Published in final edited form as:

Electrophoresis. 2000 June ; 21(10): 2080–2085. doi:10.1002/1522-2683(20000601)21:10<2080::AID-ELPS2080>3.0.CO;2-6.

Detection of a urinary biomarker for oxidative DNA damage 8-hydroxydeoxyguanosine by capillary electrophoresis with electrochemical detection

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Abstract

8-Hydroxydeoxyguanosine (8-OHdG) is present in urine as a result of oxidative DNA damage associated with age-related diseases such as cancer. In this report a method is presented for the detection of 8-OHdG in human morning urine utilizing capillary electrophoresis with electrochemical detection (CEEC). The limit of detection for a aqueous standard of 8-OHdG is 50 nM (signal to noise ratio S/N = 3). A single solid-phase extraction (SPE) step with a C18 column is used for sample cleanup and 20-fold preconcentration of the urine before analysis by CEEC. Optimized conditions for analysis of extracted urine are $E_{app} = 0.5$ V vs. Ag/AgCl with 20 mM sodium borate/20% MeOH v/v, pH 9, as the background electrolyte, and a separation voltage of 22 kV. The concentration of 8-OHdG varied from 6 to 86 nM with an average value of 42 ± 26.9 nM for four healthy female and four healthy male subjects between the ages of 23 and 43.

Keywords

8-Hydroxydeoxyguanosine; Oxidative DNA damage; Capillary electrophoresis with electrochemical detection

1 Introduction

Reactive oxygen species such as $\bullet\text{OH}$, $\text{O}_2^{\bullet-}$, and $\text{H}_2\text{O}_2\bullet$ [1] cause oxidative damage to DNA which may contribute to degenerative diseases such as cancer, heart disease, and other age-related diseases. One pathway of oxidative damage is through hydroxyl radical attack on the guanine residues of DNA [2,3]. Damaged DNA is enzymatically repaired and the 8-hydroxydeoxyguanosine (8-OHdG) produced is excreted in urine without further metabolism [2,4]. Recent reports using morning urine have found that in healthy adults the concentration of 8-OHdG varies from 2.5 to 93 nM with an average of 30 nM for nonsmokers [5]. Results from 24 h urine samples of smokers have shown that concentrations can be 50% greater than for non-smokers [4,6]. The concentration of 8-OHdG is also elevated in cancer patients or those who are undergoing chemo or radiative treatment for cancer [6–9]. 8-OHdG has been identified as a biomarker for breast cancer, malignant teratoma, gastric cancer, head and neck cancer, brain tumor, lung cancer, pancreatic cancer, and colorectal cancer [8].

While GC-MS [10] has been used for analysis of 8-OHdG, this method often requires derivatization and, as a result, may produce results that are erroneously high [11]. The most common method for 8-OHdG analysis utilizes HPLC with electrochemical detection (LCEC)

[1–4,5,6,8,12–14] which provides at least 2–3 orders of magnitude better limits of detection than optical methods. In addition, EC detection has greater selectivity with complex samples than optical techniques as a result of detection based upon the electrode potential. However, liquid chromatographic methods used previously for analysis of 8-OHdG in urine have required complex and time-consuming double and triple column switching [4,6,8] as well as multiple solid-phase extraction steps [1,4,5].

Capillary electrophoresis offers an alternative to LC as the separation is based on the charge-to-size ratio of the analyte rather than partitioning and it typically provides higher separation efficiency. Capillary electrophoresis with electrochemical detection (CEEC) allows for high efficiency separations needed for complex biological samples as well as excellent selectivity and limits of detection afforded with electrochemical detection. Lin *et al.* [15] employed CEEC for analysis of purine bases, ribonucleotides and ribonucleosides in plasma. Hadwiger *et al.* [16] demonstrated that while quantitation of isoproterenol was not possible with CE-UV because of insufficient detection limits, a detection limit of 13 nM could be achieved using CEEC. In addition, detection limits for catechol standards have been reported to be in the range of 2–5 nM [17,18]. With this in mind, a method is presented here for screening human morning urine for 8-OHdG using a single extraction step and based upon CEEC. In order to illustrate the utility of the method, urinary concentrations of 8-OHdG in human morning urine are reported for eight male and female subjects.

