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Comparison of Results from Different Laboratories in Measuring 8-oxo-2′-deoxyguanosine in Synthetic Oligonucleotides

BENTE RIIS*[a] and ESCODD[b]

[a]*Department of Clinical Pharmacology, Q7642, Rigshospitalet, Copenhagen University Hospital, Blegdamsvej 9, Dk-2100 Copenhagen, Denmark;*
[b]*European Standards Committee on Oxidative DNA damage*

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The European Standards Committee on Oxidative DNA Damage (ESCODD) was set up in 1997 to resolve methodological problems and to reach agreement on the basal level of 8-oxo-2′-deoxyguanosine (8-oxodG) in biological samples. In the present ESCODD trial 6 samples of 8-oxodG-containing oligonucleotides with different ratios of 8-oxodG/2′-deoxyadenosine (dAdo) were sent to 25 laboratories throughout Europe. The methods used were HPLC with electrochemical detection (amperometric or coulometric), GC–MS or LC–MS–MS. The LC–MS–MS and the coulometric HPLC analyses gave 8-oxodG concentrations within 53 and 73% of expected values, respectively, whereas the amperometric HPLC and GC–MS consistently overestimated the 8-oxodG concentration by several fold. As the oligonucleotides contained no 2′-deoxyguanosine (dGuo), this was not due to artificial oxidation. On the contrary, in most cases the concentrations of dAdo and thymidine (dThd), used as estimates for non-oxidised DNA bases were underestimated, though a few laboratories overestimated the lowest concentration samples containing 8 and 20 μM, respectively. In one-third of the reported results, the ratio of 8-oxodG/10^5 dAdo was within 25% of the calculated value in the oligonucleotide samples and in half of the results the coefficient of variation in duplicate samples was less than 10%. The coefficients of variation were higher for the dAdo concentrations than for 8-oxodG. Our findings strongly indicate that careful quality control must be applied to the analytical procedures for 8-oxodG and very importantly also to the procedures for non-modified 2′-deoxyribonucleosides. We recommend the use of synthetic oligonucleotides for this purpose.

Keywords: Oxidative DNA damage; 8-oxo-2′-deoxyguanosine; HPLC; GC–MS; LC–MS–MS; Standardisation

INTRODUCTION

Oxidative stress to DNA has for long been recognised as a potential cause in a variety of human diseases[1–3] and as a factor in the ageing process.[4,5] However, it has been difficult to elucidate the exact mechanisms and significance of oxidative damage. One important factor for the difficulty is the highly variable estimations of oxidative stress reported in the literature[6] and also lack of precision and accuracy in the measurement of oxidative DNA modification are problems that need to be addressed. To this end the European Standards Committee on 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 various samples. The focus was on 8-oxo-2′-deoxyguanosine (8-oxodG) since this is the most commonly measured marker of oxidative DNA damage. Further goals of ESCODD are to improve the accuracy and specificity in the measurements of 8-oxodG, and to provide standard operating procedures. The work of ESCODD consists of approximately 3 trials a year, in which samples are distributed to all contributing laboratories. The samples range from standard solutions of 8-oxodG to samples of DNA, HeLa cells or pig liver. Several reports are available.[7–9]

*Tel.: +45-3545-7647. Fax: +45-3545-2745.

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TABLE I Oligonucleotide sequences (X = 8 − oxodG)

	Residues			
	N	8-oxodG	dAdo	Sequence 5′ → 3′
Oligo ox1	22	1	6	CAT TTA CAT ATX CTT ATC ATT C
Oligo ox5	22	5	6	CAT XTA CAX ATX CTX ATC AXT C
Oligo noG	22	0	6	CAT TTA CAT ATT CTT ATC ATT C

This paper describes an ESCODD trial from October 2000 on 8-oxodG-containing oligonucleotides, where 20 laboratories submitted results from a total of 21 analyses.

METHODS

Three different HPLC-purified 22′mer oligonucleotides, called oligo ox1, ox5 and noG (Table I), were synthesised by DNA-technology (Aarhus, Denmark). Oligo ox5 included five 8-oxodG residues, oligo ox1 included one 8-oxodG residue, whereas oligo noG did not contain 8-oxodG. Their nucleotide sequences were identical except for the 8-oxodG residues that were partly or totally substituted by dThds in oligo ox1 and oligo noG. The rest of the 2′-deoxyribonucleotides in the three oligonucleotides were dAdo, dThd and dCyt so that none of them contained dGuo residues. The concentrations of the oligonucleotides were determined by measurements of the optical density (OD) at 260 nm (approximation: 1 OD_{260} = 33 μg/ml). The molecular weight was calculated from the sequence (provided by DNA-technology). Six oligonucleotide samples were prepared with defined concentrations of 8-oxodG, dAdo, and dThd (Table II) in a total volume of 15 ml. The ratios of 8-oxodG to 10^5 dAdo and to 10^5 dThd are also listed in Table II. The samples A1 and B1 were designed to test the limits of detection of the methods, as the 8-oxodG concentrations were only 1 nM. The other samples (A2, A3, B2, and B3) contained 4 nM 8-oxodG. The concentration of dAdo ranged from 8 to 400 μM and the ratio of 8-oxodG/10^5 dAdo from 1 to 50. The concentration of dThd ranged from 15 to 733 μM and the ratio of 8-oxodG/10^5 dThd from 0.6 to 27.3. Aside from knowing the accuracy and precision of the different methods, the set-up also would provide information about the effectiveness of DNA hydrolysis.

