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Review

Recent developments in analytical methodology for 8-hydroxy-2′-deoxyguanosine and related compounds

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Abstract

When biomolecules such as proteins, lipids, and DNA are subjected to oxidative attack by free radicals or other reactive species, a number of measurable biomarkers may be produced. The study of oxidative DNA damage is valuable in research concerning cancer and aging. The current review includes methodology involving various separation science techniques for the analysis of DNA oxidation biomarkers, mainly 8-hydroxy-2′-deoxyguanosine. This review will present recent analytical developments with respect to sample preparation and instrumental considerations, noting key outcomes and biological relevance where appropriate.

Keywords: Oxidative stress; 8-Hydroxy-2′-deoxyguanosine; DNA damage; Analysis

Contents

1. Introduction

The study of oxidative stress in biological systems generally involves the measurement of biomarkers that reflect damage induced from an attack by free radicals or other reactive species. Reactive species can occur from normal cellular metabolism or exogenous sources and are countered by antioxidants. When antioxidant defenses are overwhelmed, oxidative stress persists and causes damage to biomolecules, such as proteins, lipids, and DNA [1,2]. Direct measurement of reactive species and free radicals is impractical because they are short-lived, due to their highly reactive nature. Thus, biomarkers have been used to reflect the degree of oxidative damage in light of a particular clinical or research interest, i.e. disease or disorder state. Oxidative stress has been linked to neurological disorders, atherosclerosis, diabetes, cancer, and other age-related diseases [1,3,4].

Oxidative stress can lead to a variety of measurable protein modifications as well as protein carbonyl derivatives, which

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have been most commonly studied [5]. The measurement of protein markers as indicators of oxidative stress may involve several products due to the fact that there are 20 amino acids available for an oxidative attack. Therefore, there is a need for technology capable of analyzing larger numbers of protein modifications to identify which oxidative products are relevant to the desired study. In light of this need for protein modification targets, proteomic-based methods are emerging as useful techniques for exploring specific proteins as markers of oxidative damage [4,6]. The process of lipid peroxidation includes oxidative chain reactions of fatty acids, where several measurable products may be produced [3]. Among the most frequently investigated are malondialdehyde (MDA), 4-hydroxynonenal (HNE), and the isoprostanes. The prostaglandin-like isoprostanes are thought to be specific markers of lipid peroxidation since their production is non-enzymatic. F_2-isoprostanes, formed from the peroxidation of arachidonic acid, have represented the bulk of isoprostanes research [7–9]. Recently, there has been a greater focus on F_4-neuroprostanes, which originate from peroxidation of docosahexaenoic acid, the major fatty acid in the brain [10]. F_4-neuroprostanes may therefore reflect oxidative injury to nervous system tissue [11].

The role of oxidative damage to DNA is considered important in studies involving aging and the development of cancer [12,13]. An overall schematic representation of oxidative stress and DNA damage is shown in Fig. 1. Reactive oxygen species (i.e. hydroxyl radical) can alter the deoxyribose-phosphate backbone, cause DNA-protein cross-links, and modify both purine and pyrimidine bases. Repair of oxidized DNA in vivo is accomplished by glycosylases (bases) and endonucleases (deoxynucleotides). Deoxynucleotides are excreted in the urine as deoxynucleosides [1].

Guanine most readily undergoes an oxidative attack, possessing the lowest oxidation potential of the four bases. Consequently, the nucleoside 8-hydroxy-2′-deoxyguanosine (8-OHdG) is the most often studied biomarker of oxidative DNA damage [2,14]. The presence of the modified base, 8-hydroxyguanine (8-OHGua), during DNA replication can cause G:C-T:A transversion mutations. Therefore, oxidative lesions not repaired before replication can become mutagenic [15,16].

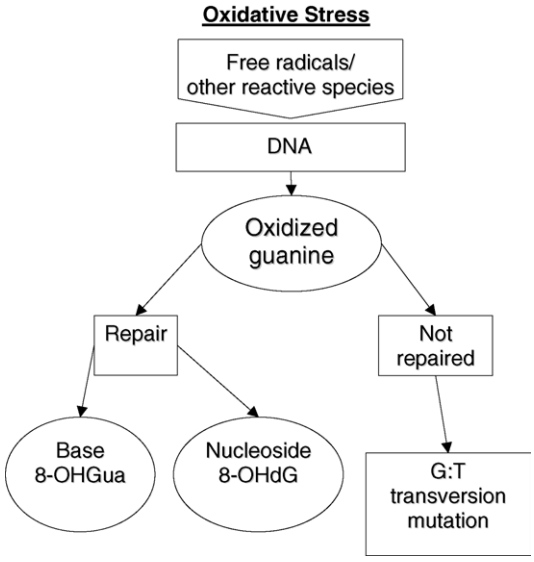

Fig. 1. Pathway of commonly measured biomarkers of oxidative stress.

In cellular DNA, detecting oxidative lesions without artificially oxidizing the normal base during sample preparation is a concern [4]. Results of 8-OHdG analysis from nuclear DNA samples such as tissue or cells are often expressed normalized to the unmodified base (8-OHdG/dG) and enzymatic DNA digestion is required to liberate and measure free 8-OHdG. Measurements of this type represent oxidative damage at the specific sampling site at the time of sampling [2].

Alternatively, analysis of 8-OHdG as a repair product in urine reflects the amount of total body oxidative DNA damage from a non-invasive sample [4]. Urinary levels of 8-OHdG in healthy human subjects have been reported at levels of approximately 10–30 nM [17-19]. Complex sample cleanup methods are often required due to interferences inherent to the urine matrix. However, once formed, 8-OHdG is a stable product and not subjected to further metabolism [2]. Furthermore, urine samples present no danger of artifactual production of 8-OHdG and do not require enzymatic digestion [19]. Table 1 summarizes urinary sample preparation procedures for methods covered in this review.

