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Chromatographic Determination of 8-oxo-7,8-dihydro-2′-deoxyguanosine in Cellular DNA: A Validation Study

P. DUEZ*, M. HELSON, T.I. SOME, J. DUBOIS and M. HANOCQ

Université Libre de Bruxelles (ULB), Institute of Pharmacy, Laboratory of Bioanalytical Chemistry, Toxicology and Applied Physical Chemistry, CP 205/1, Bd du Triomphe, B-1050 Brussels, Belgium

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Although a series of biomarkers are widely used for the estimation of oxidative damage to biomolecules, validations of the analytical methods have seldom been presented. Formal validation, that is the study of the analytical performances of a method, is however recognized as the best safeguard against the generation and publication of data with low reliability. Classical validation parameters were investigated for the determination of an oxidative stress biomarker, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) in cellular DNA, by high-performance liquid chromatography coupled to amperometric detection (HPLC-EC); this modified base is increasingly considered as a marker of oxidative damage to DNA, but many questions are still raised on the analytical methods in use. Upon a rigorous statistical evaluation of the quality criteria currently required for assays in biological media, including selectivity, linearity, accuracy, repeatability, sensitivity, limits of detection and quantification, ruggedness and storage at different stop points in the procedure, the HPLC-EC assay method is found mostly reliable.

The present validation attempt demonstrates that (i) the HPLC-EC assay of 8-oxo-dG provides consistent data allowing to reliably detect an increase of this biomarker in cellular DNA; (ii) a harsh oxidative stress does not hinder the enzymatic digestion of DNA by nuclease P1; and (iii) the analytical results must be expressed relative to the internal standard dG which significantly improves both repeatability and sensitivity. Whereas the described assay minimizes the artifactual production of the analyte from processing and storage, this cannot be totally ruled out; the true 8-oxo-dG base levels still lack a definitive assay method, which remains a considerable analytical challenge and the object of controversy.

Keywords: Biomarker, analytical validation, 8-oxo-dG, 8-OH-dG, oxidative stress, DNA damage

INTRODUCTION

Oxidative stress, that is an imbalance between cellular oxygen-derived species and antioxidant defenses, is recognized as a major cause and/or consequence of tissue damage and degeneration; free radicals are now believed to play a major role in the aging process and in a large number of human diseases.[1] Considerable interest has arisen in the development of reliable biomarkers

*Corresponding author. Tel.: 32 2 650 5242. Fax: 32 2 650 5187. E-mail: pduez@ulb.ac.be.

of exposure to pro-oxidant conditions; these allow a number of investigations, both *in vitro* and *in vivo*, in diverse fields such as cellular defenses, stress conditions and mechanisms, nutrition, genotoxicology, repair studies and environmental and human biomonitoring. The antioxidant biomarkers, both non-enzymatic and enzymatic, measure the capacity to react to oxidant conditions but give only scant information on damage undergone by the cell, tissue or organism. Such damage is actually reflected by the biomolecules, and a series of oxidative lesions have been proposed as both invasive and non-invasive biomarkers[2,3] for lipids, proteins and nucleic acids.

Nucleic acids are a major target for oxidative modifications resulting in strand breaks[4] and in a number of oxidized bases and adducts.[5–7] Although the impact of RNA oxidation has yet to be evaluated, it is now established that some DNA modifications can be highly mutagenic. Such lesions are then expected to be biomarkers, not only of exposure to pro-oxidant conditions, but also of carcinogenic risk and susceptibility.[6] Oxidative damage to DNA has been quantitated by measurements of strand breaks[4,8–11] and by the assay of modified bases with chromatographic[12–16] and immunological[17,18] techniques.

The validation of analytical methods is largely recognized as the best safeguard against the generation of unreliable data and is becoming an absolute requirement in many fields. Validation is the process by which it is established, by laboratory studies, that the performance characteristics of an analytical method meet the requirements for the intended applications.[19] Depending on the objective of the analytical procedure, the typical validation characteristics which can be considered through a statistical approach are accuracy, precision, specificity or selectivity, detection limit, quantification limit, linearity and ruggedness.[20] For the biomarkers of oxidative stress, the ruling out of artifactual production from the analytical procedure is a proposed additional validation parameter.

The steps required for applying the validation concept to a biomarker of oxidative stress to biomolecules will be illustrated through the determination of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) in cellular DNA by HPLC with amperometric detection. This lesion of the most easily oxidized base in DNA[21,22] is increasingly considered a marker of oxidative damage[23–26] and is highly mutagenic.[27–29] Although the exact steady-levels of 8-oxo-dG as evaluated by different methods are still controversial,[30] significant increases have been found after treatment with a large number of mutagens in a series of models (reviewed in Ref. [26]); the urinary excretion of 8-oxo-dG is also a recognized biomarker of exposure to mutagens for epidemiological studies.[31,32]

MATERIALS AND METHODS

Chemicals

Solutions of proteinase K (*Tritirachium album*; EC 3.4.21.14), alkaline phosphatase (calf intestine; EC 3.1.3.1) and RNA 16S- and 23S-ribosomal (*E. coli*) were obtained from Böhringer Mannheim (Germany). Nuclease P1 (*Penicillium citrinum*; EC 3.1.30.1), deoxyribonuclease I (bovine pancreas; EC 3.1.21.1), RNase A (bovine pancreas, heat-inactivated; EC 3.1.27.5), 2′-deoxyguanosine (dG), 2′-deoxyadenosine (dA), 2′-deoxycytidine (dC), thymidine (T) and 8-oxo-2′-deoxyguanosine were from Sigma (St. Louis, USA). Cell culture media and reagents were from Gibco Life Technologies (Paisley, Scotland).

Cell Culture and Exposure to UV_C and H_2O_2

Murine P388D1 leukemia cells (ATCC CCL-46) were maintained, as a cell suspension in logarithmic growth, at 37°C in a humidified atmosphere of 5% CO_2 in air, in RPMI 1640 medium supplemented with HEPES (10 mM), penicillin (100 U/ml), streptomycin (100 U/ml) and 9% fetal bovine serum.

