Short communication

# Analysis of 8-hydroxy-2′-deoxyguanosine in human urine using hydrophilic interaction chromatography with tandem mass spectrometry

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### ARTICLE INFO

### ABSTRACT

Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) is a widely used noninvasive biomarker of oxidative stress. A selective, sensitive and rapid method for determining 8-OHdG in human urine was developed using hydrophilic interaction chromatography–tandem mass spectrometry (HILIC–MS/MS) with electrospray ionization. 8-OHdG and isotopically labeled 8-OHdG (internal standard) were separated on a HILIC column with a mobile phase of 10 mM ammonium acetate: acetonitrile (1:9, v/v) within 10 min and detected by using a positive electrospray ionization interface under the selected reaction monitoring mode. The detection limits of 8-OHdG (corresponding to a signal-to-noise ratio of 3) for the HILIC-MS/MS system and the conventional method using a reversed-phase column with MS/MS were 1.0 and 26.0 fmol/injection, respectively. The proposed method makes it possible to monitor the basal level of urinary 8-OHdG from non-exposed healthy subjects and can be used for large-scale human studies.

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## 1. Introduction

Oxidative stress in an organism arises from excessive generation of reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl radicals or from depletion of antioxidants [1]. The production of ROS can be induced by both endogenous and exogenous factors [2]. While endogenous factors include physiological processes, exogenous factors include environmental sources such as smoking, diet and pollution [3]. ROS may cause oxidative damage to nucleic acids, proteins, and lipids [2]. In particular, oxidative damage to DNA has been associated with numerous pathological conditions, having both genetic and epigenetic consequences [4–6]. To understand how ROS affect normal and pathological processes, an indicator to assess oxidative stress *in vivo* is required.

The oxidized product of DNA, 8-hydroxy-2′-deoxyguanosine (8-OHdG), is the most frequently measured biomarker of the oxidative stress [7,8]. The 8-OHdG has been analyzed in various kinds of samples, such as urine, serum, peripheral blood leukocyte, and organ tissue [9]. Measurements of 8-OHdG in urine samples are especially well-suited to large-scale human studies and clinical applications because they are noninvasive [10,11]. Urinary

8-OHdG has been analyzed by several methods, such as enzyme-linked immunosorbent assay (ELISA) [12,13], high-performance liquid chromatography with electrochemical detection (HPLC-ECD) [14], gas chromatography with mass spectrometry (GC/MS) [15,16], and liquid chromatography with tandem mass spectrometry (LC/MS/MS) [17–26]. The ELISA method suffers from the problem of non-selectivity because the antibody may cross-react with other substances present in urine [12,27,28]. HPLC-ECD has often been used [29], but it suffers from possible interference from the biological matrix, incompatibility of a stable isotope labeled internal standard [16,21]. For GC/MS analysis, 8-OHdG must be purified by HPLC and derivatized before analysis [15,16,28].

LC–MS/MS has been increasingly applied to detect urinary 8-OHdG. LC–MS/MS, when combined with the isotope dilution technique, is highly selective, sensitive, and accurate, and does not require derivatization [28]. In most of previous LC–MS/MS studies, reversed-phase columns have been used to separate 8-OHdG [17–26]. As a polar compound, 8-OHdG is hardly retained on a reversed-phase column, even though aqueous mobile phases are used. Such poor retention of 8-OHdG and its insufficient separation from polar components in the matrix may lead to matrix effects, which can increase or decrease the 8-OHdG MS signal [30]. In addition, aqueous mobile phases that are used to retain polar compounds on reversed-phase columns are not suited for electrospray ionization (ESI) conditions [30]. Indeed, urinary 8-OHdG levels have even been below the detection limit of LC–MS/MS

method (7.5 fmol/injection, $S/N = 3$) [27,31]. Therefore, a more sensitive LC–MS/MS method is required to measure basal levels of urinary 8-OHdG in non-exposed healthy subjects.

