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Improved detection limit for a direct determination of 8-hydroxy-2′-deoxyguanosine in untreated urine samples by capillary electrophoresis with optical detection

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Abstract

Method for a direct determination of 8-hydroxy-2′-deoxyguanosine (8OHdG) in untreated urine samples by capillary electrophoresis with optical detection was developed. Optimisation of conditions resulted in a significant lowering of the limit of detection (LOD) by a factor of 400 as compared to our previous study. Optimum separation of 8OHdG from other urine components was achieved using the separation electrolyte containing 80 mM 2-(cyclohexylamino)ethanesulfonic acid, 9 mM LiOH (pH 8.6), and 0.1 mM cetyltrimethylammonium bromide ensuring the electro-osmotic flow inversion. In the model aqueous samples, these conditions allow separating 8OHdG and 2′-deoxyguanosine (dG) from other nucleosides/nucleotides including 2′-deoxycitidine 5′-monophosphate (dCMP), thymidine 5′-monophosphate (TMP), adenosine (A), and thymidine (T). On the other hand, 2′-deoxyadenosine 5′-monophosphate (dAMP) and 2′-deoxyguanosine 5′-monophosphate (dGMP) migrate together, and guanosine (G), 2′-deoxyadenosine (dA), 2′-deoxycytidine (dC) are transported as neutral species with the electro-osmotic flow. In the spiked urine samples, 8OHdG and dG are well separated from each other and from other urine components and exhibit a linear calibration over the concentration range of 0.1–2.0 μM for 8OHdG (LOD = 42 nM) and 0.2–5.0 μM for dG (LOD = 86 nM), but urine metabolites interfere with the determination of dCMP, TMP, A and T. Method is applicable to untreated urine samples with slightly enhanced levels of 8OHdG compared to that found in healthy individuals.

Keywords: Capillary electrophoresis; 8-Hydroxy-2′-deoxyguanosine; Detection limit; Untreated urine

1. Introduction

Oxidative damage of DNA has attracted a great deal of interest in relation to mutagenesis, carcinogenesis and aging [1,2]. One of the major products of the oxidative degradation of nucleic acid is 8-hydroxy-2′-deoxyguanosine (8OHdG) [3–7], which has been, therefore, proposed as a useful biomarker [6,7]. Recent reports on analysis of urinary 8OHdG in healthy individuals have found that the 8OHdG concentration varies from 1 to 50 nM, with a mean of, e.g., 9.1 ± 3.7 nM (27 individuals) [8], 27.2 ± 13.8 nM (2507 individuals) [9], 14.4 ± 1.1 nM (104 individuals) [10] or 29.2 ± 21.6 nM (60 individuals) [11]. Enhanced levels of urinary 8OHdG above these values have been found in various groups of patients, in particular cancer patients and those undergoing chemotherapy or radiation therapy [8,12–14].

The most common method for determination of 8OHdG in body fluids such as urine or plasma utilizes HPLC with electrochemical detection (HPLC/EC) [8–12]. Using HPLC/EC, the detection limits of 0.6 nM [8], 3.4–10.2 nM [10] or 0.9 nM [11] can be achieved, but the methods require complex and time-consuming multiple column switching [8,9] and/or multiple solid phase extraction (SPE) steps [11,12]. Recently, the LC methodology has been combined with the tandem mass spectrometry [15–17], avoiding any urine sam-

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ple pre-treatment. This novel approach allows determinations of 8OHdG in urine over the linear dynamic range 0.5–5 × 10^5 nM with the detection limit as low as 0.3 nM [15]. A drawback of the method is that the equipment is expensive. An alternative way to measure 8OHdG is by enzyme-linked immunosorbent assay (ELISA) [18–20], though the technique also detects other 8OHdG-associated molecules in addition to 8OHdG, thus, providing apparently enhanced 8OHdG concentrations [9].

