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Analysis of 8-hydroxy-2′-deoxyguanosine in urine using high-performance liquid chromatography–electrospray tandem mass spectrometry

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Abstract

8-Hydroxy-2′-deoxyguanosine (8-OHdG) is a widely used biomarker of oxidative stress in research related to DNA, protein damage as well as lipid peroxidation. HPLC–MS/MS with electrospray ionization (ESI) and the use of isotopically labelled 8-OHdG as an internal standard allows a simple quantification of 8-OHdG in urine samples. HPLC separation utilized the peak cutting technique and a 1.5 mm × 120 mm analytical anion exchange column. Novel method entails only minimal sample handling including the addition of a buffer and an internal standard followed by centrifugation before the samples are ready for analysis. The levels of 8-OHdG in human urine samples ($n = 246$) varied from 0.16 to 16.48 μg/L and the corresponding creatinine-normalized values were ranged from 0.49 to 14.27 μg of 8-OHdG/g creatinine. The correlation between the developed HPLC–MS/MS method and the existing HPLC-EC method was good with an R^2 value of 0.8707.

Keywords: DNA damage; Monitoring; Radicals

1. Introduction

Oxidative stress is a general term for the cellular damage evoked by highly reactive free radicals. The majority of the damaging radicals in cells is derived from oxygen, and these radicals attack very crucial cellular biomolecules, such as DNA, lipids and proteins [1].

There are many initiators of oxidative DNA damage, e.g. endogenous cellular metabolism, chemicals, drugs, ionizing radiation, solar light, cigarette smoke and air pollution [2]. However, DNA lesions can be repaired by base excision repair, nucleotide excision repair and/or mismatch repair mechanisms and sanitization of the nucleotide pool. The excision products are transported out of the cells and excreted into urine mainly without further metabolism. In addition to those originating from the DNA repair process, products of oxidative damage in urine can be derived from the diet and end products of lyses of death cells [3–5].

8-Hydroxy-2′-deoxyguanosine (8-OHdG) is formed from a hydroxyl radical and a deoxyguanosine residue. It is one of the most abundant products of DNA oxidation and also one of the most extensively studied oxidation lesions due to its mutagenic potential. 8-OHdG pairs with adenine in DNA replication, producing G to T transversions and this may also cause misreading of the adjacent bases [6]. 8-OHdG has been used as a common biomarker in the evaluation of oxidative stress caused by occupational exposure [7], lifestyle [8,9] and many human diseases, such as cancer, diabetes and neurological disorders [10–13].

Measurements of 8-OHdG in urine samples are challenging due to the low levels of the analytes, the complex matrix [14] and the ease of artefactual formation of 8-OHdG from deoxyguanosine (dG) during sample preparation. However,

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artefactual oxidation is much higher in DNA samples, in which the ratio of dG to 8-OHdG is higher than that found in urine [15].

Urinary 8-OHdG can be measured by using several techniques including gas chromatography–mass spectrometry (GC–MS) [16–18], liquid chromatography–mass spectrometry (HPLC–MS/MS) [15,19–21], enzyme-linked immunosorbent assay (ELISA) [22], and liquid chromatography with an electrochemical detector (HPLC-EC) [23–27]. However, electrochemical detection requires extensive sample purification before the analysis because of the presence of interfering compounds in urine. Thus, sample preparation typically includes a solid phase extraction, column-switching techniques and automated coupled-column systems. In GC–MS applications, 8-OHdG has to be derivatized to increase the volatility of the analyte. Unfortunately, derivatization considerably increases the risk of artificial formation of 8-OHdG. The immunochemical methods suffer the problem of non-selectivity because the antibody may cross-react with other substances present in urine [22].

In the present study, we describe a simple and rapid assay based on HPLC–MS/MS to detect and quantify 8-OHdG in human urine. The method does not require complex sample pretreatment as was the case in our recent papers where we analyzed 246 urine samples with the HPLC-EC method [27,28].

