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Determination of 8-hydroxy-2′-deoxyguanosine in untreated urine by capillary electrophoresis with UV detection

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Abstract

A capillary electrophoresis method with UV detection was developed for the determination of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in untreated urine samples. The calibration graph for 8-OHdG in urine is linear in the concentration range 10–500 mg/l, and the detection limit is 5 mg/l (17 μ*M*). 8-OHdG was determined in urine from oncological patients treated by radiation therapy. Its concentrations relative to creatinine were found to be in the range 10–47 μg 8-OHdG/1 mg creatinine (4–19 μmol 8-OHdG/mmol creatinine). The overall time of the analysis of a urine sample was less than 15 min.

Keywords: Hydroxydeoxyguanosine; DNA

1. Introduction

Enhanced production of reactive oxygen species (ROS), such as superoxide anion, peroxyl, hydrogen peroxide and hydroxyl radicals, leads to so-called oxidative stress [1] and, among others, oxidative DNA damage [1–3]. The extent of the oxidative DNA damage is closely related to the metabolic rate, i.e., to the consumption of oxygen [2]. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) is probably the most important and best-documented product of oxidative DNA damage in biological systems [4–7], which is the oxidative product of 2′-deoxyguanosine (dG) [8,9]. Guanine base in DNA represents the main targets of one-electron oxidants, such as the singlet oxygen [10], or the OH radical produced by ionising radiation and by the transition metal catalysed decomposition of hydroperoxides [11]. DNA photooxidation [12] is believed to proceed via the guanosine radical cation [13].

A number of analytical methods have been developed for measuring 8-OHdG in urine. This determination has the advantage in that DNA lesions from all the cells in the body are concentrated in a relatively small volume of urine, and that the procedure is non-invasive. 8-OHdG was analysed first by using multiple solid-state extractions on minicolumns preceding high-performance liquid chromatography (HPLC) separation [14,15]. The extraction can be also accomplished by using immunoaffinity columns with specific antibodies to 8-OHdG, requiring only isocratic separation [16]. In both previously reported methods, the electrochemical (amperometric) detection was used, the sensitivity of which is about three orders higher than UV absorbance. Another isocratic coupled-column HPLC has been developed also with the electrochemical detection [17–20]. Typical urinary excretion values for healthy individuals are around 15 nmol of 8-

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OHdG per 24 h, which corresponds to ca. 9 nmol/l urine and ca. 1 μmol 8-OHdG/mol creatinine [18]. The capillary electrophoresis (CE) method utilising diode-array detection [21] has been introduced for the determination of 8-OHdG in human cells [21]. Recently, the CE method combined with the solid-phase extraction and electrochemical detection has been applied to analyse the urinary 8-OHdG in healthy individuals [22].

Low detection limit for 8-OHdG (5–50 n*M* [18,22,23]) has been achieved in part by the sensitive (e.g., electrochemical) detection and, in part, by the sample preparation leading to 10-fold and more concentration of the sample. Such treatment of the sample is rather tedious and time consuming. The aim of this work was to develop a capillary electrophoresis method for the determination of 8-OHdG in untreated urine sample, which presumably should shorten time of the determination. Since avoiding the pre-concentration step leads to an increase in the detection limit, the method can be used for the analysis of urine samples with the enhanced levels of 8-OHdG that can be found in, e.g., oncological patients treated by radiation therapy.

2. Experimental

2.1. Chemicals

Aqueous solutions of carrier electrolytes and of standards were prepared by using 18 MΩ cm water from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All chemicals were of analytical reagent grade quality. Sodium tetraborate, boric acid, 8-hydroxy-2′-deoxyguanosine (8-OHdG), 2′-deoxyadenosine (dA), 2′-deoxycytidine (dC), 2′-deoxythymidine (dT) and 2′-deoxyguanosine (dG) were purchased from Sigma (St. Louis, MO, USA). Thiourea, citric acid and phosphoric acid were purchased from Fluka (Buchs, Switzerland).

2.2. Instrumentation

SpectraPhoresis 1000 (Thermo Quest, San Jose, CA, USA) equipped with an UV detector and PC 1000 System Software was employed for the determination of 8-OHdG. Separations took place in an uncoated 70 cm×75 μm I.D silica capillary.

