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Discontinuous electrophoretic stacking system for cholate-based electrokinetic chromatographic separation of 8-hydroxy-2′-deoxyguanosine from unmodified deoxynucleosides

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Abstract

The stacking and baseline-resolved separation of the oxidative damage marker, 8-hydroxy-2′-deoxyguanosine (8-OHdG), from unmodified deoxynucleosides in under 4 min is reported. Separations of 8-OHdG from 2′-deoxyadenosine, 2′-deoxycytosine, 2′-deoxyguanosine, and thymidine are accomplished using micellar electrokinetic capillary chromatography with sodium cholate. Importantly, the use of sulfate, intentionally added to the sample matrix, results in effective stacking of 8-OHdG and other analytes. This work extends electrokinetic stacking injection of neutral analytes to include deoxynucleosides. The procedure works well with either electrokinetic or hydrodynamic injection. The separation buffer and sample matrix composition were optimized to effect stacking conditions with an uncoated 50 μm fused-silica capillary. The lower limit of detection for the analytes is in the nanomolar range, and is more than an order of magnitude lower than without stacking. With 30 s (5.7 cm) electrokinetic injections, stacking and baseline separation of 8-hydroxy-2′-deoxyguanosine from the unmodified nucleosides is accomplished, even in the presence of a 400-fold excess of unmodified deoxynucleosides.

Keywords: Stacking; 8-Hydroxy-2′-deoxyguanosine; DNA damage; UV detection

1. Introduction

There is great interest in electrophoretic stacking techniques for use with capillary electrophoresis (CE) separations [1–9]. As a result of the high-efficiency rapid separations coupled with low sample volume requirements, CE has gained wide acceptance. However, a major disadvantage of CE methods that employ concentration-sensitive detection is lower sensitivity and higher limit of detection (LOD) than the corresponding approaches using high-performance liquid chromatography (HPLC). Thus, methods that preconcentrate or stack the analytes in CE are desirable. Numerous reports have addressed the fundamentals and the applications allowed by electrophoretic stacking with CE-based separation methods [1–9]. Ionic compounds can be effectively

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concentrated with field-amplified stacking [1]. Stacking of neutral analytes is slightly more complex, and requires an electrokinetic vector, such as a charged micelle or cyclodextrin, with which the neutral analyte can interact in order to attain a non-zero electrophoretic mobility [5–9]. High-salt stacking [6] and sweeping [7] are two techniques that have recently been developed to effectively stack neutral analytes. Sweeping utilizes continuous sample matrix/separation buffer conditions (i.e., the background electrolyte in the sample matrix and the separation buffer are identical) and relies on the charged micelles traversing the sample zone, allowing interaction between the analyte and the micelle. High-salt stacking utilizes a discontinuous buffer system to effect the stacking of the charged micelles at the co-ion boundary initially present at the interface between the separation and sample zones. This discontinuous system employs a high-mobility leading co-ion (e.g., chloride or sulfate) in the sample matrix [6,8,9]. When an electric field is applied, a single sharp boundary is generated between the sample matrix anion and cholate (anionic) in the separation buffer [6,8]. As the electroosmotic flow (EOF) mobilizes neutral analytes through the co-ion boundary, they are stacked in the cholate micelles. This stacking mechanism can also be invoked by electrokinetic (EK) injection of the analyte solution [9]. The advantages of EK injections are faster injections with decreasing capillary (or channel) diameter, the possibility of injections that are greater than one capillary volume, and ease of transferability to the microchip format [9].

There is considerable interest in oxygen-derived free radicals, primarily owing to the fact that these compounds have been linked with scores of diseases and clinical conditions [10–13]. These include inflammatory-immune injuries such as rheumatoid arthritis, Alzheimer's disease, Parkinson's disease, ischemia reperfusion injuries associated with strokes, myocardial infarctions, and organ transplants. Additionally, oxygen-derived free radicals have also been associated with iron overload, radiation injury, the aging process, and various disorders involving red blood cells, the lungs, the cardiovascular system, the kidneys, the gastrointestinal tract, the eyes, and skin [10–13].

