

# Development of Urinary 8-Hydroxy-2'-Deoxyguanosine (8-OHdG) Measurement Method Combined with SPE

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## Abstract

A novel separation method of urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) was developed. The C<sub>18</sub> and the strong cation-exchange (SCX) columns were employed to separate urinary 8-OHdG. The major interfering substances were removed by the consecutive processes of the C<sub>18</sub> and the SCX columns. This newly developed procedure allows researchers to quantitatively measure urinary 8-OHdG by high performance liquid chromatography-electrochemical detector (HPLC-ECD) successfully. The newly developed separation method produces the optimized procedure of 8-OHdG measurement, and followed by the evaluation of its basic performance. The optimized measurement was highly reproducible (CV = 2.0–2.9%, *n* = 10). A correlation was observed between the proposed HPLC-ECD method and the column switching method (*r* = 0.96).

## Introduction

8-Hydroxy-2'-deoxyguanosine (8-OHdG) is known as a biomarker of oxidative stress. 8-OHdG is produced by the oxidation of 2'-deoxyguanosine in cellular DNA. Recently, the concentration of 8-OHdG from patients has actively been investigated. For example, an increase in the amount of 8-OHdG was reported in patients with intestinal cancers (1), diabetes (2), or Alzheimer's disease (3). As well, its increases have been recognized in aging (4), exposure to different toxic agents (5), and environmental insults (5,6). Therefore, daily monitoring of urinary 8-OHdG is useful for the health management. However, the determination of urinary 8-OHdG has analytical difficulties because of the low concentration of the analyte and the considerable amount of interfering substances.

Various methods have been developed for quantifying urinary 8-OHdG, based on gas chromatography-mass spectrometry (GC-MS) (7), enzyme-linked immunosorbent assay (ELISA) (8), liquid chromatography-mass spectrometry (LC-MS) (9), LC-MS-MS (10–16), and HPLC-ECD which has two methods

with use of either column-switching (1,4,17–20) or solid phase extraction (SPE) (21–26). However, each method has specific difficulties. GC-MS analysis faces additional problems in the hydrolysis and derivatization steps (7). The ELISA method exhibited nearly two times higher urinary 8-OHdG concentrations than HPLC-ECD method (8,27,31). Song et al. have reported that discrepancies between the data by ELISA and HPLC-ECD method derived from urea in urine sample (31). In other words, the determination of urinary 8-OHdG by ELISA is considered less quantitative. LC-MS and LC-MS-MS systems are expensive and their operations are complicated (9–16). A HPLC-ECD using column-switching method requires a complicated HPLC system which needs another pump, a column oven, a six-port valve and so on (1,4,17–20). The SPE method is further divided into a few types; 1 step [C18 (26) or Certify (C8 + SCX) (25)], 2 steps (C18 + C18) (24), and multi-steps (C18 + C18 + silica gels) (22). These SPE methods have difficulties of inadequate removal of the interfering substances in urine.

In this study, a new SPE method has been developed by the consecutive procedure of C18 and cation exchanger SCX columns. The SPE is followed by the determination of 8-OHdG by isocratic HPLC system.

## Experimental

### Chemicals and urine samples

8-OHdG was obtained from Sigma-Aldrich, Inc. (St. Louis, MO) and Interference Check A plus was from Sysmex (Hyogo, Japan). Acetonitrile and ethanol of chromatographic grade were purchased from Wako (Osaka, Japan). The SPE resins of the C18 (ODS-AQ, Spherical Silica Gels) and the SCX were obtained from YMC Co., Ltd. (Kyoto, Japan) and Varian (Palo Alto, CA), respectively. The empty polypropylene SPE Tube was purchased from Sigma. AccuBondII C18/OH was obtained from Agilent Technologies, Inc. (Tokyo, Japan). Deionized water was produced by Milli-Q water purification system (Millipore, Bedford, MA).

Urine samples of this study were collected from volunteers. The urine samples were stored at –20°C. This study was approved by the Ethical Review Committee of AIST.

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### Measurement of urinary 8-OHdG

The SPE was performed on the original-made columns. The C18 column and the SCX columns were prepared by the packing of the empty polypropylene SPE tubes (3 mL, Supelco, St. Louis, MO) with 500 mg of ODS-AQ and 250 mg of Bond Elut SCX (Varian, Santa Clara, CA), respectively. These columns were stored in a desiccator.