For the exposure to oxidative stress, cells were harvested by centrifugation (900*g*, 2 min), washed 2 times with phosphate-buffered saline (PBS) and suspended at about 10^7 cells in 400 µl of PBS in a 30-mm Petri dish. Hydrogen peroxide was added up to 20 mM and cells were then exposed to UV_C (total output of a Sylvania G8T5 germicidal tube placed at a 5 cm distance of vessel surface; 2–15 min; ambient temperature).[33,34] Cells were immediately transferred to a 1.5 ml *Eppendorf* tube, centrifuged (900*g*, 2 min), gently overlaid with 250 µl of HM buffer (300 mM sucrose, 25 mM Tris, 2 mM $EDTANa_2H_2$, 6 mM reduced glutathione, 2 mM deferoxamine mesylate, 8 mM DL-histidine, pH 7.3) and frozen at −80°C. Some experiments were performed with a freezing buffer not containing antioxidants (H buffer, 300 mM sucrose, 25 mM Tris, 2 mM $EDTANa_2H_2$, pH 7.3) at −20°C.

DNA Extraction

Extraction and digestion were performed as described in Ref. [35] with some modifications in the buffers, storage conditions and operations schedule; all work was under reduced light. To the thawed and vortex-mixed cell suspension, 5 µl of proteinase K (75–100 µg) and 250 µl of DES buffer (1 M LiCl, 2 M urea, 50 mM Tris, 5 mM $EDTANa_2H_2$, 2% sodium dodecylsulfate, pH 8.0) are added; the mixture is then heated at 55°C for 2.5 h, extracted 2 times with 500 µl of an ice-cold mixture chloroform : isoamyl alcohol (24 : 1) and centrifuged (1800*g*, 5 min). The final supernatant, including any viscous material, is transferred to a 2-ml Eppendorf (previously washed with ethanol, water then with ethanol and room-temperature dried). After addition of 33 µl sodium acetate (3 M, pH 7.0) and 1 ml ice-cold 94° ethanol, the mixture is stirred until apparition of a white thread, cooled at −20°C for 1 h and centrifuged (10,000*g*, 5 min). The DNA pellet is washed 2 times with ice-cold 70° ethanol and then with ice-cold 94° ethanol (suspension in 500 µl solvent and centrifugation at 1800*g*, 2 min), dried under a gentle stream of nitrogen, suspended in 250 µl TE buffer (10 mM Tris, 1 mM $EDTANa_2H_2$, pH 7.4) and left to dissolve for maximum one night at 4°C.

Enzymatic Digestion of DNA and HPLC Analysis

Enzymatic digestion and chromatography are performed within the same day.

After addition of 25 µl digestion buffer (0.5 M sodium acetate, 0.11 M magnesium chloride, pH 5.1) and denaturation (100°C for 5 min, then ice-bath for 5 min), the DNA solution is incubated with 10 µl of nuclease P1 (400 U/ml in water) at 37°C for 1 h, followed by addition of 8 µl of 1 M Tris and 2 U of alkaline phosphatase and re-incubation at 37°C for 1 h. After addition of 4 µl of acetic acid 5.8 M, the solution is maintained at 4°C in the dark for maximum 6 h before chromatography; 100 µl is injected.

The HPLC system consisted of a Gilson Model 305 pump with Model 805 manometric module (Villiers le Bel, France), a Rheodyne 7125 injector (Cotati, USA), an Alltech Ultrasphere C_{18} guard column cartridge (15 × 4.6 mm i.d.) (Deerfield, USA) and a Beckman Ultrasphere C_{18} column (250 × 4.6 mm i.d.) (Berkeley, USA) maintained at 30°C by a water jacket. Two detectors were connected in series (i) a Gilson Holochrome HM UV detector set at 245 nm and 0.5 AFS; and (ii) a TL 5 A glassy carbon electrode held at +700 mV vs Ag/AgCl (B.A.S., West-Lafayette, USA) through an A.S.I. – Tacussel ED-110 electrochemical detector (Villeurbanne, France) set at 0.5 nA full scale. The working electrode was dismantled every 3–4 days and gently polished with methanol using a long fibers cleaning paper; this allows to compensate for the observed slight reduction in signal level. Some injections were performed using a Beckman Module 168 diode array UV detector, interfaced to the Gold integration system. The isocratic mobile phase is a mixture of 92.5% aqueous buffer (0.05 M NaH_2PO_4, 1 mM $EDTANa_2H_2$, pH "as is", 5.45) and 7.5% methanol, carefully degassed. The flow-rate is 1 ml/min.

Standard Solutions

A combined 8-oxo-dG and dG stock solution is prepared in water and suitably diluted, the intermediate dilutions being aliquoted in 1-ml Eppendorf and maintained at −20°C until needed. The working solution is re-prepared from more concentrated dilutions every month, aliquoted in 500 µl and also maintained at −20°C. Each solution is thawed only once, just before use.[36] The 8-oxo-dG content of dG commercial powder has been determined and adjusted for; on 2 different batches, the found level is about 1.7 mol per 10^5 mol of dG, which is far from negligible with regard to the 8-oxo-dG level in standard.

Validation Parameters and Statistical Analysis

Specificity of the HPLC Method

UV and electrochemical peaks from DNA were identified by co-injection of a stressed biological sample with authentic standards. For the four normal DNA bases, the identity and purity of UV peaks were verified with the help of the diode array detector by window evolving factor analysis. Optimal peak purity assignment conditions have been observed,[37] i.e., a data acquisition rate of 1 Hz and maximum absorbance of 0.4 AU. Cross-correlation coefficients have also been computed between 200 and 400 nm for each matched pair of peaks. The peaks from RNA were identified by comparing retention times and cross-correlation coefficients with those of the peaks obtained by digestion of a commercial ribosomal RNA. The 8-oxo-dG electrochemical peak identity and purity were checked by injecting a standard and a harshly stressed sample (H_2O_2 20 mM + UV_C 10 min) at different potentials and comparing peak ratios.

Linearity and Accuracy of the HPLC Method

The concentration vs detector response curve for both aqueous calibrators and spiked blank DNA extracts was investigated on 4 different days at 8 concentration levels for dG, T, dA and 8-oxo-dG. A bulk DNA extract was prepared from 5×10^8 non-stressed cells, aliquoted, spiked, stored at 4°C and digested as per our general protocol.