Much of oxidative damage to DNA is caused by oxygen free radicals [14–18]. The hydroxyl radical ($^{\bullet}OH$) is a highly reactive oxygen-derived free radical, and is implicated as the major culprit in oxidative damage. A large body of work on oxidative DNA damage indicates that such damage is selective [10–16], with levels of the modified base, 8-hydroxy-2′-deoxyguanosine (8-OHdG) increased in DNA exposed to $^{\bullet}OH$. This indicates 8-OHdG as a biological marker for oxidative DNA damage.

Methods employed to determine 8-OHdG include gas chromatography with mass-selective detection (GC–MS) [17,18] and HPLC with either mass spectrometric [19] or electrochemical detection [20–24]. Gas chromatographic methods require that samples be derivatized. Unfortunately, the derivatization procedures can produce quantitative results that are erroneously high [18]. In contrast, HPLC assays require no derivatization, but most require cumbersome column switching techniques, and suffer from relatively long analysis times (20 to 45 min) [19–24].

Capillary electrophoresis is an attractive alternative to HPLC, as superior resolution and shorter analysis times can often be realized. Micellar electrokinetic capillary chromatography (MEKC) and capillary zone electrophoresis (CZE) have been applied to the separation of mixtures of nucleosides [25–30], nucleotides [27,28] and, more recently, mixtures containing 8-OHdG [25,26]. Previous MEKC studies have employed sodium dodecyl sulfate (SDS) as the micellar agent [27,30]. However, the low separation voltages, required due to the high concentration of SDS needed for adequate resolution, lead to separation times on the order of 30 min. This provides little improvement in analysis time over HPLC methods. In a recent report, Weiss and Lunte have demonstrated the ability to determine 8-OHdG in urine (following solid-phase extraction) using CZE with a borate buffer system and electrochemical detection [25]. This elegant approach demonstrated enhanced sensitivity over UV detection and, despite employing capillaries up to 100 cm in length, achieved separations requiring only 10 to 25 min.

The literature indicates that neither cholate micelles nor stacking protocols have been examined for the determination of 8-OHdG. Given the structural and polarity differences between SDS and cholate, it is likely that the molecular level interactions of SDS

and cholate micelles with nucleosides will differ. Additionally, stacking of these compounds would result in enhanced sensitivity and LOD lower than achievable with EKC systems with non-stacking conditions. This is especially important for 8-OHdG because of its relatively uninteresting UV spectrum at wavelengths above 220 nm. While dG absorbs strongly at 212 and 265 nm, the spectrum for 8-OHdG has a much lower absorbance maximum centered around 265 nm. Thus, UV absorbance detection for these compounds is convenient [26,27], but, without stacking, its sensitivity is clearly inferior to electrochemical [25], and thermooptical detection [28]. Consequently, enhanced sensitivity without the need for exotic detection modes or cumbersome derivatization of the analytes, can be accomplished with stacking of the analyte(s).

By the combination of a discontinuous buffer system with EK injection, this work demonstrates the on-line stacking and separation of 8-OHdG from other unmodified deoxynucleosides using cholate micelles. This involves injecting a sample matrix containing 2′-deoxyadenosine (dA), 2′-deoxycytosine (dC), 2′-deoxyguanosine (dG), and thymidine (T) with sulfate, a high mobility anion purposely added to the sample matrix with sample plugs 2 to 8 cm in length (8–31% of the effective capillary length). Sulfate effects the concentration of the lower mobility micelle species (i.e., cholate) at the sample/separation buffer interface, with the analytes subsequently stacked in the concentrated zone of cholate micelles [9]. It is noteworthy that this work extends prior work involving high-salt stacking [8,9] to a different class of analytes. This stacking protocol allows UV absorption detection to be employed to achieve detection limits in the low μ*M* to high n*M* range for 8-OHdG, even in the presence of a 400-fold excess of the unmodified nucleosides.

2. Experimental

2.1. Chemicals

Sodium cholate, sodium sulfate, sodium chloride, 2′-deoxyguanosine, 2′-deoxyadenosine, 2′-deoxycytosine, and thymidine were obtained from Sigma (St. Louis, MO, USA). 8-Hydroxy-2′-deoxyguanosine was obtained from Calbiochem (La Jolla, CA, USA). Sodium phosphate (dibasic) was obtained from Fisher Scientific (Pittsburgh, PA, USA) and sodium phosphate (dibasic) was obtained from Mallincrodt (Paris, KY, USA). All reagents were used as received without further purification.

