Comparison between High-Performance Liquid Chromatography and Enzyme-linked Immunosorbent Assay for the Determination of 8-Hydroxy-2′-deoxyguanosine in Human Urine[1]

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Abstract

8-Hydroxy-2′-deoxyguanosine (8-OH-dG), which has been regarded as a potential marker of oxidative DNA damage induced by reactive oxygen species, was measured in human urine by a commercial ELISA using a monoclonal antibody N45.1 and by high-performance liquid chromatography (HPLC) coupled to an electrochemical detector (HPLC-ECD) to evaluate whether the ELISA system is applicable to human monitoring studies. The urine samples were collected from 120 healthy men ages 18–58 in a steel-manufacturing company. A good correlation ($r = 0.833$; $P < 0.0001$) was observed between the two methods on HPLC-purified 8-OH-dG fractions from 23 urine samples. The mean value (±SD) of 8-OH-dG (μg/g creatinine) was 5.47 (±2.97) by HPLC-ECD assay and 5.50 (±2.36) by ELISA. However, the correlation (r) between the two methods on 120 original urine samples was 0.460 [$P < 0.001$; mean value (±SD) of 8-OH-dG (μg/g creatinine) was 4.46 (±2.03) by the HPLC assay and 9.33 (±3.23) by ELISA]. ELISA estimates were about 2-fold higher than the HPLC estimates on original urine. For an unknown reason, 10% of the urine samples showed more than a 4-fold increase in value by ELISA. It is suggested that the ELISA system is applicable for comparative human monitoring studies. Prepurification of ‘samples is required to determine the absolute value of 8-OH-dG in individual urine samples by ELISA.

Introduction

ROS[3] are endogenously generated in cells and may be involved in aging and the development of diseases such as cancer and diabetes. 8-OH-dG has been regarded as a potential marker of oxidative DNA damage induced by ROS (1) and has been analyzed as a marker of cellular oxidative stress relevant to carcinogenesis (2). 8-OH-dG was increased in the DNA of target organs after treatment with carcinogens that generate ROS in animal experiments (2). In humans, higher levels of 8-OH-dG were observed in the lungs of smokers (3), the liver of chronic hepatitis patients (4), lymphocytes from cancer patients during radiotherapy (5), and mononuclear cells from diabetic patients (6).

The level of urinary 8-OH-dG has been reported to be higher in cancer patients than in healthy people (7) and higher in smokers than in nonsmokers (8), and to be affected by metabolic rate (9). The urinary 8-OH-dG has been measured by several methods such as liquid chromatography-tandem mass spectrometry (LC-MS-MS; Ref. 10), gas chromatography with mass spectrometric detection (GC-MS; Ref. 11), and HPLC-ECD (12). Recently, a monoclonal antibody specific to 8-OH-dG (N45.1) has been developed and an ELISA has been established for 8-OH-dG determination (13). Urinary 8-OH-dG levels increased in non-small cell carcinoma patients during the course of radiotherapy and small cell carcinoma patients showed higher levels of urinary 8-OH-dG than did controls (14). However, it has been reported that a commercially available ELISA kit using N45.1 monoclonal antibody yields ~8-fold higher results, on average, with a high variation, compared with the HPLC-ECD method (15). It was suggested that there may be cross-reacting substances in urine. Because it is more simple and easier to measure urinary 8-OH-dG by the ELISA method than by HPLC analysis in the aspects of equipment, analyzing time, running cost, and urine sample volume, the number of reports on urinary 8-OH-dG levels determined by ELISA has been on the increase recently. However, there remain methodological problems in urine analysis.

In this study, human urinary excretion of 8-OH-dG was determined by the commercial ELISA method, the ELISA method with HPLC prepurification, and the HPLC-ECD method to evaluate whether the ELISA system is applicable to human monitoring studies.