8-OHdG in urine samples was fractionated by the two-step SPE. Urine samples were centrifuged at 8000 rpm for 10 min. An aliquot of 0.7 mL of the supernatant was mixed with 0.7 mL of 80 mM phosphate buffer solution (pH 7.0) (buffer A) containing 4 mM EDTA and 2.8 mL of deionized water. Two milliliters of the mixture was applied to the C18 column preconditioned with 3 mL of ethanol and 3 mL of deionized water. This C18 column was washed with 6.5 mL of 10 mM phosphate buffer solution (pH 7.0) containing 2% (w/v) ethanol (buffer B), and followed by the additional wash with 0.97 mL of 10 mM phosphate buffer solution (pH 7.0) containing 8% (w/v) ethanol (buffer C). 8-OHdG was eluted with 0.6 mL of buffer C. The protocol, which used the C18 column produced with spherical silica gel, was defined as treatment (a). Next, the eluent was transferred to the SCX column preconditioned with 3 mL of ethanol, 3 mL of deionized water and 1 mL of buffer C. The SCX column was washed with 0.3 mL of buffer C, and then 8-OHdG was eluted with 0.9 mL of buffer C. The eluent was added to 0.1 mL of deionized water, and 15  $\mu$ L of this eluted mixture was injected into the HPLC–ECD system. The time needed to complete this whole procedure was approximately 3 h at 23°C.

The protocol which uses C18 column produced with irregular silica gel (AccuBondII C18/OH) was defined as treatment (b). After 2.0 mL of the mixture composed of urine, buffer A and water was applied to the preconditioned C18 column, the C18 column was washed with 5.0 mL of buffer B followed by additional washing with 0.85 mL of buffer C. 8-OHdG was eluted with 0.8 mL of buffer C.

Details of the high performance liquid chromatography (HPLC) system configuration were mentioned in Supporting Information. The Develosil C30 column (3  $\mu$ m in particle size, 250  $\times$  4.6 mm) and the Develosil C18 column (3  $\mu$ m, 250  $\times$  4.6 mm) were employed to separate 8-OHdG by HPLC. The Develosil C30 (3  $\mu$ m particle size, 1.0  $\times$  4.0 mm) was used as a guard column. The mobile phase used for isocratic elution of 8-OHdG composed of 10 mM phosphate buffer solution (pH 7.0), 5% (w/v) acetonitrile and 30  $\mu$ M EDTA. 8-OHdG was eluted at flow rate of 0.8 or 1.0 mL/min, and at column oven temperature of 27°C. 8-OHdG was detected by ESA model Coulochem III detector equipped with a guard cell (No. 5020) and a high sensitivity analytical cell (No. 5011). The potentials for electrodes guard, 1 and 2 were adjusted to 350, 0, and 300 mV, respectively, as previously described (19).

The analysis time of urinary 8-OHdG was shortened by the following improvements: The Develosil C30 column (5  $\mu$ m particle size, 250  $\times$  4.6 mm) was employed to separate 8-OHdG by HPLC. The mobile phase used for isocratic elution of 8-OHdG was composed of 35 mM phosphate buffer solution (pH 7.0), 5% (w/v) acetonitrile and 30  $\mu$ M EDTA (solvent A). The solvent for the washing steps was composed of 35 mM phosphate buffer solution (pH 7.0), 50% (w/v) acetonitrile and 30  $\mu$ M EDTA (solvent B).

The used solvents were as follows: 0–14.5 min (100% solvent A); 14.5–15.5 min (100% solvent B); 15.5–30 min (100% solvent A). 8-OHdG was eluted at a flow rate of 1.0 mL/min.

## Results and Discussion

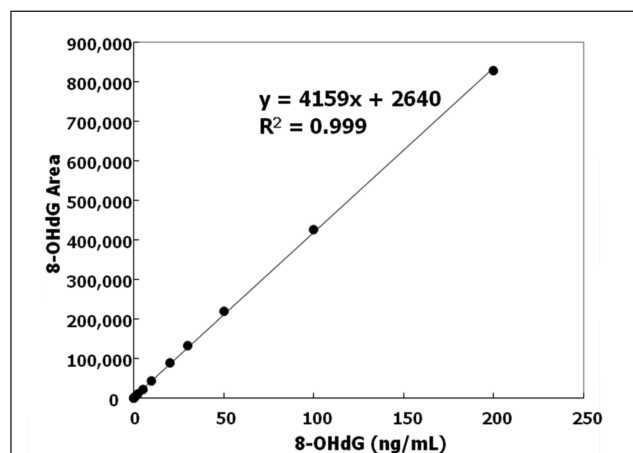
### Calibration curve for 8-OHdG

The calibration curve for 8-OHdG was investigated using standard sample of 8-OHdG. The standard sample was prepared with buffer C. The measurement was carried out at a flow rate of 0.8 mL/min and the commercially prepaced Develosil C30 (3  $\mu$ m particle size) which was employed for the analytical purpose. This result is shown in Figure 1, where the plots were averaged for ten consecutive measurement. There was relationship between the concentrations of 8-OHdG and the detected peak area. The dynamic range of this HPLC–ECD was 0.1–200 ng/mL of 8-OHdG which was separated by the SPE method developed by us. The correlation coefficient was 0.99. The lower limit of detection of 8-OHdG was 0.1 ng/mL, and the correlation of variation (CV:  $n = 10$ ) was 6.3% for 0.1 ng/mL, 3% for more than 1 ng/mL, and 1% for more than 5 ng/mL of 8-OHdG concentration, respectively. The concentrations of urinary 8-OHdG for healthy people have been reported by Nakano et al. (4), where the non-corrected value of urinary 8-OHdG partially taken in the morning was  $7.70 \pm 4.87$  (mean  $\pm$  SD) ng/mL. The urinary 8-OHdG concentrations reported in previous papers indicated the proposed SPE method should be qualified to measure urinary 8-OHdG.