In recent years, hydrophilic interaction chromatography (HILIC) has been increasingly used in LC–MS/MS for analyzing polar compounds such as metabolites in biological samples. Under HILIC conditions, the analyte interacts with a hydrophilic stationary phase and is eluted with a high concentration of organic solvent (typically acetonitrile with a small percentage of water/buffer). The highly organic mobile phase can result in increased sensitivity with ESI-MS detection [30,32].

In this study, we developed an improved LC–MS/MS method for measuring urinary 8-OHdG using a HILIC column. We found that the HILIC column provided much greater sensitivity than a conventional reversed-phase column with the same MS/MS detection conditions.

## 2. Experimental

### 2.1. Materials

The 8-OHdG and [$^{15}N_5$]8-OHdG were purchased from Sigma (MO, USA) and Cambridge Isotope Laboratories (MA, USA), respectively. HPLC grade acetonitrile was obtained from Kanto Chemical (Tokyo, Japan), and water from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals and solvents used were of an analytical grade.

### 2.2. Sample preparation

Urinary samples were pretreated as described previously with a slight modification [22,28]. After centrifugation at $2150 \times g$ for 10 min, a 100 μL aliquot of each supernatant was diluted with 900 μL of water and spiked with 10 pmol of the stable isotope labeled internal standard (IS), [$^{15}N_5$]8-OHdG. The diluted sample was subjected to solid-phase extraction using Oasis HLB cartridge (3 cc, 60 mg; Waters, Milford, MA, USA) that had been primed with 1 mL of methanol and 1 mL of water. After sample loading, the cartridge was sequentially washed with 1 mL of water. The 8-OHdG was eluted from the cartridge with 500 μL of water: acetonitrile (1:1, v/v), and evaporated to dryness using a centrifugal vacuum evaporator. The residue was redissolved in 100 μL of water: acetonitrile (1:9, v/v), and an aliquot of 20 μL was injected into the LC–MS/MS system.

### 2.3. 8-OHdG analysis by HILIC-MS/MS

#### 2.3.1. Apparatus and chromatographic conditions
The Agilent 1100 series LC system consists of a G1379A degasser, a G1312A binary pump, a G1367A autosampler, and a G1316A column oven (all from Agilent Technologies, Palo Alto, CA, USA). The chromatographic separation of 8-OHdG in the pretreated urine samples was performed on a COSMOSIL HILIC (150 mm × 2.0 mm i.d., 5 μm, Nacalai Tesque, Kyoto, Japan), kept at the temperature of 40 °C. The column was eluted isocratically with 10 mM ammonium acetate: acetonitrile (1:9, v/v) at a flow rate of 0.4 mL/min. The retention time of the analyte was optimized by varying the mobile phase acetonitrile content between 70% and 95% with 10 mM ammonium acetate and by varying the aqueous buffer concentration between 10 and 50 mM with 90% acetonitrile. The retention factor ($k$) of 8-OHdG was defined as $k = (t_R - t_0)/t_0$, where $t_R$ and $t_0$ are the retention times of the analyte and the hold-up time, respectively. Sample volumes of 20 μL were injected for each analysis. The mass spectrometric analyses were performed using an API 4000 Q-Trap tandem mass spectrometer (Applied Biosystems, CA, USA) equipped with an electrospray ionization (ESI) and operated in a positive ion mode. Sensitivity of the selective reaction monitoring (SRM) was optimized by testing with an infusion of 8-OHdG under the mobile phase condition. The mass spectrometer was operated under SRM mode of the transitions at $m/z$ 284.3 → 167.9 for 8-OHdG and at $m/z$ 289.1 → 173.0 for [$^{15}N_5$]8-OHdG (IS) with dwell times of 1000 ms. The spray voltage was maintained at 5.5 kV. Nitrogen gas was used as the collision gas (4 psi) and curtain gas (20 psi), whereas zero grade air was used as the nebulizer gas (40 psi) and heated gas (60 psi). Source temperature was set at 600 °C. The collision energy and declustering potential were set at 19 V and 71 V, respectively. Analyst software (version 1.4, Applied Biosystems) was used to control the LC–MS/MS system, and to acquire and process the data.

