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Original Contribution

MEASUREMENT OF DNA OXIDATION IN HUMAN CELLS BY CHROMATOGRAPHIC AND ENZYMIC METHODS

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Abstract—The European Standards Committee on Oxidative DNA Damage (ESCODD) was set up to resolve problems in the measurement of DNA oxidation that have resulted in varying estimates of the extent of this damage in humans. HeLa cells, sent to members for analysis, were either untreated, or treated with light in the presence of a photosensitizer to induce different amounts of 8-oxo-7,8-dihydroguanine (8-oxoGua) in DNA. Laboratories employing HPLC with electrochemical detection were able to measure the induced damage with similar efficiency; dose response gradients for seven of the eight sets of results were almost identical. GC-MS and HPLC-MS/MS, employed in three laboratories, did not convincingly detect the dose response. An alternative approach to measuring base oxidation employs the enzyme formamidopyrimidine DNA N-glycosylase (FPG) to convert 8-oxoGua to strand breaks, which are then measured by alkaline unwinding, alkaline elution, or the comet assay. Ten laboratories used this approach; five were able to detect the dose response in cells treated with photosensitizer plus light (at lower doses than for chromatographic methods, because the enzymic methods are more sensitive and less prone to spurious oxidation). Median values for 8-oxoGua (or FPG-sensitive sites) in untreated cells were 4.01 per 10^6 guanines for chromatographic methods, and 0.53 per 10^6 guanines for techniques based on FPG. © 2003 Elsevier Inc.

Keywords—DNA oxidation, Method validation, 8-Oxo-7,8-dihydroguanine, Free radicals

INTRODUCTION

The European Standards Committee on Oxidative DNA Damage (ESCODD) was set up to examine critically the different approaches to measuring base oxidation in DNA, in particular 8-oxo-7,8-dihydroguanine (8-oxoGua). Such an appraisal is necessary because of the very wide variation in estimates of the basal level of this damage; in human white blood cells, for example, over two orders of magnitude separate the highest from the lowest estimates [1]. It is now recognized that guanine in DNA is readily oxidized during preparation of samples for analysis. GC-MS is particularly prone to this artifact, when the derivatization step is carried out in the presence of oxygen at high temperature. Protocols have been revised in order to limit this problem, and it is now standard practice to include antioxidants and metal chelators during sample preparation for all chromatographic procedures (HPLC with electrochemical detection [HPLC-ECD] and HPLC-MS/MS in addition to GC-MS) [2–8].

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An alternative approach depends on the use of the bacterial DNA repair endonuclease, formamidopyrimidine DNA N-glycosylase (FPG), which creates single-strand breaks in the DNA at the sites of altered purines, including 8-oxoGua. These breaks are then measured using alkaline unwinding, alkaline elution, or the comet assay (single cell alkaline gel electrophoresis). These enzymic methods are intrinsically highly sensitive, as they are based on the ability of small numbers of DNA breaks to disrupt the physicochemical behavior of large DNA molecules. As a corollary, they detect breaks accurately only over a limited range. In the alkaline unwinding technique, the extent of denaturation over a fixed period is increased by the presence of DNA breaks, and the fractions of single- and double-stranded DNA are measured by hydroxyapatite chromatography [9]. Under alkaline elution, single-stranded DNA moves through a filter at a rate depending on the size of DNA fragments (i.e., the frequency of breaks) [10]. The comet assay measures DNA breaks by their ability to relax DNA supercoiling and allow DNA to migrate under electrophoresis, forming a comet-like image in which the % DNA in the tail reflects break frequency [11]. The DNA breakage assays are calibrated indirectly, by comparison with cells treated with ionizing radiation to induce breaks at a known dose rate (e.g., see [11]) These enzymic methods have produced the lowest estimates of background damage levels in human white blood cells [1].

ESCODD is a consortium of 27 member laboratories, supported as a Concerted Action by the European Commission. Samples of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), oligonucleotides containing defined amounts of 8-oxoGua, calf thymus DNA either as purchased or with additional oxidative damage, pig liver, and HeLa cells have been sent to all members for analysis, and the results comparing the various techniques have been reported [12–14].

In this round of ESCODD, HeLa cells treated with a photosensitizer plus visible light to induce different amounts of 8-oxoGua were distributed to the member laboratories, together with untreated cells. The photosensitizer used was Ro 19-8022, which acts on DNA predominantly by the type II mechanism, with subsequent formation of $^1O_2$ as the reactive species [15]. The reaction of $^1O_2$ with isolated and cellular DNA gives rise primarily to 8-oxoGua to the exclusion of other FPG substrates such as the formamidopyrimidine (fapy) derivatives of guanine and adenine [16,17]. Therefore, the bulk of the FPG-sensitive sites generated by photoexcited Ro 19-8022 are likely to be 8-oxoGua lesions. In control cells, however, sites recognized by FPG may include fapyGua, in addition to 8-oxoGua. (FapyAde is likely to be present at a much lower level.)

Our aim in this trial was to test the ability of different techniques to detect the dose response of induced damage, and also to establish how close the techniques now are to agreement over the basal DNA oxidation level.

MATERIALS AND METHODS

Preparation of untreated HeLa cells (sample C)

HeLa (human transformed epithelial) cells were grown to confluence in 14 80 ml flasks (Nalge Nunclon International, Roskilde, Denmark) in Glasgow-modified Eagle's Minimal Essential Medium (GMEM) with 5% fetal calf serum, 5% calf serum, supplemented with glutamine and nonessential amino acids. The cells were harvested using trypsin/versene, and the cells from each flask were transferred to a polystyrene roller bottle (850 cm^2 surface area: Corning Inc., Corning, NY, USA) in 75–100 ml medium in a 6% CO_2 atmosphere. The bottles were placed on a roller at 37°C and after 7 d the medium was changed. Two days later the cells were harvested with trypsin/versene from two bottles at a time. Medium was added to stop the reaction; the cells were transferred to sterile glass bottles and stored in a 37°C incubator until all the cells had been harvested. The cell suspensions were combined, gently inverted to mix, the total volume noted, and the cell density determined using a hemocytometer. The cells were collected by centrifuging 50 ml aliquots of the suspension at $700 \times g$, 7 min at 20°C. The cell pellet was resuspended gently in 1 ml freezing medium (GMEM containing 20% fetal calf serum, 10% DMSO). Additional freezing medium was added to a cell density of 5×10^6/ml and the cell suspension was divided into aliquots of 1.5×10^6 cells (300 μl) for the comet assay, 6×10^6 cells (1.2 ml) cells for alkaline unwinding, 10×10^6 cells (2 ml) cells for alkaline elution and 52×10^6 cells (10.4 ml) for chromatographic methods. The cells were cooled slowly to −80°C and stored at this temperature.