### Development of the SPE method for urinary 8-OHdG

#### Comparison between spherical silica gels and irregular ones

As the first step of the SPE, C18 columns were employed and their separation abilities were compared with spherical silica gels and irregular ones. Previous reports approximate measurements



**Figure 1.** Standard calibration curve for the quantitation of 8-OHdG. The curve was constructed from the peak area after injections of aliquots with an addition of 1, 2, 3, 5, 10, 20, 30, 50, 100, and 200 ng/mL of 8-OHdG. HPLC conditions: mobile phase, 35 mM phosphate buffer (pH 7.0) containing 5% (w/v) acetonitrile and 30  $\mu$ M EDTA; column oven temperature, 27°C; injection volume, 15  $\mu$ L; and flow rate: 0.8 mL/min.

of 8-OHdG by the SPE method were scarcely useful, because the peak of 8-OHdG overlapped with the peaks of interfering substances (28). Almost all these reports have used irregular silica C18 as the SPE carrier, but the study has used spherical silica C18 as the SPE carrier. This HPLC measurement was carried out at a flow rate of 0.8 mL/min using a Develosil C30 pre-packed analytical column. The chromatograms of urine samples after the SPE with the spherical silica C18 and the irregular silica C18 were shown in Figure 2A [treatment (a)] and Figure 2B [treatment (b)], respectively. As the results, the retention time of the eluted 8-OHdG was 17.5 min. The sample volumes necessary to collect more than 90% of urinary 8-OHdG were 0.6 mL for the SPE method using spherical silica C18 and 0.8 mL for the same method with irregular silica C18. The analysis time of the urine samples after extraction was 55 min in case of the spherical silica C18 and 100 min in case of the irregular silica C18. The SPE method by a column using the spherical silica C18 (Figure 2A) removed more interfering substances and the elution volume of 8-OHdG was 0.6 mL. This result was smaller than the volume (0.8 mL) of the SPE method by column with the irregular silica C18 (Figure 2B). Existing various SPE methods have been improving by the introduction of the strategy to raise retention of 8-OHdG such as a SPE method employing a column with C18/OH24. In case of the newly developed SPE method, the columns with the spherical silica C18 [carbon content: 14% (w/w)] have exhibited the sharp separation of urinary 8-OHdG.

### Secondary treatment

The secondary treatment was investigated. Interfering substances were further removed by process with use of the SCX column, followed by the separation of urinary 8-OHdG with the C18 column. This HPLC measurement was carried out at a flow rate of 0.8 mL/min and the Develosil C30 (3  $\mu$ m particle size) which was used as the analytical column. The chromatogram of the sample which had been consecutively applied to the treatment (a) and the SPE of the SCX processing is shown in Figure

2C. As the result, the urinary 8-OHdG peak was observed as a single peak, which could be clearly detected by HPLC–ECD. By an anion exchange procedure, interfering peaks have not been removed. The extraction of urine sample by gel filtration has demonstrated low reproducibility and the eluted sample has been diluted (data not shown). Martinis et al. (24) reported SPE method with Certify (C8 + SCX). Their SPE method was simple and rapid procedure because of one step SPE. However, their method allowed removal of interfering substances insufficiently, thus requiring additional removal procedure. The two steps of the SPE method allowed to remove interfering substances sufficiently, by which the eluted urine sample has contained 8% (w/v) ethanol. HPLC–ECD analysis of this eluted sample [contained 8% (w/v) ethanol] was not influenced.