#### 2.3.2. Calibration curve and validation
Calibration curves for quality control (QC) samples were obtained from the ratio of peak areas of 8-OHdG and [$^{15}N_5$]8-OHdG (IS) using 0.1 mL human urine samples from six humans spiked with 8-OHdG at final concentrations of 0.2, 5, 10, 30, 50, 100 nmol/L. Standard curves were also obtained from plotting the peak area ratio against the same six concentrations of the analyte as the spiked urine samples ($n = 6$ for each). The lower limit of quantification (LLOQ) was determined as the lowest standard on the calibration curve that gave a signal-to-noise ratio of more than ten and reached a precision of 20% and an accuracy of 80–120%. The limit of detection (LOD) was determined as the lowest concentration that gave a signal-to-noise ratio of more than three. To evaluate the intra and inter-day accuracy and precision, the stock solution of 8-OHdG was added to urine at concentrations of 10 and 50 nmol/L in 0.1 mL urine. The spiked samples, together with non-spiked samples, were analyzed using HILIC–MS/MS and 8-OHdG concentrations were calculated using a standard curve. Accuracy was expressed as the ratio of the quantified concentration to the known concentration of 8-OHdG. To evaluate the intra-day precision, the non-spiked samples and the samples spiked at the two levels were prepared five times per day. The inter-day precision was determined using five independent experiments. The precision was calculated as the relative standard deviation (RSD) (%) of the replicates.

### 2.4. 8-OHdG analysis by LC–MS/MS using reversed-phase column

On the basis of previously reported LC–MS/MS method [25], chromatographic separation of 8-OHdG and [$^{15}N_5$]8-OHdG (IS) in urine samples was performed on a XBridge $C_{18}$ column (150 mm × 2.1 mm i.d., 3.5 μm, Waters) with a guard column (XBridge $C_{18}$ column, 10 mm × 10 mm i.d., 5 μm, Waters). The elution was run isocratically with a mobile phase consisting of 10 mM ammonium acetate: methanol (19:1, v/v) at a flow rate of 0.4 mL/min. The column temperature was set at 40 °C. The mass spectrometric detection was carried out by the same SRM transitions as the HILIC mode.

## 3. Results and discussion

### 3.1. Mass spectrometry and chromatography

The full scan mass spectrum of 8-OHdG in the positive ESI mode and the fragmentation pattern of protonated molecular ion [M+H]$^+$ observed in this study were consistent with those of previous studies [17,18,24,26]. The transition from the molecular ion [M+H]$^+$ to the most intense fragment was recorded in the selective reaction monitoring (SRM) acquisition mode. The main product ions of 8-OHdG and [$^{15}N_5$]8-OHdG (IS) were $m/z$ 167.9 and 173.0 [M+H − 116]$^+$, respectively. Therefore, the [M+H]$^+$ → [M+H − 116]$^+$ transition was used in the SRM mode.
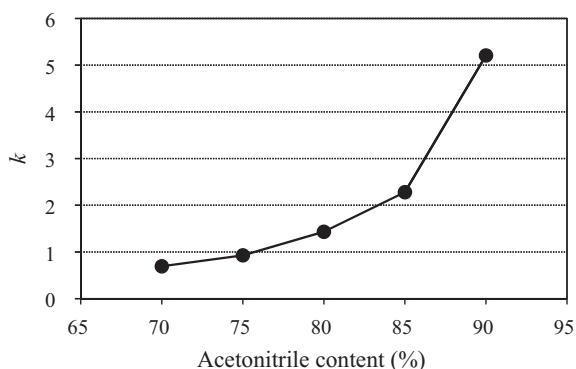
**Fig. 1.** Effect of acetonitrile content in the mobile phase on the retention of 8-OHdG.

