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Controlled Oxidation of Calf Thymus DNA to Produce Standard Samples for 8-Oxo-deoxyguanosine Analysis; Effects of Freeze-Drying, Storage and Hydrolysis Conditions

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Calf thymus DNA containing defined levels of 8-hydroxy-2′-deoxyguanosine (8-oxodG) was prepared by treatment with visible light in the presence of photosensitiser Ro 19-8022. The DNA was checked for stability; after freeze-drying, the amount of 8-oxodG did not increase during 6 weeks' storage at room temperature. However, freeze-drying itself can introduce additional oxidative damage. Two enzymic hydrolysis regimes (DNase I, phosphodiesterases I and II, and alkaline phosphatase; or P1 nuclease and alkaline phosphatase) give similar values for 8-oxodG.

Keywords: Oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine, quality control

INTRODUCTION

8-Hydroxy-2′-deoxyguanosine (8-oxodG), measured in the DNA of human white blood cells, is often regarded as a biomarker of oxidative stress. Evidence is accumulating that guanine is prone to oxidation during the preparation of samples for analysis by GC-MS or HPLC, and it is therefore crucial to minimise the opportunity for oxidation to occur. Several recent publications address this question.[1–6] We have prepared samples of calf thymus DNA containing different amounts of 8-oxodG, by treating the DNA with a photosensitiser, Ro 19-8022, and visible light – a combination that has been shown to introduce predominantly oxidised purines, of which about 75% are 8-oxoguanine.[7] The samples were distributed to laboratories taking part in a validation exercise (organised by ESCODD, the European Standards Committee for Oxidative DNA Damage). In the course of preparing this material, we have continued the examination of critical steps in the processing and storage of DNA prior to analysis of 8-oxodG content.

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MATERIALS AND METHODS

Preparation of Calf Thymus DNA Treated with Ro 19-8022

Calf thymus DNA (Sigma) was dissolved at 200 μg/ml in 10 mM Tris-HCl, 2 mM Na_2EDTA, pH 8.5. Ro 19-8022 – a generous gift from Hoffman-La Roche (Basel, Switzerland) – dissolved in 67% ethanol at 750 μM, was added to 120 and 60 ml calf thymus DNA solution to final concentrations of 5 and 20 μM respectively. A further 60 ml of DNA solution was not treated with Ro 19-8022.

All three solutions were irradiated on ice, as 10 ml aliquots in open 90 mm plastic Petri dishes, for 5 min at 33 cm from a 1000 W tungsten halogen lamp. After recombining the solutions, 6 M NaCl was added to 1.4 M final concentration. The DNA was precipitated on ice with two volumes of ethanol at −20°C. The precipitate was washed 3× with 150 ml ice-cold 70% ethanol to remove any traces of salts or photosensitiser and then dried under a stream of nitrogen at room temperature for 5 min. The DNA was dissolved in 48 ml (0 and 20 μM Ro 19-8022-treated) or 96 ml (5 μM Ro 19-8022-treated) of HPLC-grade water (Rathburn Chemicals, Walkerburn, UK) by rolling gently overnight at 4°C and then incubating in a 37°C water bath.

DNA concentrations were determined by measuring absorbance at 260 nm and volumes containing approximately 120 μg were aliquoted into 2 ml Eppendorf tubes. The open tubes were covered in Nescofilm which was then pierced with a needle. The aliquots were snap-frozen in liquid nitrogen, freeze-dried overnight (in an Edwards Modulyo freeze-drier, model EF4, chamber volume 6.5 l) and then stored at room temperature until analysis. Some freeze-dried aliquots were stored under nitrogen at room temperature. When samples were to be hydrolysed, they were dissolved for 45 min at 37°C in either 0.4 ml 10 mM Tris-HCl, pH 7.3 or 0.6 ml 40 mM Tris-HCl, pH 8.5 for 2- and 4-enzyme hydrolysis respectively.

Similar samples (i.e. calf thymus DNA solution treated with different doses of Ro 19-8022) were prepared without freeze-drying in order to check for possible artefactual oxidation during lyophilisation. In this case, the N_2-dried DNA precipitate from 2 ml calf thymus DNA solution, after treatment, was dissolved in 0.6 ml 40 mM Tris-HCl buffer, pH 8.5, by gently rolling overnight at 4°C then at 37°C for 2 h. The solution was stored under nitrogen at −80°C.

4-Enzyme Hydrolysis of DNA

Calf thymus DNA (in 40 mM Tris-HCl, pH 8.5, 150 μg/ml) was incubated with DNase I (from bovine pancreas), phosphodiesterases I and II (from *C. durissus* and calf spleen respectively), and alkaline phosphatase (from calf intestine), in the presence of $MgCl_2$ for 2 h at 37°C. (All four enzymes were obtained from Boehringer Mannheim, Lewes, UK.) The method was based on that of Richter *et al.*[8] except that one-tenth of the recommended amount of phosphodiesterase I was found to be just as effective.