The construction of the oligonucleotides was based on the idea that if dGuo was not present, artificial oxidation creating 8-oxodG could not take place. In order to test for artificial oxidation of dGuo, inclusion of an oligonucleotide with dGuo but without 8-oxodG was desirable. However, as one of the steps in oligonucleotide synthesis is treatment with 0.2 M iodide which involves oxidation, it was not possible to obtain an oligonucleotide free of 8-oxodG. This problem of getting dGuo without oxidation products is well known in our laboratory as we also see significant amounts of 8-oxodG in pure dGuo standards. We therefore strongly recommend that the 8-oxodG and dGuo standard curves are always made separately.

Analysis

The oligonucleotide samples were aliquoted and sent at ambient temperature to the 25 participating laboratories throughout Europe. Samples were recommended to be stored at −20°C upon receipt. The six samples (Table II) were to be hydrolysed and analysed by double injections on two different occasions, at least two days apart. The results were submitted as 8-oxodG/10^5 dAdo, but the laboratories were also asked to report the actual measured concentrations of dAdo and 8-oxodG. ESCODD normally recommend that the unit oxidised nucleoside/base per 10^6 unmodified nucleoside/base is used. Measurement of dThd, or the use of a molecular biology grade alkaline phosphatase without adenine deaminase activity, was recommended to solve a problem with degradation of dAdo during hydrolysis reported from some laboratories.

TABLE II Concentration of 8-oxodG, dAdo, dThd and ratios of 8-oxodG/10^5 dAdo and 8-oxodG/10^5 dThd in the oligonucleotide samples

Sample	8oxodGuo (nM)	dAdo (μM)	8-oxodG/10^5 dAdo	dThd (μM)	8-oxodG/10^5 dThd	Concentration oligo (μg/ml)
A1	1	100	1	183	0.55	111
A2	4	40	10	73	5.45	45
A3	4	400	1	733	0.55	442
B1	1	20	5	37	2.73	22
B2	4	8	50	15	27.27	9
B3	4	80	5	147	2.73	88

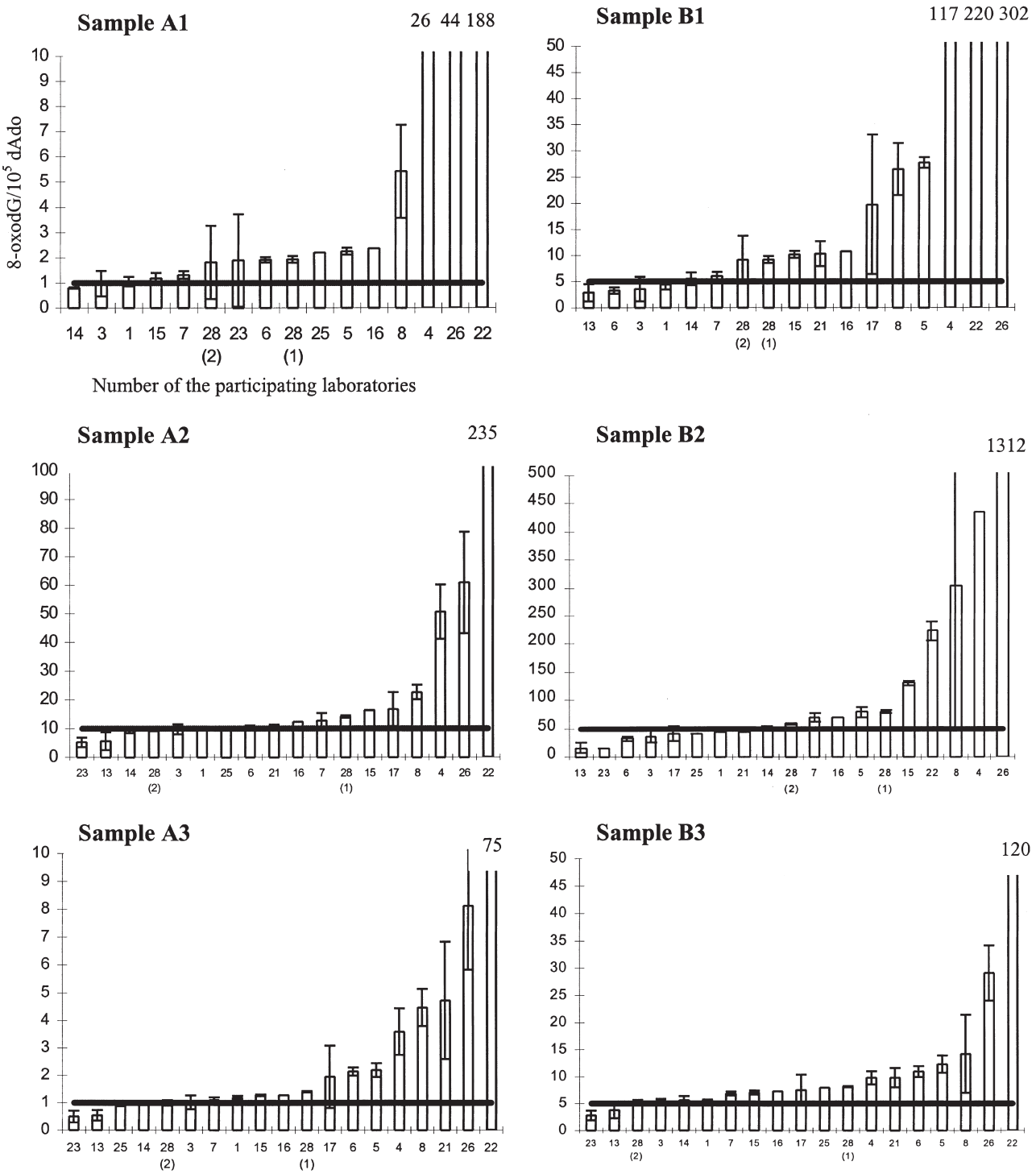

FIGURE 1 Ratios of 8-oxodG/10^5 dAdo. The solid line marks the target value. The numbers on the *x*-axis refer to the various participating laboratories. They are sorted according to the lowest values. Values that are off scale in the figure are given above the open columns.