2.3. Sample preparation

Urine samples were prepared using the first morning urine either from healthy persons (mixture sample) or from oncological patients treated by radiation therapy. After the collection, the samples were either frozen at −20 °C, or immediately processed in the following way. Fresh urine samples were centrifuged for 10 min at 2000 rpm. After 10 min, 100 μl of supernatant were taken off and diluted to 1 ml by deionised water, in order to lower the concentration of other ions possibly interfering with the CE analysis. The sample was then injected into the CE instrument. Frozen samples were processed in the same way after defrosting at room temperature.

2.4. Separation conditions

Borate buffer, 10 m*M*, pH 9.0, was used as the carrier electrolyte in all determinations. UV detection was performed at 200 nm. Samples were introduced in the hydrodynamic injection mode set to a pressure of 100 mbar with an injection time of 20 s. Applied voltage was 20 kV. Measurements were carried out at a constant temperature of 25 °C.

3. Results and discussion

3.1. Optimisation of CE conditions

Three carrier electrolytes were examined including phosphoric acid, a mixture of citric acid and Na_2HPO_4, and a mixture of sodium tetraborate and boric acid (borate buffer). Borate buffer has been previously employed because of the convenient pH range [21]. Our electrophoretic measurements have revealed that the presence of boric acid has an effect on the mobility of 8-OHdG. Fig. 1 shows the dependence of electrophoretic mobility of 8-OHdG on pH of these carrier electrolytes. The electrolyte pH was adjusted by changing the concentration of phosphoric acid, citric acid and Na_2HPO_4, or boric acid, respectively. Apparently, 8-OHdG can be analysed as a cation in acidic solutions (pH<3), while it

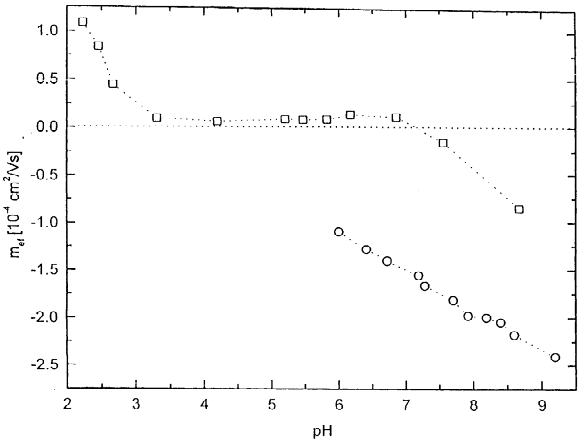

Fig. 1. Electrophoretic mobility μ_{ef} of 8-OHdG as a function of pH of the carrier electrolyte: phosphoric acid or citric acid+ Na_2HPO_4 (□), or sodium tetraborate+boric acid (○).

can be analysed as an anion in basic solutions (pH> 7.5), cf. the squares in Fig. 1. In borate buffer solutions, the anionic mobility of 8-OHdG is significantly enhanced, cf. the circles in Fig. 1, which is likely to be due to the formation of the complex of boric acid with the sugar moiety of 8-OHdG [24]. As the solution pH increases, the dissociation of proton from boric acid leads to an increase in the fraction of the anionic form of the complex, and to the linear dependence of the electrophoretic mobility on pH, cf. Fig. 1. Consequently, the use of the borate buffer with pH adjusted to 9.0 appears to be the optimum choice.

The UV absorption spectrum was measured, so as to find the maximum absorption of 8-hydroxy-2′-deoxyguanosine in borate buffer. This measurement suggests that the wavelength of 200 nm is convenient for detection.

The interference by other 2-deoxynucleotides that can be also present in urine was examined. Fig. 3 shows the electropherogram of their mixture in water. At pH 9.0, dA and dC are electrically neutral and, hence, they are not separated and the rate of their transport is determined by the electroosmotic flow (EOF). Obviously, 8-OHdG is well separated from both dT and dG, which also carry the negative charge under the conditions of the determination.