2 Materials and methods

2.1 Chemicals

8-OHdG was purchased from Sigma (St. Louis, MO, USA) and used as received. Stock solutions of 8-OHdG were stored refrigerated at 4°C when not in use. Sodium borate was obtained from Fisher Scientific (Fair Lawn, NJ, USA) and titrated to the appropriate pH either with 1 N HCl or 1 N LiOH. Zwitterionic buffers *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), 2-(*N*-morpholino)ethanesulfonic acid (MES), and 2-(*N*-cyclohexyl-amino)ethanesulfonic acid (CHES) were obtained from Sigma, and 10 mM solutions were titrated as above to the pH = pK_a of the buffer. Sodium lauryl sulfate and HPLC-grade methanol were both used as received from Fisher. Disodium ethylenediaminetetraacetic acid dihydrate (EDTA) was also purchased from Fisher and a 0.5 M solution, pH 13, was prepared by titrating with solid NaOH. All solutions were prepared with NANOpure water (Barnstead, Dubuque, IA, USA) and filtered through a 0.2 μ m syringe filter before use.

2.2 CE

For CE experiments, electrophoresis was driven by a high voltage dc (0–30 kV) power supply (Glassman High Voltage, Whitehouse Station, NJ, USA). The anodic high voltage end was isolated from the electrochemical detector in a plexiglass box with double interlocks for operator safety. Uncoated silica capillaries 65cm–100cm in length $\times$ 50 μ m ID, were obtained from Polymicro Technologies (Phoenix, AZ, USA). The capillary was flushed at the beginning of the day with 0.5 M EDTA, pH 13, for 15 min, followed by NANOpure water for 5 min, and CE background electrolyte (BGE) for 10 min. The capillary was allowed to equilibrate in BGE at high voltage for at least 10 min before experiments were performed. Between runs the capillary was flushed with EDTA for 1 min followed by run buffer for 6 min. Flushing and sample injections were performed hydrodynamically with Ar at 20 and 5 psi, respectively. Injections for 65 cm long capillaries were done for 1 s, while for capillaries of greater length injections were performed for 2 s. The CEEC system with a cast-nafion end column decoupler has been previously described [18]. Amperometric detection was performed with a 1 mm long, 33 μ m diameter carbon fiber as the working electrode, a platinum wire as the auxiliary electrode, and a BAS RE-6 or RE-4 sat. KCl Ag/AgCl reference electrode from Bioanalytical

Systems (BAS; West Lafayette, IN, USA). The carbon fiber working electrode was placed inside the decoupler 200 μm from the outlet of the capillary. An LC-4C potentiostat modified for CE (BAS) was used for amperometric detection. Between each run, the electrode was pretreated for 120 s with a $\pm 1.5\text{V}$ 100 Hz square-wave using the built-in waveform generator of the potentiostat. Experiments without addition of organic modifier to the BGE were performed with the same BGE in both the anodic buffer reservoir as well as the electrochemical cell. With addition of the organic modifier to the BGE the electrolyte in the electrochemical cell continued to be the BGE without organic modifier. Data were collected with a SP4400 Datajet integrator interfaced to a computer with the program Chromnet using a Labnet connection (Spectra Physics, San Jose, CA, USA).