2.2. Solutions

Buffers and standard salt solutions were prepared in 18 MΩ-cm (Nanopure) water, degassed via manual decompression, and filtered through a 0.2 μm syringe filter prior to use. All buffers were used within 3 days of preparation. Stock solutions (1 to 2 mg/ml) of nucleosides were prepared in 18 MΩ-cm water, and stored at 4°C until use. Analysis solutions were prepared from stock solutions on the day of use.

2.3. Capillary electrophoresis

All analyses were carried out using a Hewlett-Packard HP 3D-CE instrument interfaced to a pentium computer running HP Chemstations software. A 33 cm×50 μm I.D. unmodified fused-silica capillary (Polymicro, Phoenix, AZ, USA) was used for all analyses. Diode array UV detection was employed, with single channel monitoring at 254 and 210 nm. The separation buffers consisted of sodium cholate (60–120 m*M*) and sodium phosphate (15–30 m*M*) at a pH from 9 and 11. Sample matrixes for stacking experiments consisted of phosphate at the same concentration as the separation buffer (15–30 m*M*), and sodium sulfate at a concentration between 1 and 20 m*M*. Stacking was implemented by applying the separation potential (+15 kV at the inlet) with sulfate, a high mobility anion present in the injected sample plug. When electrokinetic injection (15 kV, 5–40 s) is employed, stacking occurs during the injection step (sample at inlet) as well as the separation step (buffer at inlet).

3. Results and discussion

Previous electrokinetic chromatography separations of nucleosides employed SDS as the micellar agent in the separation buffer [27,30]. The high SDS

concentrations (100–300 m*M*) employed in earlier reports necessitated low voltage fields (50–200 V/cm) that led to correspondingly long (30 min) separations. The use of sodium cholate as the micelle-forming species was explored. Experiments were first performed to optimize the MEKC separation conditions (with no stacking) using cholate in the separation buffer for analysis of mixtures containing 8-OHdG and the four unmodified deoxynucleosides dA, dC, dG, and T (sample dissolved in diluted running buffer). With a phosphate buffer system held constant at 20 m*M*, it was determined that sodium cholate concentrations between 50 and 100 m*M* allowed resolution of the five nucleoside species. Still higher resolution was obtained at higher cholate concentration (120 m*M*), albeit at the expense of analysis time. Representative data obtained with 80 m*M* cholate/20 m*M* phosphate are shown in Fig. 1. This data was obtained with 3 s electrokinetic injections of a mixture containing unmodified deoxynucleosides (panel A – 160 μ*M*; panel B – 40 μ*M*) and 8-OHdG (panel A – 16 μ*M*; panel B – 4 μ*M*). These non-stacking MEKC conditions allow for excellent resolution in under 5 min with an applied voltage of 15 kV (454 V/cm, 33 cm capillary length). Similar results were obtained using pressure injections. It is important to note that the injections here are on the order of 3.7 mm in length (7.3 nl), corresponding to ~1% of the total capillary volume. Longer injections with this non-stacking procedure resulted in loss of efficiency and peak capacity. Under these non-stacking conditions, the limit of detection (LOD) for 8-OHdG was 9 μ*M* (with a signal-to-noise ratio of 3).

To pursue a lower LOD, stacking procedures were investigated. Using the stacking protocol [8,9] illustrated in Fig. 2, EK injections were examined with different leading electrolytes (chloride, sulfate) added to the sample matrix (data not shown). The addition of sodium sulfate (Na_2SO_4) to the sample matrix resulted in maximal stacking of the analytes. To investigate the effect of the sample matrix sulfate concentration, differing amounts of Na_2SO_4 were added to the sample with the phosphate concentration maintained at 20 m*M* in both the sample matrix and separation buffer. The sample matrix with 20 m*M* phosphate and 10 m*M* sulfate effectively stacked the nucleosides, as evidenced by the data given in Fig. 3. There is a ~10-fold gain in sensitivity, accompanied by a slight loss in efficiency relative to analyses without stacking. Depending on the separation conditions, dC and dA (identified by analyses with individual standards and/or by spiking) were often not completely resolved from the negative baseline deflection marking the front edge of the injection plug (indicated by arrow in Fig. 3). Apparently, this is due to weak intermolecular interactions between these two analytes and the cholate micelles. However, 30 s injections give rise to well-resolved peaks and baseline resolution of dG and 8-OHdG in under 4 min. Higher concentrations of sulfate (up to 20 m*M*) result in increased migration times and enhanced resolution between the EOF marker and all peaks. For a balance between resolution and analysis time, 10 m*M* sulfate was chosen for further studies. Under these conditions, stacking results in enhanced