Materials and Methods

Urine Collection. Urine samples were collected in paper cups from 120 healthy men ages 18–58 in a steel-manufacturing company, who gave their informed consent, in the afternoon (12:45–15:30) during their periodic health examination. Aliquots of each urine sample were stored at −80°C until the 8-OH-dG analysis.

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[3] The abbreviations used are: ROS, reactive oxygen species; 8-OH-dG, 8-hydroxy-2′-deoxyguanosine; HPLC, high-performance liquid chromatography; ECD, electrochemical detection; HPLC-ECD, HPLC with ECD; ELISA, enzyme-linked immunosorbent assay.

Table 1 Urinary 8-OH-dG/creatinine levels determined by ELISA and HPLC-ECD

	8-OH-dG/creatinine (μg/g)			ELISA/HPLC
	Mean ± SD	Maximum	Minimum	
n = 23				
Original urine				2.63 ± 2.07
ELISA	10.53 ± 3.95	19.52	4.35	
HPLC-ECD	5.47 ± 2.97	11.09	1.20	
8-OHdG purified fraction				1.18 ± 0.49
ELISA	5.50 ± 2.36	9.61	2.20	
HPLC-ECD	5.47 ± 2.97	11.09	1.20	
n = 120				
Original urine				2.43 ± 1.31
ELISA	9.33 ± 3.23	19.52	3.93	
HPLC-ECD	4.46 ± 2.03	11.09	1.20	
n = 27				
Original urine (nonsmoker)				2.21 ± 0.79
ELISA	8.16 ± 2.66	14.14	4.35	
HPLC-ECD	4.18 ± 2.13	10.47	1.52	
n = 76				
Original urine (smoker)				2.57 ± 1.51
ELISA	9.91 ± 3.34	19.52	4.90	
HPLC-ECD	4.57 ± 2.03	11.09	1.20	
n = 21				
Original urine (smoker: more than 26 cigarettes/day)				2.54 ± 1.99
ELISA	10.81 ± 3.37	19.52	5.44	
HPLC-ECD	5.19 ± 2.10	8.99	1.20	

HPLC Analysis and Fractionation of 8-OH-dG. HPLC analysis was carried out by the method described previously (16). Briefly, 1.0 ml of each urine sample was defrosted, diluted with 0.5 ml of water, and acidified with 45 μl of acetic acid. The urine solution was centrifuged, and 0.75 ml of the supernatant was automatically injected into the first HPLC column [Shodex Asahipak GS-320HQ 7G (500 × 7.6 mm), 25°C], which has the functions of gel filtration-, reverse phase-, and ion exchange-column fractionation. Elution was performed using 0.1% acetic acid at the flow rate of 1 ml/min. An aliquot (100 μl) of the fraction containing 8-OH-dG (50–61 min) was automatically injected into the second HPLC column [YMC-Pack ODS-AM. (250 × 4.6 mm), 25°C]. The mobile phase was aqueous methanol (5%) containing 35 mM NaOAc and 12.5 mM citric acid (pH was adjusted to 7.5 by adding 1M NaOH solution). The flow rate was 0.8 ml/min. The HPLC system was equipped with an ECD (Coulochem II, esa; guard cell, 350 mV; channel 1, 300 mV; channel 2, 150 mV) for detection. Quantification of 8-OH-dG was done by measuring the peak areas based on calibration plots of the peak area of standard 8-OH-dG at various concentrations. The accuracy of the measurement estimated from the recovery of an added 8-OH-dG standard was 90–98%.

8-OH-dG fraction (50–61 min) was collected through the first HPLC column as mentioned above, and the solution was evaporated into dryness by a freeze dryer for the ELISA method.

Urinary creatinine was determined by a kit (Creatinine-test, Wako, Osaka).