2-Enzyme Hydrolysis of DNA

DNA dissolved in 10 mM Tris-HCl, pH 7.3 was diluted to approximately 0.3 mg/ml with the buffer. To 0.4 ml of this was added 10 μl 1 M sodium acetate, 45 mM $ZnCl_2$, pH 4.8, followed by 12 μl P1 nuclease (from *P. citrinum*, supplied by Calbiochem, Nottingham, UK) (1100 U/ml in 25 mM sodium acetate, 1 mM $ZnCl_2$, pH 4.8) and the DNA was incubated for 1 h at 37°C. Forty μl 1.5 M Tris-HCl, pH 8.0 was added and the nucleotides were incubated with 10 μl alkaline phosphatase (750 U/ml in 100 mM Tris-HCl, pH 8.0) for 30 min at 37°C.

The hydrolysate from each of the above methods was filtered through a 0.2 μm syringe filter before injection into the HPLC system.

HPLC Analysis

The DNA hydrolysate was applied to an Apex ODS 3 μm C18 (150 × 4.6 mm) column (Capital

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Analytical, London, UK) with a 20 mm pellicular LC18 guard column (Supelco, Poole, UK). The mobile phase was 50 mM potassium phosphate buffer pH 5.5 containing 8% methanol (HPLC grade, from Rathburn Chemicals, Walkerburn, UK) and the flow rate was 0.5 ml/min. A Coulochem II electrochemical detector (ESA) was used with an ESA 5021 conditioning cell and 8-oxodG was detected at 400 mV on the 5011 analytical cell. Deoxyguanosine (dG) was detected by UV at 254 nm.

RESULTS AND DISCUSSION

Identity of the Peak of 8-oxodG

HPLC does not give a definitive identification of the individual components of a mixture. However, with electrochemical detection it is possible to construct a 'voltammogram', from measurements of electrochemical signal over a range of voltages, which is distinctive for different components. Voltammograms for standard 8-oxodG, and for the putative 8-oxodG peak from HPLC of hydrolysed calf thymus DNA, with base oxidation induced by Ro 19-8022 and light, are shown in Figure 1. The patterns are identical, strongly suggesting that the peak in calf thymus DNA is solely due to 8-oxodG, but this evidence is not conclusive.

Loss of Sample during Preparation

The preparation of a standard 8-oxodG solution for distribution to participants in ESCODD (phase 2) was carried out by another laboratory, who reported that partial loss of standard had occurred, probably during or after freeze-drying (see Ref. [9]). It seems likely that in some cases the dried material detached from the tube and adhered to the film covering the tube. We also noticed, in preparing calf thymus DNA samples, that a few tubes lacked any visible DNA pellet, but did not find evidence of partial loss. We measured

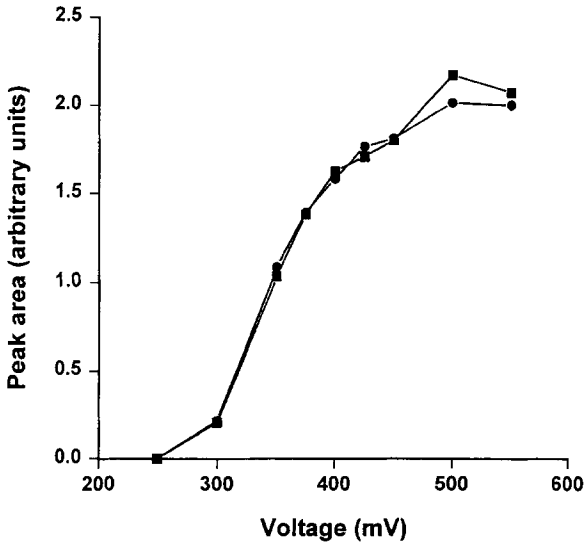

FIGURE 1 Voltammograms of standard 8-oxodG in a mixture with dG (■), and the putative 8-oxodG peak from calf thymus DNA hydrolysate (●). Calf thymus DNA was treated with Ro 19-8022 and visible light (sample B/D; see legend to Figure 3. The amount of standard 8-oxodG, and its ratio to dG, were arranged to be similar to those expected in the DNA; results were then normalised to a concentration of dG of 100 μM.

the concentration of dG in the (4-enzyme) hydrolysate from all samples of calf thymus DNA analysed in Figure 2, as an indicator of the quantity of DNA originally present. CVs for these determinations – on DNA treated with 0, 5 and 20 μM Ro 19-8022 – were 7.6%, 12.3% and 9.9% respectively. However, in the case of analysis of DNA samples, as opposed to standard, the concentration of 8-oxodG is expressed relative to dG concentration measured by UV absorbance, so loss of sample material does not affect the final result.