2. Experimental

2.1. Materials

Deoxyguanosine and 8-hydroxy-2′-deoxyguanosine were purchased from Sigma–Aldrich (Steinheim, Germany) and sodium acetate from Riedel-deHaën (Seelze, Germany). [$^{13}C^{15}N$] dG was purchased from Medical Isotope Inc. (Pelham, USA). The internal standard, [$^{13}C^{15}N$] 8-OHdG, was synthesized by Dr. Toshiaki Mori (Research Institute for Advanced Science & Technology, Osaka Prefecture University). The anion exchange resin MCI GEL CA08F was purchased from Mitsubishi Chemical Corporation (Tokyo, Japan). Acetonitrile and methanol were high-performance liquid chromatography grade (Labscan Ltd., Dublin). Acetic acid was obtained from Merck (Darmstadt, Germany). The water used was Millipore Milli-Q purity (Espoo, Finland).

2.2. Synthesis of internal standard

The internal standard of stable-isotope-labelled 8-OHdG-$^{13}C_{10}$, $^{15}N_5$ was synthesized according to the method described by Bialkowski and Kasprazak with minor modifications [29].

Briefly, deoxyguanosine-$^{13}C_{10}$, $^{15}N_5$ (20 mg) was treated with ascorbic acid (50 mg) and hydrogen peroxide (200 μL, 30%) in phosphate buffer (10 mL, 20 mM, pH 7) for 3 h at room temperature. The reaction was terminated by adding 120 μg of catalase and incubated for 30 min.

After lyophilization, water (2 mL) was added. The labelled 8-OHdG was separated with HPLC using 10 mm × 250 mm ODS column (Capcell Pac C18, Shiseido, Japan), and triethylamine acetate (50 mM, pH 7) as an elution buffer. The fraction containing labelled $^{13}C_{10}$, $^{15}N_5$ 8-OHdG was eluted after dG. The collected fraction which contain labelled 8-OHdG was lyophilized and the structure was confirmed by mass spectrometry.

Due to presence of buffer salt residues in $^{13}C_{10}$, $^{15}N_5$ 8-OHdG (ISTD) preparations, the gravimetric value was not applicable in the preparation of stock solutions in quantification experiments. Therefore, the ISTD concentration in stock solutions was determined by using a calibration graph generated from commercially available 8-OHdG. A linear calibration curve ($R^2 = 0.992$) was obtained using variable amounts of 8-OHdG ranging from 0.5 to 25 μg/L. All calibration points were determined in triplicate and the average relative standard error of all points was 6.2%. ISTD was added (10 μL) in all samples which resulted into a concentration of 9.6 to 11.01 μg/mL of ISTD in a single sample depending on the ISTD concentration in stock solutions.

2.3. Sample preparation

A total of 246 spot urine samples was collected from healthy controls and workers occupationally exposed to diesel exhaust. Urine samples were stored at −20 °C. As was reported earlier, frozen urine samples were thawed and 50 μL of urine was then mixed with the same volume of a NaOAc buffer, (130 mM, pH 4.5) containing 4% acetonitrile and H_2SO_4 (0.6 mM) (27). The pH of diluted urine samples was adjusted to below 7 to facilitate good separation of 8-OHdG from other urinary components. The sample solutions were stored in a refrigerator and after 1 h, they were centrifuged at 14,900 × g for 5 min. An aliquot (50 μL) of sample supernatant was transferred to a vial for HPLC–MS/MS analysis.

Urinary 8-OHdG was quantified by comparing the ratio between the peak area of the analyte and the internal standard. Qualitative confirmation was achieved by using the retention times of the sample and the internal standard and multiple reaction monitoring.

2.4. HPLC conditions

The HPLC system was equipped with a binary pump, a vacuum-degasser, a column compartment with a column oven and a six-port valve, an auto sampler and a control module (HP 1100 series, Hewlett Packard, Waldbrown, Germany). An HPLC pump (Jasco PU 1580, Jasco Inc., Easton, USA) was used to deliver the eluant into the mass spectrometer before and after peak cutting to ensure a constant effluent flow.