Treatment of HeLa cells with Ro 19-8022 and light for chromatographic analysis of DNA

HeLa cells were treated with visible light in the presence of the photosensitizer Ro 19-8022 (Hoffmann-La Roche, Basel, Switzerland) at a concentration of 0, 2, or 5 μM (for samples B, A, and D respectively) in PBS-G (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% glucose pH 7.4). A new supply of Ro 19-8022 was used; aliquots of a stock solution at 1 mM in 67% ethanol were stored at −20°C in the dark. The working solutions were prepared immediately before use by dilution of the stock solution with PBS-G and kept in a foil-wrapped glass bottle on ice during the experiment. The experiment was carried

out in a darkened room and all solutions were kept on ice.

For each dose of Ro 19-8022, cells were grown to confluence in 10 80 ml flasks in GMEM as above, harvested by trypsinization and the resultant cell suspensions combined. The cells were divided equally between 40 140 mm dishes (Nalge Nunclon International) in 18 ml medium. The dishes for the three doses of photosensitizer were set up at different times. After 3 d, 15 ml medium were added to each dish of cells. By day 5 the cells were confluent. Taking six dishes at a time, the cells were rinsed twice with 20 ml PBS, and 18 ml of Ro 19-8022 in PBS-G (or PBS-G alone) was added to each dish. The dishes were irradiated on aluminium trays on ice at 33 cm from a 1000 W halogen lamp (Halotone Plusline; Phillips, Eindhoven, The Netherlands) for 5 min. Two lamps were used (one lamp for three dishes), and a screen between the lamps prevented cross-irradiation. The cells were washed twice with 20 ml PBS and then gently scraped into 15 ml PBS. The cells were combined in a foil-covered glass bottle and were stored on ice until all the cells had been treated (this took approximately 2 h). They were then dispersed by gentle inversion of the bottle, aliquoted into 13 50 ml tubes and collected by centrifugation at 700 $\times$ *g* for 7 min at 4°C. The cells in each tube were dispersed gently in 1 ml freezing medium, 11 ml more freezing medium was added and the suspension was divided equally into two 15 ml centrifuge tubes. The aliquots were cooled to −80°C and stored at −80°C. One tube of samples A, B, D, and C (untreated HeLa; 10.4 ml: see above) was sent to each participant for each chromatographic method used; this was sufficient for two DNA preparations, each to be analyzed in triplicate.

Treatment of HeLa cells with Ro 19-8022 and light for FPG methods

FPG methods are more sensitive than the chromatographic methods, and have a limited detection range. The cells prepared for analysis with FPG were therefore treated with lower doses of photosensitizer, i.e. 0, 0.2, and 0.4 μM Ro 19-8022 in PBS-G (for samples F, G, and H, respectively), as well as a shorter time of irradiation (2 min).

Four 140 mm dishes of HeLa cells were set up for each dose of Ro 19-8022. The cells were grown to confluence and treated with photosensitizer and light as above. After scraping very gently into 15 ml PBS, the cells from two dishes were combined and centrifuged in two 50 ml tubes at 700 $\times$ *g* for 7 min at 4°C. The cells in each tube were dispersed in a total of 8.8 ml freezing medium as above. They were then combined and inverted gently to mix. The cell suspension was aliquoted into cryovials as follows: 0.5 ml for the comet assay, 3 $\times$ 1.1 ml for alkaline elution and 2 $\times$ 1ml for alkaline unwinding. The samples were cooled slowly to −80°C and transferred to liquid nitrogen for storage until distribution. One aliquot of samples F, G, H, and C was sent to each partner using an enzymic method, to be analyzed in triplicate, and mean background breaks subtracted to calculate net FPG-sensitive sites.

Distribution of samples

The samples were sent out on dry ice (one tube of each sample per method) in expanded polystyrene boxes. The transfer to dry ice was done very quickly to prevent any thawing of the samples. All samples that remained for quality control were kept on dry ice for 24 h to mimic postage conditions, and then stored at −80°C until use. Laboratories were asked to store the samples they received at −80°C.

DNA preparation, hydrolysis, and analysis; chromatographic methods

Various methods for isolating DNA were used by different laboratories. Details are given in Table 1. (Note that numbers identifying laboratories in tables and figures do not correspond to the order of ESCODD members listed as authors.) Different buffers, with and without the antioxidant desferal, were employed. Incubations with SDS, Rnase, and protease were at different temperatures and for various times. Some laboratories used chloroform/3-methyl-1-butanol to partition DNA. Precipitation of DNA with ethanol or 2-propanol was generally done in the presence of a salt, and different methods were used to dry and subsequently dissolve the DNA.

Details of hydrolysis conditions are given in Table 2. For HPLC-ECD, DNA was hydrolyzed enzymically with P1 nuclease and alkaline phosphatase, or with phosphodiesterase II at acidic pH followed by phosphodiesterase I and alkaline phosphatase (laboratory 3). Separation of DNA hydrolysate, 8-oxodGuo, and dGuo standards on a C18 column was followed by coulometric electrochemical detection of 8-oxodGuo, and UV detection of dGuo. For analysis with GC-MS, samples were hydrolyzed to bases with 60% formic acid at 130°C for 30 min. Bases were derivatized with bis(trimethylsilyl)trifluoroacetamide at room temperature for up to 2 h, under argon or nitrogen. One laboratory used HPLC followed by mass spectrometry detection. Nucleosides were separated on a C18 column and injected into a triple quadrupole mass spectrometer for identification and quantitative analysis using the so-called multiple reaction-monitoring mode [18].