2.3 Sample preparation

Urine samples were collected from the first morning urine of healthy male and female smokers and nonsmokers between the ages of 23 and 43. After collection, the samples were either frozen whole or divided into fractions and frozen individually at -20°C . For analysis, samples were brought to room temperature in a water bath followed by vortexing. Portions for extraction were then filtered through a 0.2 μm PES syringe filter before solid-phase extraction (SPE). Supelco C18 (6 mL, 500 mg C18) columns were used for SPE. A 12-port vacuum manifold (Supelco, Bellefonte, PA, USA) was used with the SPE column to supply vacuum to the cartridge. The cartridge was pretreated with 3 mL of 100% MeOH followed by 3 mL of water and then 2 mL of urine was applied. The column was then flushed with 7 mL of water and the urine sample was eluted with 2 mL of 20 or 30% MeOH. The extract was evaporated to dryness under Ar and reconstituted with 100 μL of water to produce a 20-fold concentration of the sample. After vortexing, the sample was split into two 50 μL fractions with one fraction spiked with 1 μL of 1 mM 8-OHdG. Extractions of standards were performed similarly with no pH modification or splitting of samples after reconstitution in filtered Nanopure water.

3 Results and discussion

3.1 Hydrodynamic voltammogram of 8-OHdG

Figure 1 shows a hydrodynamic voltammogram (HDV) for a 1 μM 8-OHdG aqueous standard determined by CEEC. The current increases rapidly with potential after 0.25 V vs. Ag/AgCl with a half wave potential at *ca.* 0.35 V. A decrease in slope to a diffusion-limited plateau is observed near 0.5 V. Results presented here agree well in shape and half-wave potential with those reported previously by LCEC [3].

3.2 SPE

The recovery of 8-OHdG from the SPE method was determined with 25 and 50 nM 8-OHdG aqueous standards. Peak currents were compared to a 5-point calibration curve from 0.05 to 1 μM 8-OHdG to determine the recovery of 8-OHdG. Using 20% methanol in the final elution step the recovery was 86 ± 6.9 ($N=3$) and 59.5 ± 12 ($N=2$) for 25 and 50 nM aqueous standards, respectively. The observed concentration dependence may be due to incomplete elution of the analyte at higher concentration with the low organic percentage in the solvent. Increasing the methanol concentration to 30%, the extraction recovery was no longer concentration-dependent and recoveries of 99 ± 1.4 ($N=2$) and $96 \pm 0\%$ ($N=2$) were obtained for 25 and 50 nM standards. Previous reports indicated that the recovery of 8-OHdG in urine may not be as high as that in aqueous standards [5]. In order to determine the recovery of 8-OHdG in urine, triplicate 2 mL samples of urine were spiked with 50 μL of 1.5, 10 and 50 μM 8-OHdG before extraction. A recovery of 56% was found to be constant over the concentration range of 63 nM– 1.25 μM 8-OHdG using urine from several donors.

3.3 Optimization of the separation

Previous results indicated that borate buffers could be used successfully for the separation of 8-OHdG from DNA extracts [19] using CE with UV detection. However, zwitterionic buffers are known to exhibit lower electrophoretic currents than borate or phosphate buffers [16]. The buffer electrophoretic noise has a critical effect on the limits of detection in a CEEC system. The detector noise increases as the electrophoretic current increases [16]. With this in mind, various buffers were investigated for their effect on the electrophoretic current and noise in the system, as well as their effect on the separation.

The electrophoretic current is a function of the concentration of the buffer as well as the buffer type [16,20–22]. Our goal was to find a BGE that produced the lowest electrophoretic current (and therefore the lowest noise) while allowing an acceptable separation. Table 1 lists the buffers, the electrophoretic current and noise obtained with each. Data for noise studies were collected from the first 5–10 min of the run. Zwitterionic buffers (CHES, HEPES, MES) exhibited electrophoretic current and noise that was an order of magnitude or more lower than borate buffers. However, no resolution of 8-OHdG from matrix interferences was observed with all three of these buffers at pH values from 5.5 to 9.5 (Fig. 2). Therefore, a borate buffer was used to achieve the separation although it does not provide the optimal electrochemical detection characteristics.

