Urinary 8-Oxo-7,8-dihydro-2'-deoxyguanosine Values Measured by an ELISA Correlated Well with Measurements by High-Performance Liquid Chromatography with Electrochemical Detection

Rie Yoshida, Yasutaka Ogawa, and Hiroshi Kasai

Abstract

Measurement of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) has recently become more popular as a means of assessing oxidative stress in the human body. Although using high-performance liquid chromatography with electrochemical detection (HPLC-ECD) is a reliable method of measuring 8-oxo-dG, easier and simpler alternative methods may be preferred, if they are quantitatively accurate. The ELISA method is the likeliest candidate for a useful alternative. Anti-8-oxo-dG monoclonal antibody N45.1 has been shown to have better specificity for 8-oxo-dG than other anti-8-oxo-dG antibodies, but the urinary 8-oxo-dG values measured by ELISA using N45.1 have been claimed to only weakly correlate with the values obtained by HPLC-ECD. Because the commercial ELISA kit has been improved, we compared the urinary 8-oxo-dG values measured by the ELISA with the values obtained by HPLC-ECD. We sampled the urine of 72 healthy Japanese individuals and measured their urinary 8-oxo-dG levels by the ELISA with appropriate controls and by HPLC-ECD. When X was defined as the values of 8-oxo-dG measured by HPLC-ECD, and Y was defined as the values of 8-oxo-dG measured by the ELISA, simple regression analysis showed the most likely relationship to be Y = 1.83X + 0.8. The correlation coefficient was 0.88, which indicated a good correlation between X and Y. These results show that the ELISA can be applied to studies comparing relative urinary 8-oxo-dG values among several groups, if the studies do not require determination of the exact concentration of 8-oxo-dG in urine.

Introduction

Because 8-oxo-dG is known to represent one of the major forms of oxidative DNA damage, many researchers have measured 8-oxo-dG in tissues or urine as a marker of useful oxidative stress (1). Measurement of urinary 8-oxo-dG has recently become more popular as a means of assessing oxidative stress in humans because urinary 8-oxo-dG probably reflects the level of oxidative DNA damage in the body as a whole (2); however, the source of urinary 8-oxo-dG has not been clarified. Although several different techniques are available for measuring 8-oxo-dG, HPLC-ECD has been widely used (2, 3) since the work of Floyd et al. (4) and Kasai et al. (5) in 1986. Although the HPLC-ECD method is one of the most reliable methods of measuring 8-oxo-dG, it demands a high technical level, and it takes time to measure large numbers of urinary specimens (6, 7), especially in population-based studies. ELISA (3) may be a very convenient and useful alternative method of measuring urinary 8-oxo-dG, if its specificity and accuracy are acceptable.

Yin et al. (8) found a good correlation between ELISA and HPLC-ECD measurements of 8-oxo-dG in DNA, but immuno-affinity purification of hydrolysates was required before the ELISA. Cooke et al. (6) adapted a commercially available version of the ELISA kit to measure 8-oxo-dG in calf thymus DNA and obtained almost the same range of values as measured by HPLC, but they did not report any correlation coefficient or the results of a regression analysis. The ELISA kit used by Cooke et al. contains anti-8-oxo-dG monoclonal antibody N45.1, whose specificity for 8-oxo-dG has been confirmed (9, 10). Monoclonal antibody N45.1 has better specificity for 8-oxo-dG (tested for 8-oxo-dG/2'-deoxyguanosine, 8-oxo-dG/8-oxo-G, and 8-oxo-dG/7,8-dihydroguanine) than other 8-oxo-dG antibodies (11). Although 8-oxo-G (oxidation of guanine in RNA at C-8 position) demonstrated some cross-reactivity with N45.1, it required 2 orders higher concentration than 8-oxo-dG (10). Urinary 8-oxo-dG has been measured with this antibody in several studies (12, 13), but the correlation between the values obtained by ELISA and those obtained by HPLC-ECD was not acceptable (13). Since then, the ELISA kit containing N45.1 has been improved, and a new version is now commercially available. Saito et al. (14) obtained acceptable results with the improved ELISA kit in measurements of urinary 8-oxo-dG by a recovery test in which several concentrations of 8-oxo-dG solution were added and by a dilution test in which urine samples were diluted severalfold with PBS. However, no results of comparisons between urinary 8-oxo-dG values measured by the improved ELISA and by HPLC-ECD have ever been reported.