Table 1. Variations in Methods Used for Preparing DNA for Chromatographic Analysis

Partici-pant	Method	Purging of solutions	Antioxidant/ chelator in extraction buffer	Detergent to lyse cells	SDS treatment*	RNase treatment*	Protein digestion*	Chloroform/ 3-methyl-1-butanol extraction	Precipitation of remaining protein with salt	DNA precipitated with:	Salt added	Removal of excess alcohol
1	HPLC-ECD	No	1 mM DF	Triton X-100	37°C 10 min	37°C 30 min	37°C 30 min	Yes	Yes	Ethanol	NaCl	Stream of N_2
3	HPLC-ECD	No	0.1 mM DF	Triton X-100	RT, 5 min	50°C 15 min	37°C 1 h	No	No	2-Propanol	NaI	Letft in air
3	HPLC-MS/MS	No	0.1 mM DF	Triton X-100	RT, 5 min	50°C 15 min	37°C 1 h	No	No	2-Propanol	NaI	Left in air
5	HPLC-ECD	No	5 mM DF	Triton X-100	37°C 10 min	37°C 30 min	37°C 30 min	Yes	Yes	Ethanol	NaCl	Stream of Ar
6	HPLC-ECD	No	5 mM DF	Triton X-100	37°C 10 min	37°C 30 min	37°C 30 min	Yes	Yes	Ethanol	NaCl	Stream of N_2
14	HPLC-ECD	No	0.1 mM DF		50°C 15 min	50°C 15 min	37°C 1 h	No		2-Propanol	NaI	DNA fibres dragged out from alcohol
15	HPLC-ECD	No; Chelex treated	1 mM DF	Tween-20	None	None	None	Yes	Yes, if any	2-Propanol	GTC	Pipette
16	HPLC-ECD	No	1 mM DF, 3 mM reduced GSH, 4 mM his, 1 mM DTT	Triton X-100	37°C 30 min	37°C 30 min	37°C 1 h	Yes	No	Ethanol	None	Stream of N_2
16	GC-MS	Yes; nitrogen	1 mM DF, 3 mM reduced GSH, 4 mM his, 1 mM DTT	Triton X-100	37°C 30 min	37°C 30 min	37°C 1 h	Yes	No	Ethanol	None	Stream of N_2
17	HPLC-ECD	No	0.1 mM DF	Triton X-100	37°C 10 min	37°C 1 h	37°C 2 h	No	Yes	Ethanol	Ammonium acetate	Paper tissue
22	GC-MS	No	0.1 mM DF	Triton X-100	None	50°C 15 min	37°C 1 h	No	No	2-Propanol	None	Vacuum centrifugation

* Temperature and time. DF = desferal (deferoxamine mesylate); DTT = dithiothreitol; GSH = glutathione; GTC = guanidine thiocyanate; his = histidine; RT = room temperature.

Table 2. Variations in Methods Used for Hydrolysis and Subsequent Processing of DNA

Participant	Method	Method for dissolving DNA	Treatment of hydrolysate	Hydrolysis	Use of labeled 8-oxodG (GC/MS)
1	HPLC-ECD	Tris buffer, 2 h at 37°C, overnight at 4°C	None	P1 nuclease/alkaline phosphatase	
3	HPLC-ECD	0.1 mM DF, 5 min at RT		Phosphodiesterase II/phosphodiesterase I + alkaline phosphatase	
3	HPLC-MS/MS	0.1 mM DF, 5 min at RT		Phosphodiesterase II/phosphodiesterase I + alkaline phosphatase	
5	HPLC-ECD	Tris buffer, 2 h at 37°C, overnight at 4°C	None	P1 nuclease/alkaline phosphatase	
6	HPLC-ECD	Tris buffer, overnight at 4°C	None	P1 nuclease/alkaline phosphatase	
14	HPLC-ECD	Water, vigorous agitation	Chloroform extraction	P1 nuclease/alkaline phosphatase	
15	HPLC-ECD	Water with TEMPO/catalase	Chloroform extraction	P1 nuclease/alkaline phosphatase	
16	HPLC-ECD	Acetate buffer with $ZnCl_2$, 5 min at RT	Filtration (5 kDa cut-off)	P1 nuclease/alkaline phosphatase	
16	GC-MS	Deionized water, 1 h at RT	Hydrolysate lyophilized for 18 h, then derivitization with BSTFA and acetonitrile under nitrogen for 2 h at 23°C. No additives to prevent oxidation.	Vacuum centrifugation for 1 h prior to hydrolysis with 60% formic acid at 130°C for 30 min	(M + 4) 8-oxoGua
17	HPLC-ECD	Tris buffer, 5 min at RT	None	P1 nuclease/alkaline phosphatase	
22	GC-MS	0.1 mM DF	Vacuum centrifugation for 2 h, then derivatisation with BSTFA + 1% TMCS under argon for 30 min at 23°C. No additives to prevent oxidation	Vacuum centrifugation for 1 h prior to hydrolysis with 60% formic acid at 130°C for 30 min	(M + 5) 8-oxoGua

RT = room temperature; BSTFA = bis(trimethylsilyl)trifluoroacetamide; DF = desferal (deferoxamine mesylate); DTT = dithiothreitol; GSH = glutathione; his = histidine; TEMPO = 2,2,6,6-tetramethylpiperidine-*N*-oxyl; TMCS = trimethylchlorosilane.

Enzymic methods

The three methods used to measure DNA breaks have been described in the Introduction. Eight laboratories used the comet assay, one used alkaline elution, and one alkaline unwinding. All the laboratories employing the comet assay used a crude extract of FPG prepared in the Rowett Research Institute. The enzyme used in the alkaline unwinding and alkaline elution methods was obtained from Serge Boiteux (CNRS-CEA/Fontenay aux Roses, France).

Quality control—HPLC analysis

DNA was isolated from tubes of A, B, C, and D before distribution and then at regular intervals over the time allowed for analysis. Two separate preparations were performed on each tube of cells yielding 150–250 μg DNA. The DNA samples were prepared and analyzed by HPLC-ECD as described [19] except that the two-enzyme method of DNA hydrolysis (with P1 nuclease and alkaline phosphatase) was employed.

Quality control—FPG methods

Prior to distribution, and at two-weekly intervals over the period allowed for analysis, aliquots of samples C, F, G, and H were analyzed by the comet assay. The cells were thawed quickly at 37°C, centrifuged at 200 $\times$ *g* for 3 min at 4°C and the pellet was dispersed in 400 μl RPMI, 10% heat-inactivated fetal calf serum. Fifteen microliter aliquots (for each gel) were added to 1 ml PBS and centrifuged again. The cell pellets were resuspended in low melting point agarose (GibcoBRL, Paisley, UK) and applied to a microscope slide ready for the comet assay [11]. Standard lymphocytes, from a pool that was aliquoted and stored at −80°C, were run as a control with each comet assay. These showed expected levels of FPG-sensitive sites and background damage on each day of analysis.