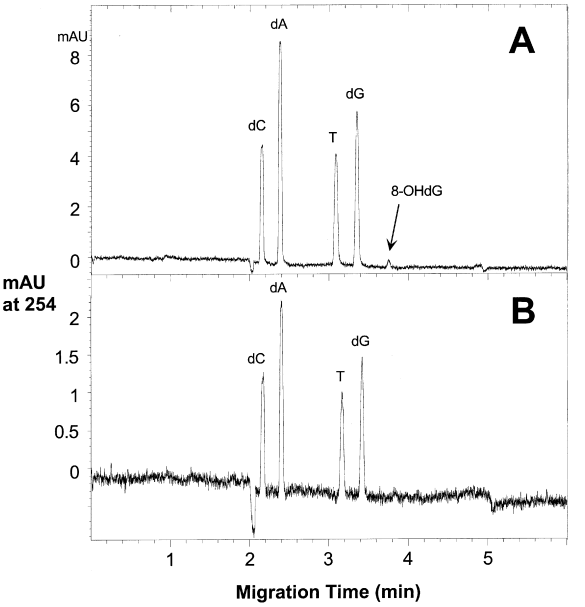

Fig. 1. MEKC separations (no stacking) employing cholate micelles for separation of deoxynucleosides at two different concentrations: (A) 160 μ*M* (8-OHdG at 16 μ*M*) and (B) 40 μ*M* (8-OHdG at 4 μ*M*). Separation conditions: 33 cm capillary (24.5 cm effective length)×50 μm I.D. unmodified fused-silica capillary, 3 s 15 kV injection, 15 kV separation (70 μA), 80 m*M* cholate, 20 m*M* phosphate, pH 11.0, sample in diluted (80%) running buffer. Peak identity: dA: 2′-deoxyadenosine, dC: 2′-deoxycytosine, T: thyamine, dG: 2′-deoxyguanisine, 8-OHdG: 8-hydroxy-2′-deoxyguanisine.

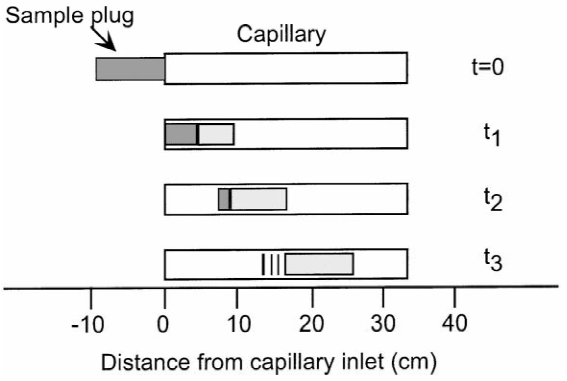

Fig. 2. Schematic diagram of stacking process with electrokinetic injection. At time t_0, a plug of sample which also contains a high mobility anion (sulfate) is introduced into the capillary. At time t_1, the separation buffer containing the anionic micelle forming species is returned at the inlet and the separation potential is applied, the co-ion boundary moves more slowly than the sample matrix. At t_2, the sample plug has partially passed (via EOF) the co-ion boundary, and the analytes from the front of the injection plug have been stacked into the concentrated plug of cholate micelles. By time t_3 the original sample plug has fully passed the co-ion boundary and the separation of the stacked analytes proceeds as with normal MEKC separations.