Because measurement of urinary 8-oxo-dG has become a popular means of evaluating the level of oxidative stress in the human body, it seemed worthwhile to determine whether the improved ELISA kit is a good alternative for measuring urinary 8-oxo-dG. In the present study, we measured urinary 8-oxo-dG by HPLC-ECD and with the improved ELISA kit and compared these two urinary 8-oxo-dG measurements.
Materials and Methods

Urine Specimens. For a preliminary test, we obtained urine specimens from eight Japanese volunteers (age, 36.6 ± 6.4 years). To compare the 8-oxo-dG values by HPLC-ECD and with the improved ELISA kit, we obtained urine specimens from 72 Japanese individuals, 60 males (age, 40.0 ± 11.5 years) and 12 females (age, 32.0 ± 11.0 years). All subjects agreed to anonymously donate urine sample and gave their written informed consent. None of them had a history of cancer, and they had not been exposed to any chemicals suspected of generating reactive oxygen species in the workplace. The urine specimens were collected in the morning, taking great care not to expose them to UV radiation in lighting. All of the specimens were immediately frozen and maintained at −80°C until the HPLC-ECD or ELISA analysis.

Measurement of 8-oxo-dG by HPLC-ECD. The urinary 8-oxo-dG levels were determined as described previously (15). Briefly, the urine specimen was injected into the first HPLC
column [ShodexAsahipak GS-320HQ 7G (500 × 7.6 mm); elution, 0.1% acetic acid]. An aliquot (100 µl) of the fraction containing 8-oxo-dG (50–61 min) was automatically injected into the second HPLC column [YMC-Pack ODS-AM (250 × 4.6 mm); elution, aqueous methanol (5%) containing 35 mM NaOAc, 12.5 mM citric acid (pH 7.5)] by an autosampling injector (231 XL; Gilson), and 8-oxo-dG was measured by an EC detector (Coulonick II, ESA; guard cell, 350 mV; channel 1, 300 mV; channel 2, 150 mV).

Measurement of 8-oxo-dG by ELISA. Thawed urine specimens were centrifuged at 4000 × g for 10 min, and the supernatant was used to measure 8-oxo-dG with a competitive ELISA kit named 8-OH-dG Check (Japan Institute for the Control of Aging, Fukuroi, Japan or Genox Corp., Baltimore, MD). The specificity of monoclonal antibody N45.1 used in the kit was confirmed in the previous study (9, 10). All materials in the ELISA kit were kept at 4°C until the day of the experiment. A diagram of the ELISA method is shown in Fig. 1. The plate layout used in our experiment is shown in Fig. 2. According to the protocol provided by Japan Institute for the Control of Aging, the outermost wells were not used for measurement due to concerns about possible edge effects (Fig. 2); however, the same volume (50 µl) of water was added into the outermost wells to maintain the uniform temperature within the wells. All standard solutions, i.e., S1–S6 (0.5, 2, 8, 20, 80, and 200 ng/ml), and the urine specimens were prepared in triplicate (Fig. 2). In blank well (line H), 50 µl of water were also added instead of samples, and the same procedure was performed before adding chromatic solution (Fig. 2). C1, C2, and C3 were urinary controls (high, medium, and low levels of 8-oxo-dG in urine, respectively) to confirm whether each experiment proceeded properly (Fig. 2). As a preliminary test, we measured 8-oxo-dG levels of the samples from eight volunteers with the ELISA kit to select the controls. The preliminary test was done three times on different days. The 8-oxo-dG levels of samples ranged from 3.0 to 23.3 ng/ml. We selected three samples, one with high 8-oxo-dG levels (about 20 ng/ml), another with medium levels (about 10 ng/ml), and the last with low levels (about 3 ng/ml), as controls C1, C2, and C3, respectively. Experiments were performed according to the instructions in the kit, except for the use of our three controls and changing plate layout slightly.