Capillary electrophoresis (CE) has been a rapidly growing separation technique that may be useful for many biomedical and clinical applications. CE with optical detection has been used first for detection of 8OHdG in human hematopoietic cells with the detection limit of 50 nM in an aqueous sample [21]. A comparable detection limits of 20 nM [13] and 50 nM [22] in the CE analysis of the model aqueous samples were achieved using the electrochemical detection, while the direct analysis of untreated urine samples by the CE with optical detection had a much higher detection limit of 17 μM [14]. These methods have been applicable in urine analysis of healthy individuals only after an SPE treatment and a preconcentration of the purified urine samples by a factor of 10 [13] or 20 [22]. On the other hand, the limit of detection of 17 μM [14] was sufficient to determine 8OHdG in urine collected from the oncological patients treated by radiation therapy.

Following our previous study [14], we have continued in developing a direct and low-cost CE method for the determination of urinary 8OHdG, which would be applicable in clinical practice. We shall show that the urine pre-treatment is not necessary to reach a low detection limit, but an optimisation of the separation buffer system composition can lead to its significant improvement. Essentially, our strategy has been based on the use of 2-(cyclohexylamino)ethanesulfonic acid (CHES)/LiOH buffer [23], replacing the borate buffer employed in the previous work [14]. A low mobility of ions of the buffer system has enabled us to increase the buffer concentration, and thereby the buffer capacity. We have assumed that it should be then possible to inject a higher amount of the analyte and/or to avoid its diluting, which was a necessary step in the previous method [14], as well as to carry out the analysis at higher electric field intensity without much increasing the release of the Joule heat causing the zone broadening. Actually, owing to a higher value of the Kohlrausch regulation function at a higher buffer concentration [24], the analytes can be expected to separate in narrower zones, which should facilitate the analysis of complex mixtures like urine. The direction and the rate of the electro-osmotic flow (EOF) represent other aspects of the CE analysis to consider [24]. As compared with the previous analysis [14], we have used the buffer system containing cetyltrimethylammonium bromide (CTAB), and a new CE instrument equipped with a diode-array detector (DAD). The introduction of CTAB leads to the inversion of EOF, so that EOF has the same direction as the anions of nucleotides migrating towards the anode. Besides, the adsorption of CTAB on the capillary wall can be expected to protect its surface from the adsorption of urine components [25]. A significant lowering of the detection limit was made possible probably by the direct injection of urine samples without dilution, a more efficient separation and an improved sensitivity to noise ratio using DAD.

Measuring nucleosides/nucleotides other than 8OHdG is presently of low clinical significance in relation to the oxidative DNA damage. However, owing to their similar molecular structure, they can interfere with the analysis of 8OHdG (Fig. 1). Therefore, we have also examined a series of guanosine, thymidine, adenosine and cytidine derivatives, with a particular attention paid to the closest 8OHdG analogue, 2′-deoxyguanosine (Fig. 1).

2. Materials and methods

2.1. Chemicals

2-(Cyclohexylamino)ethanesulfonic acid (99%) and LiOH (98%+) were purchased from Fluka and Aldrich, respectively. Cetyltrimethylammonium bromide (99%), 8-hydroxy-2′-deoxyguanosine (water content 4.9%), 2′-deoxyguanosine (dG, 99–100%), 2′-deoxyadenosine (dA, 99–100%), 2′-deoxycytidine hydrochloride (dC, 99%), guanosine (G, 98%), thymidine (T, 99%), adenosine (A, 99%), cytidine (C, 99%), 2′-deoxyguanosine 5′-monophosphate sodium salt (d-GMP, 98%), 2′-deoxyadenosine 5′-monophosphate sodium salt (d-AMP, 99%), 2′-deoxycitidine 5′-monophosphate (d-CMP, 98%) and a thymidine 5′-monophosphate disodium salt (TMP, 99%) were purchased from Sigma. The molecular structure of 8OHdG and dG is shown in Fig. 1. Milli-Q deionised water (Millipore, Bedford, USA) was used for preparation of 1 mM stock solutions of nucleosides and nucleotides, which were stored in a refrigerator at 5 °C.