Homoscedasticity was checked for by the Bartlett's χ^2 test and, when needed, data were log transformed prior to linearity testing (lack of fit test[38]).

For accuracy evaluation, the blank levels were estimated for each base from the spiked DNA regression curve as the x-intercept and subtracted from found levels. The Bartlett's χ^2 test was applied to verify homoscedasticity of accuracies between concentration levels; the probability for the F-ratio "variance between concentration levels" to "variance within concentration levels" was then computed to ascertain that the variations of observations between the concentration levels are due to experimental errors. The mean recovery was then computed along with its confidence interval for a probability level of 0.05.

Precision of the Whole Analytical Procedure

This parameter has been investigated on blank samples (unstressed cells) and on cells stressed at 3 different levels; cells were exposed to 20 mM H_2O_2 with different UV_C irradiation times (5, 10 and 15 min), aliquoted and stored at −20°C under H buffer. All samples were analyzed on 4 different days in triplicates (3 repetitions of the complete analytical procedure, including extraction). After the first 2 days, the electrode was polished with methanol to verify that the electrode surface effect on the raw electrochemical signal presents no influence on the analytical results. The data were analyzed for dG, 8-oxo-dG and the ratio 8-oxo-dG per 10^5 dG by a 2-way ANOVA with repetition, considering 2 random effects, "*sample* (*concentration*)" and "*day*"; the within-day and total ("between-day") variations for the whole analytical procedure were computed.

Sensitivity of the Whole Analytical Procedure

A sensitivity relevant to routine measurements was estimated, that is the electrochemical detector raw signals were corrected by the signal of an external 8-oxo-dG standard (average of 4 injections) so to modulate the day-to-day differences in electrochemical response. As there is no possibility to introduce different known 8-oxo-dG levels in cell DNA,[30] the concentration parameter was estimated from the data of the "Precision" section, considering that the first triplicate analytical run accurately assayed the various concentrations. This is a likely assumption comforted by the fair repeatability of the analytical procedure. The total variability and the curve "concentration" vs "detector response" were then computed from the data of the following 3 days to estimate sensitivity.

Lower Limits of Detection and Quantitation

The lower limit of detection was classically defined as the concentration yielding a signal-to-noise ratio of 3. The lower limit of quantification was estimated at the lowest blank level we found in biological samples (100 fmol on-column).

Ruggedness of the HPLC Method

The ruggedness was investigated through a factorial plan testing for 4 parameters. Experimental variation (2 levels) of the following parameters, susceptible of variation in routine work and possibly critical, have been realized: (i) DNA content in 8-oxo-dG (cells exposed to 20 mM H_2O_2 with 5 and 15 min UV_C irradiation); (ii) methanol content of the HPLC mobile phase (7.0% and 8.0%); (iii) column temperature (25°C and 35°C); (iv) quantity of DNA digested and injected (DNA from 10^7 and 2×10^7 cells). A series of 8 analyses allowed to test 4 parameters, the triple interaction of the first 3 parameters being affected to the fourth ("aliasing"). Confidence intervals were computed for the value "effect of the parameter" at a probability level of 0.05.

Stability of Analytes

The analytical process being quite extensive, it cannot be performed within a single day run; possible stop points in the technique have, consequently, been investigated along with their effect on oxidized dG levels: (i) storage between sample preparation and DNA extraction; (ii) storage between DNA extraction and enzymatic digestion; and (iii) storage between enzymatic digestion and HPLC analysis.

RESULTS

HPLC Conditions

Figure 1 demonstrates typical UV and EC chromatograms for a stressed cell sample (20 mM H_2O_2, UV_C 10 min). The optimum potential for the electrochemical analyses (+700 mV) was determined from the response curve "current intensity" vs "applied potential" for a standard of 8-oxo-dG at 2 concentration levels.

Specificity of the HPLC Method with Regard to Matrix Constituents

Regarding the UV chromatogram, the diode array principal component analysis (Figure 1(b)) demonstrates no interference in the peaks for G, dG, T, A and dA. The peaks for C, U and dC are not mono-components; they either co-elute or elute in the injection peak. The 8-oxo-dG electrochemical peak identity and purity data are presented in Table I.

Linearity Study

A linear relationship "detector response" vs concentration was demonstrated in the investigated ranges (Table II, slope significance and lack of fit tests) for 8-oxo-dG, dG, T and dA, both for