We examined the effect of the acetonitrile concentration in the mobile phase of the HILIC column on the retention time of the analytes. The column was operated under isocratic elution conditions using acetonitrile concentration between 70 and 95%, at the interval of 5%. The retention factor ($k$) increased with the increasing acetonitrile concentration (Fig. 1). The high content of acetonitrile increases the hydrophilic interactions between the analytes and the stationary phase [33]. At 95% acetonitrile, 8-OHdG was retained for over 15 min on the column and its peak shape was broad. The retention time of charged analytes can be affected by adding salt to the mobile phase due to the electrostatic interactions between the column and analytes [33,34]. For a salt, we selected ammonium acetate because of its solubility in concentrated acetonitrile solutions, and its volatility at the ion source. The effect of the buffer concentration in the mobile phase on the retention of the analyte was investigated in the range 10–50 mM. Increasing the buffer concentration only slightly decreased the retention and did not influence the peak shape (data not shown). Finally, the analytes were separated by isocratic elution with 10 mM ammonium acetate: acetonitrile (1:9, v/v). The analyte and the internal standard eluted within 10 min using the optimized mobile phase (Fig. 2, A-1 and A-2).

### 3.2. Calibration curve and validation

The calibration curve for the standard compound was linear ($r^2 > 0.999$) for concentrations in the range of 0.2–100 nmol/L (LLOQ: 0.2 nmol/L), which covers the lower range of the reported levels of 8-OHdG in human urine [29], and the slope was $0.00630 \pm 0.00026$ (mean ± S.D. RSD, 4.1%).

Representative SRM chromatograms for the analyte and the internal standard of a urine sample from a non-smoker showed that the physiological components of the urine did not interfere with the identification and quantification of the analytes in the chromatograms (Fig. 2, B-1 and B-2). The matrix effect on the mass spectrometric response was evaluated by comparing the slope of the calibration curve with the slope obtained in the presence of urine matrix. Six curves were obtained using six different urine samples, each spiked with six different amounts of 8-OHdG. The mean slope was $0.00655 \pm 0.00043$ (mean ± S.D., RSD, 6.6%), which was almost identical to the mean slope obtained with the standard solutions. This clearly showed that the matrix did not affect the calibration curve. Therefore, 8-OHdG was quantified by using the calibration curve obtained from the standard solution.

The precision and accuracy of 8-OHdG determination in human urine with the HILIC–MS/MS system were examined by adding two different known amounts of 8-OHdG to a urine sample. The RSDs of the intra-day precision assay ($n = 5$) were in the range 2.3–2.6%, and those of the inter-day assay ($n = 5$) were in the range 2.1–4.0% (Table 1). The accuracy values (%) of the intra-day study and the inter-day assay were in the range 96–102%. Both intra and inter-day precision and accuracy values were satisfactory for determining 8-OHdG in human urine.