Measurement of cellular integrity

The percentage of cells permeable to trypan blue was measured on thawed aliquots of samples C, F, G, and H. An equal volume of 0.14% trypan blue in PBS was added to the aliquot; cells taking up trypan blue and cells negative for the dye were counted using a hemocytometer.

To test the integrity of cells after harvesting (either by scraping or by trypsinization) and freezing, six 60 mm dishes containing equal numbers of HeLa cells in GMEM were incubated for 2 d, by which time the cells were subconfluent. Cells from three dishes were harvested with 1 ml versene/trypsin; 3 ml medium was added to stop the reaction and the cells were pelleted at 700 $\times$ *g* for 7 min at room temperature. The cell pellet was dispersed in freezing medium at a density of 5 $\times$ 10^6/ml (see above) and frozen slowly to −80°C. Cells from the other three dishes were harvested by gentle scraping with a Cell Scraper (Corning Inc.) into 4 ml fresh medium. The cell suspensions were centrifuged, and the cells were made up in freezing medium and frozen as above. After 24 h, the six samples of cells were thawed and prepared as for the comet assay except that they were re-suspended in PBS rather than LMP agarose. The number of cells was counted on a hemocytometer.

RESULTS

Quality control

Because the range of detection with the enzymic methods is much lower than that of the chromatographic techniques, different samples containing induced 8-oxoGua were produced for these two approaches. Samples B, A, and D were treated with 0, 2, and 5 μM Ro 19-8022, respectively before irradiation with a halogen lamp for 5 min; these samples were for analysis by HPLC-ECD, GC-MS, and HPLC-MS/MS. Samples F, G, and H received 0, 0.2, and 0.4 μM Ro 19-8022 and 2 min of irradiation and were used in the enzymic procedures. Sample C (untreated HeLa cells) was common to both chromatographic and enzymic methods. Several times during the period of 5 weeks set for analysis, aliquots of each sample were analyzed by the distributing laboratory. Figure 1 shows the dose response curves obtained by HPLC-ECD with coulometric electrochemical detection (samples B, A, and D) and with the comet assay (samples F, G, and H). In each case the dose response was linear. Calibration of HPLC-ECD is by reference to standard 8-oxodGuo and dGuo solutions. Calibration of the comet assay is indirect, by comparison with the effect of ionizing radiation on cellular DNA, making use of information in the literature concerning the frequency of DNA, breakage by a certain dose of radiation [11].

Comets were analyzed by visual scoring. One hundred comets for each sample were divided into five classes according to the intensity of the comet tail; undamaged DNA with no comet tail, corresponds to class 0, and the highest level of damage, where very little DNA remains in the comet head, is classed as 4. Treatment with Ro 19-8022 from 0 to 0.4 μM resulted in a gradual shift of comets to higher classes, but with little increase in the number in class 4 (Fig. 2). Thus there is no indication that the assay is saturated by the doses we have used. Saturation would lead to underestimation of enzyme-sensitive sites.

Cellular integrity was estimated on the samples used for enzymic analysis. Trypan blue uptake is an indicator of disruption of cell membranes. Uptake of the dye was

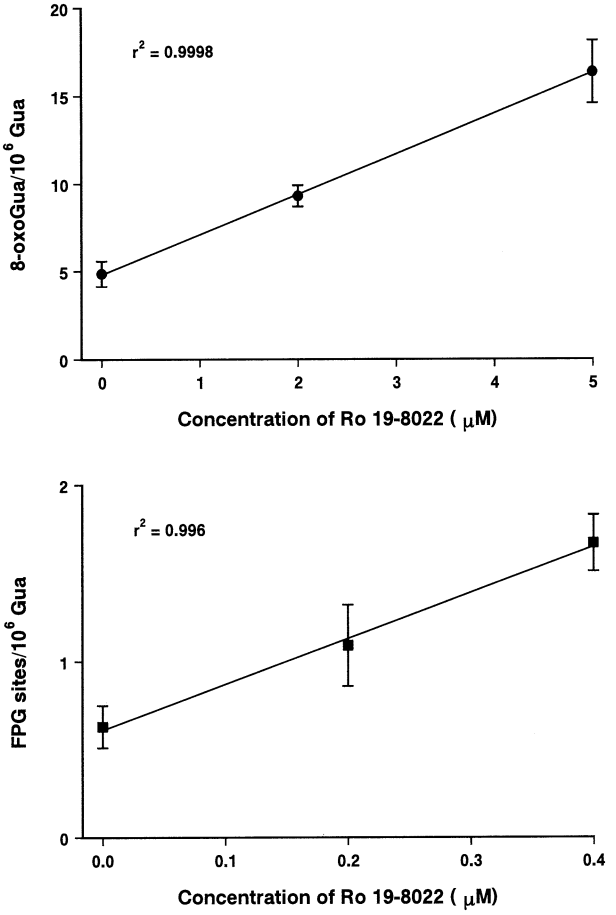

Fig. 1. Quality control. Samples of HeLa cells treated with Ro 19-8022 and light were analyzed several times during the period allowed for analysis; means and SD are shown. Top: HPLC-ECD; cells were irradiated for 5 min. Bottom: comet assay with FPG; cells were irradiated for 2 min. (Data obtained on one predetermined occasion during the quality control series were contributed to the datasets on which Figs. 3 and 4 are based.)