2.2. Instrumentation

Urine samples were analysed using HP^{3D}CE system (Agilent Technologies, Waldbronn, Germany) equipped with a built-in photometric diode-array detector (DAD) and controlled by the ChemStation CE software. Separation took place in an uncoated fused-silica capillary (64 cm length, 75 μm i.d. × 375 μm o.d., Silica Tubing & Optical Fibers, SR) at the controlled temperature of 25 °C. Before its first use, the capillary was conditioned by washing with 1 M NaOH for 10 min, deionised water for 10 min, and finally with the separation buffer for 15 min. The capillary was washed between each two CE runs with 0.1 M NaOH for 2 min, and then with the separation electrolyte for 4 min. Separation was performed in the anodic mode using the applied voltage −30 kV.

The built-in DAD enables to measure the absorption spectrum of the samples and hence to optimise the optical detection by choosing the proper light wavelength. The absorption was measured relative to that of the separation buffer back-

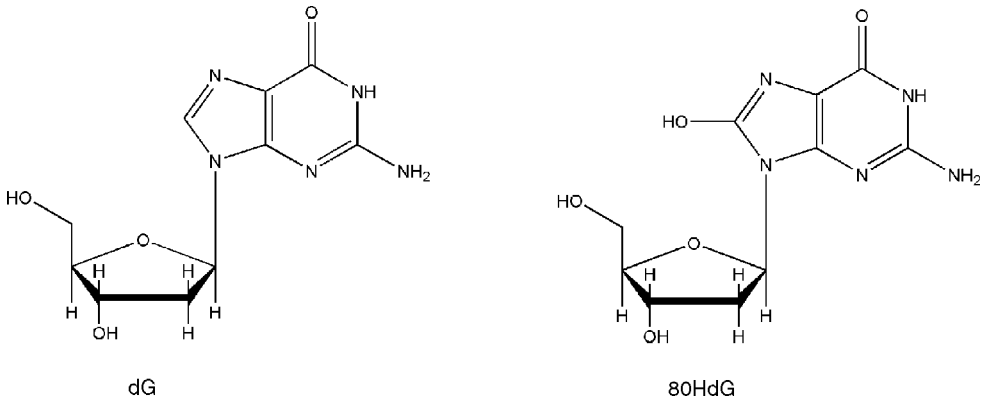

Fig. 1. Molecular structure of 8OHdG and dG.

ground. The background subtracted UV spectrum of 8OHdG and dG is shown in Fig. 2. Major absorption, maximum for 8OHdG, is observed at 204 nm, which was used for the detection of 8OHdG, and also dCMP, TMP, T a A. On the other hand, dG shows a well-developed maximum only at 254 nm, which was then used for its detection. The measurements at different wavelength in one run was not a complication, because the built-in DAD made it possible to perform the analysis at different wavelengths simultaneously.

Samples were introduced with the help of hydrodynamic injection. Typical injection pressure and times range are from 25 to 100 mbar (2.5–10 kPa) and 0.5 to 20 s, respectively, which corresponds to volume ranging from 12.5 mbar s (1.25 kPa s) to 2000 mbar s (200 kPa s).

2.3. Urine sample preparations

Urine samples were collected from 10 healthy individuals, including seven females and three males in the age of 45–75 year, and from five oncological patients treated with radiation therapy in the Hospital Kralovske Vinohrady in Prague. The samples were stored at −20 °C. After defrosting, 1 mL of the sample was centrifuged for 5 min at 1200 × *g*. The supernatant was filtered using a micro-porous Nylon filter (0.45 μm), and injected directly into the CE instrument.