### 3.3. Comparison of the HILIC column with a reversed-phase column

The instrumental detection limit of 8-OHdG measured by the HILIC–MS/MS was 1.0 fmol/injection (signal-to-noise ratio, $S/N > 3$). In contrast, the detection limit achieved with a reversed-phase column and the same detection system was 26.0 fmol/injection under optimal instrumental conditions. Our detection limit of 1.0 fmol
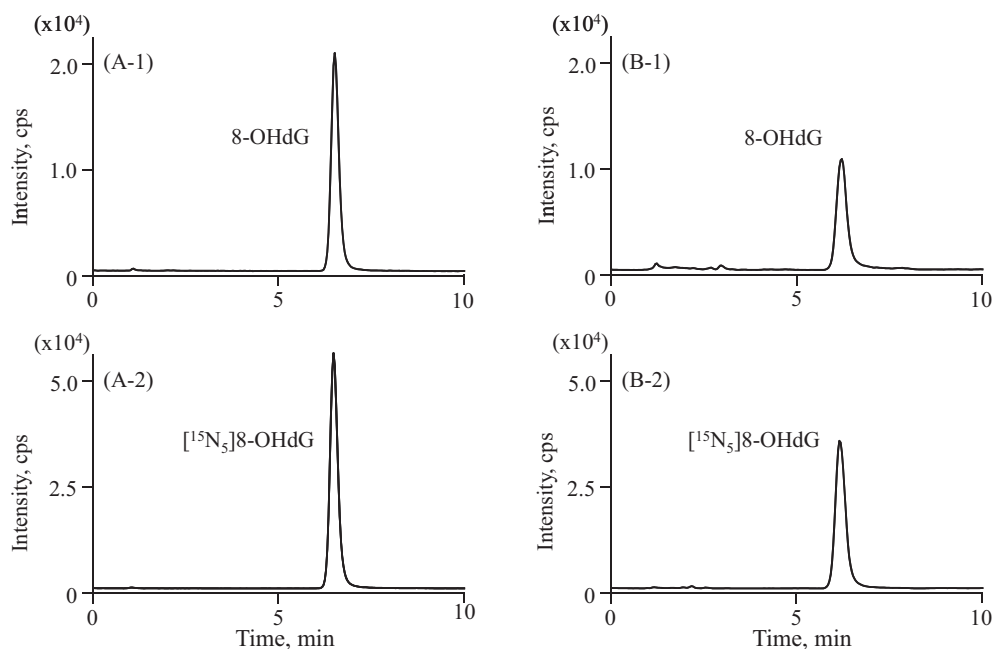


**Fig. 2.** Representative SRM chromatograms (transition: $m/z$ 284.3 → 167.9 for 8-OHdG and $m/z$ 289.1 → 173.0 for the internal standard ([$^{15}$N$_5$]8-OHdG)) of a standard solution corresponding to 200 fmol 8-OHdG/injection (A) and a non-smoker urine sample (B) (A-1 and B-1 panels for 8-OHdG; A-2 and B-2 panels for the internal standard).

**Table 1**
Precision and accuracy in the determination of 8-OHdG in urine samples.

| | Intra-day assay ($n = 5$) | | | Inter-day assay ($n = 5$) | | |
|---|---|---|---|---|---|---|
| Added amounts (pmol/mL urine) | 0 | 10 | 50 | 0 | 10 | 50 |
| Found ± SD (pmol/mL urine) | 17.5 ± 0.4 | 27.0 ± 0.7 | 68.5 ± 1.6 | 16.8 ± 0.4 | 26.9 ± 0.7 | 67.5 ± 2.7 |
| RSD (%) | 2.3 | 2.6 | 2.4 | 2.1 | 2.8 | 4.0 |
| Accuracy (%) | – | 96 | 102 | – | 101 | 101 |

was considerably lower than the detection limits reported for other previous LC–MS/MS methods: 20 fmol [17], 7 fmol ($S/N = 3$) [18], 5 fmol ($S/N = 4$) [19] and 7.5 fmol/injection ($S/N = 3$) [27,31]. The low detection limit of method enables the measurement of basal levels of urinary 8-OHdG in non-exposed healthy subjects that were not quantified in the previous report [27,31]. Furthermore, the analysis can be completed in 10 min and does not require washing and re-equilibrating the column, which makes it well suited for continuous analyses.

## 4. Conclusions

HILIC-MS/MS provides a selective, sensitive and rapid method for determining 8-OHdG in human urine. The method has acceptable linearity, accuracy, and precision, and is more sensitive than previously described LC–MS/MS methods that have been used in reversed-phase columns. The proposed HILIC–MS/MS method is well suited for large-scale human studies and clinical studies, and would also be applicable to analysis of 8-OHdG in not only urine but also other biological fluids such as plasma, serum and saliva, and in tissue.

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