Table 1
Urine sample preparation procedures

Sample Size	Pretreatment	Analysis time or throughput	Separation or assay	Ref.
30 μL	Dilute and inject[a]	34 min run time	HPLC-EC	[35]
2 mL	Double SPE	25 min chromatogram shown	HPLC-EC	[19]
50 μL	Dilute and inject[a]	∼45 min chromatogram shown	HPLC-EC	[36]
10 mL	Single SPE	12 min chromatogram shown	HPLC-EC	[37]
Unclear	Single SPE	Unclear	CE-EC	[18]
1 mL	Single SPE	Unclear	CE-EC	[39]
2 mL	Single SPE	Unclear	CE-EC	[40]
100 μL	Dilute and inject	<15 min total analysis	CE-UV	[42]
0.8–3.2 mL	Single SPE	<10 min run time	GC/MS	[46]
3 mL	Single SPE	∼50 samples/day throughput	LC/MS/MS	[47]
15 mL, 100 μL injection	Untreated	15 min chromatogram shown	LC/MS/MS	[50]
50 μL	Untreated	3.5–4 h per 18 samples total analysis	ELISA	[b]

[a] Automated valve-initiated column switching.
[b] "New 8-OHdG Check" from Japan Institute for the Control of Aging, Fukuroi, Shizuoka (www.jaica.com/biotech/e).

In addition to DNA repair products as biomarkers of oxidative damage, the enzymes responsible for repair may be studied. For example, OGG1 (8-oxoguanine glycosylase 1) has been detected in human cells and shown to suppress transversion mutations caused by 8-OHGua [16]. Monitoring DNA repair enzymes may also provide useful biomarkers because a decreased repair capacity could result in an elevated frequency of mutations from oxidized guanine [2].

Analytical aspects of lipid peroxidation and oxidative stress biomarkers have been recently reviewed [20–22] along with the analysis of DNA adducts by separation methods [23]. The aim of the current review is to focus on progressive analytical developments for the analysis of DNA oxidation biomarkers, with emphasis on 8-OHdG.

Analytical approaches for biomarkers of oxidative damage include the following: (1) ^{32}P and fluorescent methods of labeling of nucleotides; (2) separation techniques such as high performance liquid chromatography, gas chromatography, and capillary electrophoresis; and (3) immunoassays. Key objectives have focused on achieving sensitive detection levels and improving sample preparation procedures. The normal levels of 8-OHdG in biological matrices (low nanomolar) have necessitated to use of capable detectors such as laser-induced fluorescence, electrochemical, and mass spectrometry.

2. Labeling methods with direct measurement

2.1. Radioacitve labeling with ^{32}P

Highly sensitive ^{32}P-postlabeling methods have been developed for the analysis of 8-OHdG adducts, measured as monophosphate nucleotides in biological samples. ^{32}P-postlabeling methods involve the incorporation of a radioactive label into nucleotides digested from DNA and monitoring the radioactivity as a quantitative measurement [24]. Ziesig et al. [25] reported a method intended to achieve sufficient sensitivity for the analysis small samples of blood, tissue, and needle biopsies. In this work, DNA from 5 μg calf thymus samples was enzymatically digested to nucleoside 3′-monophosphates and then enriched by HPLC separation, along with 8-OHdG standard solutions in water. The HPLC system used a 5-mM ammonium formate buffer at pH 2.5 as an isocratic mobile phase, two C_{18}-bonded phase columns connected in series, and a radioactivity detector. After enrichment, samples were ^{32}P-postlabeled at the 5′ site and treated with nuclease P1 to hydrolyze the 3′ phosphate groups, yielding nucleoside 5′-monophosphates. The samples were then isocratically separated on the same HPLC system as above, but with the buffer at pH 3.5. The 5′-monophosphates were less polar than the 3′-, 5′-bisphosphates and therefore better resolved under the reverse phase conditions. Additionally, the use of the two C18 columns in series improved the resolution of the ^{32}P-HPLC separation by increasing the efficiency. The limit of detection was reported as 0.1 8-OHdG per 10^5 dG for 1 μg of DNA. Similarly, Gupta and Arif [26] improved ^{32}P-postlabeling analysis of 8-OHdG by employing a thin layer chromatography (TLC) sample enrichment prior to labeling. Two-directional polyethyleneimine-cellulose TLC was used after the ^{32}P-postlabeling step for quantitation. The method was able to detect less than 1 8-OHdG per 10^7 nucleotides. The basal level of 8-OHdG in various rat tissue samples was demonstrated to be 0.75 ± 0.15 to 1.17 ± 0.28 8-OHdG per 10^6 nucleotides. Table 2 summarizes detection limits for various methods used to assess oxidative DNA damage.

Table 2
Summary of recent analytical methods for 8-OHdG or related DNA damage markers

Analyte	Approach	Matrix	LOD	Ref.
8-OHdG	^{32}P-labeling	Calf thymus DNA	0.1 per 10^5 dG	[25]
8-OHdG	^{32}P-labeling	Calf thymus/rat tissue DNA	<1.8 per 10^7 nucleotides	[26]
dAMP[a]	CE-LIF	Calf thymus DNA	2 Adducts per 10^6 nucleotides	[31]
8-OHdG	HPLC-EC	Neat standard	25–74 pM	[35]
8-OHdG	HPLC-EC	Aqueous standard	0.9 nM	[37]
8-OHGua	HPLC	Aqueous standard	0.6 nM	[37]
8-OHGua	HPLC	Human urine	80 nM	[37]
8-OHdG	CE-EC	Aqueous standard	50 nM	[18]
8-OHdG	CE-EC	Aqueous standard	20 nM	[39]
8-OHdG	CE-EC	Aqueous standard	4.3 nM	[40]
8-OHdG	CE-UV	Aqueous standard	0.85 μM	[42]
8-OHdG	CE-UV	Human urine	17 μM	[42]
8-OHdG	CE-UV	Aqueous standard	450 nM	[43]
8-OHdG	GC/MS-SIM	Human urine	2.5 nM	[46]
8-OHdG	LC/MS/MS-MRM	Aqueous standard	0.7 nM	[47]
8-OHdG	LC/MS/MS-MRM	Pure compound	10 fmol	[48]
8-OHdG	LC/MS/MS-MRM	Salmon testes	1 per 10^8 nucleotides	[49]
8-OHdG	LC/MS/MS-MRM	Human urine	3.5 nM	[50]
8-OHdG	LC/MS/MS-MRM	Neat standard	25 fmol on column	[51]
8-OHdG	LC/MS-SIM	Calf thymus DNA	35 fmol per 1 μg	[52]
8-OHGua	GC/MS-SIM	Calf thymus DNA	3 fmol on column	[52]
8-OHdG	LC/MS/MS-MRM	Aqueous standard	85 pM	[54]

[a] LOD reported for dAMP, but technology was also used for 8-OHdGMP.