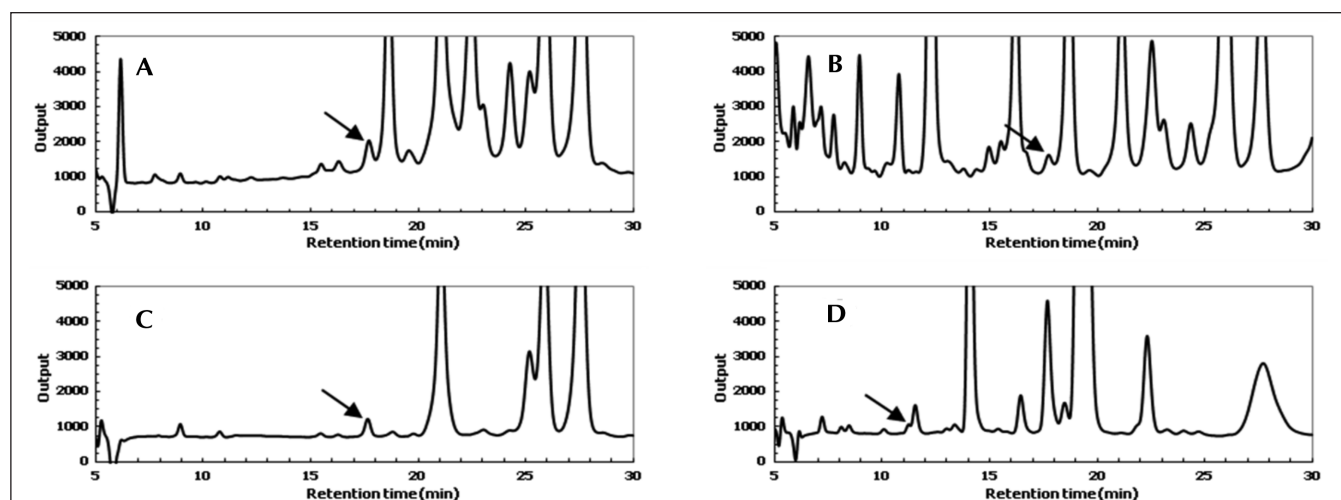
The pretreatment took 3 h. However, the method can carry out the pretreatment of many samples simultaneously, because it is based on solid phase extraction in free-fall which enables the separation at the same time.

The chromatogram of urine sample which was treated by the two-step SPE using the Develosil C18 as the analytical column was shown in Figure 2D. 8-OHdG was eluted at 11.6 min, and then it overlapped with other substance peaks. As the results, the column used with the Develosil C30 was the best performance as the analytical column for the determination of urinary 8-OHdG by HPLC–ECD.

### Analysis of urinary 8-OHdG

#### *Simultaneous reproducibility and the day-to-day reproducibility*

The simultaneous reproducibility and the day-to-day reproducibility were investigated. These measurements were carried out at a flow rate of 0.8 mL/min and the Develosil C30 was used as the analytical column. When four urine samples were simultaneously analyzed ten times, the simultaneous reproducibility of these CV values was in the range of 2.0–2.9% by the SPE method. The CV value at 5.8 ng/mL of 8-OHdG concentration



**Figure 2.** HPLC–ECD chromatograms of urine sample, after the pretreatment by SPE procedures as follows: (A) SPE procedure by the spherical silica C18, (B) SPE procedure by the irregular silica C18, and (C) and (D) consecutive SPE of the treatment (a) and the SCX processing. Regarding A–C, the Develosil C30 (3  $\mu$ m particle size) HPLC column was used for HPLC–ECD, as well as regarding (D) the Develosil C18 (3  $\mu$ m particle size) HPLC column was used. HPLC conditions for the 4 chromatograms are the same as follows: mobile phase: 35 mM phosphate buffer (pH 7.0) containing 5% (w/v) acetonitrile and 30  $\mu$ M EDTA, column oven temperature: 27°C, injection volume: 15  $\mu$ L, flow rate: 0.8 mL/min.

was 2.3% ( $n = 10$ ) [Figure S1 (Appendix: Supporting Information)]. This result is satisfactory in comparison with CV [= 12.5% ( $n = 5$ )] at 55 nM (15.6 ng/mL) of 8-OHdG concentration by the SPE method of Germadnik et al. (23). When other four urine samples were analyzed for five days (1 sample/day,  $n = 6$ ), these CV values were 4.2–7.2% (Figure S2, see Appendix). The values of the day-to-day reproducibility were higher than the values of the simultaneous reproducibility.

Lengger et al. have investigated separation of urinary 8-OHdG by the column switching method and they have reported good results (19). In their paper, the CV value of simultaneous reproducibility and that of day-to-day difference reproducibility was 2.2% and 4.0%, respectively. These results were nearly equivalent to the results by the SPE method.

#### Recovery of added 8-OHdG in urine, lower detection limit, and effect of interfering substances

The recovery of added 8-OHdG in urine was investigated. This measurement of 8-OHdG was carried out with use of the analytical column with Develosil C30 at a flow rate of 0.8 mL/min. The recoveries of the added 8-OHdG (1.5–15 ng/mL) in urine were shown in Table I. Averages of the recoveries were 84–114 %. This result was decisively high compared with 45.8% by the SPE method of Germadnik et al. (23) and was equivalent to results of the previous papers using SPE method (70–98%) (24–26). When the concentration of added 8-OHdG in urine was less than 1.5 ng/mL, the recoveries showed the tendency to increase. In case of the column switching method reported by Lengger et al. (19), the recovery of added 8-OHdG was 100% (6.2–37.9 nM). These results were nearly equivalent to the results by the SPE method.