Separation of 8-OHdG was carried out in a 1.5 mm × 120 mm analytical anion exchange column. The anion exchange resin, MCI GEL CA08F (7 μm, polystyrene-type resin with quaternary ammonium group, acetate form), was packed in-house. Chromatographic separation of dG and 8-OHdG ensured that any possible auto-oxidation of dG to 8-OHdG in the ionization process did not result in an overestimation of the levels of 8-OHdG. The retention times were around 7 and 11 min for dG and 8-OHdG, respectively. Special steps or actions, such as working in an inert atmosphere to prevent auto-oxidation during

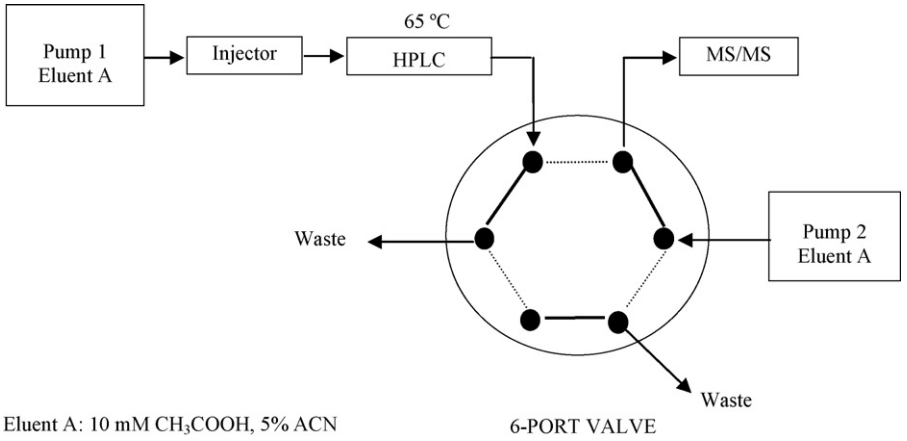

Fig. 1. Schematic presentation of the analytical device used in this exercise. A six-port valve was connected to the HPLC pumps, columns and to a MS/MS instrument which was used to detect urinary 8-OHdG.

sample preparation were not taken [19,20]. An isocratic elution was applied (CH_3COOH 10 mM and acetonitrile 5%). Separation was carried out at 65 °C with a flow rate 50 μL/min. A 4-min fraction that contained 8-OHdG and ISTD was transferred to a mass spectrometer utilizing a peak cutting technique. The injection volume was 10 μL except in the case of extremely dilute samples when 20 μL injections were used. The column-switching apparatus is schematically shown in Fig. 1. After every 100 urine samples, the analytical column was washed by applying the back-flush technique and isocratic elution (NH_4Ac, 500 mM and 30% acetonitrile) to clean the column. The performance of the analytical device was monitored by analyzing 8-OHdG standard (5 μg/L) or water between the samples.

2.5. ESI-MS/MS

Mass spectral analysis was carried out by using a Quattro LC triple quadrupole mass spectrometer in a positive ion mode (Micromass, Milford, United States). The instrument was optimized by using a syringe pump infusing at a flow rate of 50 μL/min of 8-OHdG (100 μg/L) into a spectrometer (Harvard apparatus, Holliston, United States).

The multiple reaction monitoring (MRM) acquisition mode was applied and the transition from the molecular ion $[MH]^+$ to the most intense fragment was recorded. The transitions were 284/168 for 8-OHdG, 268/152 for dG and 299/183 for [$^{13}C^{15}N$] 8-OHdG. The limit of quantification was determined by using a value three times the background noise. The daughter ion originates from the cleavage of the N-glycosidic bond with the transfer of a hydrogen atom from the sugar moiety. The mass spectrometer was controlled and data were processed with MassLynx software (Micromass, Milford, United States).

