relating to measurement of duplicate samples B and D: CV calculated on the basis of all 6 determinations (triplicate injections for two identical samples), or on the basis of the mean determinations for the two samples B and D. (Exceptions: *based on single injections for B and D; **based on duplicate injections for B and D.) For each method, laboratories are listed in order of CVs calculated on the basis of six replicates. The definition of ability to detect dose response is given in the text.

DISCUSSION

The tendency of certain GC-MS procedures to report values above the expected concentration of 8-oxodG in the standard sample probably indicates a calibration problem. (The same two laboratories reported high values in the calf thymus DNA experiment.) All the GC-MS laboratories used an internal standard of 8-oxogua labelled with heavy isotope (8-oxogua M + 4). It is possible that the need to hydrolyse the 8-oxodG sample to 8-oxogua introduces an anomaly. CVs calculated on the basis of the triplicate determinations of this sample are below 10% for most HPLC methods, but tend to be higher with GC-MS.

The aim with the calf thymus DNA samples was to test ability to distinguish different levels of damage, to identify a dose response, and to recognise the identical nature of samples B and D. A failure to detect the increase from 0 to 5 μM would imply insufficient sensitivity for detection of 8-oxodG in normal human cellular DNA (which seems to be at a level about 10× less than that in calf thymus DNA). In fact, fewer than half of the procedures tested were able to detect the dose response; successful methods tended to be those with low CVs (relatively high precision).

CVs range from less than 4% to over 50%, whether calculated on the basis of replicate analyses of B and D or of the mean values for B and D. A high CV may result from problems encountered on the day the analyses were carried out, and evaluating specific laboratories on this basis alone would be inappropriate. In addition, a high CV may simply reflect lack of experience with the technique. This consideration applies to the non-standard methods; two of the three failed to detect the dose response and all suffer from high CVs. They therefore cannot yet be recommended in place of conventional procedures, although LC-MS-MS has potential advantages, since it can resolve problems of identity and has a potentially high throughput of samples.

HPLC methods showing acceptable CVs for the calf thymus DNA assay all employ coulometric detection. Amperometric detection is known to be less sensitive, and it seems from these results that it is not appropriate for measuring low background levels of damage. Two laboratories used 4-enzyme rather than 2-enzyme hydrolysis prior to HPLC analysis. The results they obtained were similar to each other – and also similar to results with the 2-enzyme method used in parallel in one of these laboratories. There is no reason to recommend one method over the other. The detailed comparison of the two approaches is given in Ref. [3]. Two HPLC laboratories, instead

of digesting DNA to nucleosides with enzymes, carried out formic acid hydrolysis of DNA to bases – the method employed for GC-MS. HPLC analysis of bases did not show a dose response and so this approach cannot at present be recommended.

Freeze-drying is still routinely employed in GC-MS sample preparation though it has been found to cause oxidation of dG.[4] Ethanethiol is now used by most of the laboratories employing GC-MS, to guard against oxidation during derivatisation. Following previous concern over the elevated temperature employed for the derivatisation reaction, most laboratories now carry out derivatisation at room temperature. Judging by the results of the calf thymus DNA analysis (Figure 2), these precautions do not guarantee consistent results. Of the two procedures which detected the dose response and were closest to the reference values, one employed prepurification of 8-oxodG and derivatisation at 130°C, while the other carried out derivatisation at room temperature.

The importance of CV is illustrated in Figure 5. If a difference exists between two groups of samples, the ease with which this difference will be detected depends on the extent of the difference and on the reliability of the assay (i.e. the CV). Figure 5 shows the number of sample determinations required to demonstrate a difference between two different groups of samples. It is clear that an increase in CV from 10% to 20% is very costly in terms of the increase in numbers of samples per group that will be needed in order to demonstrate a fairly small difference between two groups. The aim with any method should be to achieve the lowest possible CV by eliminating analytical variation.

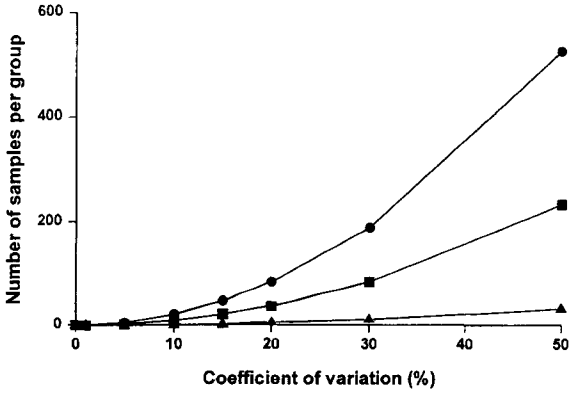

FIGURE 5 The influence of variability (CV) on the number of sample determinations required to demonstrate a difference between two different groups of samples. The difference is set at 10% (●), 15% (■) or 40% (▲).

Conclusions

To summarise, the criteria for an assay to be used in human studies to measure oxidative DNA damage are:

(1) that the method should have a CV of $< 10\%$ based on replicate injections of the same sample (a measure of precision);
(2) that, as a measure of reproducibility, the method should have as low a CV as possible (preferably $< 5\%$, certainly $< 10\%$) based on values obtained from identical samples (each value the mean of replicate injections);
(3) that the method should recognise differences between experimentally induced 8-oxodG at levels close to those found in nature (a measure of sensitivity).

When these aims have been met, the methods can then be refined to measure accurately DNA oxidation in biological material.

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List of ESCODD Participants

A.R. Collins (Chairman), J. Brown, M. Bogdanov, J. Cadet, M. Cooke, T. Douki, C. Dunster, J. Eakins, B. Epe, M. Evans, P. Farmer, C.M. Gedik, B. Halliwell, K. Herbert, T. Hofer, R. Hutchinson, A. Jenner, G.D.D. Jones, H. Kasai, F. Kelly,

A. Lloret, S. Loft, J. Lunec, M. McEwan, L. Möller, R. Olinski, I. Podmore, H. Poulsen, J.-L. Ravanat, J.F. Rees, F. Reetz, H. Shertzer, B. Spiegelhalder, R. Turesky, R. Tyrrell, J. Viña, D. Vinicombe, A. Weimann, B. de Wergifosse and S.G. Wood.

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