Analytical Procedures

Of the 25 laboratories, 20 submitted results, one of them using two different methods, giving a total of 21 analytical procedures. Seventeen laboratories used high performance liquid chromatography with coulometric detection (HPLC/c), 1 used high performance liquid chromatography with amperometric detection (HPLC/a), 1 used liquid chromatography coupled to a tandem mass spectrometer (LC–MS–MS), and two laboratories used gas chromatography with mass spectrometry detection (GC–MS) for the analyses. Of the 5 laboratories not submitting results, 3 laboratories

TABLE III Concentrations of 8-oxodG (nM) and dAdo (μM) in the six oligonucleotide samples. CVs are calculated from the means of duplicate determinations of two replicates

		A1				A2				A3			
Part	Method	8-oxodG	CV (%)	dAdo	CV (%)	8-oxodG	CV (%)	dAdo	CV (%)	8-oxodG	CV (%)	dAdo	CV (%)
1	HPLC/c	1.05	2.71	92.11	3.67	3.78	11.60	35.00	12.52	4.95	2.72	386.96	2.22
4	HPLC/a	18.95*	0.37	74.85*	0.09	10.05*	4.93	20.10*	13.37	8.75*	20.20	263.10*	0.00
5	HPLC/c	0.54*	5.24	23.78*	1.31	MP		MP		1.98	11.47	95.56	1.94
6	HPLC/c	1.41	6.52	73.51	5.79	3.02	1.40	28.70	0.25	2.47	7.59	115.00	0.61
8	HPLC/c	2.66	7.83	50.62	4.69	2.98	19.74	13.25	21.91	7.14	22.74	156.36	4.96
13	HPLC/c	ND		87.63	16.58	2.20	48.21	41.16	20.98	1.83	25.18	318.30	11.33
14	HPLC/c	0.71	1.00	88.23	1.72	3.33	5.20	35.45	0.40	3.56	1.29	362.20	4.12
21	HPLC/c	MP		MP		1.19	8.32	11.11	13.56	2.45	18.80	59.93	61.00
25	HPLC/c	0.34†		15.35†		0.98†		9.72†		0.98†		110.00†	
26	GC/MS	44.93	22.59	102.30	0.00	22.70	28.66	37.20	0.00	31.43	26.66	386.70	0.00
28 (1)	HPLC/c	1.55	5.93	79.29	1.06	4.92	2.45	34.68	0.37	4.80	0.66	340.07	0.57
28 (2)	LC–MS/MS	1.53	67.23	82.05	7.24	3.18	12.25	33.80	10.25	3.20	13.69	312.97	7.16
Expected		1		100		4		40		4		400	
		B1				B2				B3			
1	HPLC/c	0.94	13.20	19.59	2.06	4.11	6.37	8.38	0.12	4.40	7.07	72.07	5.73
4	HPLC/a	10.35	17.08	8.78	4.43	8.65*	4.09	ND		4.18	56.74	41.65	53.14
5	HPLC/c	3.17	5.87	11.44	1.89	2.96*	5.26	3.77*	6.57	5.52	1.99	48.14	0.65
6	HPLC/c	0.55	14.27	16.63	0.21	3.82	5.37	11.63	12.71	4.84	15.78	44.75	25.76
8	HPLC/c	1.33	2.13	5.19	17.89	2.18	29.84	1.25	101.66	3.11	17.15	25.05	51.07
13	HPLC/c	0.73	22.81	26.99	28.90	2.53	21.00	17.61	34.71	2.53	28.28	73.15	17.87
14	HPLC/c	1.33	0.80	24.80	12.83	4.29	15.16	8.83	22.03	3.95	13.52	69.83	2.18
21	HPLC/c	3.34	21.17	34.03	43.22	6.28	30.85	13.34	24.23	1.75	11.75	17.81	6.31
25	HPLC/c	NA		NA		1.04	17.68	2.51	18.59	NA		NA	
26	GC/MS	47.88	42.17	15.87	0.00	43.63	3.16	3.33	0.00	22.13	6.23	76.00	0.00
28 (1)	HPLC/c	1.63	5.85	17.67	0.74	5.69	2.61	7.12	1.04	5.57	1.27	68.02	0.33
28 (2)	LC–MS/MS	1.45‡	63.40	15.48‡	4.80	2.95	33.56	5.13	36.56	3.53	13.04	64.70	6.23
Expected		1		20		4		8		4		80	

* Indicates value from one replicate only (duplicate injections). † Indicates the concentration of one injection only. ‡ Indicates that the two replicates are from different mailings of the sample. ND: not detected; NA: not analysed; MP: mechanical problem; NES: not enough solution.