The source temperature was 80 °C and the cone voltage was 19 V. Nitrogen (98% purity) generated using a Whatman gas generator (Whatman International Ltd., Kent, England) was used as a nebulizer gas (260 °C), a desolvation gas (260 °C) and a cone gas. The capillary voltage of the mass analyzer was 2750 V, the collision gas (argon) pressure was 7.5×10^{-4} mbar and the collision energy was 12 eV. The voltage of the electron multiplier was 600 V and the dwell times of the monitored reactions were 0.5 s.

The early eluting components were diverted to waste and a 4-min fraction (from 9 to 13 min) containing 8-OHdG and ISTD was introduced into the mass spectrometer. The column-switching method was not able to entirely produce contaminant free fractions and some impurity components were indeed present in the sample fraction. Column contamination was followed by monitoring the appearance of additional peaks on the chromatograms close to the peak of the internal standard. The cone of the spectrometer was cleaned at the same time as the column by using methanol and formic acid in the ultrasonic cleaning procedure.

3. Results and discussion

8-OHdG binds strongly in an anion exchange column and a high elution temperature (65 °C) facilitated elution of a large proportion of urinary components to elute before the analyte. 8-OHdG and the corresponding isotopically labelled compound

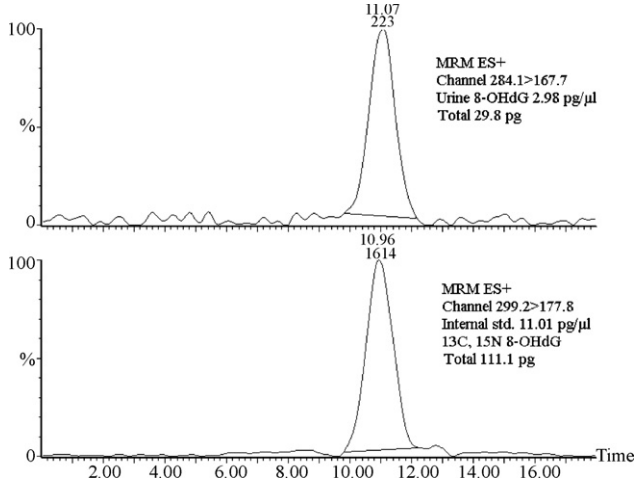

Fig. 2. MRM analysis of a urine sample of exposed worker. The upper trace shows the signal derived from urinary 8-OHdG (2.98 μg/L) and the lower trace shows the signal derived from the internal standard (11.11 μg/L).