Neutral and basic borate buffers were used to study the effect of buffer pH on the separation. While BGE pH values of 7.5 and above resulted in resolution of the 8-OHdG from matrix interferences, 7.5 is out of the buffer range of borate (pK_a 9.24). Therefore, borate buffer at pH 9 was used for analysis of 8-OHdG in urine for quantitation. The electrophoretic current for 20 mM sodium borate, pH 9, was 25 μ A with 9 pA noise and is higher than for the zwitterionic buffers. To decrease the electrophoretic current, lower ionic strength borate buffer was investigated. Decreasing the concentration of sodium borate from 20 to 10 mM reduced the current from 20 to 8 μ A (Table 1). However, the resolution deteriorated below baseline with a reduction of the BGE ionic strength.

With longer capillaries the resistance is greater and the electrophoretic noise and current decreased. The length of the capillary was varied from 65 cm up to 100 cm and the electrophoretic current and separation observed. The average electrophoretic current for an 89 cm long capillary was 12.6 μ A with electrophoretic noise of 2 pA. The noise in this system rivals that when zwitterionic buffers are used with the shorter capillary. In addition, this length offers acceptable resolution and short analysis time compared to longer capillaries.

An 89 cm $\times$ 50 μ m ID capillary with 20 mM sodium borate, pH 9, BGE was used for analysis of morning urine extracts of eight people. While the peak belonging to 8-OHdG was resolved in five extracts (Fig. 3), it was partially or completely unresolved from matrix interferences in three out of the eight urine extracts run. One method available to enhance the separation of complex samples is the use of organic modifiers. The migration time, migration order, and selectivity can all be affected by acetonitrile or methanol [23–25]. In addition, organic modifiers may change the polarity, conductivity, and viscosity of the BGE as well as the zeta potential. Methanol increases the viscosity of the BGE slowing the EOF [26,23]. Therefore, methanol was used as the organic modifier in order to decrease the EOF and increase the resolution of the separation.

The percentage of methanol added to 20 mM sodium borate BGE was varied from 5 to 20% and the effect on the separation observed. One of the extracts that was unresolved with the aqueous BGE was used in these experiments. As the percentage of organic modifier increased, the separation was enhanced until the peak for 8-OHdG was resolved from the neighboring matrix interferences. With an increase to 20% methanol in the BGE, the migration time of the

8-OHdG standard doubled, while the EOF decreased by 50% from 6.6 to $3.6 \times 10^{-4} \text{ cm}^2/\text{Vs}$. The electrophoretic current and noise also decreased, to $10 \text{ }\mu\text{A}$ and 1 pA , respectively.

A comparison between the separation using the BGE and the modified BGE with the same urine extract is shown in Fig. 4. In the case of the borate BGE (Fig. 4A) the peak attributed to 8-OHdG is a shoulder on the larger concentration matrix interference that has a migration time similar to 8-OHdG under these conditions. Addition of 20% methanol to the BGE allows for resolution of 8-OHdG from the matrix interferences including changes in migration order. Figure 4B shows the peak for 8-OHdG as a separate peak resolved from the rest of the matrix.

3.4 Analysis of 8-OHdG in human urine by CEEC

Liebich *et al.* [27,28] have used 3-deazauridine as an internal standard for UV detection of 8-OHdG in urine. Therefore, compounds similar in structure and behavior to 8-OHdG such as 3-deazauridine, deoxyguanosine, and 1-hydroxyisoguanine were investigated as internal standards. Unfortunately, these compounds were undetectable, oxidized at potentials much greater than 1 V vs. Ag/AgCl , or were unstable. Analysis of 8-OHdG by LCEC commonly does not include an internal standard and relies on migration time and spiking with genuine 8-OHdG [5,6,8,12].

The identity of the peak was determined in this study by comparison of the migration times, spiking and peak current ratios. Calibration was performed with a five-point calibration curve from 0.1 to $1 \text{ }\mu\text{M}$ with correlation coefficient greater than 0.99 . Table 2 lists the concentration of 8-OHdG from four normal healthy females and four healthy males determined by CEEC using BGE with organic modifier. The urinary concentration of 8-OHdG varied from 6.3 to 85.5 nM with an average concentration of $42 \pm 25.4 \text{ nM}$ for eight subjects. The average interday and intraday coefficients of variation were 2.6 and 6.1% , respectively.