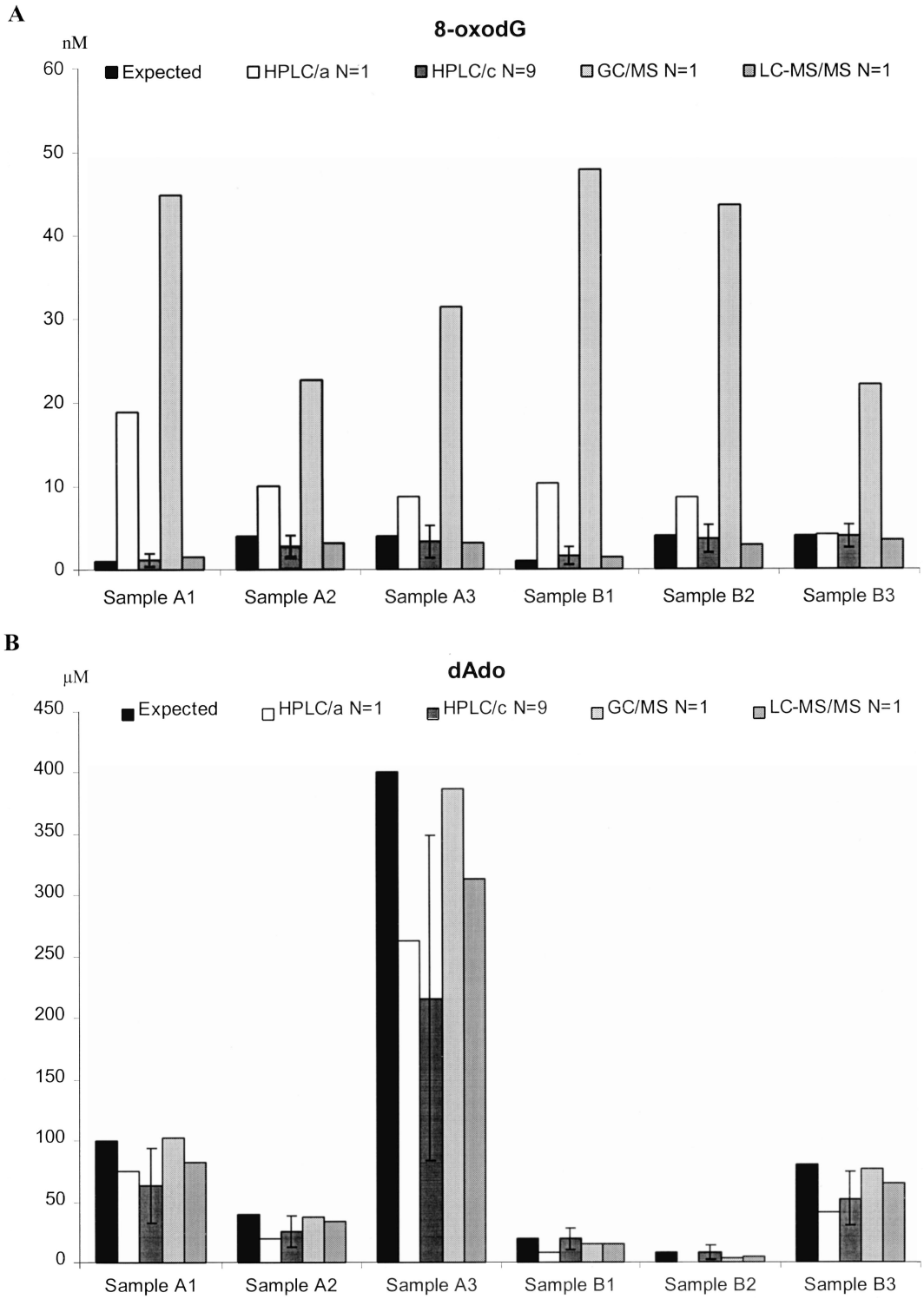

FIGURE 2 Concentrations of 8-oxodG (A) and dAdo (B) arranged according to the method used. The values are means of duplicate determinations of two replicates, though for the HPLC/c-method the averages of means from 8 laboratories with the standard deviations shown by bars. The black columns show the expected values for each oligonucleotide. The concentrations of 8-oxodG are given in nM, and of dAdo in μM.

(1 HPLC/c, 1 HPLC/a, and 1 LC–MS–MS) did not give reasons and 2 (1 HPLC/c and 1 GC/MS) reported mechanical or technical problems. Of the 20 laboratories submitting results on the 8-oxodG/10^5 dAdo (or dThd) ratio, 12 laboratories also gave the measured concentrations of 8-oxodG and dAdo. 9 laboratories submitted dThd results.

The analytical procedures are only briefly outlined. For further details on the specific methodologies

used, we refer to published literature by the individual members.

HPLC–ECD

The oligonucleotides were hydrolysed with nuclease P_1 and alkaline phosphatase (laboratories no. 1–7, 12–21, 23, 28(1,2)), or with nuclease P_1, alkaline phosphatase and T1 ribonuclease (laboratory no. 25), or deoxyribonuclease I, spleen exonuclease, snake venom exonuclease and alkaline phosphatase (laboratory no. 8). The hydrolysate and standards of dAdo, dThd and 8-oxodG were in all cases separated on C18 columns, though of various types and dimensions, followed by electrochemical detection (coulometric or in one case amperometric (laboratory no. 4)) of 8-oxodG, and UV detection of dAdo and/or dThd.