After adding primary and secondary antibody, we incubated the plate at 37°C for 1 h using Incubator IC400 (Yamato, Japan). Incubator IC400 circulates warm air by thermal convection, and temperature was controlled at 37 ± 1°C. To maintain the same plate temperature for each experiment, we selected one location in the incubator for every experiment. The absorbances of the wells were measured at 450 nm using Wellreader sme 3400 (Iwaki, Japan). A calibration curve with concentrations of the standard 8-oxo-dG solutions (in logarithm) versus the absorbances (in linear) was constructed for each experiment and used to calculate the 8-oxo-dG concentrations in urine specimens.

Statistical Analysis. CVs were used to compare the variances of the values of 8-oxo-dG measured by HPLC-ECD and ELISA. Pearson’s correlation coefficient and regression analysis were used to test the correlation between the 8-oxo-dG values measured by HPLC-ECD and by ELISA.

Results
For all experiments, one plate was assayed per day. To confirm the reliability of the ELISA method using the kit, intra-plate variability of 8-oxo-dG values of three controls was calculated. The CV of C1, C2, and C3 was 16.34%, 11.97%, and 1.31%, respectively (n = 3 in the same plate). To confirm inter-day variability, three controls were assayed on 30 different days within a 3-month period. A histogram of the 8-oxo-dG measurements of each control (30 times each) is shown in Fig. 3. The median, mean value, SD, and CV of each control are also calculated (Table 1). Because the 8-oxo-dG values in each control were not distributed normally (Fig. 3, C1-a, C2-a, or C3-a), the median of each control was selected as the representative value. Percentiles of three controls are shown in Fig. 3 (C1-b, C2-b, and C3-b). According to these percentile figures, values exceeding 27.5, 12.0, and 4.75 ng/ml, which correspond to C1, C2, and C3, respectively, were regarded as incorrect values. The borders of incorrect control values were 25%, 23%, and 55% higher than the median (representative value) of each control, respectively. We decided that we should regard the experiment as unsuitable if any of the controls in each experiment were 25% higher or lower than the corresponding representative value.
For appropriate selection on each experiment, each experiment included three controls arranged in the microtiter plate as shown in Fig. 2. 8-oxo-dG in 72 samples measured by both the ELISA and HPLC-ECD was analyzed within 40 days. Table 2 shows the urinary 8-oxo-dG values measured by HPLC-ECD and ELISA. Although the 8-oxo-dG value measured by the ELISA was 2-fold higher than the value measured by HPLC-ECD, the CV of the ELISA was similar to that of HPLC-ECD. Fig. 4 shows the scatter plots and regression line for the relation between the 8-oxo-dG values measured by ELISA and by HPLC-ECD. When $X$ was defined as the 8-oxo-dG values (ng/ml) measured by HPLC-ECD, and $Y$ was defined as the 8-oxo-dG values measured by the ELISA, simple regression analysis showed the most likely relationship to be $Y = 1.83X + 0.8$. The correlation coefficient was 0.88, which indicated a good correlation between $X$ and $Y$.

**Discussion**

Although the measurement of urinary 8-oxo-dG by HPLC-ECD is reliable, it demands a high technical level and takes a relatively long time (6, 7). Because the technique of the ELISA is easier than that of HPLC-ECD, the ELISA would be the preferred alternative method for measuring urinary 8-oxo-dG, if the correlation with HPLC-ECD were acceptable. In the present study, the urinary 8-oxo-dG values measured with a commercially available ELISA kit were compared with the values obtained by the generally accepted HPLC-ECD method. The results showed a good correlation between the urinary 8-oxo-dG values obtained by HPLC-ECD and those obtained by the ELISA.