Since β radiation from ^{32}P may oxidize normal nucleotides, artifactual production of 8-OHdG in the presence of the dG is possible, and thus separation of the two prior to ^{32}P-postlabeling could be fundamental. Thus, sample enrichment addresses the nonspecific oxidation artifact by removing the normal nucleotides.

2.2. Fluorescent labeling

Fluorescence postlabeling assays have been used to detect biomarkers of DNA damage, offering a safer alternative to ^{32}P-postlabeling methods. Sharma et al. [27] applied HPLC separation with dansyl chloride postlabeling for the analysis of 8-OHdG-5′-monophosphate in calf thymus after X-irradiation. For a 100-μg DNA sample, the detection limit was 1 adduct per 10^6 normal nucleotides. Fluorescein isothiocyanate (FITC) labels have also been used for the analysis normal and modified nucleotides and compared to the dansyl chloride label using high performance liquid chromatography [28]. The labeling process involves generating a phosphorimidazolidate from the nucleotide, which when treated with either ethlyenediamine (EDA) or hexamethyldiamine (HAD) forms a 5′-phosporamidate. Both dansyl chloride and FITC will react with the free amino group to yield fluorescent nucleotide conjugates. It was determined that the dansyl chloride-labeled was the better label in terms of the chromatographic separation. The fluorescein isothiocyanate-labeled nucleotides were difficult to resolve due to interferences from excess labeling reagents.

More recently, capillary electrophoresis with laser-induced fluorescence (CE-LIF) has been employed for the study of DNA adducts [29,30]. Schmitz et al. [31] have developed a CE-LIF method for studying a range of DNA adducts as carcinogenesis biomarkers, including 8-OHdG-3′-monophosphate (8-OH-dGMP). The fluorescent label, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionylethylenediamine (BODIPY FL EDA), was conjugated to the phosphate group of deoxynucleoside-3′-monophosphates, after DNA hydrolysis. The detection limit for this method was reported to be 2 DNA adducts per 10^6 normal nucleotides, but an improvement to 1.4 adducts per 10^7 normal nucleotides was proposed through focusing samples via electrostacking.

Although labeling methods to detect DNA damage are highly sensitive, they often require multiple reaction steps to form detectable nucleotide conjugates. These reactions and additional purification procedures can add to the preparation and analysis time, and in the case of ^{32}P labels, pose a health hazard.

3. Separation methods

3.1. High performance liquid chromatography

One of the more commonly used methods of measuring 8-OHdG levels involves HPLC separation with coulmetric or amperometric electrochemical detection (HPLC-EC). Recent methodology focuses on simplifying and expediting sample cleanup procedures for urine and reducing artifactual oxidation for DNA digests [32–34]. These issues have been addressed by employing various solid phase extraction (SPE) columns, immunoaffinity columns, and column-switching techniques [17,35,36]. The latter methodology captures a fraction corresponding to 8-OHdG on the first column, which is then eluted to and chromatographically separated on the second column. A number of columns have been used for this technique, including anion and cation exchange and reverse phase columns such as C_8 and C_{18}.

A thorough 1-year study was presented by Bogdanov et al. [35] for analyzing 8-OHdG in a variety of biological matrices using a unique and highly specialized HPLC-EC coulometric method. An injected sample fraction containing 8-OHdG was eluted from a C_8-bonded silica column in a mobile phase of pH 6.4 0.1 M lithium acetate with 4% methanol and valve-switched to two treated porous carbon columns in series, where the mobile phase was pH 3.3 0.1 M lithium acetate with 4.5% acetonitrile. Next the peak was eluted in the same mobile phase with the addition of adenosine at 1.5 g/L. The adenosine caused the release of 8-OHdG from the carbon columns and the final separation step was completed on a C_{18}-bonded phase silica column. The system relied upon specially treated carbon columns, which showed unique selectivity for nitro-substituted aromatics and purines. Thus, the serial carbon columns retained 8-OHdG while interferences were eluted. This selectivity hindered the use of an internal standard, as it would have needed to mimic 8-OHdG throughout the separation process. It was therefore suggested that an isotope-labeled internal standard may prove useful. It was also noted that although sample pre-treatments by various solid-phase extraction (SPE) columns (anion, cation, C_{18}, C_8, and immunoaffinity) simplified the chromatograms for HPLC-EC methods, several interfering peaks were still present in urine with varying degrees for each patient tested.

The importance of randomly collected urine samples versus 24-h urine collection in human subjects was recently evaluated using HPLC-EC [19]. The samples were subjected to two SPE treatments with C_{18}-OH cartridges and then isocratically separated on two C_{18}-bonded analytical columns in series in a mobile phase consisting of pH 3.5 50 mM phosphate buffer, 2.5% acetonitrile, and 1% methanol. Additionally, urinary creatinine was measured for the samples using a commercially available kit (Merck). Detection was achieved with an amperometric detector including a glassy carbon working electrode at 0.6 V versus a Ag/AgCl reference electrode. Even after two SPE steps urine matrix contaminants were still present and dependent upon creatinine level. Spot urine samples versus 24-h urine samples were tested for non-smokers and smokers. While no statistical difference in 8-OHdG levels was found in the subjects for spot urine collections, non-smokers demonstrated a significantly lower level of 8-OHdG than smokers for 24-h urine samples. Two subjects were tested for 24-h urine 8-OHdG levels over 10 days and the intra-individual variations were 37 and 57%. This study suggests that one should consider intra-individual variations, creatinine levels, and the method of sample collection when interpreting results for 8-OHdG levels in urine.