Effect of Freeze-Drying

In addition to a physical loss of material during the freeze-drying step, oxidation of guanine may occur, as has been reported previously.[6] Overnight freeze-drying of our calf thymus DNA samples had a substantial effect, increasing the amount of 8-oxodG by up to about 2-fold (Figure 3).

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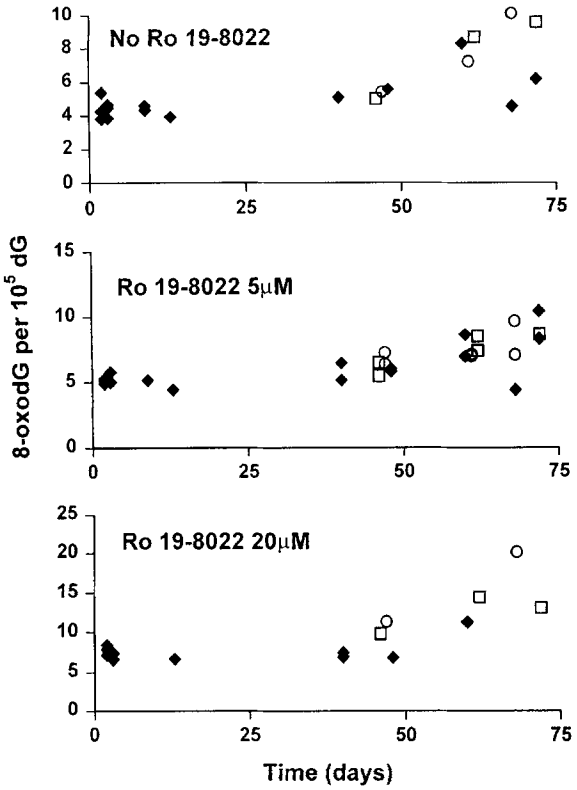

FIGURE 2 Yield of 8-oxodG from DNA treated with different concentrations of Ro 19-8022 and visible light (5 min). Samples were stored for the time shown before hydrolysis. (◆) samples stored in air and hydrolysed by the 4-enzyme method; (□) samples (4-enzyme hydrolysis) stored in nitrogen; (○) samples hydrolysed by the 2-enzyme method.

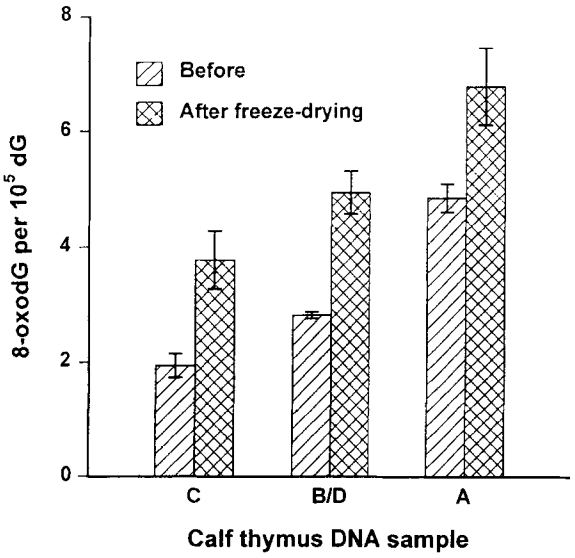

FIGURE 3 Effect of overnight freeze-drying on yield of 8-oxodG. Sample C is of untreated DNA; B/D is from the same batch of DNA, but treated with 5 μM Ro 19-8022 and light to induce a low level of 8-oxodG; A (treated with 20 μM Ro 19-8022) contains a higher level of damage. Bars indicate SD.

Comparison of Methods of Enzymic Hydrolysis

There are two alternative methods for hydrolysing DNA to doxyribonucleosides prior to HPLC analysis; one employing two enzymes (most commonly P1 nuclease/alkaline phosphatase), the other four. The 4-enzyme method is routinely used in our laboratory, and we run daily standard curves of dG and 8-oxodG dissolved in HPLC-grade water. We have confirmed that the peak area of a standard amount of 8-oxodG (or dG) is identical, whether it is dissolved in HPLC-grade water or dissolved in the Tris-buffer used for the hydrolysis and incubated with enzymes and Mg^{++}. Therefore, we can safely estimate concentrations of 8-oxodG after 4-enzyme hydrolysis by comparison with the standard curve determined with water as solvent. However, when dG and 8-oxodG are made up in the 2-enzyme buffer and incubated with the enzymes and Zn^{++}, the peak area for dG is the same as when dissolved in water, whereas the peak area of 8-oxodG shows an increase of $14 \pm 0.1\%$ over that of 8-oxodG in water (mean of nine separate measurements) – presumably a consequence of the difference in ionic strength/pH. When comparing the yields of 8-oxodG from 2-enzyme and 4-enzyme hydrolysis, the appropriate adjustment must be made. Allowing for this, when the 2-enzyme and 4-enzyme methods were applied to samples of the same untreated calf thymus DNA, yields of 8-oxodG were not significantly different (results not shown).