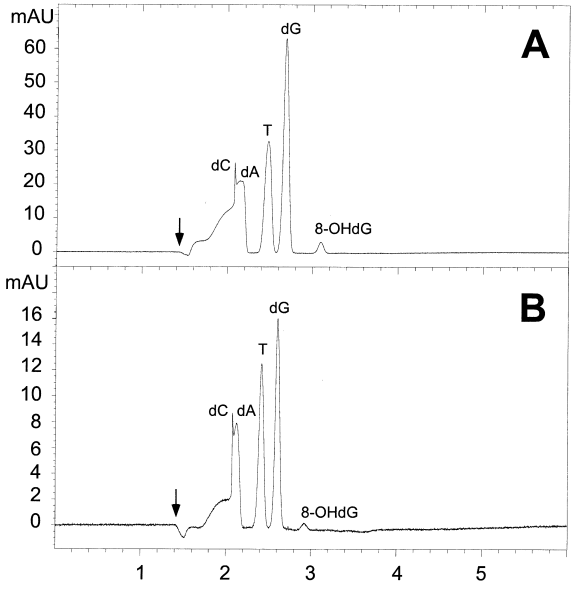

Fig. 3. MEKC with stacking afforded by 10 m*M* sulfate/20 m*M* phosphate in sample matrix. Analytes, capillary and separation buffer as in Fig. 1. 30 s, EK injection at 15 kV.

sensitivity over the conventional MEKC method (Fig. 4). At a concentration of 4 μ*M*, 8-OHdG is not clearly discernable from baseline without stacking. However, analysis of the same sample with the stacking protocol results in a peak with signal to noise of 20 to 1, lowering the LOD for 8-OHdG to 750 n*M* with these conditions. Under the same conditions, the LODs for the deoxynucleosides T and dG are 350 and 300 n*M*, respectively, owing to the higher extinction coefficients of these compounds.

In an attempt to resolve dA and dC, the separation buffer content was re-examined. A higher concentration of sodium cholate in the separation buffer can give rise to slightly higher resolution, but at a cost of longer analyses and higher system current. Electropherograms for cholate concentrations between 60 and 120 m*M* (with the concentrations of phosphate in the buffer and sulfate in sample matrix scaled proportionally to maintain stacking conditions) are shown in Fig. 5. Apparently, the carbonyl function-

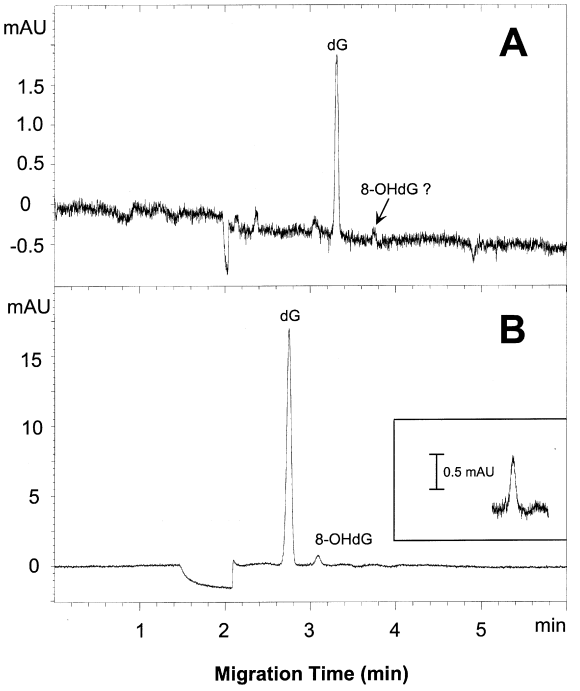

Fig. 4. Direct comparison of MEKC without (A) and with (B) sample stacking for analysis of 40 μ*M* dG, 4 μ*M* 8-OHdG. Arrow in A indicates the expected location of the 8-OHdG peak. Inset in B shows expanded view of the 8-OHdG peak. Separation conditions as in Figs. 1 and 3, respectively.

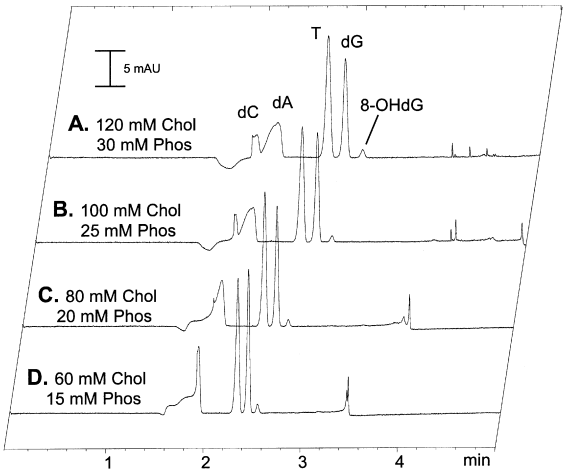

Fig. 5. Effect of cholate concentration on stacking and subsequent separation of a mixture of deoxynucleosides. Separation buffers as indicated in figure, sample matrix with same phosphate (monobasic) concentration as separation buffer, with sulfate at same relative concentration: (A) 7.5 m*M*, (B) 10 m*M*, (C) 12.5 m*M*, (D) 15 m*M*. Peak identification as given in A. Analyte concentration: 8-OH dG at 4 μ*M*, all others at 40 μ*M*.