Kasai [36] has identified nine methods for urinary 8-OHdG analysis and explained why each is not widely accepted for routine analysis. Briefly, it was noted that immunoaffinty columns

used for sample enrichment gave inconsistent recovery and were not commercially available. The main criticism of HPLC-EC methods employing multiple column switching techniques was that the timing of the 8-OHdG fraction elution changed on different days and was dependent on the sample injected. Methods using mass spectrometry required an isotopically-labeled internal standard, which was commercially unavailable. Finally, an enzyme-linked immunosorbent assay produced higher results as compared to HPLC-EC. Along with those observations, a new method was introduced for automated analysis of 8-OHdG in urine by HPLC-EC utilizing two separation columns and valve-switching. The ribonucleoside 8-hydroxyguanosine (8-OHG) was added to urine samples during preparation as a marker peak. The samples were injected onto a guard column and the first column, both packed with anion exchange resin and maintained at 65 °C, in a mobile phase of 0.3 mM sulfuric acid containing 2% acetonitrile. The eluent from the first column was monitored using a UV detector at 254 nm to record the 8-OHG marker peak. Based on the elution time of the marker, a fraction containing 8-OHdG was collected and injected onto a second column of C_{18}-bonded polymer-coated silica maintained at 40 °C. The mobile phase for separation on the second column was pH 6.7 10 mM phosphate buffer, 5% methanol, and 100 μL/L of an antiseptic reagent. Since 8-OHdG eluted 4–5 min after the marker, this system was able to precisely collect the desired fraction for further separation. The combination of the anion exchange column and the 65 °C temperature for the first column allowed most urinary contaminants to pass through the system before 8-OHdG. Selectivity was further enhanced by the use of dual electrochemical channels (280 and 350 mV), which enabled recording of peak height ratios for 8-OHdG. The total analysis time for one sample was approximately 1 h.

The simultaneous detection of the nucleoside 8-OHdG and base 8-OHGua was demonstrated in the presence of uric acid [37]. Uric acid poses a potential interference with 8-OHGua due to similarities in structure and is present in urine at approximately 0.5 mM. A one-step SPE method was developed for sample cleanup and C_{18} cartridges with endcapped and non-endcapped silanol groups were evaluated. An excess of uric acid (0.6 mM) was spiked into standard solutions of 8-OHdG and 8-OHGua prior to SPE to optimize the separation conditions. The procedure was carried out with, and without, the addition of uricase to enzymatically degrade uric acid. After SPE, samples were isocratically separated on a C_{18}-bonded silica guard and analytical column using a mobile phase of 50 mM phosphate buffer (pH 6.1), 2 mM KCl, and 6% methanol. The signal was monitored by both photodiode array and electrochemical detection, with HPLC-EC providing superior sensitivity. The detection system used a glassy carbon working electrode, a stainless steel auxillary electrode, and an in situ Ag/AgCl reference electrode. The working potential was 0.5 V versus the in situ Ag/AgCl reference electrode. Prior to SPE, the 8-OHGua peak eluted on the shoulder of the uric acid peak. The SPE treatment alone removed 97% of uric acid, although the remaining 3% still produced an appreciable signal. SPE of the uricase-spiked solutions completely removed uric acid and did not affect 8-OHdG or 8-OHGua. The recovery of 8-OHdG post-SPE compared to pre-SPE was 65% and increased to 71% with the uricase addition. It was also determined that while the endcapped SPE cartridge removed more urine interferences, more 8-OHGua was also removed. The limit of detection for standard solutions of 8-OHdG and 8-OHGua in phosphate buffer was 0.9 and 0.6 nM, respectively. When a calibration curve was constructed by spiking urine with 8-OHGua, the limit of detection was increased to 80 nM. This reflects the increased background from the urine matrix.

Bolin et al. [38] have developed a novel DNA extraction method for analyzing 8-OHdG and the repair enzyme 8-oxoguanine DNA glycosylase 1 (Ogg1) from the same sample. Mouse cerebral cortex tissues were separated into two equal portions for separate sample preparations. Samples were subjected to a DNA glycosylase extraction procedure that yielded a supernatant containing the repair enzyme. The supernatant was exposed to a double-stranded probe incorporated with 8-OHdG. The ability of the mouse enzyme mOgg1 to repair the oxidized lesion was considered a measurement of enzyme activity. Activity was expressed as the amount of cleaved substrate per total substrate. To measure 8-OHdG, DNA in the remaining tissue sample was extracted, purified, and subjected to enzymatic hydrolysis. The extracted DNA was hydrolyzed into deoxynucleotides by digestion in sodium acetate, followed by separate incubations with nuclease P1 and alkaline phosphatase. The samples were then lyophilized and reconstituted in a mobile phase of 100 mM acetate buffer (pH 5.2) with 4% methanol. Both 8-OHdG and 2-deoxyguanosine (dG) were analyzed and levels were reported as a molar ratio of 8-OHdG to dG. The system involved isocratic high performance liquid chromatography separation on a reverse phase analytical column with an electrochemical detector capable of measuring multiple voltages as separate channels. Calibration and measurement of 8-OHdG was calculated using peak areas from a 250 mV channel, while dG was measured from the sum of peak areas in the 850, 890, and 900 mV channels. Channel voltage was important for optimal response, particularly for 8-OHdG. For voltages greater than 250 mV, interferences were present that would co-elute with the 8-OHdG peak. A second sample portion was prepared as above, but with the addition of the antioxidant 2,2,6,6,-tetramethlypiperidine-*N*-oxyl (TEMPO). The use of TEMPO did not result in a significant reduction in the levels of 8-OHdG, indicating that artifactual production was minimized during the sample preparation procedure. This method allows for the measurement of both 8-OHdG and repair enzyme activity, making it possible to study the relationship between the two from a single sample.

3.2. Capillary electrophoresis

Capillary electrophoresis (CE) has been applied as a separation approach for the analysis of 8-OHdG as an alternative to HPLC methods, which may involve column switching techniques [18,39]. Greater separation efficiency is expected with capillary electrophoresis, which is favorable for 8-OHdG analysis in complex biological matrices where interferences may complicate the assay [40]. However, there are inherent disadvantages in capillary electrophoresis concerning concentration

sensitivity and detection limits, particularly for UV detection. The small volumes used for detection in CE result in lowered concentration sensitivity and reduced limits of detection when compared to HPLC. Sample concentration techniques and sensitive detection modes have been employed for the analysis of 8-OHdG using capillary electrophoresis [41]. Detection modes used in CE include electrochemical (CE-EC), UV (CE-UV), and laser-induced fluorescence of the labeled nucleotide (discussed above).