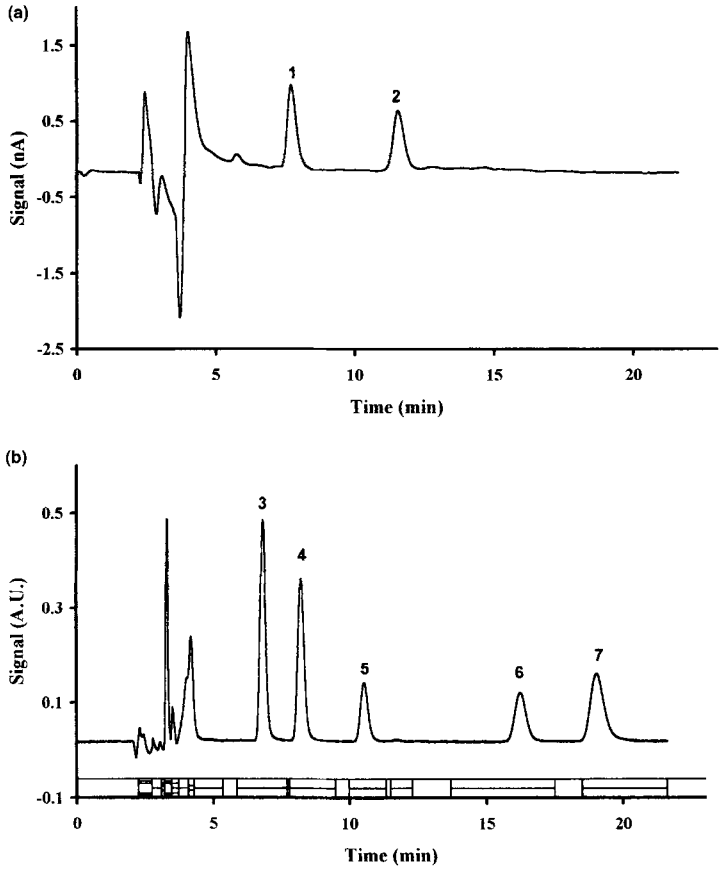

FIGURE 1 Chromatograms of enzymatically digested nucleic acids (P388D1 cells stressed by 20 mM H_2O_2 + UV_C 10 min). C_{18} column (5 µm; 250 × 4.6 mm i.d.) with guard column; mobile phase, aqueous buffer (0.05 M NaH_2PO_4, 1 mM EDTA-Na_2H_2, pH "as is", 5.45) : methanol (92.5 : 7.5); 1 ml/min 30°C; UV (diode array) and amperometric detectors in series. (a) Amperometric detection; 700 mV vs Ag/AgCl; 2 nAFS. Peaks: 1 = peak from RNA, presumably 8-oxo-7,8-dihydro-guanosine; 2 = 8-oxo-dG. (b) UV detection; 245 nm, 0.5 AUFS. Peaks: 3 = guanosine; 4 = 2′-dG; 5 = T; 6 = adenosine; 7 = 2′-dA. The plot under the chromatogram is a real-time visual display of peak homogeneity as measured through diode array detection. The number of horizontal lines represent the number of components detected by an algorithm based on window evolving factor analysis; a single line indicates homogeneous peaks.

TABLE I Identification of 8-oxo-dG in oxidatively stressed cells: peaks ratio at different potentials (3 injections for each potential; in brackets, range)

Ratio (mV)	Authentic 8-oxo-dG	Stressed cells (H_2O_2 + UV_C 10 min)
700/650	1.11 (1.07–1.14)	1.15 (1.12–1.20)
700/600	1.45 (1.38–1.51)	1.44 (1.43–1.50)
700/550	3.00 (2.89–3.12)	3.03 (2.76–3.24)
700/500	4.00 (3.83–4.63)	4.09 (3.61–5.49)
650/600	1.31 (1.22–1.40)	1.27 (1.20–1.32)
600/550	2.47 (2.40–2.60)	2.16 (1.98–2.34)
600/500	3.40 (3.16–3.87)	3.05 (2.58–3.97)
550/500	1.38 (1.28–1.54)	1.41 (1.21–1.87)

aqueous calibrators and spiked DNA. The slopes are parallel, justifying the use of aqueous standards for daily work. For this study, spike was at the earliest possible analytical step; as extractable 8-oxo-dG could not be accurately introduced into cell lysates, spiking was performed on DNA before the enzymatic digestion step. DNA solutions are, however, quite viscous and the aliquoting of solutions to be spiked was difficult. As the endogenous levels of the normal DNA bases are high relatively to spikes, unavoidable small variations in aliquoted DNA are reflected in

TABLE II Linearity study

Compound Investigated range (spike per 100 µl injected)	8-oxo-dG 0.2–32 pmol	2′-dG 0.3–41 nmol	T 0.3–41 nmol	2′-dA 0.3–41 nmol
Aqueous solutions				
n	32	32	32	32
Determination coefficients r^2	0.9904	0.9976	0.9980	0.9971
Spiked DNA solutions				
n	32	28[a]	32	32
Determination coefficients r^2	0.991	0.975	0.941	0.963
p for the parallel slopes t-test[b] (aqueous solutions vs spiked DNA)	0.479	0.638	0.583	0.358
p for the Bartlett χ^2-test (homoscedasticity)	4.07×10^{-9}	0.701	0.716	0.559
Eventual transformation	log–log	No	No	No
Slope ± standard deviation[c] (RSD%)	1.020 ± 0.013 (1.3%)	5238 ± 164 (3.1%)	1576 ± 72 (0.5%)	2994 ± 107 (3.6%)
Intercept ± standard deviation[c] (RDS %)	4.529 ± 0.013 (0.3%)	59 655 ± 2662 (4.5%)	25 112 ± 1485 (5.9%)	52 637 ± 2390 (4.5%)
p for the slope significance F-test[c]	3.38×10^{-36}	2.20×10^{-22}	5.26×10^{-20}	4.83×10^{-23}
p for the lack of fit F-test[c]	0.990	0.322	0.226	0.364

[a]Higher tested spike peaked over detector limits and was not considered. [b]For all p-values, the significance limit was considered as 0.05. [c]Computed after the eventual transformation.

spiked DNA calibration curves and determination coefficients. This problem is much less apparent for 8-oxo-dG, the endogenous levels being similar to the lowest spikes.

Accuracy Study

Table III presents the data for the accuracy study; the mean relative recovery is around 100% for all tested compounds. Whereas the slight bias observed for 8-oxo-dG is statistically significant at the level 0.05 (0.2–6.3%), it is negligible, the upper limit of the confidence interval being lower than SD (8%), that is the precision of the method.

The relatively high standard deviations observed for dG, T and dA and the bias for dG come from the method precision but mainly from the dificulty inherent to DNA aliquoting discussed here above.

Precision of the Whole Analytical Procedure

Table IV details the components of the variance for the precision data. The dG precision was not influenced by either of the investigated factors, *"stress level"* or *"analysis day"*. On the other hand, the DNA 8-oxo-dG content allowed to differentiate the stress levels of the different samples without influence of the factor *"analysis day"*. When computing the results as "8-oxo-dG per 10^5 dG", the variation due to DNA extraction is strongly reduced (3% total RSD), dG playing the role of an internal standard. With this increased precision, a slight interaction of day × sample can be evidenced ($p = 0.044$) for 8-oxo-dG; a close examination of the data reveals that this interaction is entirely due to the blank (unstressed sample), its level increases slightly but steadily according to storage before analysis (8-oxo-dG per 10^5 dG ranged: day 1, 0.83–1.03; day 2, 0.88–1.12; day 3, 0.87–1.33; day 4, 1.08–1.24; $n = 3$).

Figure 2 evidences the precision of the analytical procedure relatively to the 8-oxo-dG levels induced by the applied stress conditions.