Prieme et al. (13) evaluated an ELISA including the same monoclonal antibody (N45.1) and reported 8-fold higher results on average as compared with HPLC-ECD. The correlation between the urinary 8-oxo-dG values obtained by HPLC-ECD and the ELISA was weak ($r = 0.42$; Ref. 13). The difference between our results and the results of the experiments performed by Prieme et al. (13) may be attributable to the use of different versions of the ELISA kit. We used the improved version of the kit, which differs somewhat from the old version used by Prieme et al. (product information from Japan Institute for the Control of Aging), for example, in the amount of 8-oxo-dG-protein conjugate coated on the plate, the concentration of the primary monoclonal antibody, the buffer contents, and the type of chromatic solution. Thus, changes in the quality of the kit may be one reason why we obtained a better correlation between the HPLC-ECD and the ELISA compared with the results of Prieme et al. (13). Another reason for the difference may be our use of three controls with appropriate concentrations and our strict selection of reliable data. The study of Prieme et al. (13) did not mention the use of appropriate controls in their experiment.

We obtained a good correlation between the results of HPLC-ECD and the ELISA; however, the 8-oxo-dG values obtained by the ELISA were still nearly 2-fold higher than the values obtained by HPLC-ECD. Although the monoclonal antibody N45.1 has high specificity for 8-oxo-dG (9–11), there may be other unknown substances in urine that react with the antibody. There is evidence that the same values were obtained by the ELISA and HPLC-ECD if urine was passed through the first HPLC-ECD column, and the 8-oxo-dG fraction was col-

### Table 1

<table>
<thead>
<tr>
<th>Median (ng/ml) Mean values (ng/ml) SD CV (%)</th>
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<tr>
<td>Medians (ng/ml)</td>
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<tr>
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</tr>
<tr>
<td>C1 21.93</td>
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<tr>
<td>C2 9.77</td>
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<td>C3 3.06</td>
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**Fig. 3** Histograms of the 8-oxo-dG values of the controls (C1, C2, and C3) and their percentiles. C1-a, histogram of 8-oxo-dG values of C1; C1-b, percentile of 8-oxo-dG values of C1; C2-a, histogram of 8-oxo-dG values of C2; C2-b, percentile of 8-oxo-dG values of C2; C3-a, histogram of 8-oxo-dG values of C3; C3-b, percentile of 8-oxo-dG values of C3.
Biomarkers Prev. 11, 4 H. Kasai, unpublished data.


4 H. Kasai, unpublished data.

Table 2  Urinary 8-oxo-dG values measured by HPLC-ECD and with an ELISA kit

<table>
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<tr>
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<th>Maximum (ng/ml)</th>
<th>Minimum (ng/ml)</th>
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<tr>
<td>HPLC (n = 72)</td>
<td>5.65 ± 2.77 (49.03)</td>
<td>11.85</td>
</tr>
<tr>
<td>ELISA (n = 72)</td>
<td>11.13 ± 5.77 (51.84)</td>
<td>31.60</td>
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Table 2 continued

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2.08 ng/ml, which were within our range of 8-oxo-dG values by the ELISA (2.08–31.60 ng/ml). Thus, good temperature control is critical. Whereas the kit instructions suggest that the outermost wells should not be used, our data (data not shown), in which we measured the absorbance of the same sample in all wells, indicate that it is not a problem. In addition, strict selection of appropriate data using values in assay controls should also improve accuracy.

In view of the good correlation between the 8-oxo-dG values measured by HPLC-ECD and the ELISA, the ELISA can at least be applied to studies comparing relative urinary 8-oxo-dG values in molecular epidemiological studies to assess the risk of cancer or other diseases from environmental chemicals. The choice between HPLC-ECD and the ELISA may depend on the apparatus, analytical techniques, or manpower that each laboratory can afford. Although expensive instrumentation is needed for HPLC-ECD analysis, this method is recommended when more precise and accurate measurements are required.

In summary, we have demonstrated that the ELISA may be applicable to epidemiological studies because of its correlation with the HPLC-ECD method. However, further efforts to improve the assay accuracy and precision are needed.

References


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