ELISA for Urinary 8-OH-dG. Urine samples were centrifuged at 2000 × *g* for 15 min and 50 μl of the supernatant was used for the determination of 8-OH-dG with a commercial ELISA kit (New 8-OHdG check, Japan Institute for the Control of Aging, Fukuroi, Shizuoka). The determination range was 0.5–200 ng/ml. The monoclonal antibody, N45.1, with an established specificity (13), was used as a primary antibody. The values from each urine sample were calculated based on calibration sigmoid plots of absorbance (492 nm) of standard 8-OH-dG at various concentrations, by fitting a logistic curve using a computer analysis. Day-to-day variation was less than 10%.

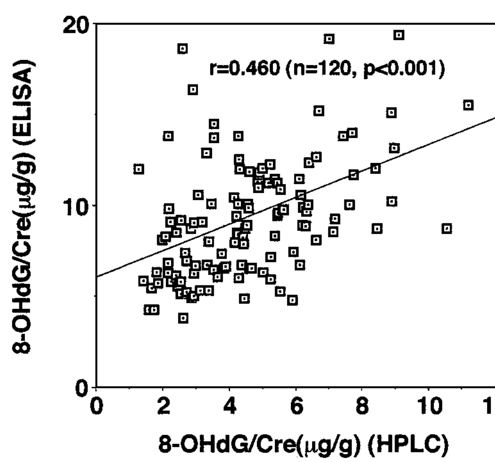

Fig. 1. Correlation between the two methods, HPLC and ELISA, on human urinary 8-OH-dG levels of 120 volunteers (men).

Statistical Analysis. Data were shown as 8-OH-dG/creatinine (μg/g). The correlation between the HPLC and the ELISA estimate was tested by a simple linear regression analysis.

Results

As shown in Table 1, the mean value (mean ± SD) of 8-OH-dG in 120 urine samples was 4.46 ± 2.03 μg/g creatinine by HPLC analysis and 9.33 ± 3.23 μg/g creatinine by the ELISA method,

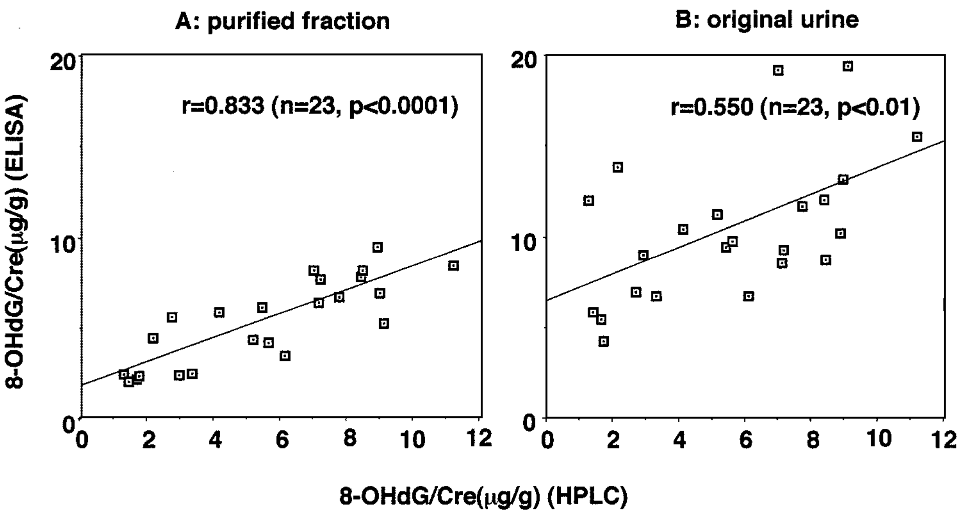

Fig. 2. Correlation between the two methods, HPLC and ELISA, on 8-OH-dG level of 23 human original urine samples (*B*) and their 8-OH-dG purified fractions (*A*).

the ELISA estimate being ~2-fold higher than the HPLC estimate. The increase of the mean value of urinary 8-OH-dG in smokers, especially in heavy smokers (more than 26 cigarettes/day), was shown by both methods. The correlation between the two methods was 0.460 ($P < 0.001$; Fig. 1). The ratio of ELISA:HPLC values ranged from 0.84 to 10.08 with a mean of 2.43 ± 1.31 (mean ± SD). Ten % of the urine samples showed a >4-fold higher ratio.