The dilution linearity of urine samples for the SPE method was observed. The lower detection limit was determined as follows; value of a certain dilution step (mean  $- 3SD$ ) > value of next dilution step (mean  $+ 3SD$ ). These lower detection limits

were 0.4–0.6 ng/mL (Figure S3, see Appendix). This result had high sensitivity compared with 5.0 ng/mL reported in previous papers using SPE method (24,26). Furthermore, the proposed method could measure urinary 8-OHdG without evaporation, although SPE method reported by Matayatsuk et al. (26) needed evaporation. The evaporation of the samples which were eluted by SPE method impairs the precision of these data.

The effects of interfering substances were investigated by the addition of interfering substances to urine samples. Practically added substances were ascorbic acid (20–100 mg/dL) and Interference Check A containing bilirubin F (2.1–18.2 mg/dL), bilirubin C (2–20 mg/dL) and hemoglobin (48–487 mg/dL). These added substances did not affect the 8-OHdG measurement in urine (Figure S4, see Appendix). As the results, the sample pre-treatment proposed in this paper was confirmed to remove almost all interfering substances. Therefore, it is thought that the measurable range of 8-OHdG concentrations for the SPE method is fairly wide. The good performance given by the SPE method is considered due to the omitted evaporation, which was necessary in existing methods.

#### Correlation between the proposed HPLC–ECD method combined with the SPE and the column switching method

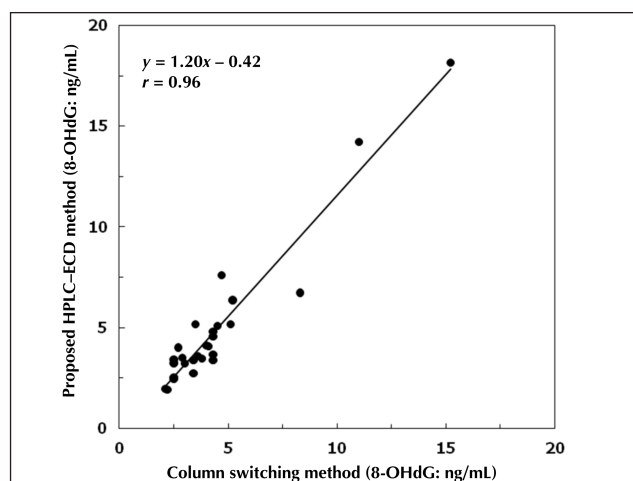
A correlation was observed between the proposed HPLC–ECD method and the column switching method reported by Nakano et al. (4) The HPLC measurement was carried out at a flow rate of 1.0 mL/min and the Develosil C30 (5  $\mu$ m particle size) was used as the analytical column. The column switching method was carried out at SRL, Inc., (Tokyo, Japan). The result is shown in Figure 3. A linear correlation was observed between the proposed method and the column switching method ( $r = 0.96$ ). 8-OHdG measurement range was 1.9–18.2 ng/mL in the urine samples. In this result, the approximate straight line was  $y = 1.2x - 0.42$  (this method was higher than the column switching method). This result exhibits that urine sample containing high 8-OHdG concentration affect the approximate straight line.

**Table I. Recoveries of the Added 8-OHdG (1.5–15 ng/mL) in Urine ( $n = 2$ )\***

	Add 8-OHdG (ng/mL)						Mean (%)
	0	1.5	3	6	9	15	
<i>Urine 1</i>							
8-OHdG <sup>†</sup>	27.7	29.1	30.6	34.6	35.4	41.7	
Recovery (%)		90.2	94.5	114.2	85.6	92.8	95.4
<i>Urine 2</i>							
8-OHdG <sup>†</sup>	11.3	12.6	13.7	16.3	19.1	23.8	
Recovery (%)		85.7	79.6	83.9	86.8	83.5	83.9
<i>Urine 3</i>							
8-OHdG <sup>†</sup>	6.1	7.9	8.8	12.2	13.6	17.8	
Recovery (%)		120.8	91.3	102.7	84.4	78.4	95.5
<i>Urine 4</i>							
8-OHdG <sup>†</sup>	11.7	13.8	15.1	18.0	21.2	27.1	
Recovery (%)		142.6	113.9	104.7	105.3	102.7	113.9

\* HPLC conditions: mobile phase, 35 mM phosphate buffer (pH 7.0) containing 5% (w/v) acetonitrile and 30  $\mu$ M EDTA. Column oven temperature, 27°C. Injection volume, 15  $\mu$ L, flow rate: 0.8 mL/min. HPLC prepacced column, Develosil C30 (3  $\mu$ m particle size).

<sup>†</sup> (ng/mL,  $n = 2$ )



**Figure 3.** Correlation between the proposed HPLC–ECD method combined with the SPE and the column switching method ( $n = 27$ ). HPLC conditions: mobile phase: 35 mM phosphate buffer (pH 7.0) containing 5% (w/v) acetonitrile and 30  $\mu$ M EDTA, column oven temperature: 27°C, injection volume: 15  $\mu$ L, flow rate: 0.8 mL/min, and HPLC prepacced column: Develosil C30 (3  $\mu$ m particle size).