Weiss and Lunte [18] have developed a CE-EC method for the detection of 8-OHdG in urine using a single step sample cleanup. Urine samples were extracted using a C_{18} SPE column treatment, which concentrated the sample 20-fold prior to injection. Samples (2 mL) were added to the SPE columns, washed with water, and eluted in a methanol/water solvent. Extracts were dried and reconstituted in 100 μL of water to concentrate the samples. The samples were then split into two 50 μL portions, one spiked with 8-OHdG. The 8-OHdG peak in the matrix was confirmed by comparison with the 8-OHdG-spiked sample portion. The amperometric detection system was comprised of a platinum wire auxiliary electrode, a KCl Ag/AgCl reference electrode, and a carbon fiber working electrode housed in a cast-nafion end column decoupler. Analysis parameters were optimized to achieve the best separation of 8-OHdG from matrix peaks in first morning urine samples. The choice of background electrolyte (BGE) was optimized for the reduction of noise/electrophoretic current and quality of separation for the 8-OHdG in the matrix. It was noted that increases in electrophoretic current of the BGE would cause an increase in the amount of detector noise and negatively affect the limit of detection. Therefore, an ideal BGE would have the lowest amount of electrophoretic current while providing adequate separation. Zwitterionic buffers provided lower noise, but poor separation versus a borate buffer. Capillary length was varied and it was demonstrated that longer capillaries gave a lower noise. An organic modifier (methanol) was added to the BGE and found to enhance the separation of 8-OHdG from the matrix, although the run time increased due to slowed electrosmotic flow. The final optimized CE-EC conditions included a BGE of 20 mM borate/20% methanol, pH 9, with an 89 cm × 50 μm uncoated silica capillary and an applied potential of 0.5 V. The limit of detection for an aqueous standard was 50 nM and recovery in the urine matrix was 56% over a concentration range of 63 nM to 1.25 μM. The detection limit in real urine samples was not reported.

Mei et al. [39] developed a CE-EC method with end-column amperometric detection and a sample focusing technique to enhance the concentration sensitivity of urinary 8-OHdG. Sample cleanup was performed with C_{18} and C_{18}/OH SPE cartridges and a 10-fold concentration was obtained prior to injection. C_{18}/OH cartridges were chosen as the final sample cleanup because better recoveries were observed versus C_{18} cartridges. It was proposed that hydrogen bonding of the OH group of 8-OHdG with the C_{18}/OH packing allowed for selective retention. The working electrode was a laboratory-constructed carbon fiber microcolumn electrode and the reference electrode was a saturated calomel electrode (SCE). The samples were focused using a dynamic pH junction, a technique that employs different pH values for the sample and BGE zones. If an analyte has appropriate functional groups, it will travel with different velocities in the sample and BGE. The sample buffer was maintained at pH 6.5, where 8-OHdG exists as a neutral compound. The pH of the 30 mM borate buffer BGE was varied to optimize the focusing of 8-OHdG. The highest peak current and number of theoretical plates was observed at a BGE pH of 8.15; however, in urine 8-OHdG was not separated from the matrix interferences. A final BGE pH of 9.12 gave the best separation in urine, but pH values of 8.5 and 9.18 yielded poor separations. Therefore, this method was very sensitive to minor pH changes of the BGE. Other final parameters included a 75 cm × 25 μm uncoated fused-silica capillary and a detection potential of 0.8 V versus the SCE. The limit of detection was 20 nM and urinary recovery was 99.36% for a concentration range of 10–100 nM. The utility of this method for urinary detection of 8-OHdG was demonstrated for healthy individuals and cancer patients (unspecified cancer) in morning urine. The average concentration of 8-OHdG in cancer patients was significantly higher than in healthy subjects, 35.26 ± 27.96 nM versus 13.51 ± 5.08 nM average, respectively.

Yao et al. [40] applied CE with end-column amperometric detection and sample focusing for the determination of urinary 8-OHdG in smokers and non-smokers. The group has reported the carbon fiber microcolumn electrode previously employed was both complicated to fabricate and short-lived. As an alternative, a carbon fiber microdisk electrode was used as the working electrode. Whereas the carbon fiber electrode was inserted into the separation capillary, the carbon fiber microdisk electrode is simply placed at the end of the capillary. The auxiliary electrode was a platinum wire and the reference was a SCE. Samples were prepared with a single step SPE using C_{18}/OH cartridges and a 20-fold concentration was achieved. Urinary creatinine levels were also determined for reporting purposes. Separation conditions were optimized with respect to response and resolution of 8-OHdG from sample matrix interferences. The dynamic pH junction focusing method was applied, with the samples at pH 6.5 and the borate BGE at pH 9.10. The capillary was an 85 cm × 25 μm uncoated fused-silica capillary and the detection potential was 0.45 V versus the SCE. A detection limit of 4.3 nM was measured for an aqueous standard and a calibration curve from 20 nM to 10 μM was linear. Overall, smokers showed a significant increase in 8-OHdG over non-smokers, both in units of nM and μg/g of creatinine. Those smoking less than 10 cigarettes per day showed no difference from non-smokers for 8-OHdG levels expressed as nM concentrations, but a significant increase was observed for 8-OHdG expressed per grams of creatinine.

Although electrochemical detection provides a sensitivity about three orders of magnitude greater than UV detection, some groups have applied the latter for analysis of 8-OHdG. As mentioned earlier, due to the low concentration sensitivity associated with capillary electrophoresis, sample concentration or enhanced sensitivity detectors are required to detect low levels. Kvasnicova et al. [42] have used a CE-UV method for untreated urine in oncological patients treated with radiation therapy. The limit of detection was found to be 17 μM in urine

and concentrations were determined by the method of standard addition. This method was reported to be suitable for measuring levels of 8-OHdG where high levels are anticipated, such as the oncological patients treated with radiation. Strein et al. [43] developed a micellar electrokinetic capillary chromatography method with UV detection and electrophoretic sample stacking to improve the concentration sensitivity. Sulfate was added to sample injection plugs and served as a high mobility anion to allow for stacking. Cholate micelles were formed from the addition of sodium cholate to the separation buffer. The pH of the separation buffer was 11 and the samples were at pH 4.0. Without sample stacking, the limit of detection for 8-OHdG in aqueous standard solutions was 9 μM. For a 40-s electrokinetic injection with detection at 254 nm, a 450-nM limit of detection was achieved using the stacking technique. The authors propose that future endeavors will include the analysis of biological samples with this methodology for capillary and microchip formats.

