

# Simultaneous Determination of 8-hydroxydeoxyguanosine, a Marker of Oxidative Stress, and Creatinine, a Standardization Compound, in Urine

Hiroshi KASAI\*, Peter SVOBODA, Sayumi YAMASAKI and Kazuaki KAWAI

Department of Environmental Oncology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

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**Abstract:** Recently, H. Kasai reported an automatic, precise method of 8-hydroxydeoxyguanosine (8-OH-dG) analysis in urine by high performance liquid chromatography coupled to an electrochemical detector (HPLC-ECD). It is based on a cleaning-up step by anion-exchange chromatography and a further purification step using reverse phase chromatography before detection by the ECD. In this communication, we report a method for the simultaneous determination of 8-OH-dG and creatinine, an internal standard for normalizing the excretion of 8-OH-dG in urine.

**Key words:** 8-hydroxydeoxyguanosine, Creatinine, Urine

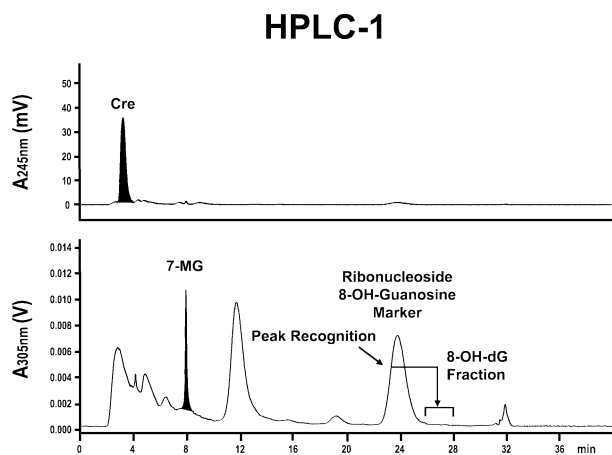
8-Hydroxydeoxyguanosine (8-OH-dG, 7,8-dihydro-8-oxodeoxyguanosine, 8-oxo-dG) is an oxidation product of dG, a component of DNA<sup>1</sup>. Extensive studies over the past three decades have established its biological significance, since 8-OH-dG is produced in cellular DNA, its repair systems, such as OGG1 and MYH, are ubiquitously present in various organisms, and the accumulation of 8-OH-dG in OGG1, MYH double knockout mouse lung DNA correlated well with cancer induction<sup>2</sup>. The urinary 8-OH-dG level is a good indicator of oxidative stress in vivo. Various environmental pollutants such as polyaromatic hydrocarbons<sup>3</sup>, metal fumes<sup>4</sup> and fly ash<sup>5</sup> increased the level of urinary 8-OH-dG. Cancer and other oxygen radical-related disease patients have high urinary 8-OH-dG levels<sup>6, 7</sup>. The 8-OH-dG levels in urine and lymphocyte DNA are also well correlated, and the 8-OH-dG/creatinine values of 24 h urine samples and overnight urine (early morning urine) show a good correlation<sup>8</sup>.

8-OH-dG has been analyzed by ELISA, HPLC-ECD and LC-MS-MS methods. The ELISA method is most often used for urinary 8-OH-dG analysis, because an analysis kit

is commercially available. However, some discrepancies have been observed between the data obtained by the ELISA- and HPLC-ECD-methods, although they have a statistically significant correlation<sup>9, 10</sup>. The 8-OH-dG levels determined by ELISA were about 2-fold higher than those obtained by HPLC-ECD, and the reproducibility of the ELISA method was poor, as compared to the HPLC-ECD method. A comparison of the analyses of urinary 8-OH-dG by an isotope-dilution liquid chromatography with electrospray tandem mass spectrometry (LC-MS-MS) method and the ELISA method also revealed that the former is more reliable than the latter<sup>11</sup>.

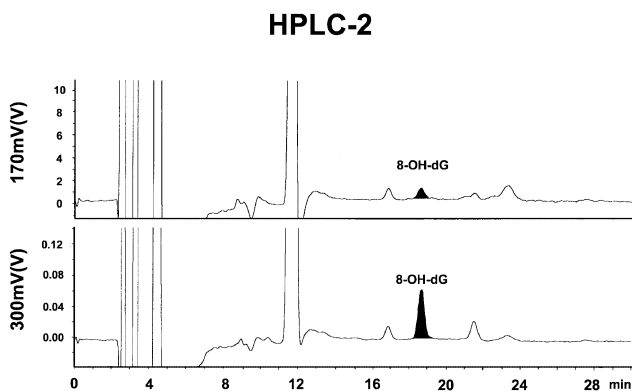
In our method of urinary 8-OH-dG analysis<sup>12</sup>, an anion exchange column (MCI GEL CA08F) (polystyrene-type resin with quaternary ammonium group, sulfate form) is used for a urine pre-purification step (HPLC-1), which removes most of the other components in the sample. The commercially available MCI GEL CA08F anion exchange resin (chloride form) was changed to the sulfate form, and was manually packed into a empty column by pushing with a thin wire, or by forming a slurry according to the method of Scott and Lee<sup>13</sup>. In HPLC-1, the 8-OH-dG fraction is accurately collected based on the elution position of ribonucleoside 8-

\*To whom correspondence should be addressed.