Time of CE analysis and the separation efficiency depends on temperature, the applied voltage, the mode of injection and the injection time. In the temperature range 25–45 °C, the dependencies of both the electrophoretic mobility of 8-OHdG and the electroosmotic mobility on temperature are linear (correlation coefficient 0.999), but the latter dependence has a higher slope. This indicates that the thermostat is capable of maintaining efficiently the temperature of the separating column, but the separation of 8-OHdG from the neutral components of the urine sample can deteriorate with the increasing temperature. Therefore, the temperature of determination was set to the lowest value, i.e., 25 °C. In the range of the applied voltage 5–25 kV, the migration times of 8-OHdG varied from 26.4 to 5.1 min. Thus, the application of a higher voltage, e.g., 20 kV, enables a relatively fast determination of 8-OHdG with plausible separation efficiency, cf. Fig. 2. Samples were introduced in the hydrodynamic injection mode. Since the instrument does not allow changing the injection pressure (100 mbar), we examined the effect of the injection time (5–30 s) on the height and width of the peak of 8-OHdG. While the peak asymmetry does not vary significantly, the peak width exhibits a steeper increase then the peak height when the injection time is increased above 25 s. In order to avoid the deterioration of the signal resolution, the samples were introduced with the injection time of 20 s.

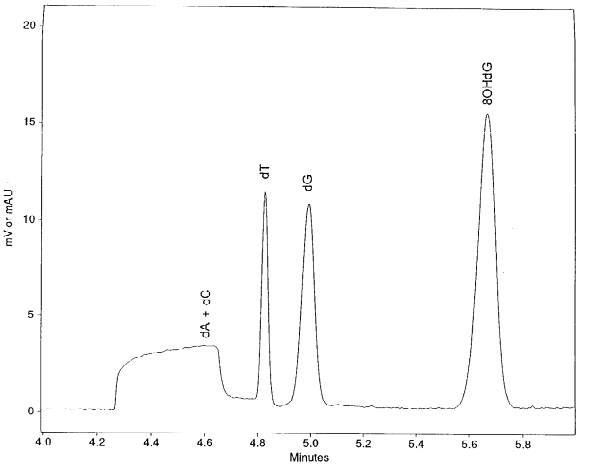

Fig. 2. Electropherogram of the mixture of 2′-deoxynucleotides (10 mg/l) in water: 8-hydroxy-2′-deoxyguanosine (8-OHdG), 2′-deoxyadenosine (dA), 2′-deoxycytidine (dC), 2′-deoxythymidine (dT) and 2′-deoxyguanosine (dG).

3.2. Calibration graphs of 8-hydroxy-2′-deoxyguanosine

The calibration graphs for the determination of 8-OHdG were constructed from the measured peak area S versus the concentration c of 8-OHdG. Standard solutions of 8-OHdG were prepared from the 10-fold diluted urine samples, which did not contain the measurable amount of 8-OHdG. In the concentration range 1-50 mg/l (i.e., 10–500 mg/l of the undiluted urine sample), the calibration graph is linear. The linear regression yields the line S(a.u.)= $17.3\times10^3+9.09\times10^3c$ (mg/l), with a correlation coefficient of 0.999 and a standard deviation of 4.43×10^3. The calibration graph in water is a similar plot with the linear regression line S(a.u.)$=3.23\times10^3+9.02\times10^3c$ (mg/l), a correlation coefficient of 0.999, and a standard deviation of 4.19×10^3. An almost identical slope of these plots (the slope ratio 1.007) indicates that the determination in urine is not affected by the systematic error arising from the separation efficiency of the method. The relative standard deviations in the peak area of 8-OHdG in water and 10-fold diluted urine between days were 7.5 and 3.8%, respectively.

Migration times for 8-OHdG and thiourea (EOF marker) are given in Table 1. Under the conditions of the determination, both thiourea and creatinine are uncharged species, the transport of which is controlled by the electroosmotic flow. The analysis of a series of urine samples suggested that, for an identification of the 8-OHdG peak based on its migration time, it is necessary to employ the method of the standard addition. The reason is that the migration time depends on the ionic strength, which is different for various urine samples.

Detection limit was estimated as the concentration that gives the signal equal to three times the noise level. The calibration graphs yield the detection limit of 0.25 mg/l (0.85 μM) or 0.5 mg/l (1.7 μM) for water or the 10-fold diluted urine, respectively. Both limits are comparable with those reported for the injected standard samples [22]. However, the latter limit corresponds to the detection limit of 5 mg/l (17 μmol/l) of the undiluted urine, which is 350 times higher than the limit of ca. 50 nmol/l attained in the CE analysis that is combined with the solid-phase extraction (SPE) [22]. The difference in the sensitivity between the SPE–CE and the CE method developed in this work is associated with the ca. 20-fold concentration of the urine sample upon the SPE [22], as compared with 10-fold dilution of the sample prior to its introduction in the present method. Thus, the present method avoids the tedious and time-consuming urine pretreatment, but its application is limited to samples with the enhanced levels of 8-OHdG.