Electrochemical detectors serve as sensitive alternatives to optical methods for the measurement of 8-OHdG using capillary electrophoresis. Sample concentration methods have also been used to increase sensitivity, including sample preparation treatments and electrophoretic stacking or focusing techniques. It may be concluded that ultraviolet detection is a poor choice for the analysis of 8-OHdG by CE where monitoring low nM concentrations is required.

3.3. GC or LC coupled to mass spectrometry

Mass spectrometry (MS) coupled with either HPLC or gas chromatography (GC) has provided a sensitive means of detection for oxidative DNA damage, capable of providing structural information. Unlike HPLC methodology, samples must be derivatized before analysis by GC/MS, a procedure that has been evaluated for potential artificial oxidation products. Recent reviews have detailed the role of HPLC/MS methods for oxidative stress biomarkers [44], as well as MS measurements specifically for products of oxidative DNA damage [45]. For 8-OHdG LC/MS/MS methods, the ion at *m/z* 168 is commonly monitored and represents a loss of the deoxyribose moiety from the molecular ion $[M + H]^+$ at *m/z* 284.

A new GC/MS method was developed for urinary 8-OHdG with a single step sample cleanup intended for high-throughput analysis and large-scale studies [46]. Creatinine levels in urine were measured using a commercially available kit (SIGMA Diagnostics) and the creatinine concentrations were used to adjust the volume of urine for the 8-OHdG assay. The amount of urine was increased with the degree of decreasing creatinine amounts. This procedure accounted for intra- and inter-day variability in urine concentration. An isotopically-labeled 8-OHdG analog, [^{18}O]8-OHdG, was added to samples as an internal standard. Samples were acidified with formic acid and eluted through hydrophilic-lipophilic balance reversed-phase SPE cartridges (Waters Oasis). After freeze-drying, the samples were solubilized and then derivatized at room temperature in bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane. GC/MS was performed using selective ion monitoring (SIM) and subject results were calculated as the ratio of the *m/z* 383 8-OHdG ion to the *m/z* 385 [^{18}O]8-OHdG ion. A calibration curve was linear from 2.5 to 200 nM and the limit of quantification was 2.5 nM. Variability was evaluated for different samples taken within the same day ($n = 5$) and on five different days. Results were normalized to creatinine levels and the coefficient of variation (CV) was less than 9% for both inter- and intra-day 8-OHdG amounts. Two of the 14 samples collected were not measured because of interferences present at the *m/z* 383 channel. In fact, 10–20% of the samples contained co-eluting contamination with 8-OHdG for the 1000 samples evaluated during method development. The same interferences would usually not appear on different sampling days for the same subject and therefore were determined not to be subject-specific. Random sample urine measurements were taken over 7 days for four subjects and the CV was found to be 9.0–22.1%. H_2O_2 and/or dG were added to urine to show whether or not artifactual oxidation occurred using the method. The presence of urinary H_2O_2 did not significantly increase 8-OHdG levels. Moreover, samples spiked with only dG also had no significant effect on 8-OHdG levels, indicating that artifactual production did not occur to a significant degree during the derivatization step.

Urinary 8-OHdG has been measured with a single SPE procedure and LC/MS/MS [47]. After SPE extraction on LiChrolut EN cartridges (Merck), samples were injected onto a C_{18}-bonded phase column, with gradient elution into a triple stage quadrupole MS and detected by multiple reaction monitoring (MRM) of ions transitioning from *m/z* 284 to *m/z* 168. The mobile phase was a 10 mM acetate buffer (pH 4.3) with a gradient of methanol from 1 to 80% and was pumped at 0.2 mL/min. The limit of detection for aqueous standards was 0.2 ng/mL (0.7 nM) and urine results were expressed normalized to creatinine concentrations. An important observation of this work was the production of 8-OHdG from oxidation of dG in the electrospray ion source. The method adequately separated dG from 8-OHdG chromatographically, a necessity considering the artificial oxidation. The method relied on external standards for calibration. Intra-individual (variation in same subject over different measurements) and inter-individual (variation between different subjects) variation of human urinary 8-OHdG concentrations was measured at six time-points over 5 days and found to be about 70% for both.

Frelon et al. [48] measured several products of DNA damage from γ-radiation treatment, including 8-OHdG, in cellular and isolated DNA. LC/MS/MS with electrospray ionization was used in the MRM mode. For 8-OHdG, the *m/z* transition of 284.1 → 168.0 was monitored and the *m/z* 289.1 → 173.0 transition was monitored for the [$^{15}N_5$]-8-OHdG internal standard isotope. Six oxidized nucleosides were eluted from a C_{18}-bonded silica gel column using a gradient of acetonitrile in 2 mM formate buffer at 0.2 mL/min. Evaporation of the electrospray was enhanced with the introduction of methanol to the column outlet. Using the positive detection mode the limit of detection for 8-OHdG was reported as 10 fmol. The method was capable of analyzing 200 μg of cellular DNA and 10 μg of isolated DNA. The use of the isotopically-labeled internal standard and monitoring of specific MRM transitions enabled selective detection of multiple DNA lesions, including 5,6-

dihydroxy-5,6-dihydrothymidine, 5-hydroxy-2′-deoxyuridine, 5-(hydroxymethyl)-2′-deoxyuridine, 5-formyl-2′-deoxyuridine, 8-oxo-7,8-dihydro-2′-deoxyadenosine, and 8-OHdG. HPLC-EC was also used to detect 8-OHdG using isocratic separation on a C_{18}-bonded silica gel column with coulometric detection at two channels, 200 and 450 mV. A 10-fold increase in sensitivity was claimed when using the LC/MS/MS method versus the HPLC-EC.

Churchwell et al. [49] have detected four oxidative stress-induced DNA adducts in human and rat liver using a LC/MS/MS method. The system used electrospray ionization in the positive mode, monitoring MRM transitions of m/z 284 → 168 for 8-OHdG and m/z 289 → 168 for the isotope-labeled internal standard, [$^{13}C_5$]-8-OHdG. DNA isolation procedures were all performed below 4 °C to reduce artifactual 8-OHdG production; however hydrolysis was performed at 37 °C. After DNA isolation and hydrolysis, samples were injected onto a C_{18}-bonded silica column trap with a 10 mM pH 7 acetate solution at 0.75 mL/min to retain the adducts and wash contaminants to waste. The desired sample plug was back flushed to a C_{18}-bonded silica analytical column via valve-switching to a gradient of 5–50% methanol in water at 0.2 mL/min and then eluted to the mass spectrometer. The limit of detection for 8-OHdG was determined in DNA from commercial salmon testes as <1 pg, which represents 1 adduct per 10^8 normal nucleotides. There was a discrepancy in the amounts of 8-OHdG in DNA from rat livers depending on the storage, although no antioxidants were added to the stored samples.