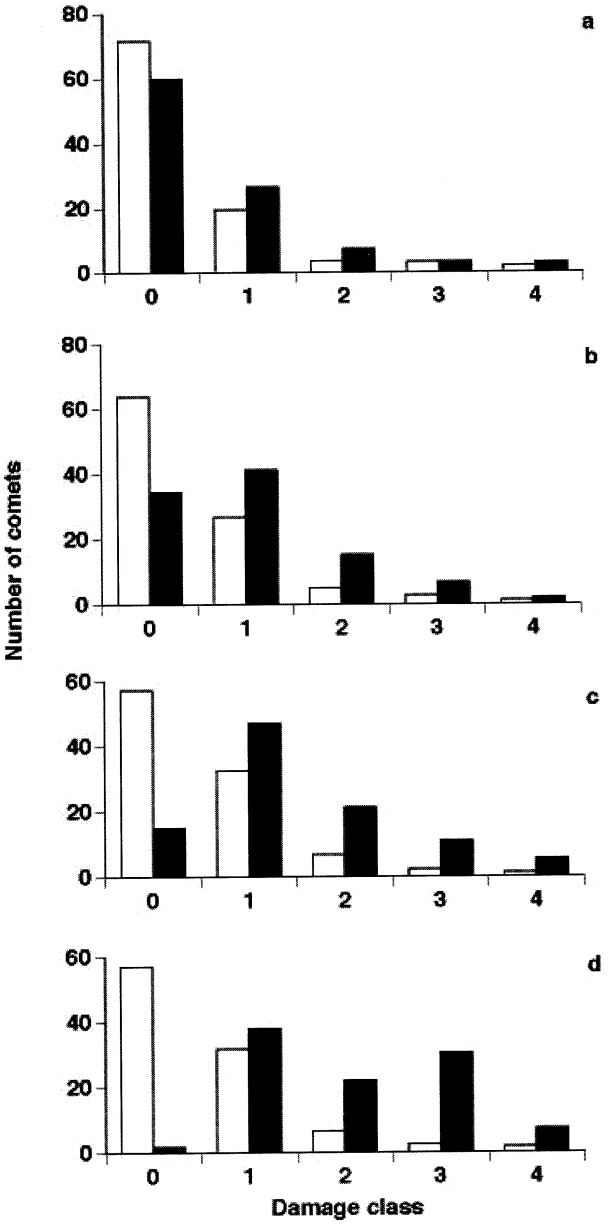

Fig. 2. Test of ability of comet assay to detect FPG-sensitive sites quantitatively over range of concentrations of Ro 19-8022. The distribution of HeLa comets (y-axis; total 100) between classes 0 to 4 (x-axis) is shown, with increasing oxidative damage. (For explanation of this classification, see Results.) From top to bottom: (a) sample C, untreated; (b) sample F, treated with light only; (c) sample G, treated with light and 0.2 μM Ro 19-8022; (d) sample H, treated with light and 0.4 μM Ro 19-8022. Open columns are from cells incubated with buffer alone; solid columns are from cells incubated with FPG.

much greater in samples F, G, and H, harvested by scraping (82, 84, and 81%, respectively) than in sample C, harvested with trypsin/versene (19%). Loss of heavily damaged cells during subsequent processing might distort the estimation of damage to DNA, if membrane damage is reflected in DNA damage. To test whether trypan blue uptake indicates cell fragility, aliquots of HeLa cells harvested by scraping and trypsin/versene were frozen and thawed as for the comet assay. Subsequent counting showed that there was a slight difference in cell numbers depending on whether they had been scraped or trypsinized, with 25% fewer cells recovered after scraping.

Detection of dose response: chromatographic methods

Eleven sets of results were returned: one using HPLC-MS/MS, two using GC-MS, and eight using HPLC-ECD. The medians calculated from all these results for samples A, B, and D are shown in Table 3, and indicate a reasonably linear dose response. Mean values within 25% of the median were reported by five, two, and six laboratories for samples A, B, and D, respectively. The individual results, with median values, are shown in Fig. 3. If we define ability to detect the dose response simply as an ability to detect the differences between B and A

Table 3. Median Values for 8-oxoGua (or FPG-Sensitive Sites) in Cell Samples Treated with Different Doses of Ro 19-8022 + Light and Analyzed by Chromatographic (B,A,D) or Enzymic (F,G,H) Methods

Sample	Ro 19-8022 concentration (μM) $\times$ time (min)	8-oxoGua (FPG sites) per 10^6 Gua
B	0	5.60
A	10	10.37
D	25	19.78
F	0	0.63
G	0.4	0.88
H	0.8	0.92

and between A and D as positive, then 8 of 11 were successful. However, the "successful" GC-MS result is a very shallow dose-response compared with the median, and the other GC-MS determinations, as well as the single HPLC-MS/MS result, were unsuccessful. Only one HPLC-ECD method did not detect the dose response. All the HPLC-ECD methods used coulometric detection.

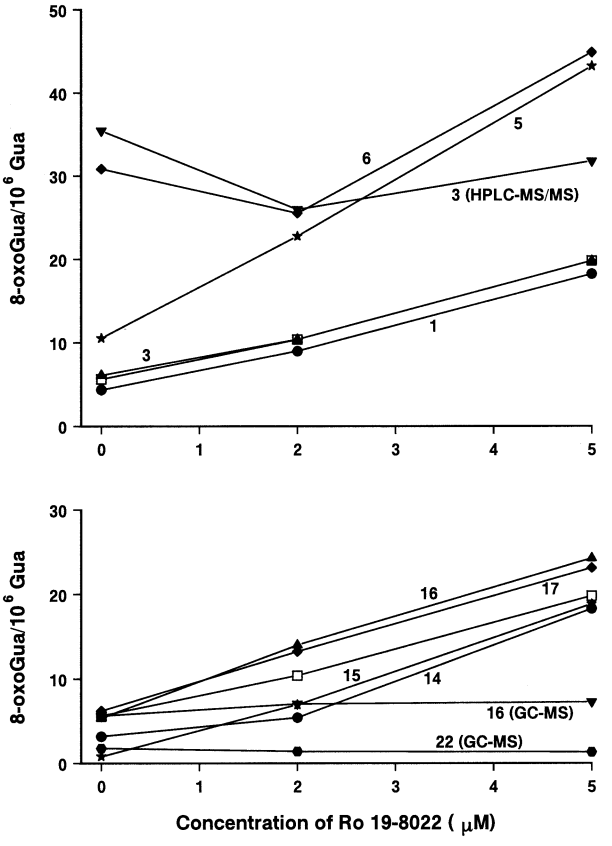

Fig. 3. Detection of dose response: chromatographic methods. HeLa cells were treated with different concentrations of Ro 19-8022 plus light, DNA isolated and hydrolyzed, and 8-oxoGua estimated by GC-MS or HPLC-MS/MS as indicated, otherwise by HPLC-ECD. Results (in most cases, means from two DNA preparations from a single sample, analyzed in triplicate) are displayed on two charts for convenience. Solid symbols represent individual laboratories, identified by numbers; median values are also shown (open squares).