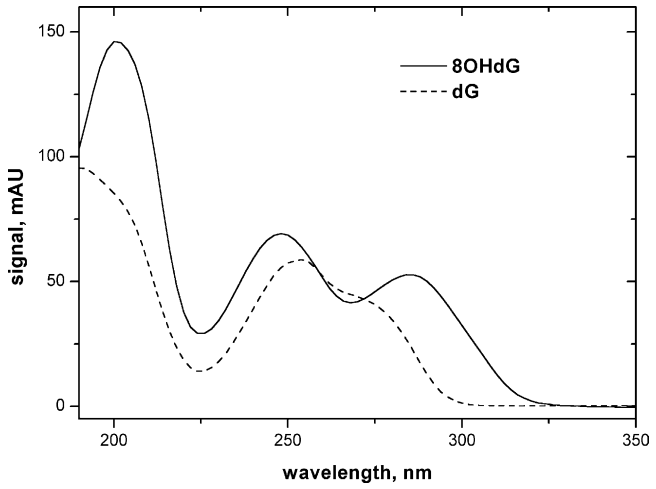

Fig. 2. UV spectrum of 20 μM 8OHdG (A) and 20 μM dG (B) in 80 mM CHES/LiOH, pH 8.6, containing 0.1 mM CTAB.

3. Results and discussion

3.1. Optimisation of the separation electrolyte composition

CHES/LiOH buffer system containing 0.1 mM CTAB was used as the separation electrolyte. The introduction of CTAB leads to the inversion and the rate lowering of the EOF, so that EOF has the same direction as the anionic nucleosides/nucleotides migrating towards the anode. Experimentally measured EOF was $-12.5 \pm 0.2 \times 10^{-9}\,m^2\,V^{-1}\,s^{-1}$. Optimisation of the buffer composition included the variation of the CHES concentration (20–80 mM) and of pH (8–9). We have chosen purposely Li^+ as the counter-ion to CHES for its low mobility as compared to that of Na^+ or K^+. As mentioned above, a lower mobility of the buffer ions allows increasing the buffer concentration, which does not influence the released Joule heat leading to a peak broadening. For the same reason it is possible to inject an enhanced amount of undiluted urine. Enhanced buffer capacity is probably responsible for a suppression of fluctuations of the electric current, as compared with the analysis of much more diluted aqueous model samples.

Optimisation of pH of the separation electrolyte is of principal significance with respect to the analysis of 8OHdG, the electrophoretic mobility of which depends on pH due to the weakly dissociating groups present in the molecule. Apparently, 8OHdG can be analysed as the cation at pH <3, while it can be analysed as an anion at pH >7.5 [14]. The solution pH of 80 mM CHES was adjusted by adding 1 M LiOH. Fig. 3 shows the electropherograms at three different pH values pointing to a strong pH effect. Owing to a large number of metabolites in urine, it was rather difficult to identify the component that interferes with 8OHdG in Fig. 3A. Nevertheless, this interference becomes insignificant upon adjusting pH, with the best separation being achieved

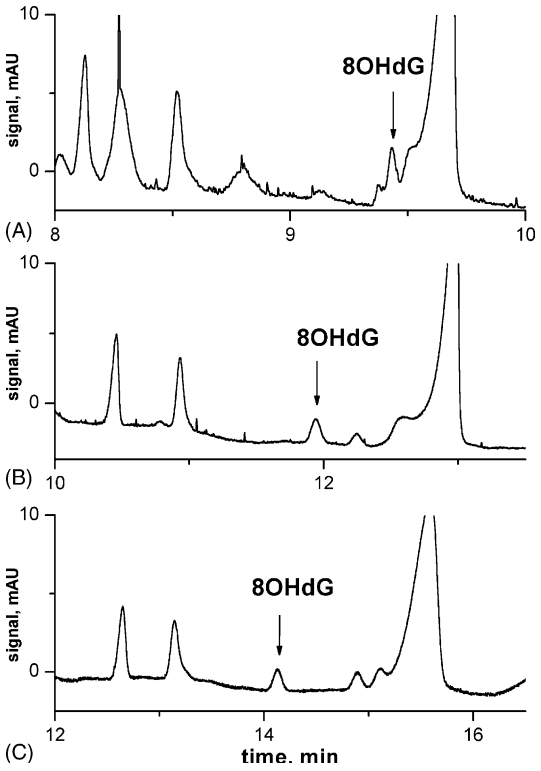

Fig. 3. Electropherograms of an untreated urine samples spiked with 10 μM 8OHdG in different pH values: (A) pH 9.0, voltage −30 kV/electric current −27 μA; (B) pH 8.8, voltage −30 kV/−26 μA; and (C) pH 8.6, voltage −30 kV/−24 μA. Other conditions: buffer system 80 mM CHES/LiOH containing 0.1 mM CTAB, hydrodynamic injection 120 mbar s, optical detection at 204 nm.