Urinary 8-OHdG was measured by LC/MS/MS with atmospheric pressure chemical ionization (APCI) in subjects in order to study the effect of the intake of tomato concentrate on 8-OHdG levels [50]. Twelve healthy subjects submitted 24-h urine at 0, 7, and 21 days after tomato concentrate supplementation. The sample preparation involved acidification and centrifugation of the urine prior to injection. Isocratic separation was achieved on a C_{18}-bonded silica column in a mobile phase of water containing 10% methanol and 0.025% trifluoroacetic acid, with the first 8 min eluting to waste. Data was collected in both SIM and MRM modes while the source temperature was 180 °C and the corona discharge was set to 6 kV. For the single ion monitoring mode the m/z 284 ion was monitored, as identified in standards. In urine, matrix interferences complicated the use of the ion at m/z 284 and the limit of detection was ∼10 ng/mL. In the MRM mode, the ion at m/z 168 was monitored and no matrix interferences were found. A calibration curve from 1 to 50 ng/mL was injected and the limit of detection was found to be ∼1 ng/mL (3.5 nM). The mean 8-OHdG levels in subjects taking tomato concentrate was reduced from an initial value of 9.4 to 3.8 ng/mL after 21 days.

Singh et al. [51] have used an immunoaffinty column with LC/MS/MS in the MRM mode for the analysis of 8-OHdG in DNA samples. Calf thymus DNA was prepared for analysis as an unmodified control and modified with methylene blue and halogen light. The methylene blue treatment was used to oxidize the guanine base present in the DNA samples and provide a sample with elevated 8-OHdG versus the control. The isotopically-labeled [$^{15}N_5$]-8-OHdG internal standard was added to 50 μg DNA samples, which were then enzymatically digested to deoxynucleosides. Standards and digested samples were purified on an immunoaffinity column containing the monoclonal 1F7 antibody to 8-OHdG. The purified fractions were separated using isocratic elution on a microbore C_{18}-bonded phase silica column at 50 μL/min in a mobile phase of 0.1% acetic acid with 10% methanol. The calf thymus DNA samples were also analyzed using the system without the immunoaffinty step. Electrospray ionization with positive ion MRM was employed to monitor 8-OHdG transitions of m/z 284 → 168 and [$^{15}N_5$]-8-OHdG internal standard transitions of m/z 289 → 173. Three additional deoxynucleosides other than 8-OHdG were present and confirmed with primary standards. A large peak was observed for 2′-deoxyadenosine (dA) in the multiple reaction monitoring (MRM) mode at m/z 168, an interfering compound potentially formed from an adduct with methanol from the mobile phase. Introducing the immunoaffinity column for sample purification effectively removed unmodified deoxynucleosides, yielding a single peak for 8-OHdG in the ion chromatogram. For 5 μg of immunoaffinity purified DNA, the level of 8-OHdG in the control was 28.8 ± 1.2 per 10^6 normal nucleotides. The method initially included a narrowbore HPLC C_{18}-bonded phase silica column, which when replaced with the microbore column provided a limit of detection of 25 fmol on-column for 8-OHdG. It was observed that the reduced column inter diameter produced a more concentrated 8-OHdG peak in the electrospray source. The combination of the 8-OHdG antibody in the immunoaffinity column coupled with LC/MS/MS and the use of an isotope-labeled internal standard was reported to provide both specificity and accuracy of the method.

The determination of 8-OHdG and the base 8-OHGua were applied to compare LC/MS and GC/MS in the SIM mode [52]. For LC/MS SIM analysis, DNA was isolated from calf thymus samples and subjected to hydrolysis by four enzymes, liberating nucleosides of the four bases. Electrospray ionization in the positive mode was used to monitor 8-OHdG and the internal standard [^{18}O]8-OHdG at m/z 168 and 170, respectively. For GC/MS SIM analysis, the oxidized base was excised from sample DNA by hydrolysis with formic acid and, for comparison, *Escherichia coli* Fpg protein. After hydrolysis, samples were converted to trimethylsilyl derivatives, with the 8-OHGua derivative at m/z 455. The limit of detection using LC/MS for a 1-μg sample injection of DNA was ∼ 35 fmol for 8-OHdG. GC/MS analysis of a 0.1-μg sample injection resulted in a limit of detection of approximately 3 fmol for the trimethylsilyl derivative of 8-OHGua. The concentrations of 8-OHdG by LC/MS and 8-OHGua by GC/MS in calf thymus samples were similar. This suggested that the derivatization step necessary for GC analysis did not contribute artifactual oxidized products. The use of Fpg protein instead of formic acid for DNA hydrolysis does not excise intact bases from the DNA samples. Hence, the Fpg protein hydrosylates did not contain guanine and could not artifactually form the oxidized base. Hydrolysis by the Fpg protein and formic acid gave similar results for 8-OHGua, further supporting the formic acid hydrolysis of DNA for GC/MS. It was concluded that the analysis of 8-OHdG by LC/MS and 8-OHGua by GC/MS provided comparable results from calf thymus DNA samples.

4. Immunoassays

Enzyme-linked immunosorbent assays (ELISA) have received significant attention as an alternative means for the analysis of 8-OHdG due to the simplicity of the assay versus HPLC-EC and LC/MS/MS methods [53,54]. In fact, both polyclonal and monoclonal antibodies are commercially available. The monoclonal antibody N45.1 has a high degree of specificity for 8-OHdG and has been used in the ELISA procedures described below [55].