These findings are relevant to the comparison of 2-enzyme and 4-enzyme methods described in Ref. [9], in which we (as laboratory number 9) reported rather higher levels of 8-oxodG with the 2-enzyme method, after applying the 14% correction to the standard curve (compare lines 9a and

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9b in Figure 3(b) of that paper). There are several possible explanations:

(a) The reported 2-enzyme analysis was carried out on day 47, towards the end of the 55-day period allowed by ESCODD for these assays to be carried out. It is possible that oxidation occurred towards the end of this period (but see below).
(b) The 4-enzyme method may not be detecting all the 8-oxodG present in DNA oxidised by Ro 19-8022 and light – especially at higher levels of damage.
(c) For the 4-enzyme hydrolysis method, it has been shown[6] that the proportion of 8-oxodG present in dG does not increase when the dG is incubated with DNA, enzymes and Mg^{++}; nor does it increase during subsequent storage for several hours at 4°C. The possibility of additional oxidation occurring during the 2-enzyme hydrolysis has not been examined, but the similar results obtained with two or four enzymes on control untreated DNA argues against it.
(d) Results reported in the ESCODD study (including our trial of the 2-enzyme hydrolysis) are based on single samples for each Ro 19-8022 concentration – in contrast to a very large number treated with four enzymes; it is therefore always possible that an anomalous result was obtained by chance.

Stability of DNA during Storage

The 55-day period allowed for analysis was chosen arbitrarily. At intervals throughout this period, and beyond, we assayed 8-oxodG in freeze-dried samples of DNA stored at room temperature in air, or in some cases in nitrogen. Figure 2 shows that the level of 8-oxodG detected, in untreated or Ro 19-8022-treated DNA, is remarkably constant up to about day 50, but later results are more erratic, as additional oxidation of guanine seems to occur. Storage under nitrogen does not protect against oxidation.

Treatment of DNA with Ro 19-8022 and Light: Dose Response

Data obtained from the DNA samples stored in air, up to day 55, were pooled to produce the Ro 19-8022 dose response curve shown in Figure 4. The few runs involving 2-enzyme hydrolysis were excluded. This curve is shown in the figures in Ref. [9] in which methods are compared for their ability to detect these relatively small increases in oxidative damage in DNA. While we are not claiming that the values for 8-oxodG are absolute (since, after all, they depend on reference to a standard curve), the linearity of the dose response and the low standard deviation indicate that this line is a reasonable reference line against which the ability of other methods to detect the linear dose response can fairly be judged.

Conclusions

Clearly, care must be taken at various stages in the preparation of samples of DNA for analysis of 8-oxodG. The partial loss of standard 8-oxodG, apparently a problem experienced by others, can be avoided. Freeze-drying over a period of hours

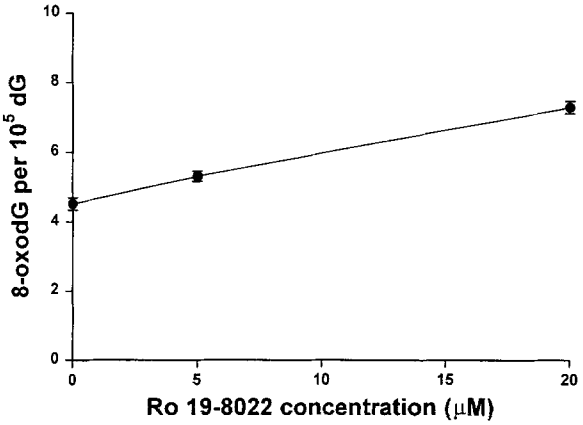

FIGURE 4 Dose response for formation of 8-oxodG by Ro 19-8022 with visible light. Only the data obtained from 4-enzyme digestion, after storage in air, within the 55-day measurement period set for analysis of DNA were used. Bars indicate SD.

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is confirmed as a serious cause of oxidation of guanine in the sample; it should be avoided if possible or kept to a minimum time. In the comparison of 2-enzyme and 4-enzyme hydrolysis procedures, the observed difference in values for 8-oxodG concentration is small compared with differences between ostensibly identical techniques used in different laboratories, and there is no reason to suppose that 2-enzyme hydrolysis is better than 4-enzyme hydrolysis or vice versa. The amount of 8-oxodG in freeze-dried DNA samples does not increase during storage at room temperature for at least six weeks. Treatment with Ro 19-8022 and visible light provides a very convenient method for introducing oxidative damage.

Acknowledgements

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