Table 1
Migration times ($t_m\pm$SD) and the relative standard deviations RSD for 8-OHdG and EOF in water and urine, concentration of 8-OHdG and thiourea: 50 mg/l

	Water		Urine	
	t_m (min)	RSD (%)	t_m (min)	RSD (%)
8-OHdG	6.006±0.235	3.9	6.484±0.269	4.2
EOF	4.720±0.288	6.1	5.082±0.118	2.3

3.3. Determination of 8-OHdG in urine of oncological patients

The present method was used to determine the urinary 8-OHdG from five randomly selected oncological patients treated by the radiation therapy based on the linear energy transfer. The 10-fold diluted fresh midstream void urine samples were analysed by the method of standard addition. Fig. 3 shows the electropherograms without and with the addition of the standard 8-OHdG solution (20 mg/l), which made it possible to identify 8-OHdG in the sample and to evaluate its concentration. In order to reduce the diuresis effect, creatinine was determined in all urine samples, and the measured concentrations of 8-OHdG were related to the amount of creatinine found. Relative 8-OHdG concentrations in irradiated patients were found in range 10–47 μg 8-OHdG/1 mg creatinine (4–19 μmol 8-OHdG/mmol creatinine).

4. Conclusions

It is shown that capillary electrophoresis can be used for a fast determination of 8-hydroxy-2′-deoxyguanosine in untreated urine samples. Owing to a

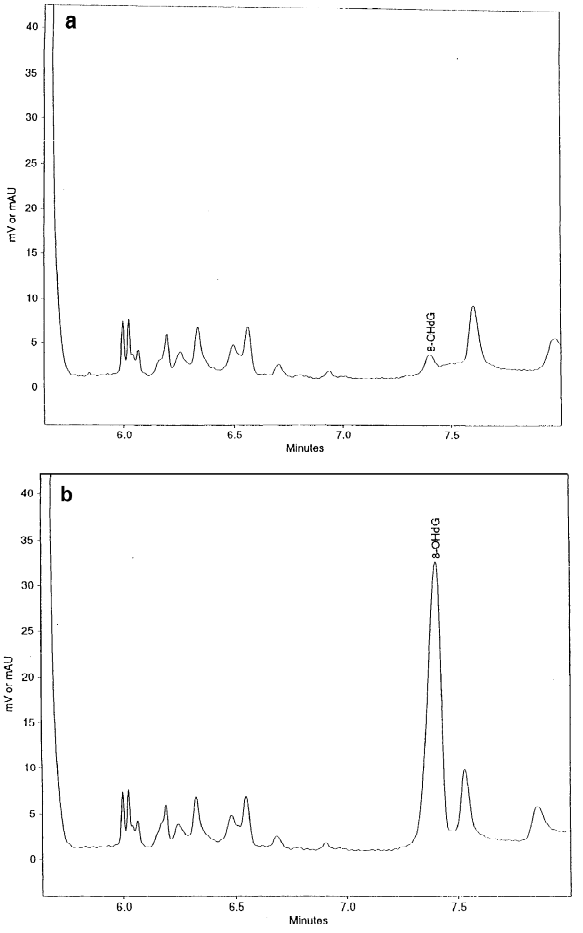

Fig. 3. Electropherograms of the 10-fold diluted urine sample of an irradiated patient before (top), and after (bottom) the standard addition of 20 mg 8-OHdG/l: 10 m*M* borate buffer, pH 9.0, UV detection at 200 nm, hydrodynamic injection (100 mbar), injection time 20 s, applied voltage 20 kV, temperature 25 °C.

higher detection limit, as compared to that attained with the HPLC or CE analysis of the pretreated urine, the present method is applicable in the analysis of urine samples with enhanced levels of 8-hydroxy-2′-deoxyguanosine. Such levels can be found in urine from the oncological patients treated by radiation therapy.

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