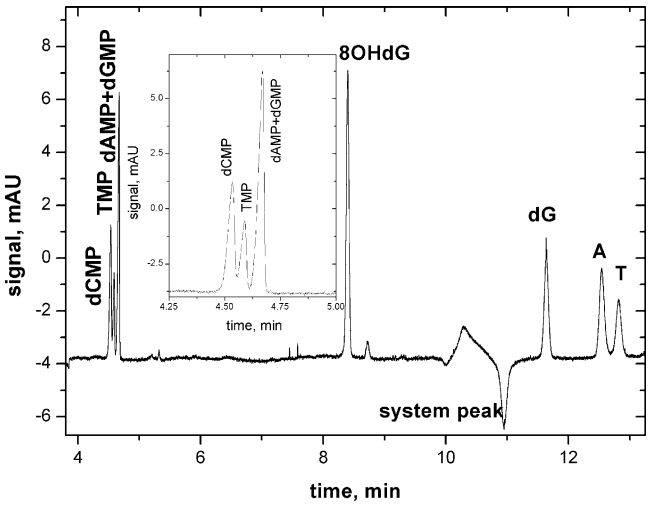

Fig. 4. Electropherogram of a mixture of 5 μM nucleotides and nucleosides in water: 8-hydroxy-2′-deoxyguanosine (8OHdG), 2′-deoxyguanosine (dG), adenosine (A), thymidine (T), 2′-deoxycytidine 5′-monophosphate (dCMP), 2′-deoxyadenosine 5′-monophosphate (dAMP), 2′-deoxyguanosine 5′-monophosphate (dGMP) and thymidine 5′-monophosphate (TMP). Inset: zoomed for nucleosides on time scale 4.25–5.00 min. Conditions: 80 mM CHES/LiOH, (pH 8.6) containing 0.1 mM CTAB, hydrodynamic injection 1000 mbar s, separation voltage/current −30 kV/−18.5 μA and optical detection at 204 nm.

at pH 8.6 (Fig. 3C). The absence of the interfering component at this pH value has allowed increasing the hydrodynamic injection from the initial value of 120 mbar s (12 kPa s) up to 2000 mbar s (200 kPa s) maintaining full separation of 8OHdG down to the baseline. The optimum composition of the separation electrolyte was found to be 80 mM CHES, 9 mM LiOH, 0.1 mM CTAB and pH 8.6.

3.2. Analysis of nucleosides and nucleotides in an aqueous sample

For the sake of comparison, the optimised composition of the separation electrolyte has also been examined under the model aqueous conditions. Fig. 4 shows the separation of dCMP, TMP, 8OHdG, dG, A and T in an aqueous sample. As it can be seen from Fig. 4 dAMP and dGMP migrate together, while G, dA and dC are transported as neutral species with the electro-osmotic flow and are not detected. Limits of detection (LOD, $S/N = 3$), linear dynamic ranges and calibration curves were determined for the individual nucleosides and nucleotides with the results summarized in Table 1 . Migration times and peak areas for 8OHdG and dG in the aqueous samples are given in Table 2. These data suggest that the analysis of nucleosides/nucleotides in an aqueous sample is quite reproducible, and that 8OHdG is well separated from dG. It is noteworthy that LOD = 34 nM and the linear dynamic range 0.1–20 μM 8OHdG is comparable to that found in the aqueous samples containing a mixture of nucleosides in 20 mM borate buffer, pH 9.5 [21].