Sensitivity of the Whole Analytical Procedure

Sensitivity was computed in units of both 8-oxo-dG and 8-oxo-dG per 10^5 dG (Table V); the influence of signal correction by the internal

TABLE III Accuracy study

	8-oxo-dG		2′-dG		T		2′-dA	
	Spiked level (pmol)	Relative recovery (mean ± SD) (%)	Spiked level (nmol)	Relative recovery (mean ± SD) (%)	Spiked level (nmol)	Relative recovery (mean ± SD) (%)	Spiked level (nmol)	Relative recovery (mean ± SD) (%)
	31.45	103 ± 6	41.16	(Overpeaked)	40.51	92 ± 9	40.51	99 ± 9
	24.19	101 ± 9	31.66	99 ± 6	31.17	92 ± 14	33.61	94 ± 12
	17.97	100 ± 7	23.52	109 ± 11	23.15	109 ± 6	24.97	102 ± 5
	11.79	99 ± 8	15.43	106 ± 13	15.19	104 ± 24	16.39	99 ± 18
	5.24	101 ± 9	6.86	120 ± 17	6.75	120 ± 34	7.28	108 ± 27
	1.05	107 ± 7						
	0.52	112 ± 7						
n	28		16		20		20	
p^a for Bartlett χ^2-test (homoscedasticity)	0.998		0.497		0.056		0.117	
p^a for the *F*-test ratio "variance between-levels" to "variance within-levels"	0.214		0.242		0.289		0.789	
Mean relative recovery (%) ± SD	103 ± 8		109 ± 13		103 ± 21		100 ± 15	
Mean recovery confidence interval (%)	100.2–106.3		101.5–115.7		93.5–113.0		93.3–107.3	

[a]For all *p*-values, the significance limit was considered as 0.05.

TABLE IV Precision of the whole analytical procedure: 2-way ANOVA with repetition; 2 random factors (*"sample"* and *"day"*) (4 different samples have been extracted and analyzed in triplicate on 4 different days)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio (*F*)	p^a	RSD% (within-day)	RSD% (total)
dG							
Day	3	6.70	2.23	1.02	0.429^{NS}	9.05	9.06
Sample	3	2.85	0.95	0.43	0.735^{NS}		
Day × sample (interaction)	9	19.71	2.19	1.12	0.379^{NS}		
Residual (error)	32	62.75	1.96				
Total	47	92.01					
8-oxo-dG							
Day	3	0.31	0.10	1.07	0.410^{NS}	7.27	7.30
Sample	3	314.04	104.68	1066.50	8.5×10^{-12}**		
Day × sample (interaction)	9	0.88	0.10	1.17	0.347^{NS}		
Residual (error)	32	2.69	0.08				
Total	47	317.93					
8-oxo-dG per 10^5 dG							
Day	3	4.48	1.61	1.30	0.333^{NS}	2.90	3.01
Sample	3	12697.41	4232.47	3407.02	4.6×10^{-14}**		
Day × sample (interaction)	9	11.18	1.24	2.26	0.044*		
Residual (error)	32	17.63	0.55				
Total	47	12731.06					

[a]NS, non-significant; *significant; **highly significant.

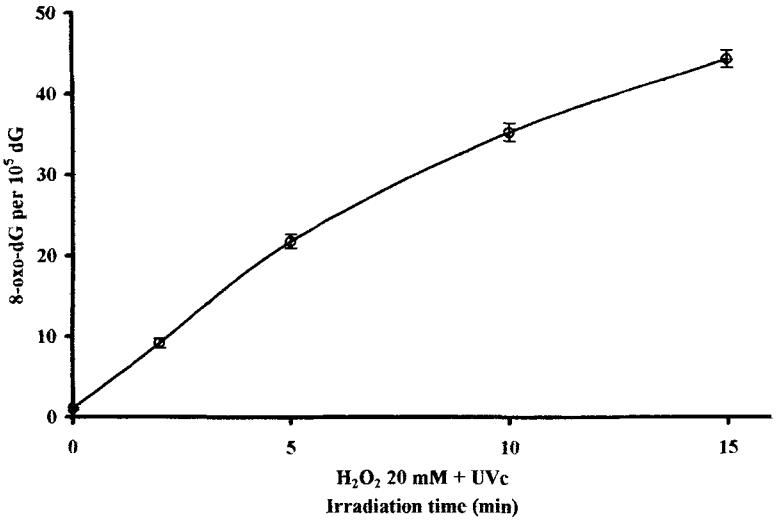

FIGURE 2 Relationship between stress (time of UV_C irradiation in presence of 20 mM H_2O_2) and 8-oxo-dG DNA content. Data from the precision study; mean ± analytical standard deviation; $n = 12$ except for time 2 min ($n = 3$).

TABLE V Sensitivity of the whole analytical procedure

Concentration expression (x)	Regression *"signal"* vs *"concentration"* ($n = 36$)			Sensitivity[a]	Statistically significant sensitivity[b]
	Total variance on signal	Determination coefficient (r^2)	Slope		
8-oxo-dG (pmol)					
Electrochemical signal (y) is:					
• corrected by external standard (8-oxo-dG)	8.03×10^{-3}	0.9829	0.285	0.31 pmol 8-oxo-dG	1.65 pmol 8-oxo-dG
• corrected by external standard (8-oxo-dG) and internal standard (dG)	8.76×10^{-14}	0.9963	2.44×10^{-6}	0.12 pmol 8-oxo-dG	0.64 pmol 8-oxo-dG
8-oxo-dG per 10^5 dG					
Electrochemical signal (y) is:					
• corrected by external standard (8-oxo-dG)	8.03×10^{-3}	0.9833	0.042	2.13 8-oxo-dG per 10^5 dG	11.15 8-oxo-dG per 10^5 dG
• corrected by external standard (8-oxo-dG) and internal standard (dG)	8.76×10^{-14}	0.9970	3.61×10^{-7}	0.82 8-oxo-dG per 10^5 dG	4.31 8-oxo-dG per 10^5 dG

[a] Sensitivity $= S_E/b$ (minimal variation of analyte concentration yielding a detectable variation of signal). [b] Sensitivity $= [t_{(1-\alpha/2;N-1)} + t_{(1-\beta;N-1)}] * S_E * \sqrt{2} * (1/b)$ (minimal variation of analyte concentration yielding a statistically significant variation of signal).

standard dG (from UV detection) was also investigated. Statistically significant sensitivity was 1.65 injected pmol of 8-oxo-dG, corresponding to 11.15 8-oxo-dG per 10^5 dG. When the electrochemical signal was corrected for DNA recovery through the dG signal, the sensitivity was sensibly reduced to 0.64 injected pmol of 8-oxo-dG, or 4.31 8-oxo-dG per 10^5 dG.