LC–MS–MS

Nucleosides separated by liquid chromatography on a C18 column were eluted into a triple quadropole mass spectrometer for identification and quantitation of the analytes.[10–13] Isotope dilution with heavy labelled internal standards was used for quantification.[10–12]

GC–MS

Samples were hydrolysed to bases with 60% formic acid at 130°C for 30 min (laboratory no. 22) or at 140°C for 45 min (laboratory no. 26). Bases were derivatised with bis(trimethyl-silyl)trifluoroacetamide at room temperature under argon for 45 min/2 h (in no. 26 with ethane–thiol present to prevent oxidation).

RESULTS

The results are presented with regard to accuracy and precision. Accuracy is defined as the degree of conformity with the target value without consideration to variability. Precision relates to the variability of the results without consideration to the target value.

Accuracy

Figure 1 shows the 8-oxodG/10^5dAdo ratios obtained for the six distributed oligonucleotides samples. The solid horizontal line marks the expected target value for each sample. The numbers on the *x*-axis represent the participating laboratories. They are sorted according to the reported result, with the lowest result to the left. Many laboratories reported values close to the target value, whereas a few repeatedly overestimated the ratio. The fraction of results above the expected level were highest for the samples A1 and B3 (88 and 90%, respectively). Looking at the average of the reported results for the ratio of 8-oxodG/10^5 dAdo for all oligonucleotide samples from each laboratory/method, one third of the results were within 25% of the expected value, and half of the results were within 50%. Seventy six percent of the results were above the expected values with maximums of 23 (sample B1) to 188 (sample A1) times the expected values in the six samples. The two GC–MS methods (no. 22 and 26) and the amperometric HPLC detection (no. 4), in particular, clearly overestimated the 8-oxodG/10^5 dAdo level in all the samples (Fig. 1).

The reported concentrations of 8-oxodG and dAdo are shown in Table III. When they are examined according to the methods used, the relative performance of the methods can be compared (Fig. 2A). The HPLC/c (average data) and LC–MS–MS methods produced 8-oxodG results within 73 and 53% of the expected values, respectively, with the highest deviations for the A1 and B1 samples. With the HPLC/a method an 8-oxodG concentration of up to 20 fold higher than the expected value was found, whereas the GC–MS methods measured 5–50 fold more 8-oxodG than expected (Table III). The reason for this could be contamination during sample preparation or during analysis possibly from the injection port or elsewhere in the system. Analysis of the noG oligonucleotide did not suggest a general problem with carry-over contamination neither by HPLC–ECD nor by GC–MS. Another explanation could be problems with the 8-oxodG standard curve. Laboratory no. 26 reported that the standard solutions were stored at working concentrations. This emphasises the importance of only storing standards at sufficiently high concentrations such that they can be routinely checked by absorbency. A previous ESCODD publication[7] also indicated that consistent over- or under-estimations seen in a few laboratories, most likely is explained by errors in the preparations of standards. Inclusion of heavy-isotope-labelled internal standards in the GC–MS method are suggested to bring more consistency to the results of the different laboratories.

Figure 2B shows that part of the overestimation of the 8-oxodG/dAdo ratios was also due to lower dAdo values than expected. In particular, the HPLC methods resulted in up to 50% less dAdo than expected except from samples B1 and B2, which had the lowest dAdo concentrations (Table III).

After the first run, one laboratory reported that the alkaline phosphatase and/or nuclease P_1 used for DNA hydrolysis might be contaminated with adenine deaminase activity. The participants were therefore asked to measure dThd as well as dAdo if possible, and also to run hydrolysed dAdo standards

TABLE IV Percent chance of peak area on the chromatograms upon incubation of standard samples with hydrolysis enzymes. Abbreviations of the methods are explained in analytical procedures

Lab.	Method	MBG	dGuo	8-oxodG	dAdo	dThd	Incubations
1	HPLC/c	N	0	2.3	−7.7	−2.4	N
5	HPLC/c	N	9.7	ND	7.4	−7.4	N
6	HPLC/c	N	−10	−19	−13	−5.7	Y
7	HPLC/c	N	3	3	−10	ND	N
8	HPLC/c	Y	−6	0	12	−6	N
12	HPLC/c	NR	0	0	−96.2	0	N
14	HPLC/c	N	ND	ND	−100	ND	NR
26	HPLC/c	N	−1	24	−4	−1	Y
28 (1)	HPLC/c	Y	−4.5	7.6	5.6	−6.8	N
28 (2)	LC/MS–MS	Y	−2.7	−3	−2.2	−6.9	N
	Avg. MGB		−4.40	1.53	5.13	−6.57	
	SD		1.65	5.46	7.11	0.49	
	Avg. others		0.28	2.06	−31.93	3.30	
	SD		6.38	15.25	45.68	3.14	

MGB: whether or not molecular biology grade alkaline/acid phosphatase was used; Incubation: whether or not standards were incubated with hydrolysis enzymes in the oligonucleotide experiments; ND: not done; NR: not reported; SD: standard deviation.

to test dAdo degradation in their system. Only 9 laboratories submitted dThd data, all using the HPLC/c method, *i.e.* with UV detection of dThd. The ratio of 8-oxodG/10^5 dThd varied from 0.2 to 5.2 fold relative to the expected values in the 51 submitted results. Eight out of nine ratios were higher than expected. When the concentration of dThd was calculated from the reported ratios of 8-oxodG/10^5 dThd and the concentrations of 8-oxodG (possible in 4 cases), 96% of the results were below the expected concentration of dThd in the oligonucleotides.