As the next step, the proposed HPLC–ECD was compared to LC–MS–MS known as the qualitative detection method of the measurement of 8-OHdG with high sensitivity and selectivity (10–16). The proposed HPLC–ECD showed the sensitivity of ~ 0.4 ng/mL, CV of simultaneous reproducibility of 2.2–2.9%, day-to-day difference reproducibility of 4.2–7.2%, and the recovery of added 8-OHdG of 84–114 %, and LC–MS–MS did the sensitivity of approximately 0.2 ng/mL, CV of simultaneous reproducibility of around 10%, day-to-day difference reproducibility of 9.9% (12) and 24.7% (11), and the recovery of added 8-OHdG of  $98.6 \pm 2.8\%$  (10) and  $83.2 \pm 5.3\%$  (12). In the results, good reproducibility was obtained by the method and other results were almost similar between the two methods. LC–MS–MS combined with the column switching method was reported by Hu et al. (13), which exhibited superior performances in all respects (sensitivity: 5.7 pg/mL, reproducibility: less than 5%, recovery: 99–102% and measurement time: 10 min). However, this measurement is quite expensive and complex. On the contrary, the results indicated the proposed HPLC–ECD method of less expensive provided the satisfactory quantitative analysis of urine samples.

#### Shortening analysis time by HPLC measurement

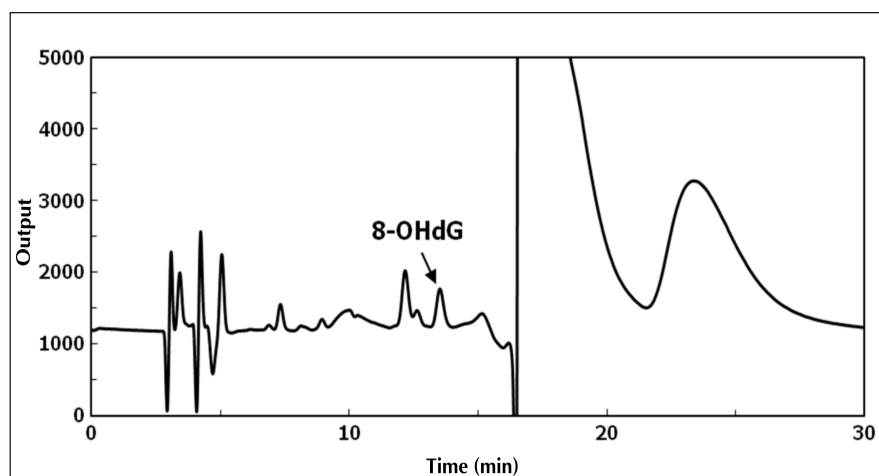
The analysis time by HPLC measurement was shortened by the HPLC method which included washing process using 50% (w/v) acetonitrile in 35 mM phosphate buffer solution (pH 7.0) containing 30  $\mu$ M EDTA. This HPLC measurement was carried out at a flow rate of 1.0 mL/min and the Develosil C30 (5  $\mu$ m particle size) was used as the analytical column. Washing process was carried out to flow washing solution [50% (w/v) acetonitrile in 35 mM phosphate buffer solution (pH 7.0) containing 30  $\mu$ M EDTA] from 10.5 min to 11.5 min. The obtained chromatogram was shown in Figure 4, indicating 8-OHdG was eluted at 13.5 min. The analysis time by this HPLC method was 30 min. As the result, HPLC measurement was able to shorten from 60 min to 30 min thereby the adding cleaning-process. In addition, the HPLC measurement adding cleaning-process demonstrated

high reproducibility when the standard 8-OHdG during continuous measurement of several urine samples was measured [peak area: CV = 0.70% ( $n = 11$ ), Figure S5, see Appendix]. It indicated that the ECD sensitivity was not affected by this cleaning-process. Therefore the method had the capacity to measure 48 samples in one day. The developed SPE method is suitable for epidemiological investigation than the column switching method, because the column switching method takes 1 h for 1 sample. Moreover, the proposed HPLC measurement does not require the regulation of detailed analytical conditions by isocratic elution. The proposed 8-OHdG measurement method is a simple system. Relationships between urinary 8-OHdG concentrations and disease, risk assessment, smoking, and health promotion were mostly reported based on the analyses by the column switching method or ELISA method (30). Because the proposed method shows equivalent performances to the previously described methods it was decided that the method is accessible to investigate the relationship between 8-OHdG concentration and disease and so on.

Finally, the proposed SPE method can carry out the pretreatment of many samples simultaneously, because it is based on solid phase extraction in free fall which enables the separation within the same time. In addition, this SPE method was simple, because urinary 8-OHdG was separated in free fall (no need to use a pump), the sample eluted by this method does not need to be evaporated and this sample was directly analyzed by HPLC–ECD (the sample was diluted two-fold). As a result, it can treat many samples at the same time.