3.3. Analysis of 8OHdG and dG in urine samples

Under the same conditions as above, 8OHdG is well separated in the spiked urine samples, but urine metabolites in-

Table 1
Parameters of calibration curves for determination in water solution containing 80 mM CHES/LiOH, 0.1 mM CTAB, pH 8.6, as evaluated from three independent measurements at each of five different concentrations

Compound	λ (nm)	LOD (nM)	Dynamic range (μM)	Slope[a] (mAU min μM^{-1})	Intercept[a] (mAU min)	R
8OHdG	204	34	0.1–20	6.58 (0.01)	−0.16 (0.11)	0.99999
dG	254	88	0.2–20	2.57 (0.01)	−0.22 (0.09)	0.99999
dCMP	204	98	0.2–20	2.29 (0.02)	−0.35 (0.22)	0.99992
TMP	204	201	0.2–20	1.12 (0.00)	−0.11 (0.06)	0.99998
A	204	49	0.2–20	4.56 (0.13)	−0.70 (1.32)	0.99921
T	204	78	0.2–20	2.88 (0.07)	−0.85 (0.71)	0.99943

[a] S.D. in parenthesis.

Table 2
Migration times and peak areas for 8OHdG and dG in the aqueous and spiked urine samples (in parentheses) containing 80 mM CHES/LiOH and 0.1 mM CTAB (pH 8.6), hydrodynamic injection 1000 mbar s (100 kPa s), as evaluated from five independent measurements

Compound	Migration time (min)	Peak area (mAU min)[a]
8OHdG	8.505 ± 0.104 (9.103 ± 0.075)	6.6 ± 0.3 (4.6 ± 0.1)
dG	12.967 ± 0.185 (14.853 ± 0.193)	2.7 ± 0.2 (2.8 ± 0.2)

[a] At concentration 1 μM.

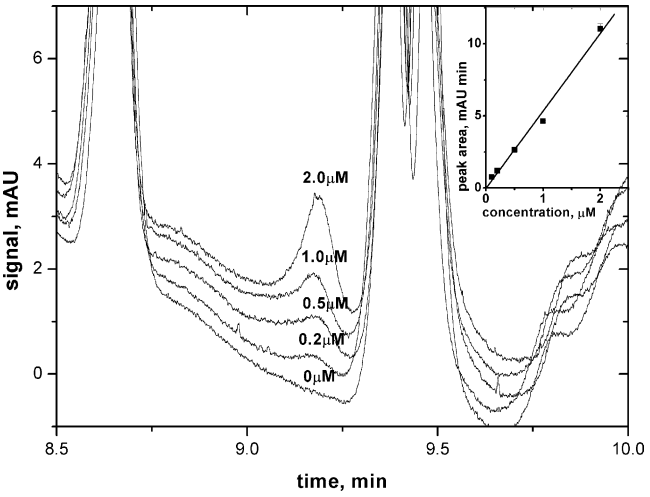

Fig. 5. Electropherogram of an untreated urine sample spiked with 8OHdG at concentrations shown by numbers on curves. Conditions: 80 mM CHES/LiOH (pH 8.6) containing 0.1 mM CTAB, hydrodynamic injection 1000 mbar s, separation voltage/current −30 kV/−23.5 μA, optical detection at 204 nm. For the sake of clarity, the electropherograms for individual 8OHdG concentrations are purposely shifted up by 0.5 mAU.

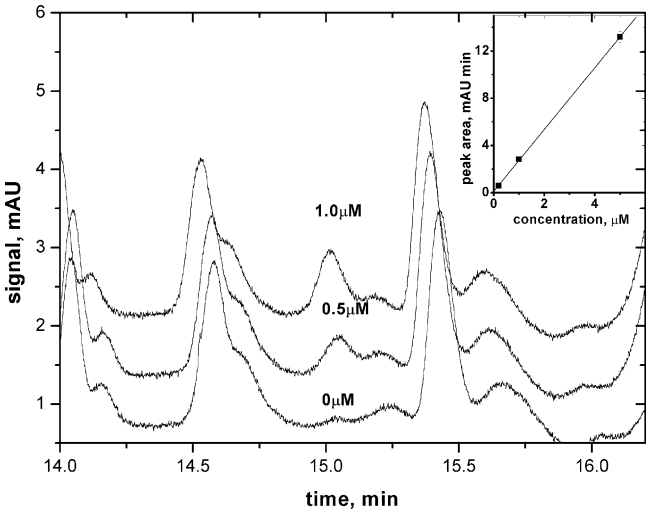

Fig. 6. Electropherogram of an untreated urine sample spiked with dG at concentrations shown by numbers on curves. Conditions: 80 mM CHES/LiOH (pH 8.6) containing 0.1 mM CTAB, hydrodynamic injection 1000 mbar s, separation voltage/current −30 kV/−23.5 μA, optical detection at 254 nm. For the sake of clarity, the electropherograms for individual dG concentrations are purposely shifted up by 0.5 mAU.