Ten laboratories submitted data on the percent change of peak area when standards (dGuo, 8-oxodG, dAdo, and dThd) were incubated with the hydrolysis enzymes (Table IV). Only three methods used molecular biology grade enzymes. The average change of peak area of dAdo for those three were +5.1% (SD 7.1), whereas the change in peak area when using non-molecular biology enzymes varied from −100 to +7.4% change, indicating none or negligible adenine deaminase activity in the molecular biology grade enzyme. For dGuo, 8-oxodG, and dThd the differences on average between the two groups were insignificant, as was the change of peak area, though the standard deviations were rather high. Only two of the ten laboratories routinely incubated the standards with the hydrolysis enzymes.

Precision

The precision of the results is presented as the coefficient of variation (CV), which describes the variability by expressing the standard deviation as a percentage of the mean. The CV is calculated from the means of duplicate determinations of the two replicates.

The maximum coefficient of variation of the ratio of 8-oxodG/10^5 dAdo in all of the six samples was 97%. About half of the total of 97 results had a CV of duplicate analysis below 10% while one out of ten showed a CV above 50%. Samples A3 and B1 showed the highest CVs with only one-third and one-fifth below 10%, respectively. In sample A1, in which the 8-oxodG concentrations was as low as B1, 3 out of 5 had a CV below 10%. The observed variances were significantly different (Levenes test of homogeneity of variance; $P < 0.0001$) and *post hoc* analysis revealed that the most deviant variances in most of the oligonucleotide samples, were from laboratories no. 4, 22 and 26, though in samples B1 and B2 also from no. 8 and 17 ($P < 0.05$).

The CVs on the 8-oxodG/10^5 dThd ratios spanned a wider range and went up to 114%, although two-thirds had a CV below 10%. One out of ten presented a CV above 50%. The variances were significantly different (Levenes test; $P < 0.0001$), with significantly deviant variances found in laboratories no. 8 (4 samples), 23 (2 samples) or 3 (1 sample) ($P < 0.05$).

For the 8-oxodG concentrations, the CVs ranged from 0.4 to 67% (Table III). The variances of the concentrations were significantly different in all samples (Levenes test; $P < 0.01$). The proportion of CVs below 10% was highest in sample A1 (three-quarters below 10%), but overall slightly less than half of the reported 8-oxodG concentrations had a CV below 10%. One in twenty were above 50%. Also for the dAdo concentrations the variances were different. Levenes test of homogeneity of variances were significant at $P < 0.0001$ in all six samples. The dAdo concentrations showed CVs going up to 102% (Table III). Three out of five had a CV below 10% and only one in fourteen of the CVs were above 50%. There was no overall correlation of the CVs of 8-oxodG versus dAdo ($R^2 = 0.12$) (Fig. 3). However, looking at the HPLC/c data alone, grouped in three groups according to the value of CV for 8-oxodG, it is clear that the highest values were connected with the highest CV values from the dAdo data, and that many high value points belonged to three