However, a good correlation ($r = 0.833$; $P < 0.0001$) was observed between the two methods on HPLC-purified 8-OH-dG fractions from 23 urine samples (Fig. 2*A*). The mean value of 8-OH-dG in the purified fraction from 23 urine samples was 5.47 ± 2.97 μg/g creatinine (mean ± SD) by HPLC analysis and 5.50 ± 2.36 μg/g creatinine by the ELISA method. The interindividual variation by each method was 9.2-fold (1.20–11.09 μg/g creatinine) and 4.4-fold (2.20–9.61 μg/g creatinine), respectively. The ELISA estimate was almost the same as the HPLC estimate (Fig. 2*A*). The mean value of 8-OH-dG in 23 original urine samples was 5.47 ± 2.97 μg/g creatinine (mean ± SD) by HPLC analysis and 10.53 ± 3.95 μg/g creatinine (mean ± SD) by the ELISA method, respectively. The interindividual variation was ~9.2 fold (1.20–11.09 μg/g creatinine) by the HPLC analysis and ~4.4-fold (4.35–19.52 μg/g creatinine) by the ELISA method. The ELISA estimate was about 2-fold higher than the HPLC estimate (Table 1). The correlation between the two methods was 0.550 ($P < 0.01$; Fig. 2*B*).

Discussion

In this study, the ELISA estimate was similar to the HPLC estimate on purified 8-OH-dG fractions from 23 urine samples. A good correlation ($r = 0.833$; $P < 0.0001$) was observed between the two estimates. It has been reported that the mean value of 8–0H-dG in human urine samples determined by HPLC-ECD (this study), HPLC-ECD (another group; Ref. 17), gas chromatography with mass spectrometric detection (11), and HPLC-ELISA (this study) was 5.47 ± 2.97 (mean ± SD), 3.68–3.96, 3.33–3.95, and 5.50 ± 2.36 (mean ± SD) μg/g creatinine, respectively. These results indicate that the monoclonal antibody N45.1 used for a commercial ELISA kit is quite specific for 8-OH-dG.

However, the ELISA estimate on 120 original urine samples (9.33 ± 3.23 μg/g creatinine) was ~2-fold higher than the HPLC estimate (4.46 ± 2.03 μg/g creatinine). A lower correlation was observed between the two estimates compared with the values on the purified 8-OH-dG fractions. Ten % of urine samples showed a high ratio of ELISA-estimates:HPLC-estimates (>4-fold). These results suggest that the monoclonal antibody N45.1 used for a commercial ELISA kit is not sufficiently specific to detect 8-OH-dG in urine without prior HPLC purification and that crude urine samples contain cross-reacting substances, modified forms of 8-OH-dG, other structurally related compounds, or inhibitors for competition with the N45.1 antibody. There is a possibility that oligonucleotides, as a result of nucleotide excision repair, and glucuronides/sulfates, as a result of metabolic conjugation, are excreted into the urine. Because it has been reported that this antibody, N45.1, recognizes both the modified base and deoxyribose structure (13), N45.1 may react with modified forms of 8-OH-dG or mimicking substances. The reason for the high ELISA estimates remains unknown.

The urinary 8-OH-dG level obtained by ELISA in crude urine in this study was 9.33 ± 3.23 μg/g creatinine. Because it was 17.1 (12) and 19.4 ± 8.5 (14) μg/g creatinine in the previous reports, the new kit used in this study appears to be somewhat more accurate. It should be noted that the urinary 8-OH-dG level was ~2-fold higher and that 10% of the urine samples showed a very high value by the ELISA method. The results shown in this study suggest that the ELISA system is applicable for comparative human monitoring studies, although HPLC prepurification of samples is required to determine the absolute value of 8-OH-dG in individual urine samples by ELISA.

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