Shimoi et al. [53] compared an HPLC-EC method and ELISA for quantitative analysis of urinary 8-OHdG. The HPLC analysis consisted of automated sample injection onto a first column, which had reverse phase, ion exchange, and gel filtration characteristics. The isocratic elution used a mobile phase of 0.1% acetic acid at 1.0 mL/min. A 100-μL fraction containing 8-OHdG was then automatically injected onto a C_{18}-bonded phase endcapped silica column and isocratically eluted using 35 mM acetate and 12.5 mM citric acid (pH 7.5) with 5% methanol. Detection was achieved with an electrochemical detector and dual channel monitoring at 150 and 300 mV. A commercial ELISA kit containing the N45.1 antibody was used for urine samples with absorbance detection at 492 nm and a determination range of 0.5–200 ng/mL. The ELISA was also carried out using urine that was purified by the first column using the HPLC-EC system. All results were normalized to μg 8-OHdG/g urinary creatinine. When using the HPLC-purified samples, the HPLC-EC and ELISA methods provided similar mean values and were reasonably well correlated ($r = 0.833$). For unpurified urine however, the ELISA results were two-fold higher than those from the HPLC-EC method and the correlation was reduced to $r = 0.550$. The authors suggested that untreated urine contained cross-reacting species with the ELISA antibody and there was a need to purify the samples. Urine may contain modified forms of 8-OHdG or other species that are structurally similar to 8-OHdG and would compete for antibody recognition.

Hu and colleagues compared an LC/MS/MS method to a commercial ELISA kit for analysis of 8-OHdG from urine of workers exposed to polyaromatic hydrocarbons (PAH) [54]. The LC/MS/MS analysis used an isotopically-labeled internal standard, [$^{15}N_5$]-8-OHdG. Samples were extracted with a C_{18}-bonded SPE cartridge treatment and injected onto a polyamine-bonded silica gel HPLC column at 0.3 mL/min using isocratic elution in 0.1% formic acid with 80% acetonitrile. Positive mode electrospray ionization enabled monitoring of ions from m/z 284.1 → 168.0 for 8-OHdG and m/z 289.1 → 173.0 for [$^{15}N_5$]-8-OHdG. The calibration curve of aqueous standards was linear from 0.75–12.02 ng/mL and the instrumental limit of detection was 0.024 ng/mL (85 pM). The ELISA kit employed the N45.1 monoclonal antibody and the standard curve was from 0.5 to 200 ng/mL, with absorbance detection at 450 nm. For both methods of analysis, 8-OHdG/creatinine levels in urine were higher in workers exposed to PAHs versus controls. The difference was only statistically significant for the LC/MS/MS method and, moreover, the ELISA results were about two-fold greater for all samples. Again, cross-reactive components in crude urine were cited as the chief reason for insufficient specificity of the ELISA assay.

Both of the papers discussed above used the "New 8-OHdG Check" from the Japan Institute for the Control of Aging (Fukuroi, Shizuoka, Japan). Instructions for use and details of the kit can be found on the company website (www.jaica.com/biotech/e). For one kit, the time of analysis is listed as 3.5–4 h with a capacity of 18 samples run in triplicate. The current price is $795 per kit. A similar kit with the same concentration range (0.5–200 ng/mL) is available at this time for $795 from Oxis International, Inc., Portland, OR (www.oxisreserach.com). A more sensitive ELISA kit is available from the Japan Institute for the Control of Aging called "Highly Sensitive 8-OHdG Check". This kit measures from 0.125 to 10 ng/mL and is currently priced at $762. However, unlike the other kits, the primary antibody reaction requires an overnight incubation. Thus, the increased sensitivity comes with an increased time of analysis.

5. Concluding remarks

Methods for the analysis of 8-OHdG as both a repair product in biological fluids or liberated from intact DNA have been presented. The sample matrix and choice of measurement can significantly affect the interpretation of clinical data. The decision to monitor 8-OHdG as the repair product or digested from DNA samples should be relevant to the study of interest. For example, the presence of 8-OHdG in urine should represent the amount of repaired oxidized guanine, with increased levels indicative of increased oxidative stress in general. This assumption only holds true if the repair capacity itself has not been compromised [19]. Lovell et al. [56] demonstrated that free 8-OHdG levels were lower in cerebral spinal fluid for Alzheimer's disease (AD) patients as compared to control subjects. The levels of 8-OHdG from intact DNA in the same samples however, were significantly elevated for the AD patients versus controls. These results indicated that repair of oxidized DNA bases had declined, and therefore the measurement for the free repair product would not be elevated. It may therefore be important for future clinical studies to analyze 8-OHdG from intact DNA and as the free repair product to monitor the balance between oxidative damage and repair.

Several separation approaches are available for a variety of matrices for the analysis of oxidative damage to DNA. Nucleotides of 8-OHdG have been measured with ^{32}P and fluorescent labeling techniques. Although these methods offer high sensitivity, they require additional reaction steps to form the labeled conjugates and can also be hazardous (^{32}P). HPLC with electrochemical detection is one of the most commonly used analytical methods for 8-OHdG. However, these approaches often use complex column switching and require thorough sample cleanup, particularly for urine. Capillary electrophoresis provides high separation efficiencies with small sample sizes, but also has low concentration sensitivity and reduced limits of detection. Sensitive detection options such as electrochemical and laser-induced fluorescence detectors have improved the sensitivity for the CE analysis of 8-OHdG. Mass spec-

trometry with HPLC or GC has proven to be both sensitive and selective for monitoring DNA oxidation. The MS methods covered have employed the use of isotopically-labeled internal standards, which required laboratory preparation. Immunoassays and immunoaffinity chromatography have been used for analysis of 8-OHdG with selective antibodies. Although tests such as ELISA offer simplicity, all immunoassay-based methods are limited by the selectivity of the antibody. It has been shown that the sample matrix may contain interferences capable of competing for antibody recognition. ELISA methods allow for small sample sizes and the total time of analysis is only about 4 h for standards, blanks, and 18 samples. However, the throughput becomes limited with the use of more sensitive kits.

As the importance of investigating biomarkers of oxidative stress has increased, novel methods of analysis have been explored to facilitate their measurement. In that regard, electrochemical biosensors have been developed as simple and inexpensive devices for the measurement of oxidized DNA [57,58]. Another approach involves simultaneously measuring different types of oxidative stress biomarkers in the same sample. Recent methods have been developed for the determination of lipid and DNA damage markers [59] and also for the analysis of protein and DNA oxidative damage [60]. Evaluating multiple biomarkers may help a researcher understand if a specific oxidative pathway is more pronounced for a particular condition under research.

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