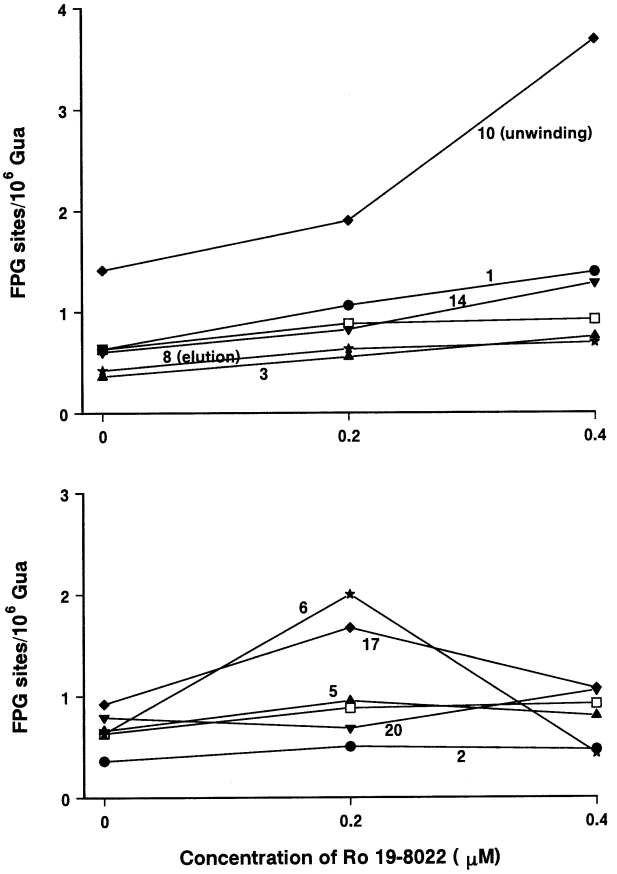

Fig. 4. Detection of dose response: enzymic methods. HeLa cells treated with different concentrations of Ro 19-8022 plus light were lysed and the DNA digested with FPG to reveal altered purines. These were then estimated as strand breaks by the comet assay except where otherwise indicated. Results (means of triplicate analyses from a single sample) shown on the upper chart are from laboratories demonstrating a dose response; those on the lower chart failed to detect the dose response. Solid symbols represent individual laboratories, identified by numbers; median values are also shown (open squares).

Detection of dose response: enzymic methods

The damage induced by Ro 19-8022 in the samples analyzed by enzymic methods was at a far lower level than in the samples used for chromatographic analysis. Ten sets of results were submitted (Fig. 4): one determination using alkaline unwinding, one by alkaline elution, and eight using the comet assay. The medians for samples F, G, and H are given in Table 3 and graphically represented in Fig. 4. Mean values within 25% of the median were reported by 5, 4, and 5 laboratories, respectively. While 9 out of 10 detected the increase from 0 to 0.2 μM Ro 19-8022, only five (including both alkaline unwinding and alkaline elution) detected the dose response over the complete range of doses to 0.4 μM.

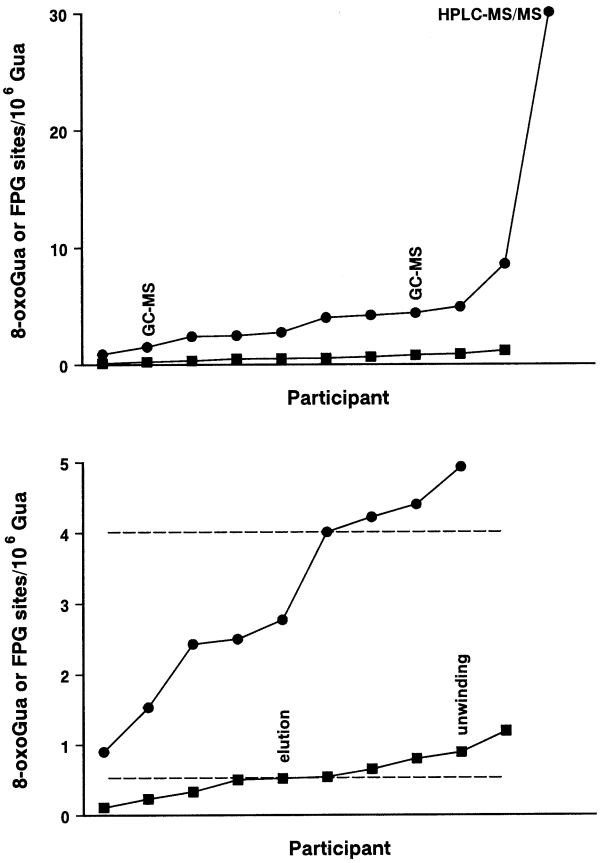

Fig. 5. Background level of 8-oxoGua or FPG sites, estimated by chromatographic (●) and enzymic (■) approaches. In the upper panel, all data are shown. In increasing order of 8-oxoGua determinations, the values from chromatographic assays (HPLC-ECD except where indicated otherwise) correspond to laboratories 15, 22, 5, 6, 14, 17, 16 (HPLC), 16 (GC-MS), 1, 3 (HPLC), and 3 (HPLC-MS/MS). Enzymic determinations correspond to laboratories 14, 1, 20, 3, 8, 17, 6, 5, 10, and 2. In the lower panel, the highest chromatographic values are omitted and the y-axis scale expanded to show the results of the enzymic methods in detail. Enzymic determinations other than by the comet assay are specified here. Horizontal lines represent median values for each approach.

Estimating the background level of 8-oxoGua

Sample C (untreated HeLa cells) was measured by both chromatographic and enzymic methods. The median value for HPLC-ECD, GC-MS, and HPLC-MS/MS is 4.01 8-oxoGua per 10^6 Gua, and the range is between 0.90 and 30.37, i.e., 34-fold. Excluding a single very high result obtained by HPLC-MS/MS, the range is about 10-fold. The enzymic methods produced a median of 0.53 8-oxoGua per 10^6 guanines, with a range from 0.11 to 1.19, i.e., 11-fold. Four of eleven chromatographic methods and 4 of 10 FPG-based methods gave mean values within 25% of the respective median. The values returned are shown in Fig. 5, in increasing order, and separated according to whether a chromatographic or enzymic method was used.