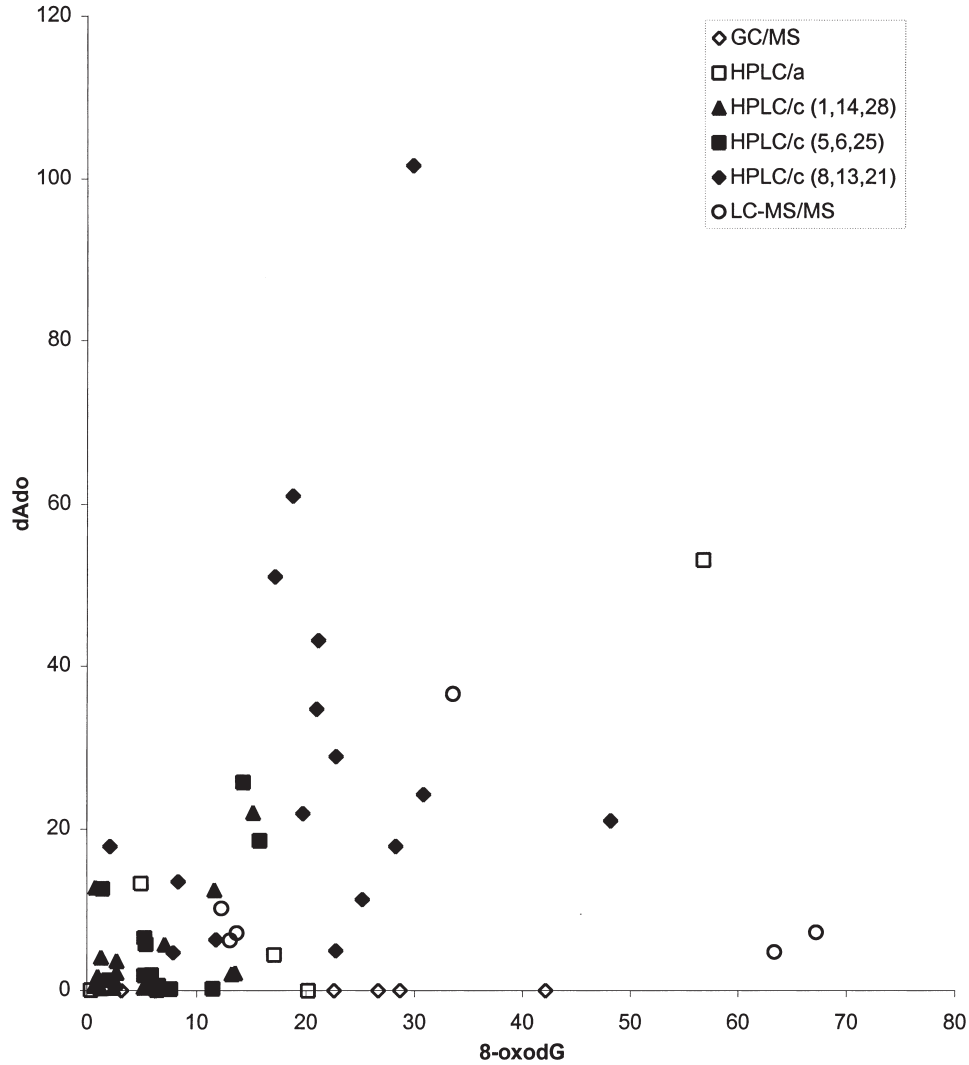

FIGURE 3 The coefficients of variation of the 8-oxodG concentrations plotted against the corresponding CV's of the dAdo concentrations. The HPLC/c methods are grouped with laboratories no. 1, 14 and 28 having low CVs, no. 5, 6, and 25 having medium CVs and no. 8, 13, 21 having generally high CVs. The CVs are calculated from the means of duplicate determinations of the two replicates.

laboratories only. Single outliers might explain two of the high CV points. The LC–MS–MS method showed CVs of dAdo around 5–10%, however for one oligo it reached 37% (Table III). For 8-oxodG the CV in all samples exceeded 10% but was about 65% in two cases. One of these may have been due to the use of a new aliquot of the oligonucleotide sample on day two of analysis, while the other was due to a single outlier. The GC–MS method (data from one laboratory only) showed high CVs from the 8-oxodG data in 4 of the oligonucleotide samples (A1, A2, A3, and B1) ranging from 23 to 42%, but CVs of 3 and 6% for the last two samples (B2 and B3). For the 6 dAdo concentrations variances of zero were reported, which must be due to a single analysis instead of duplicate determinations. The amperometric HPLC method reported CVs within 20% except in one sample (B3), where both CV values exceeded 50%. It must be noted though, that in samples A1, A2, A3, and B2, the CV was only based on duplicate data from a single day, due to mechanical or technical problems. In sample B2, dAdo was not detected with the HPLC/a method.

DISCUSSION

In the first round of ESCODD,[9] the initial 8 contributing laboratories received a lyophilised standard 8-oxodG, a 20′ mer 8-oxodG-containing oligonucleotide, a sample of lyophilised calf thymus DNA, and a sample of frozen pig liver tissue for analysis. The results showed coefficients of variation of up to 63% and estimates differing between laboratories by up to 137 fold for both the calf thymus DNA and pig liver. For the standard 8-oxodG samples 9 out of 10 methods reported values within 50% of the expected value (12.6 nM). Only 30% of the results were above the target. The CVs ranged from 0.7 to 43% with 6 out of 10 below

10%. For the oligonucleotide sample only 1 out of 8 results was within 50% and 7 of 8 results were higher than expected. However, the 8-oxodG-containing oligonucleotide also contained dGuo and moreover was diluted 5 times in a dGuo-containing oligonucleotide, which probably accounts for the overestimation (as explained under methods). The results were given as nmol 8-oxodG per mg oligonucleotide calculated from its extinction coefficient, but this method itself should not contribute to the higher levels. CVs varied from 1.9 to 62%, with two thirds below 10%.

For the next trials more laboratories were invited, and the analytical tasks were simpler, as the samples consisted of four samples of calf thymus DNA with different levels of induced damage and freeze dried standards of 8-oxodG. These were later replaced by solutions as some laboratories had difficulties in reconstituting the freeze-dried material. The analysis of the calf thymus DNA samples resulted in coefficients of variation of up to 55% and many methods were unable to detect induced damage in the DNA.[8] For the standard solutions about half of the reported results were within 25% of the expected value of 6.9 nM; however, four-fifths of the laboratories reported values higher than the calculated concentration of the standard sample. Results from 22 methods were reported. The CV calculated from the means of triplicate determinations of three replicates varied from 1.2 to 23%. In four out of five laboratories the CVs were lower than 10%. The CVs tended to be higher for GC–MS than for LC–MS–MS and HPLC.[8]

When we compare the 8-oxodG to dAdo ratios in the present study with the earlier oligonucleotide data, the improvement is obvious. Instead of one-eighth within 50% of the target value, we now see half of the data within 50% of the target value. Also fewer of the present results were higher than the expected value. This can, however, be explained by the design of the initial oligonucleotide sample, and the resulting underestimation of the target value. The CVs span a wider range in the present material as expected when the number of observations increase, but the percentage of methods with a CV below 10% are approximately the same.

If the reported concentrations of 8-oxodG in the present six oligonucleotide samples are compared with the standard solutions from the previous trials of ESCODD, the fraction of results within 50% of the expected value declined from 90% (first trial) and 77% (second trial) to 57% in this round. Half of the present 8-oxodG concentrations were higher than the target value compared to 30 and 82% in the previous trials, respectively. The results must be seen in the light of the enhanced requirements for the procedures, as the concentrations of 8-oxodG in the oligonucleotide samples were significant lower than the concentration of 8-oxodG in the standard solutions in the previous trials (1 and 4 nM vs. 12.6 nM (first trial) or 6.9 nM (second trial)). The present CVs showed a wider range, with fewer CVs below 10% as compared to the two earlier trials. Again the low concentration of 8-oxodG in the oligonucleotides close to the limit of detection is a possible explanation for this.