ality at the 6 position on the purine ring with dG, but not with dA (and at the 4 position on the pyrimidine ring with T but not with dC) is critical for stronger interaction with cholate, probably acting as a hydrogen bond acceptor. If resolution of dC and dA is necessary, higher cholate concentrations can be used. For a balance of speed and resolution of 8-OHdG from dG, 80 m*M* cholate was found optimal. The trends observed with higher cholate concentrations in the separation buffer could also be obtained by manipulating the sulfate concentration in the sample matrix (data not shown). This is not surprising, as the character of a local zone of cholate at the sample/buffer interface depends upon both the initial sodium cholate concentration and the stacking efficiency, which is presumably effected by sulfate. Further attempts to improve the resolution involved evaluating the effect of an organic modifier (acetonitrile) as an additive to the cholate/phosphate separation buffer. In general, this was found to be deleterious (data not shown), resulting in earlier migration times, a loss of resolution, and a narrowing of the separation window. Thus, the use of an organic modifier was not found to be appropriate for this work.

As part of the method optimization, the pH of the separation and sample matrixes were individually examined. With separation buffers ranging in pH from 9 to 11, the use of lower pH buffer was associated with rapid loss of resolution with all peaks co-migrating or only being partially resolved from other components migrating with EOF. In general, buffer with a higher pH provided higher resolution and improved peak height for the deoxynucleosides. The resolution between T and dG was maximal at pH 10, perhaps owing to the acid–base chemistry of these analytes (pK_a values at 9.8 and 9.4, respectively). Regardless, pH 11.0 yielded the optimum resolution between dG and 8-OHdG and was therefore selected for further use. It was not surprising that the pH of the sample matrix was also important; lower sample pH generally resulted in enhanced detectability of the deoxynucleosides. This may be related to favorably biased injection of cations, pH-mediated stacking [31,32] of the analytes during the injection step or, perhaps, both. With the separation buffer at pH 11 and the sample at pH 4.0, 30 s EK injections resulted in a detection limit for 8-OHdG of 750 n*M* (with a signal-to-noise ratio of 3). Longer injections and/or higher sulfate concentrations in the sample matrix were found to decrease the LOD well into the nanomolar domain, but with losses in separation power for lower k' analytes, and higher analysis times, respectively.

In order to test the quantitative analytical utility of this optimized stacking system, the linearity of response with variation in the injection time and the analyte was investigated. Using 160 μ*M* dG and 16 μ*M* 8-OHdG, 10 to 40 s EK injections were performed (Fig. 6). These injections corresponded to 7.8 to 31.1% of the effective capillary length, respectively. These represent relatively large injections by conventional CE standards, but rather modest injections compared to those recently reported with high salt stacking of steroids in SDS micelles [9]. For 16 μ*M* 8-OHdG, both peak area ($y=0.494x+0.90$, $r^2=0.998$) and peak height ($y=0.0764x+0.85$, $r^2=0.9997$) were linear with injection time (data not shown). At higher concentrations, the peak height drops below the linear trendline, probably owing to saturation of the local cholate zone. As expected, the migration times with EK injection are shifted by the injection time. That is, because the ion boundary is formed at the onset of injection, stacking and sepa-