terfere with the determination of dCMP, TMP, A and T. Electropherograms of the untreated urine samples spiked with various concentrations of 8OHdG is shown in Fig. 5. It is to be noted that the remarkable difference between the migration times of 8OHdG between Fig. 3C and 5 is due the large difference in the hydrodynamic injection 120 mbar s (Fig. 3C) and 1000 mbar s (Fig. 5). The origin of the system peak can be related to the so-called eigenmobility of separation electrolytes containing two and more co-ions [26,27]. In the present system, these co-ions are represented by CHES and bromides. Since the dG concentration in urine can be comparable to that of 8OHdG, an attention was also paid to the efficient separation of dG. Electropherograms of the urine samples spiked with various concentrations of dG are shown in Fig. 6. A very good reproducibility of the migration times and peak areas was analogous to that observed in the model aqueous mixtures (Table 2).

Calibration curves were linear over the concentration range of 0.1–2.0 μM for 8OHdG and 0.1–5.0 μM for dG (Figs. 5 and 6). Detection limits corresponding to the signal-to-noise ratio $S/N = 3$ have been found to be 42 nM for 8OHdG and 86 nM for dG, which are the values comparable with those obtained for the aqueous samples. Parameters of the calibration curves in urine are summarized in Table 3. Although the migration times for the same hydrodynamic injection are somewhat longer in urine samples than in water (Table 2), the CE analysis can be completed within ca. 15 min. As it can be seen from Fig. 5, 8OHdG level in the unspiked urine is below the noise level of 0.1 mAU. Based on the linear regression to the calibration graph in Fig. 5, this level corresponds to 11 nM 8OHdG, which is about the concentration in urine of healthy individuals found using more sensitive method [8–11,17].

3.4. Comparison with the previous method

Separation conditions in the previous [14] and the present study are compared in Table 4. In order to suppress the interference from other metabolites, 8OHdG has been previously determined in 10 times diluted urine samples with LOD of 1.7 μM (i.e., 17 μM in undiluted urine) [14]. A comparison of the separation conditions suggests that the improved LOD is essentially due to the enhanced ratio of the sensitivity to noise by a factor of 30 (in water) or 50 (in 10 times diluted urine). LOD has been lowered by a comparable factor of 25 (in water) and 40 (in 10 times diluted urine), while for undiluted urine samples this factor makes 400. The 120-fold higher sensitivity (slope of the calibration curve) in the present study is likely to be in part due to a more efficient separation of 8OHdG from other component, and in part due to the difference in the UV detection in the previous and the present study. By using DAD, the noise is enhanced 2–5 times, but

Table 3
Parameters of the calibration curves for determination in urine containing 80 mM CHES/LiOH, 0.1 mM CTAB, pH 8.6, as evaluated from three independent measurements at each of five different concentrations

Compound	λ (nm)	LOD (nM)	Dynamic range (μM)	Slope[a] (mAU min μM^{-1})	Intercept[a] (mAU min)	R
80HdG	204	42	0.1–2.0	5.37 (0.31)	−0.02 (0.32)	0.99946
dG	254	86	0.2–5.0	2.61 (0.03)	−0.14 (0.08)	0.99994

[a] S.D. in parenthesis.