## Conclusion

The SPE method combined with the consecutive procedures of the C18 and the SCX column effectively removed interfering substances in urine and this SPE method was able to elute urinary 8-OHdG. HPLC–ECD measurement using Develosil C30 column (3  $\mu$ m particle size) as the analytical column was well suited to both separate and detect urinary 8-OHdG compared with many C18 columns reported. In addition, this SPE method was simple, because urinary 8-OHdG was separated by free fall (a pump need not be used), the sample eluted by this method does not need to be evaporated, and this sample was directly analyzed by HPLC–ECD (the sample was diluted two-fold). As a result, it can treat many samples at the same time. Forty-eight samples were measurable for 1 day, because the analysis time by the proposed HPLC–ECD method was 30 min per sample. Thus, the SPE method is suited epidemiological investigation than column switching method (column switching method takes 1 h for 1 sample). The SPE method is accessible to investigate relationship between 8-OHdG concentration and disease and so on.



**Figure 4.** Chromatograms of urine sample using the HPLC method which contained washing process using 35 mM phosphate buffer (pH 7.0) containing 50% (w/v) acetonitrile and 30  $\mu$ M EDTA. HPLC conditions: mobile phase: 35 mM phosphate buffer (pH 7.0) containing 5% (w/v) acetonitrile and 30  $\mu$ M EDTA, column oven temperature: 27°C, injection volume: 15  $\mu$ L, flow rate: 1.0 mL/min, and HPLC prepacked column: Develosil C30 (5  $\mu$ m particle size).

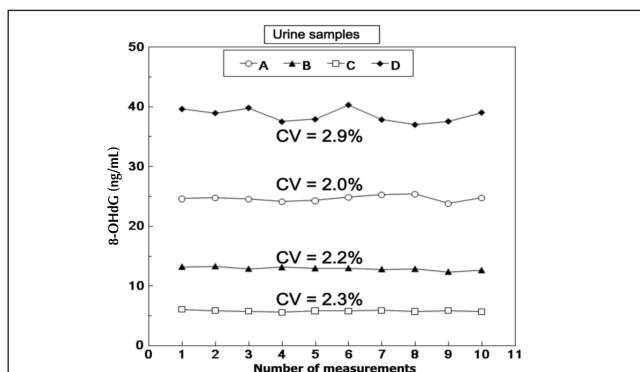
In the future, 8-OHdG concentrations in other vital samples will be investigated by the proposed method. Especially, 8-OHdG concentrations in serum are quite low compared with urinary 8-OHdG concentrations. Therefore, 8-OHdG concentrations in serum are rarely reported. We will study the relationship between 8-OHdG concentrations and disease and so on by the proposed method.

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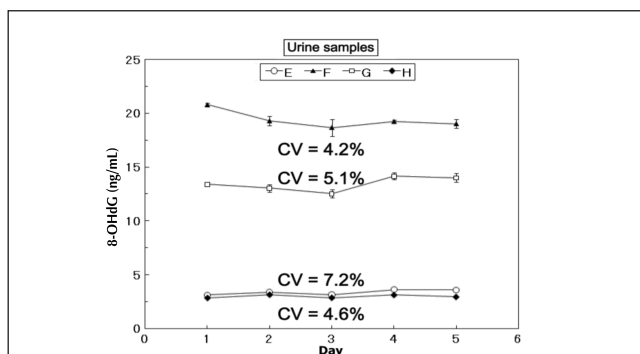
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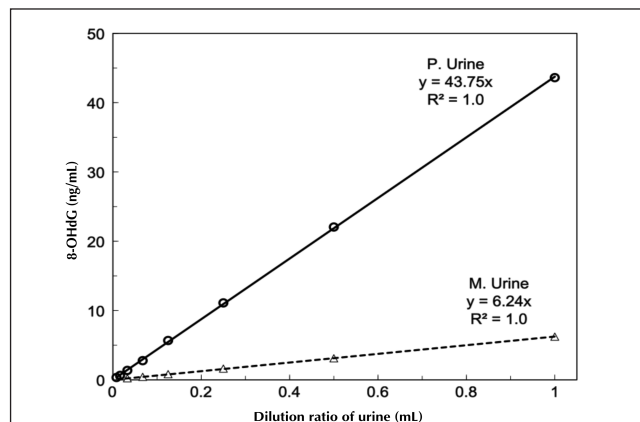
## Appendix: Supporting Figures