Table 4. Analysis of Dose Response Curves from Fig. 3

Method	Laboratory	Gradient	Intercept
HPLC-ECD	1	2.80	3.98
	3	2.77	5.61
	5	6.54	10.20
	6	3.07	26.52
	14	3.13	1.65
	15	3.63	0.36
	16	3.75	5.77
	17	3.37	6.30
GC-MS	16	0.31	5.91
	22	−0.08	1.67
HPLC-MS/MS	3	−0.53	32.27

Gradients of curves and intercepts on the y-axis were calculated by regression analysis. Units are 8-oxoGua/10^6 Gua for the intercept, and 8-oxoGua/10^6 Gua/μM Ro 19-8022 for the slope.

DISCUSSION

Measurement of background oxidative damage

Sample C, containing untreated HeLa cells, was used for estimation of the background level of 8-oxoGua in DNA. The median values of 4.01 and 0.53 8-oxoGua per 10^6 Gua for chromatographic and enzymic methods, respectively, are very similar to the results of an earlier round of ESCODD [14]. The median values in that exercise were 5.23 and 0.79 8-oxoGua per 10^6 Gua, respectively. In each trial, the medians of chromatographic and enzymic determinations differed by a factor of about 7. (The range of values in the earlier trial was far wider, especially for the chromatographic methods, and the improvement in the present trial can be attributed to nonparticipation of those reporting the highest values or, in the case of one laboratory, a markedly lower value obtained in the present round.)

Measurement of induced oxidative damage; chromatographic methods

To induce additional oxidative DNA damage, we incubated cells with different concentrations of the photosensitizer Ro 19-8022 and irradiated them with visible light. The ability to detect a dose response is a crucial test of the accuracy of an analytical method, and a prerequisite for use of the assay in biomonitoring. All but three of the chromatographic analyses shown in Fig. 3 succeeded in this, according to the simple criterion applied (detection of increases over the whole dose range). The slopes of the dose response curves, calculated by regression analysis, are shown in Table 4, together with the y-axis intercepts. The agreement over estimates of slope among laboratories using HPLC-ECD is impressive; a factor of only 2.4 separates the highest and the lowest, and seven of the eight values are within ± 15% of the median (3.25 8-oxoGua/10^6 Gua/μM Ro 19-8022). In contrast, mass spectrometric methods seem to be unable to detect a

significant slope. However, there is no consensus over the intercept, which ranges from 0.36 to 32.27 8-oxoGua per 10^6 Gua. The conclusion seems inescapable: HPLC-ECD (with coulometric detection) is capable of measuring induced 8-oxoGua in cellular DNA with great accuracy, but estimation of the background level of damage is still hampered by the occurrence of very variable amounts of oxidation during sample preparation.

Measurement of induced oxidative damage; enzymic methods

Compared with HPLC-ECD, the enzymic methods proved less capable of detecting the dose response; only half of the laboratories using this approach were successful. The levels of base oxidation for enzymic analysis were much lower, to match the higher sensitivity but limited detection range of these methods. In the case of the comet assay, the limited range results from the fact that once all of the DNA is in the comet tail, introducing more breaks into the DNA has no effect. That this saturation effect was not a problem in the present trial is illustrated by the comet distributions in Fig. 2. Comets resulting from incubation with buffer alone (reflecting strand breaks and alkali-labile sites) show little change over the range of Ro 19-8022 concentrations used, indicating that, as expected for an agent that generates predominantly $^1O_2$, few of these lesions are formed [16,17]. Comets from nucleoids incubated with FPG show a steady shift towards higher classes of damage with increasing concentration of Ro 19-8022, but even at the highest concentration, most comets are in class 3 or below. In a calibration experiment with X-irradiation to induce DNA breaks, the relationship between comet class and DNA breaks is linear up to class 3 [20]. There is, therefore, no danger of underestimating oxidized bases when net FPG-sensitive sites are calculated by deducting the comet score with buffer alone from the score with FPG. The results of the quality control experiment (Fig. 1, bottom) confirm that linearity can be achieved over this range of Ro 19-8022 concentrations, and a similar dose response over a slightly wider range of Ro 19-8022 concentrations was recently reported [21].

The sensitivity of these methods would be severely reduced if additional strand breaks were introduced during processing of the samples. It is commonly assumed that, if cells are "nonviable" by the simple trypan blue exclusion test, they cannot give reliable results in the DNA breakage assays. In fact, trypan blue exclusion is only a test for integrity of the cell membrane and does not predict whether cells will survive or not. Scraping of cells from the culture dish clearly causes more membrane damage than does trypsinization, but this does not necessarily result in significant cell death or DNA damage. Scraped or trypsinized HeLa cells, if replated and incubated for 24 h, show the same degree of recovery as assessed by the number of attached cells (C. M. Gedik, unpublished).

Other potential problems with the enzymic approach have been enumerated [14]; they include the fact that FPG is not specific for 8-oxoGua but also recognizes fapy derivatives of purines. Whether the concentration of these lesions in untreated cellular DNA is sufficient to lead to significant overestimation of damage with the enzymic methods is an important question. The report already mentioned, from one of the ESCODD partners [21], compares the dose response curves for HPLC-ECD and the comet assay with FPG, measuring damage induced by Ro 19-8022 in HeLa cells; care was taken to ensure that the conditions in which the cells were kept during treatment were identical. The slopes of the curves were almost identical, indicating that the two approaches are, in fact, equally efficient at detecting this damage. (Because of the large number of samples that had to be prepared for the present ESCODD study, it was impossible to maintain the same conditions, and so a comparison of slopes between chromatographic and enzymic methods was not attempted.) The fact remains that, in this trial, there was considerable variation between laboratories, in estimates of background levels of damage as well as induced 8-oxodGuo. The need for standardization and interlaboratory validation of the comet assay, and other enzymic methods, is now widely recognized and is being addressed.

Conclusions

Considerable progress has been made towards the accurate measurement of oxidative DNA damage in human cells. Using HPLC-ECD, we have successfully measured moderate levels of 8-oxoGua induced experimentally in HeLa cells, with very low variation between laboratories. Mass spectrometry-based methods have not reached an acceptable standard; further comparisons with HPLC-ECD using identical cellular DNA samples (eliminating the influence of the DNA extraction process) may identify the reason for their failure. While HPLC-ECD can accurately measure induced damage, it still gives very variable estimates of the background level of DNA oxidation. The enzymic methods give a similarly wide range of values for background damage, varying over an order of magnitude, but the median value is 7.5 times lower than the median value for chromatographic methods.