Lower Limits of Detection and Quantitation

For 8-oxo-dG, the lower limit of detection (signal-to-noise ratio = 3) was 0.05 injected pmol (50 fmol); the lower limit of quantification (RSD = 20%; $n = 8$) was 0.1 injected pmol (100 fmol). Preliminary experiences have demonstrated that these limits can be improved several folds using a CC-5 cross-flow cell (B.A.S., West-Lafayette, USA) fitted *before* the UV detector.

Although these data are of the same order as those encountered in the literature (detection limits, 20,[39,40] 40,[41] 70,[42] 250 fmol[43]), some authors present impressive limits that we could not reach (1.76 fmol detected, with[36] and without[44] a noise-reduction algorithm).

Ruggedness

From Table VI, the only significant parameter is "stress level" (confidence interval not recovering the value "0"); the method is then rugged against small variations in the parameters investigated, that is methanol content in the mobile phase, column temperature and DNA amount digested.

Stability of Analytes

Table VII demonstrates the effect of suspending the analytical process on measured 8-oxo-dG levels. Prolonged storage at any of the different steps increases the artefactual oxidation of dG. Notably, storage between enzymatic digestion and chromatography for more than one day should be avoided, even with −20°C freezing; HPLC can however be performed on the day of digestion with an excellent stability (Table VIII). After extraction, precipitation and washing, the DNA fibers must be completely dissolved in TE buffer before enzymatic digestion; as this dissolution step may require several hours, the extract can be left at 4°C overnight which allows HPLC injection on the day of digestion. This storage should however not be prolonged for more than 2 days.

Whereas the analysis steps (extraction, digestion and HPLC) can be easily performed within 2 days, experimental planning usually makes it necessary to store the biological samples for some time before extraction. Data from Table VII demonstrate that storage for more than 2 days in buffer H at −20°C increases the 8-oxo-dG levels in

TABLE VI Ruggedness of the whole analytical procedure

Sample	Parameters				Interactions			Analytical result 8-oxo-dG/10^5 dG
	A Stress level[a]	B Methanol % in mobile phase[b]	C Column temperature[c]	D DNA amount[d]	AB	AC	BC	
1	−1	−1	−1	−1	1	1	1	13.70
2	1	−1	−1	1	−1	−1	1	49.91
3	−1	1	−1	1	−1	1	−1	14.73
4	1	1	−1	−1	1	−1	−1	51.85
5	−1	−1	1	1	1	−1	−1	15.72
6	1	−1	1	−1	−1	1	−1	53.05
7	−1	1	1	−1	−1	−1	1	13.91
8	1	1	1	1	1	1	1	45.66
Parameter effect	17.80	−0.78	−0.23	−0.81	−0.59	−0.53	−1.52	
Confidence interval								
From:	1.79	−16.80	−16.25	−16.83	−16.60	−16.55	−17.54	
To:	33.82	15.24	15.78	15.21	15.43	15.49	14.50	
Statistical significance ($p = 0.05$)	S	NS	NS	NS	NS	NS	NS	

[a] ($H_2O_2 + UV_C$) for 5 and 15 min; [b] 7% and 8%; [c] 25°C and 35°C; [d] DNA from 10^7 cells and from 2×10^7 cells.

TABLE VII Influence of storage at different points during the analytical procedure

	Storage before extraction (Buffer H at −20°C)	Storage between extraction and digestion (Buffer TE at 4°C)	Storage between enzymatic digestion and HPLC injection (Digestion mixture at −20°C)	8-Oxo-dG per 10^5 dG (range; $n=3$)
Unstressed cells	Overnight	Overnight	No[a]	0.83–1.03
	Overnight	Overnight	1 day	0.69–0.95
	Overnight	Overnight	3 days	1.48–3.19
	Overnight	2 days	No	0.88–1.12
	Overnight	4 days	No	1.00–1.35
	2 days	Overnight	No	0.87–1.33
	2 days	4 days	No	1.08–1.27
	6 days	Overnight	No	1.81–2.13
	8 days	Overnight	No	1.65–2.09
Stressed cells (5 min $H_2O_2 + UV_c$)	Overnight	Overnight	No	24.9–25.3
	Overnight	2 days	No	25.6[b]
	2 days	Overnight	No	24.1–25.5
	2 days	4 days	No	24.6–25.8
	6 days	Overnight	No	25.1–26.4
	8 days	Overnight	No	25.7–26.7

[a] "No" means HPLC analysis on the same day as enzymatic digestion, with in-between storage at 4°C. [b]Only one value available.

TABLE VIII Influence of storage between enzymatic digestion and HPLC. Data from one representative experiment

Time between enzymatic digestion completion and injection (h)	Aqueous standard (signal ratio 8-oxo-dG/dG)		Stressed sample (signal ratio 8-oxo-dG/dG)	
	Storage at 4°C	Storage at 25°C	Storage at 4°C	Storage at 25°C
0	8.59	8.59	1.83	1.83
2	8.65	8.46	1.85	1.86
4	8.56	8.55	1.82	1.80
6	8.50	8.57	1.84	1.82
8	8.74	8.70	1.82	1.87
Average	8.61	8.57	1.83	1.84
SD	0.09	0.08	0.01	0.03
RSD (%)	1.05	0.99	0.67	1.58

blank samples; a similar trend is also observed in stressed samples but to a lesser degree. Trials with storing at −80°C and addition of antioxidants (glutathione, deferoxamine and histidine), at doses found to drastically reduce the blank level in the analysis of fibroblasts DNA,[45] could not improve stability (after 3–4 days storage, 8-oxo-dG/10^5 dG = 1.6 ± 0.3; $n=7$; a very large increase in level, compared to Table VII data).

We came to realize that all these data were obtained by "careless" addition of the storage buffer to centrifugation-packed cells, provoking some cell dispersion within the buffer. In fact, cells completely suspended in buffer by vortexing and stored for one night at −80°C are already severely oxidized; this effect cannot be prevented by antioxidants (Table IX). However when packed cells (2000*g*, 2 min) are *gently* overlaid with buffer HM and stored at −80°C, sensibly reduced levels of oxidized dG were measured (Table IX). The best protection for DNA is probably to remain packed in cells during storage.