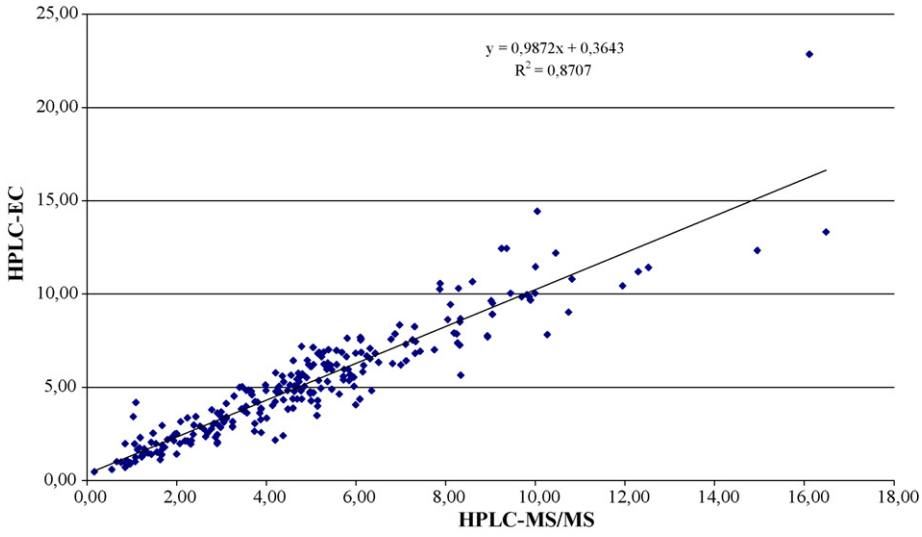

Fig. 3. The scatter plot presentation of the 246 urine samples demonstrating the good correlation ($y = 0.9872x + 0.3643$, $R^2 = 0.8707$) between the two assays, HPLC–MS/MS (x-axis) and HPLC-EC (y-axis).

had the same retention volume. If no sample preparation was used, then the column became contaminated with the many unknown components present in urine. Column contamination was clearly observed and it was noticed to lead to a shorter retention time for dG and 8-OHdG in the contaminated column compared to their elution from a conditioned column. In a uncontaminated column, the retention times of dG and 8-OHdG were close to 7 and 11 min, respectively. This fact needs to be taken into consideration if a peak cutting technique is to be applied. A typical MRM mode chromatogram of a urine sample is presented in Fig. 2. The limit of quantification of 8-OHdG measured by HPLC–MS/MS in the MRM mode was 0.16 μg/L.

The levels of 8-OHdG in human urine samples ($n = 246$) varied from 0.16 to 16.48 μg/L, and the corresponding values in creatinine-normalized samples required from 0.49 to 14.27 μg of 8-OHdG/g creatinine. The corresponding ranges using HPLC-EC were 0.49–22.85 μg/L and 1.18–9.42 μg of 8-OHdG/g creatinine. The mean value of 8-OHdG was 4.85 μg/L ±2.85 SD determined by HPLC–MS/MS compared to 5.15 μg/L ±3.02 SD by HPLC-EC. The correlation between the EC and MS/MS methods was calculated using 246 urine samples and a good correlation coefficient was achieved ($R^2 = 0.87$). The scatter plot presentation of the values of all 246 samples is shown in Fig. 3.

The use of a 2.1 mm ID C18 column and an appropriate flow rate rapidly decreased the sensitivity of the mass spectrometer due to the column contamination. When we replaced the conventional-sized column (ID 2.1 mm) with a capillary column (Inertsil ODS-3, 75 μm × 15 cm) good sensitivity (0.005 μg/L limit of detection) was achieved and numerous urine samples could be analyzed without jeopardizing the sensitivity of the mass spectrometer. Finally, we used a method with a flow rate of 200 nL/min and a six-port valve to carry out the peak cutting to direct a fraction of the HPLC effluent to the mass spectrometer. Gradient elution was used to separate 8-OHdG from interfering components in urine. Even though good sensitivity was achieved with the method, 8-OHdG could not be detected directly from urine samples. Therefore, we performed a solid phase extraction step during the sample preparation step and were able to reduce the levels of co-eluting components which suppressed the signal of the analyte in ESI.

By using the peak cutting technique, it was possible to further reduce but not completely remove the undesired compounds present in the fraction directed to the mass spectrometer. The anion exchange column permitted the use of a switching device which separated 8-OHdG from the interfering impurities in urine samples without extensive sample preparation. In that way, consumption of the valuable internal standard was also minimized. The assay proved to be reliable, fast, simple and sensitive enough to detect 8-OHdG also from diluted urine samples. The risks of artificial oxidation were minimized and MS/MS detection enabled us to use isotopically labelled internal standards to measure losses during sample preparation. Urine is a complex matrix containing numerous endogenous compounds and metabolites [14] with considerable inter-individual differences. Differences in the levels of compounds between individuals may lead to differences in suppression in mass spectrometry and this can result in errors in quantification.

As a conclusion, we have developed an accurate and reliable HPLC–MS/MS method which can be used for routine determination of urinary 8-OHdG in a wide linear range of the assay (0.5–25 μg/L). The MS/MS data are in good agreement with the HPLC-EC data.

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