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REFERENCES

[1] Collins, A.; Cadet, J.; Epe, B.; Gedik, C. Problems in the measurement of 8-oxoguanine in human DNA. Report of a workshop, DNA Oxidation, held in Aberdeen, UK, 19–21 January, 1997. *Carcinogenesis* **18:**1833–1836; 1997.

[2] Nakajima, M.; Takeuchi, T.; Morimoto, K. Determination of 8-hydroxydeoxyguanosine in human cells under oxygen-free conditions. *Carcinogenesis* **17:**787–791; 1996.

[3] Kvam, E.; Tyrrell, R. M. Artificial background and induced levels of oxidative base damage in DNA from human cells. *Carcinogenesis* **18:**2281–2283; 1997.

[4] Gedik, C. M.; Wood, S. G.; Collins, A. R. Measuring oxidative damage to DNA; HPLC and the comet assay compared. *Free Radic. Res.* **29:**609–615; 1998.

[5] Helbock, H. J.; Beckman, K. B.; Shigenaga, M. K.; Walter, P. B.; Woodall, A. A.; Yeo, H. C.; Ames, B. N. DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. *Proc. Natl. Acad. Sci. USA* **95:** 288–293; 1998.

[6] Hofer, T.; Möller, L. Reduction of oxidation during the preparation of DNA and analysis of 8-hydroxy-2'-deoxyguanosine. *Chem. Res. Toxicol.* **11:**882–887; 1998.

[7] Hofer, T.; Möller, L. Optimization of the workup procedure for the analysis of 8-oxo-7,8-dihydro-2'-deoxyguanosine with electrochemical detection. *Chem. Res. Toxicol.* **15:**426–432; 2002.

[8] Ravanat, J.-L.; Douki, T.; Duez, P.; Gremaud, E.; Herbert, K.; Hofer, T.; Lasserre, L.; Saint-Pierre, C.; Favier, A.; Cadet, J. Cellular background level of 8-oxo-7,8-dihydro-2'-deoxyguanosine: an isotope based method to evaluate artefactual oxidation of DNA during its extraction and subsequent work-up. *Carcinogenesis* **23:**1911–1918; 2002.

[9] Hartwig, A.; Dally, H.; Schlepegrell, R. Sensitive analysis of oxidative DNA damage in mammalian cells: use of the bacterial Fpg protein in combination with alkaline unwinding. *Toxicol. Lett.* **88:**85–90; 1996.

[10] Epe, B.; Hegler, J. Oxidative DNA damage: endonuclease fingerprinting. *Methods Enzymol.* **234:**122–131; 1994.

[11] Collins, A. R.; Dušinská, M.; Gedik, C. M.; Štětina, R. Oxidative damage to DNA: do we have a reliable biomarker? *Environ. Health Perspect.* **104**(Suppl. 3):465–469; 1996.

[12] ESCODD. Comparison of different methods of measuring 8-oxoguanine as a marker of oxidative DNA damage. *Free Radic. Res.* **32:**333–341; 2000.

[13] ESCODD. Inter-laboratory validation of procedures for measuring 8-oxo-7,8-dihydroguanine/8-oxo-7,8-dihydro-2'-deoxyguanosine in DNA. *Free Radic. Res.* **36:**239–245; 2002.

[14] ESCODD. Comparative analysis of baseline 8-oxo-7,8-dihydroguanine in mammalian cell DNA, by different methods in different laboratories: an approach to consensus. *Carcinogenesis* **23:**2129–2133; 2002.

[15] Will, O.; Gocke, E.; Eckert, I.; Schulz, I.; Pflaum, M.; Mahler, H.-C.; Epe, B. Oxidative DNA damage and mutations induced by a polar photosensitizer, Ro 19-8022. *Mutat. Res.* **435:**89–101; 1999.

[16] Ravanat, J.-L.; Di Mascio, P.; Martinez, G. R.; Medeiros, M. H. G.; Cadet, J. Singlet oxygen induces oxidation of cellular DNA. *J. Biol. Chem.* **275:**40601–40604; 2000.

[17] Ravanat, J.-L.; Saint-Pierre, C.; Di Mascio, P.; Martinez, G. R.; Medeiros, M. H. G.; Cadet, J. Damage to isolated DNA mediated by singlet oxygen. *Helv. Chim. Acta* **84:**3702–3709; 2001.

[18] Frelon, S.; Douki, T.; Ravanat, J.-L.; Pouget, J. P.; Tornabene, C.; Cadet, J. High-performance liquid chromatography—tandem mass spectrometry measurement of radiation-induced base damage to isolated and cellular DNA. *Chem. Res. Toxicol.* **13:**1002–1010; 2000.

[19] Wood, S. G.; Gedik, C. M.; Vaughan, N. J.; Collins, A. R. Measurement of 8-oxo-deoxyguanosine in lymphocytes, cultured cells, and tissue samples by HPLC with electrochemical detection. In: Barnett, Y. A.; Barnett, C. R., eds. *Aging methods and protocols*. Totowa, NJ: Humana Press Inc.; 2000:171–178.

[20] Collins, A. R.; Dušinská, M. Oxidation of cellular DNA measured with the comet assay. In: Armstrong, D., ed. *Oxidative stress biomarkers and antioxidant protocols*. Totowa, NJ: Humana Press Inc.; 2002:147–159.

[21] Gedik, C. M.; Boyle, S. P.; Wood, S. G.; Vaughan, N. J.; Collins, A. R. Oxidative stress in humans: validation of biomarkers of DNA damage. *Carcinogenesis* **23:**1441–1446; 2002.

ABBREVIATIONS

ESCODD—European Standards Committee on Oxidative DNA Damage
fapy—formamidopyrimidine
FPG—formamidopyrimidine DNA N-glycosylase
GMEM—Glasgow-modified Eagle's Minimal Essential Medium
8-oxoGua—8-oxo-7,8-dihydroguanine
8-oxodGuo—8-oxo-7,8-2'-dihydrodeoxyguanosine
HPLC-ECD—HPLC with electrochemical detection