TABLE IX Influence of cells storage before extraction (unstressed cells)

Pre-freezing treatment	Pre-extraction storage[a]		8-oxo-dG per 10^5 dG (range)	*n*
	Freezing conditions	Time		
Vortexing in storage buffer	Buffer H at −80°C	Overnight	2.29–2.78	3
	Buffer HM[b] at −80°C	Overnight	2.31–2.50	3
Centrifugation packing and gentle overlaying with storage buffer	Buffer HM[b] at −80°C	Overnight	0.42	1
		2 days	0.28–0.70	2
		6 days	0.53–0.67	2
		7 days	0.61–0.75	2
		9 days	0.23–0.45	2
		15 days	0.33–0.47	2

[a]Between extraction and digestion, storage at 4°C in buffer TE, overnight; HPLC analysis on the same day as enzymatic digestion, with in-between storage at 4°C. [b]Buffer HM is buffer H supplemented with 6 mM reduced glutathione, 2 mM deferoxamine and 8 mM histidine.

DISCUSSION

Analysis of 8-oxo-dG in Cellular DNA

8-oxo-dG has been assayed by several analytical methods, including HPLC coupled to electrochemical[12] and mass detections, GC-MS,[13] capillary electrophoresis,[14,46] immunological techniques,[17,18,47,48] immunocytology,[49] fluorescence[15] or ^{32}P post-labeling.[16] The results have been reported as nmoles 8-oxo-dG per mg DNA or, most often, as 8-oxo-dG per 10^5 dG. Alkaline elution,[8] alkaline unwinding,[9] electrophoresis[10] and single cell gel electrophoresis (SCGE or "comet" assay)[11,50] after treatment with glycosylases specific for oxidative lesions have also been described. These latter techniques have shown that the real 8-oxo-dG base levels could probably be largely inferior to the levels found by other methods; this led to the conclusion that the DNA extraction and/or digestion steps might generate artifactual 8-oxo-dG from dG, apparently through a Fenton reaction.[36] HPLC-EC has been the most widely used technique for the 8-oxo-dG assay since more than 10 years and a number of publications have investigated possible optimal conditions for its application (reviewed in Ref. [51]). The method applied in this work has been retained after experimental evaluation of the following points: (i) a comparison of chloroform : isoamyl alcohol extraction with the recently proposed "pronase" method;[36,52] the latter method demonstrated no advantage, either in terms of recovered DNA, artifact formation or extraction speed; (ii) an inefficiency of RNase treatment during cell digestion; as RNA bases do not interfere in chromatography, a RNase treatment was finally deemed unnecessary; (iii) introduction of DNase I in the nuclease P1 digestion step and a comparison of digestion pH (5.4 and 7.0), according to Ref. [53]; in our hands, none of these factors influenced artifact formation or 8-oxo-dG recovery; (iv) HPLC conditions; most of the published methods separate the DNA bases on a 5 µm C_{18} reverse polarity column with mobile phases containing 5–10% methanol and slightly differing in buffer compositions; an isocratic 0.05 M phosphate and 1 mM EDTA buffer (pH "as is") with 7.5% methanol allowed an excellent resolution for dG and 8-oxo-dG with a total analysis time of only 26 min.

Validation Study for 8-oxo-dG in Cellular DNA

The study was undertaken on cell samples that have been severely stressed (20 mM H_2O_2 plus UV_C). These harsh conditions were a deliberate choice; they are known to produce numerous oxidative base lesions but also DNA–protein

adducts and dipyrimidine lesions, cyclobutane dimers and 6–4 photoproducts. This allowed to investigate the ability of the HPLC method to assay 8-oxo-dG and dG in such badly damaged DNA so to assess its suitability for the study of diverse carcinogens effects. We found, as previously reported,[34,54] that exposure to H_2O_2 alone, up to 20 mM, does not induce significant increase in 8-oxo-dG DNA content.

Application of Validation Parameters to 8-oxo-dG

Specificity

The specificity (also termed selectivity) of an analytical method is its ability to measure accurately and specifically the analyte in the presence of the components such as matrix, impurities or degradants which may be expected to be present.[19,20] For biomarkers of biomolecules, it is generally not possible to prepare a reconstituted matrix not containing the analyte; the study of specificity then entirely relies on the detector's selectivity and, depending on the detection method, the biomarker's chromatographic peak homogeneity is more or less easy to assess.

In our conditions, no interference from the cell matrix is expected as the extraction procedure is quite specific for nucleic acids; the RNA bases do not interfere in the dG and 8-oxo-dG peaks. For UV detection, the diode array study based on window evolving factor analysis, an efficient algorithm developed from principal component analysis,[37] demonstrates that the G, dG, T, A and dA peaks are essentially monocomponents, even for a severely stressed sample. For amperometry detection, the ratio of signals acquired at different potentials was compared between standards and stressed samples. This selectivity assessment technique has however many limitations: (i) it can hardly detect less than 5–10% impurities in a peak; (ii) it is largely unpractical for low levels of analytes such as 8-oxo-dG in unstressed cellular DNA; (iii) it requires large amounts of biological material. Given the peak identity and purity data for stressed samples (Table I), we can however conclude that, even if the low peak observed in blank samples were not entirely pure, the component appearing upon the applied stress is 8-oxo-dG. More efficient selectivity assessment could theoretically be performed from hydrodynamic voltamograms generated through the recent technology of coulometric electrode arrays; our preliminary trials with this detector have however demonstrated that, for low levels of analytes, the distribution of potentials among several electrodes rapidly buries the signal under background noise.

Linearity and Accuracy

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Linearity refers to the overall system response.[38] The accuracy is the closeness of test results obtained by the analytical method to the true value.[19] To take into account most of the analytical parameters, protocols recommend to spike the analytes as high as possible in the procedure. Hence our decision to spike normal bases and 8-oxo-dG before the enzymatic digestion step; this clearly presented a problem of DNA aliquoting reproducibility, more apparent for the normal bases detected in UV, due to their high endogenous levels as compared to spikes. The method achieves linearity and reasonably acceptable accuracy even under these unfavorable experimental conditions.