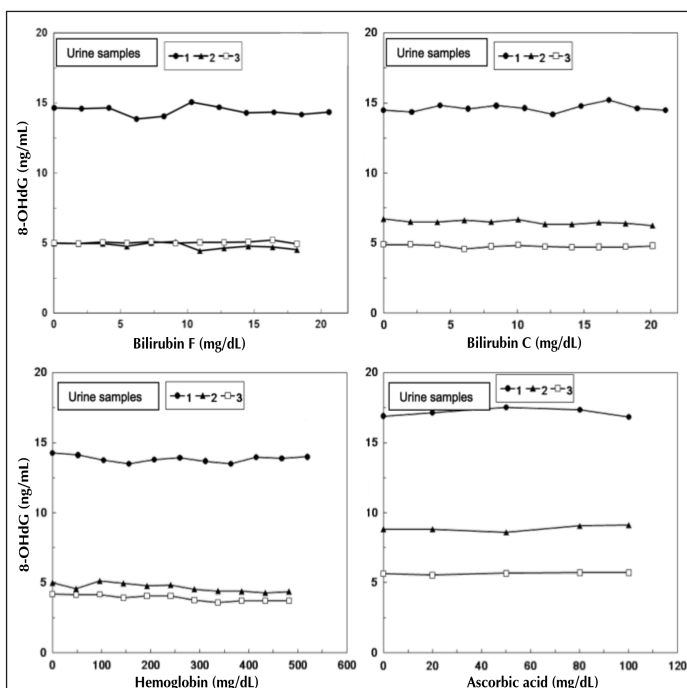
**Figure S1.** Simultaneous reproducibility of urine samples pretreated by our developed SPE method ( $n = 10$ ). HPLC conditions: mobile phase, 35 mM phosphate buffer (pH 7.0) containing 5% (w/v) acetonitrile and 30  $\mu$ M EDTA; column oven temperature, 27°C; injection volume, 15  $\mu$ L; flow rate, 0.8 mL/min; and HPLC prepacked column, Develosil C30 (3  $\mu$ m in particle size).



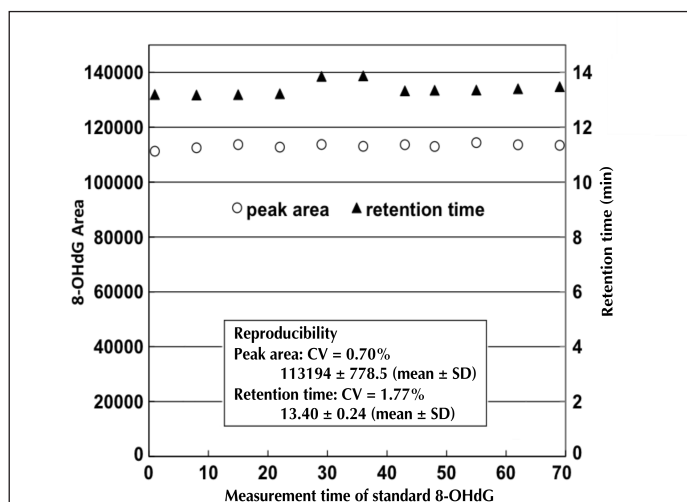
**Figure S2.** Day-to-day reproducibility of urine samples pretreated by our developed SPE method (1 sample/day,  $n = 6$ ). HPLC conditions: mobile phase, 35 mM phosphate buffer (pH 7.0) containing 5% (w/v) acetonitrile and 30  $\mu$ M EDTA; column oven temperature, 27°C; injection volume, 15  $\mu$ L; flow rate, 0.8 mL/min; and HPLC prepacked column, Develosil C30 (3  $\mu$ m particle size).



**Figure S3.** Dilution linearity of urine samples for our developed SPE method. HPLC conditions: mobile phase, 35 mM phosphate buffer (pH 7.0) containing 5% (w/v) acetonitrile and 30  $\mu$ M EDTA; column oven temperature, 27°C; injection volume, 15  $\mu$ L; flow rate, 0.8 mL/min; and HPLC prepacked column, Develosil C30 (3  $\mu$ m particle size).



**Figure S4.** Effects of interfering substances that were added to urine samples for our developed SPE method. Ascorbic acid (20–100 mg/dL), bilirubin F (2.1–18.2 mg/dL), bilirubin C (2–20 mg/dL), and hemoglobin (48–487 mg/dL) were added to urine samples. HPLC conditions: mobile phase, 35 mM phosphate buffer (pH 7.0) containing 5% (w/v) acetonitrile and 30  $\mu$ M EDTA; column oven temperature, 27°C; injection volume, 15  $\mu$ L; flow rate, 0.8 mL/min; and HPLC prepacked column, Develosil C30 (3  $\mu$ m particle size).



**Figure S5.** Reproducibility of standard 8-OHdG during continuous measurement of urine samples by HPLC-ECD method adding cleaning-process. The standard 8-OHdG were measured for respective 5–6 urine samples. This measurement was continuously carried out from 1 to 69 samples. HPLC conditions: mobile phase, 35 mM phosphate buffer (pH 7.0) containing 5% (w/v) acetonitrile and 30  $\mu$ M EDTA; washing solution, 50% (w/v) acetonitrile in 35 mM phosphate buffer solution (pH 7.0) containing 30  $\mu$ M EDTA; washing process, flowing from 10.5 min to 11.5 min; column oven temperature, 27°C; injection volume, 15  $\mu$ L; flow rate: 1 mL/min; and HPLC prepacked column, Develosil C30 (3  $\mu$ m particle size).