Table 4
Comparison of the separation conditions in the previous [14] and present study

Parameter	Present study	Previous work [14]
Separation buffer	80 mM CHES + 9 mM LiOH + 0.1 mM CTAB, pH 8.6	10 mM $Na_2B_4O_7$, pH 9.0
Voltage/current	−30 kV/−23 μA	20 kV/25 μA
EOF	Anodic, −12.5 m^2 V^{-1} s^{-1}	Catodic, +76.6 m^2 V^{-1} s^{-1}
Capillary length (i.d.)	64 cm (total), 75 μm	70 cm (total), 75 μm
Hydrodynamic injection	1000 mbar s (100 kPa s)	2000 mbar s (200 kPa s)
Injection plug length	27.2 mm	50.2 mm
Detected wavelength	204 nm	200 nm
Sensitivity in water	6.58 mAU min μM^{-1}	9.02 × 10^3 μAU s mg^{-1} L (0.043 mAU min μM^{-1})
Noise	0.1 m AU	0.02 mAU
LOD in water	34 nM	0.85 μM
Sensitivity in urine	5.37 mAU min μM^{-1}	9.09 × 10^3 μAU s mg^{-1} L (0.043 mAU min μM^{-1})
Noise	0.1 mAU	0.04 mAU
Urine dilution	No	10 times
LOD in urine	42 nM	1.7 μM (17 μM)[a]

[a] Recalculated for undiluted urine.

the sensitivity increases ca. 120 times. The significant criterion for lowering LOD is especially the ratio of these factors, but the absolute values of sensitivity and noise are quite unsubstantial.

The improved method was tested in the analysis of urine samples collected from five oncological patients. In three patients, the urinary 8OHdG levels were below LOD; in other two patients 8OHdG could be reliably detected at a concentration of 100 and 390 nM. The separation of 8OHdG in urine of an oncological patient is compared with that in the spiked urine of a healthy individual in Fig. 7 . Considerably lower 8OHdG levels compared to those previously found [14] are likely to be due to a difference in the radiation therapy. Patients examined in the present study were treated with the focused rather than dispersed radiation leading to a lower load and disorder of the organism.

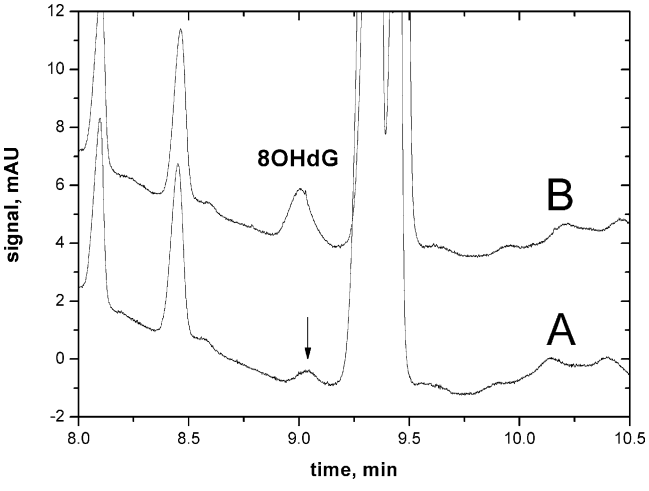

Fig. 7. Electropherograms of an untreated urine sample of irradiated patient, concentration of 8OHdG 390 nM (A) and of the same sample spiked with 2 μM 8OHdG (B). Conditions: 80 mM CHES/LiOH (pH 8.6) containing 0.1 mM CTAB, hydrodynamic injection 1000 mbar s, separation voltage/current −30 kV/−23.5 μA, and optical detection at 204 nm. For the sake of clarity, the two electropherograms are purposely shifted with respect to each other.

4. Conclusions

Optimisation of conditions for the CE analysis with the optical detection using DAD resulted in a significant lowering of the limit of detection of 8OHdG in untreated urine down to values reported for the CE method applied to the model aqueous samples [13,21,22]. We attempted to avoid time consuming and more expensive pre-treatment of the urine samples using the solid-phase extraction and pre-concentration, which however turns out to be necessary to obtain reliable data for healthy individuals. At the moment, the application can be foreseen in the analysis of untreated urine samples with the enhanced 8OHdG levels, such as those encountered in urine of oncological patients treated by radiotherapy. Further improvement in the sensitivity is likely to be possible using the electrochemical detection.

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