One important point is that the variation in this trial (max. 234 fold difference between the highest and lowest values in the six samples) as well as the other trials, is well below the variation in the published literature on 8-oxodG/dGuo in biological samples, which is as high as 60,000 fold.[6] Furthermore, the very high values are limited to a small number of laboratories. One major reason for this relatively good performance may be improved standardisation procedures as well as the limited sample preparation (hydrolysis only) and simplified sample constituents. This supports the notion that treatment of the samples before hydrolysis is a critical step in the measurement of 8-oxodG in biological samples, and that the procedures of sample handling must be carefully controlled.

The ability of the majority of the HPLC/c and LC–MS–MS methods to measure the target value of 8-oxodG, indicates that the enzymatic hydrolysis methods perform correctly. This is in accordance with the substrate specificity of the widely used nuclease P_1[14] for essentially all phosphodiester bonds in synthetic as well as native DNA.

All laboratories using enzymatic hydrolysis measured too low concentrations of dAdo in most of the oligonucleotide samples compared to the target values, though a few laboratories overestimated the level in the low concentration samples (8–40 μM dAdo). While some laboratories consistently found 15–20% less dAdo than expected, others reported more irregular underestimation of up to 85%, noticeably in the high concentration samples (80–400 μM). The concentration of dThd obtained by calculation from the data of 8-oxodG/dThd ratios and the 8-oxodG concentrations from 4 laboratories also showed a significant underestimation of dThd ranging from 20 to 80%. One explanation for this might be a destructive enzymatic digestion of the 2′-deoxyribonucleosides. The present experiments of incubating standards with the hydrolysis enzymes (Table IV) showed that the laboratories experienced varying changes of peak area of dAdo from +12 to −100%, depending on the types of enzymes used. For dThd, Table IV shows that on average not more than −6.6% can be explained by enzymatic hydrolysis in the oligonucleotide samples. Another explanation of the underestimation of dAdo and dThd could be a deviation from linearity of the UV absorbency with the concentration in the high concentration standards. However, when we

determined the slope on a standard curve from the low values (10–150 μM) and extrapolated to 1000 μM we did not see any deviation from the normal linear standard curve of dGuo, dAdo or dThd. Thus we must conclude that the laboratories to a varying degree underestimate dAdo and dThd when using HPLC with UV detection or LC–MS–MS. We therefore emphasise that the issue of standardising the measurements of oxidative damage in DNA relative to unmodified 2′-deoxyribonucleosides is not only focused on the levels of 8-oxodG, but also includes quality controls of the 2′-deoxyribonucleoside measurements.

The GC–MS method using formic acid hydrolysis gave dAdo results in accordance with the expected value except in two low concentration samples (8 and 20 μM dAdo), that were underestimated by 60 and 20%, respectively.

To test for artificial oxidation in future trials, the mean of several measurements of 8-oxodG in a dGuo oligonucleotide could be used as the true value, but the information about the level of oxidation during the hydrolysis step would be lost. Another possibility is to incubate a double stranded dGuo-containing oligonucleotide with the purified repair glycosylase OGG1 prior to hydrolysis, as OGG1 specifically excises the oxidised guanine base, and thereby removes the 8-oxoGuas. Thus any 8-oxodG detected after a transient incubation with OGG1 would be a result of artificially oxidation of dGuo during sample handling. However, the approach would not work for the GC–MS method, as this method measures the base 8-oxoGua instead of the deoxynucleoside and thus would also detect the OGG1 released bases. A third possibility to be considered in forthcoming experiments is the availability of cellular DNA that has been specifically oxidised with a chemical source of [^{18}O] singlet oxygen.[15] Thus, [^{18}O]-8-oxodG which is generated within the cell nucleus can be used as internal standard for optimisation of conditions of DNA extraction and subsequent work-up before LC–MS–MS or GC–MS analysis as it is possible by mass spectrometry to measure both labelled and unlabelled 8-oxodG. However, this scheme is not applicable to the HPLC–ECD methods.

CONCLUSIONS

Consequences of oxidative stress are most likely important issues in relation to many disease states, and to normal health and ageing. However, this is very difficult to conclude from published data as the levels of damage in various tissues and cells differ by several orders of magnitude. The ubiquitous oxidative environment *in vivo* and *ex vivo* makes it very likely that artefacts are induced during sample preparation but also part of the variation may be related to the analytical method used. Careful handling, omission of oxidising reagents and/or addition of antioxidants can reduce this problem,[16] but there is still a need for a further consensus in the measurement techniques and conditions between laboratories. ESCODD is studying only one oxidative modification in DNA, but there might be a need of similar initiatives for other lesions and also in relation to other vulnerable macromolecules *i.e.* lipids and proteins, if proper standards and protocols can be found.

The use of synthetic oligonucleotides is recommended as a method of quality control of 8-oxodG measurements. Oligonucleotides containing 8-oxodG are commercially available, and the target concentration of 8-oxodG can easily be calculated from the concentration and known sequence of the oligonucleotide. As with every other standard, the quality of the oligonucleotide should be checked with time of storage as the stability of the modified DNA is not assured. We will once again emphasise that the oligonucleotides should not contain dGuo, until a methodology is achieved whereby artificial oxidation during the oligonucleotide synthesis is avoided or by which oxidised nucleosides can be removed.

The advantage of the standardising program is, besides avoiding controversies, that data on oxidative DNA damage in the future will be more easily comparable and therefore will help us to elucidate the health effects and consequences of oxidative DNA damage. Further work in ESCODD and similar initiatives are warranted.

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