4 Concluding remarks

This method allows detection of 8-OHdG in urine with a simple one-step extraction without the complex and time-consuming column switching needed for LC analysis. Previous analyses of urine by CEEC have relied on dilution [29,30] to remove interferences for detection of creatine, uric acid, carbohydrates and other urinary metabolic markers at micromolar concentrations. In addition, where SPE was used in conjunction with UV/Vis detection [27, 28] urinary concentrations of the nucleosides continued to be in the micromolar to millimolar range.

Acknowledgements

This work was supported in part by NIH grant R01GM44900. The authors gratefully acknowledge the assistance of David Pivinski. DJW acknowledges the support from the National Cancer Institute Training Grant T32CA09424.

Abbreviations

8-OHdG	8-hydroxydeoxyguanosine
CEEC	capillary electrophoresis with electrochemical detection
LCEC	HPLC with electrochemical detection

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Figure 1.

Hydrodynamic voltammogram for 1 μM 8-OHdG. Electrophoretic conditions: 89 cm $\times$ 50 μm ID capillary; background electrolyte, 20 mM sodium borate, pH 9; electrophoresis voltage, 22 kV; injection, 5 psi, 2 s. The 8-OHdG sample was in water.

Figure 2.

Electropherograms of a morning urine extract with BGE (A) 10 mM HEPES, pH 5.5; (B) 10 mM MES, pH 7.5; (C) 10 mM CHES, pH 9.5. An arrow denotes the expected position of 8-OHdG. Conditions as in Fig. 1 with a 65 cm $\times$ 50 μ m capillary and $E_{app} = 0.5$ V vs. Ag/AgCl.

Figure 3. Electropherogram of a spiked (dashed trace) and unspiked (solid trace) morning urine extract. The inset shows an expanded portion of the electropherogram with an arrow denoting the 8-OHdG peak. Conditions are as in Fig. 1 with $E_{app} = 0.5$ V vs. Ag/AgCl.

Figure 4. Electropherogram of a morning urine extract with (A) 20 mM sodium borate, pH 9, and (B) 20 mM sodium borate/20% methanol v/v, pH 9, BGE. Other conditions as in Fig. 1.

Table 1

Electrophoretic current and buffer characteristics

Buffer	pH	CE current (μ A)	Electrophoretic noise (pA)
20 mM Na-borate	9.0	25.0	9
20 mM Na-borate	7.5	20.0	7
20 mM Na-borate ^a	9.0	12.6	2
10 mM Na-borate	7.5	8.3	3
10 mM CHES	9.3	2.6	0.1
10 mM HEPES	7.5	2.8	2
10 mM MES	5.5	1.5	2

Conditions: All values are for experiments run at 20 kV with the same 65 cm $\times$ 50 μ m ID fused-silica capillary used for each experiment

^aExperiments were run with a 89 cm $\times$ 50 μ m ID capillary at 22 kV.

Table 2

Comparison of 8-OHdG concentrations in morning urine of healthy human males and females

Female	[8-OHdG] (nM)	Male	[8-OHdG] (nM)
#1	$26.9 \pm 3.2 (N = 7)^a$	#1	$54.0 \pm 9.4 (N = 3)$
#2	$60.5 \pm 1.4 (N = 4)$	#2	$18.1 \pm 2.0 (N = 3)$
#3	$48.2 \pm 2.7 (N = 3)$	#3	$6.2 \pm 0.85 (N = 3)$
#4	$36.8 \pm 2.8 (N = 3)$	#4	$85.5 \pm 26.7 (N = 3)^b$

^a *N* is number of extractions performed; each extraction was analyzed twice.

^b $\frac{1}{2}$ -1 Pack/day smoker.