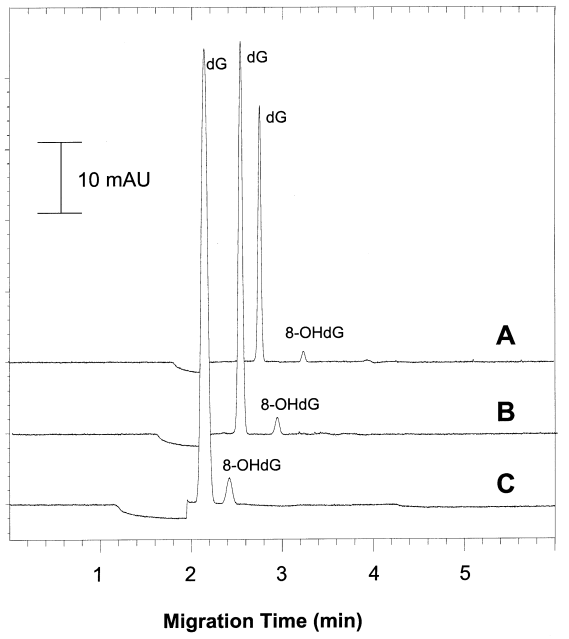

Fig. 6. Effect of injection time on resultant electropherograms for 160 μ*M* dG, 16 μ*M* 8-OHdG. 15 kV EK injections for (A) 10 s, (B) 20 s, and (C) 40 s. Separation conditions as in Fig. 3.

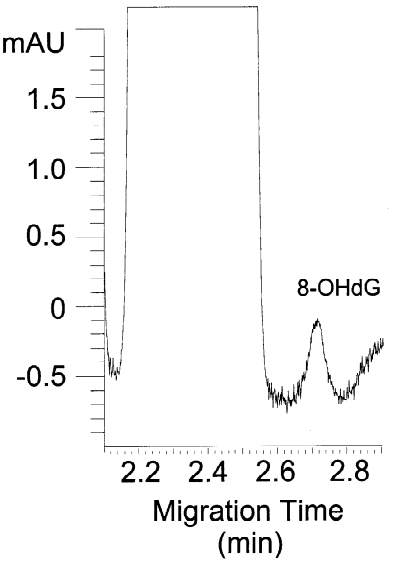

Fig. 7. Detection of 2.5 μ*M* 8-OHdG in the mixture containing 850 μ*M* dG. Separation conditions as in Fig. 3.

ration are occurring simultaneous with injection, shortening the perceived separation time. Peak area ($y=0.759x+0.757$, $r^2=0.98$; 30 s EK injection) and peak height ($y=0.155x+0.124$, $r^2=0.990$; 30 s EK injection) were also linear for 8-OHdG concentration between 2 and 16 μ*M* in a mixture containing 10-fold higher concentration of dG. Linearity similar (response with injection time) or superior (response with concentration) to that obtained with 8-OHdG was obtained for dG and T with both pressure and EK injections. The concentration sensitivity for dG was higher than that for 8-OHdG, as would be predicted from the UV spectra of these two compounds. For 40 s injections and detection at 254 nm, the LOD for 8-OHdG was determined to be 450 n*M*. Monitoring the signal at shorter wavelengths (200–210 nm) gave similar results with slightly enhanced sensitivity for 8-OHdG, but a nearly identical LOD.

Finally, we investigated the ability of this method to determine low μ*M* concentrations of 8-OHdG in mixtures containing a large excess of unmodified deoxynucleosides, a scenario likely to be encountered with real biological samples. An expanded view of the area of interest from an electropherogram of 2.5 μ*M* 8-OHdG in a mixture of highly concentrated (500–900 μ*M*) dA, dC, T and dG is presented in Fig. 7. Although the signal for 8-OHdG is small, the ability of this stacking MEKC protocol for the determination of low levels of 8-OHdG in the presence of unmodified deoxynucleosides at much higher concentration is apparent.

4. Conclusions

We have shown that sodium cholate is capable of resolving 8-OHdG from mixtures of unmodified deoxynucleosides, and that stacking of the analytes, afforded by the addition of sulfate to the sample matrix, increases the sensitivity of the MEKC protocol by an order of magnitude. This work extends the use of salt, intentionally added to the sample matrix to effect stacking of neutrals (a.k.a. high-salt stacking) to a new analyte system. While sensitivity and LOD with UV detection will likely always be inferior to that obtained with electrochemical detection, this work represents an important first step in addressing the short comings of CE with UV detection, as well as the speed of analysis for the determination of 8-OHdG. Finally, the method pre-

sented here offers an LOD for 8-OHdG in the n*M* range with analysis times under 4 min. Further efforts are focused on using the approach described herein, both in capillaries and on microchips, with biological fluids in order to assess the ruggedness of this approach with real samples.

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