Precision

The precision (repeatability) of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample;[19] the repetitions allow investigation of within-day and total precisions. The

precision analysis should be performed at several concentration levels of the analyte.

For 8-oxo-dG, there are practically no precision data published; a previous study[55] on blank samples revealed inter and intra-assay coefficients of variation of about 14%, but no real statistical analysis has been undertaken. In the present study, an excellent repeatability (3% total RSD) was found for 8-oxo-dG when dG is used as an internal standard. Considering that most of the variation encountered in the assay of the bases comes from the DNA extraction step, an overall variation of about 7–9% in DNA recovery can then be estimated, this recovery being independent of stress or analysis day; this indicates that the various modifications introduced in DNA by the harsh oxidative and UV treatment do not hinder digestion by the nuclease P1.

Sensitivity

Sensitivity is the minimal variation of analyte concentration giving a detectable variation of signal. A method is said to be sensitive if small changes in concentration cause larger changes in the response function.[56] From the slope (b) of a "detector signal vs concentration" graph and the estimation of signal total variance, hence standard deviation (S_E), the sensitivity of the whole analytical procedure is computed:

$$\text{Sensitivity} = S_E/b. \quad (1)$$

The statistically significant sensitivity, that is the minimal variation of analyte concentration giving a significant variation of signal was computed as per Ref. [57] (α, bilateral; β, unilateral):

$$\text{Sensitivity} = [t_{(1-\alpha/2;N-1)} + t_{(1-\beta;N-1)}] * 2^{1/2} * S_E * (1/b). \quad (2)$$

The expression of the 8-oxo-dG content as a ratio of dG considerably improves both the precision and the sensitivity of the analytical method; due to the problems of accurately dispensing and diluting viscous DNA solutions, it is advisable to rely on dG signal rather than on UV spectroscopy to compensate for variable DNA recoveries.

Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions. Ruggedness is normally expressed as the lack of influence on test results of operational and environmental variables of the analytical method.[19]

In the present study (Table VI), the 8-oxo-dG level is the only factor affecting the analytical results; the method can then be considered rugged vs small variations in the experimental conditions.

Stability and Artifactual Formation of Analyte

For the biomarkers of oxidative stress, the ruling out of this biomarker artifactual production from the storage and analytical procedure is absolutely indispensable to investigate; this demonstration however would require that 8-oxo-dG true base levels be established by a definitive method, which still remains a considerable analytical challenge and the object of heavy controversy.[58,59]

Artifactual formation of 8-oxo-dG during storage A too long storage of samples at any step of the process may induce dG oxidation and is probably a major source of trouble; it should be noted that papers in the literature hardly ever specify storage time and conditions. The analysis time schedule proposed in this work was demonstrated satisfactory in minimizing artifacts formation.

Generation of the analyte through storage has been previously demonstrated for 8-oxo-dG[51,60] but no time schedule has been proposed to reduce this phenomenon. Metal chelation with deferoxamine has been proposed to increase stability of

digested nucleosides[61] but no data are available over 48 h. Some authors have recommended to perform individual digestion extemporaneously prior to each HPLC injection, but this is certainly excessive; HPLC is best performed on the day of digestion with an excellent stability (up to 8 h) of digested solutions stored at 4°C or 25°C.

Artifactual formation of 8-oxo-dG during samples work-up Further work is certainly needed to clarify the exact level of 8-oxo-dG in blank samples and the mechanisms of its formation in the course of storage and extraction. Trivial operations may inadvertantly induce 8-oxo-dG formation, which can be difficult to evidence. We could for example observe a contamination of digested solutions by metal ions released from an aged HPLC syringe tip; this resulted in a rapid and severe increase of 8-oxo-dG that could be monitored by repeated injections of the solution. A recent study[62] concluded that oxidation of dG during work-up could be reduced by (i) the use of chemicals low in transition metals; (ii) a cold work-up procedure; (iii) limited incubation time; and (iv) addition of a nitroxide antioxidant in all steps. The first three recommendations are already part of our current protocol that results in blank levels similar to Ref. [62]. DNA extraction by the NaI chaotropic method was recently demonstrated[51] to reduce blank levels by a factor of about 40 as compared to a phenol method. The authors claim a more consistent DNA recovery than with other methods, less artifactual oxidation and faster extraction; a validation of these procedures is however not yet available and the real impact of the method on the levels of detected 8-oxo-dG is controversed.[63] DNA recovery is indeed variable with the current method we use, but this variability is compensated for by the use of dG as an internal standard. As discussed from repair endonuclease-based tests, artifactual oxidation exact levels may be much more severe than the level reductions yielded from the chaotropic method and the problem still needs considerable testing. It should be emphasized that if the endonuclease techniques approach the real blank levels, these will be far inferior to the present detection or quantification limits of electrochemical techniques, however considerable these limits may be.

CONCLUSIONS

The analytical method for the assay of 8-oxo-dG in the DNA of suspension culture cells has been validated for the first time from the points of view of selectivity, linearity, repeatability, accuracy, ruggedness, analyte stability and limits of detection and quantification.

The identity and purity were verified for the dG and 8-oxo-dG peaks in severely stressed samples; it was demonstrated that stress level does not hinder the digestion of DNA by nuclease P1. The analytical results must be expressed relatively to the internal standard dG which significantly improves both repeatability and sensitivity. The method is robust versus small variations in HPLC conditions and the analysis time schedule proposed in this work was demonstrated satisfactory in minimizing artifactual production of the analyte from the process.

The limitations of the method were underlined, including the difficulty to assess the 8-oxo-dG peak specificity at low concentration levels and the problematics of trivial artifact formation. Based on the presented data, but reminding these limitations, we conclude that the analytical method is mostly reliable; the HPLC-EC assay of 8-oxo-dG provides consistent data allowing to reliably detect an increase of this biomarker in cellular DNA, provided that adequate blank controls are included in each run to monitor for eventual artifact formation.

A further method validation, that is inter-laboratory validation, is one of the objectives of the recently established European Standards Committee on Oxidative DNA Damage. Published preliminary data[64] have revealed huge

differences between teams measuring 8-oxo-dG and demonstrate that a consensus validated method yielding data coherent between laboratories is really needed.

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