**Fig. 1.** Detection of creatinine and 7-methylguanaine by anion exchange chromatography (HPLC-1).

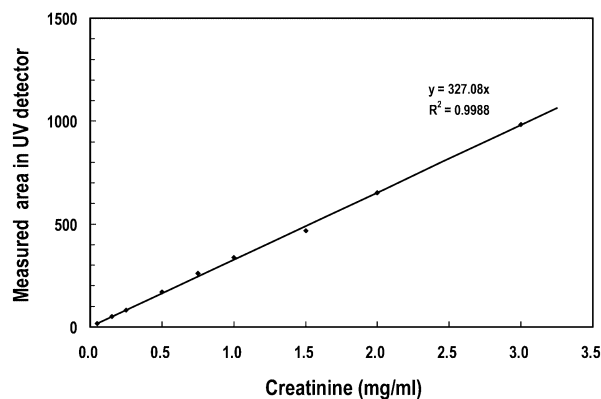
A human urine sample was mixed with the same volume of a dilution solution containing the ribonucleoside marker, 8-hydroxyguanosine. A 20  $\mu$ l aliquot of the diluted urine sample was injected into HPLC-1 (MCI GEL CA08F, 7  $\mu$ m, 1.5  $\times$  120 mm, solvent A, 50  $\mu$ l/min, 65°C), via the guard column (1.5  $\times$  40 mm), and the chromatograms were recorded by a Gilson UV detector (UV/VIS-155 with 0.2 mm light path cell) at 245 and 305 nm. The 8-OH-dG fraction was collected, depending on the relative elution position from the peak of the added marker, 8-OH-G, and was automatically injected into the HPLC-2 column. Solvent A: 2% acetonitrile in 0.3 mM sulfuric acid.



**Fig. 2.** Detection of 8-OH-dG by reverse-phase HPLC (HPLC-2).

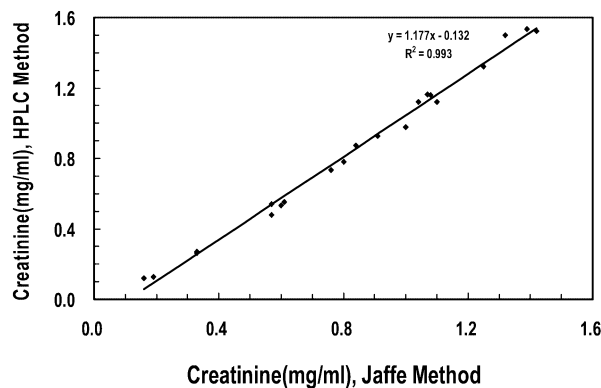
The 8-OH-dG fraction was fractionated by the HPLC-2 column (Shiseido, Capcell Pak C18, 5  $\mu$ m, 4.6  $\times$  250 mm, solvent B, 1 ml/min, 40°C). The 8-OH-dG was detected by a Coulochem II EC detector (ESA, USA) with a guard cell (5020) and an analytical cell (5011) (applied voltage: guard cell, 350 mV; E1, 170 mV; E2, 300 mV). Solvent B: 10 mM sodium phosphate buffer (pH 6.7), 5% methanol, plus an antiseptic Reagent MB (100  $\mu$ l/l).

hydroxyguanosine, added as a marker (Fig. 1). Furthermore, by monitoring two ECD channels with different applied voltages, for example, 170 and 300 mV, the 8-OH-dG peaks appear with a specific ratio of peak heights, as shown in



**Fig. 3.** Standard curve for 8-OH-dG analysis.

Various amounts of Cre were added to a urine sample, which was diluted two-fold with the marker-containing solution, and then a 20  $\mu$ l aliquot was injected into the HPLC-1 column. The increased area of the Cre peak was plotted against the amount of added Cre.



**Fig. 4.** Correlation between Cre levels obtained by the HPLC method and those by the Jaffe method.

**Fig. 2.**

Creatinine (Cre) is frequently used as an internal standard for normalizing urinary 8-OH-dG. We attempted to analyze both 8-OH-dG and Cre simultaneously in the HPLC-1 step with our method. It has been reported that Cre elutes in a very early fraction from an anion exchange column (Aminex A-27, Bio-Rad Lab.USA), which has the same chemical structure as MCI GEL CA08F<sup>14)</sup>. We also found that Cre eluted in an early fraction in the HPLC-1, and overlapped with two other urinary components, one with UV absorption at a wavelength shorter than 240 nm, and the other with a UV max at 260 nm. When we chose 245 nm for monitoring in HPLC-1, and used a thinner UV cell (0.2 mm light path), we successfully detected the Cre peak, as shown in Fig.1, because the Cre concentration in urine is extremely high. It

was also possible to measure 7-methylguanine, another possible standardization compound in urine, by monitoring 305 or 245 nm in addition to Cre<sup>15</sup>.

For the determination of urinary Cre, we first prepared a standard curve by injecting urine samples containing various amounts of Cre. We observed linearity up to 3 mg Cre/ml (the actual amount in the injection was 15 µg) (Fig. 3). We analyzed 21 human urine samples from a stock used in a previous study<sup>16</sup>. The urinary Cre concentration determined by the present method and the Cre data previously determined by a commercial laboratory (BML Corp., Kitakyushu, Japan), using a colorimetric method (Jaffe method), were compared. As shown in Fig.4, we confirmed that our measurements of Cre by HPLC-1 correlated well with the measurements obtained by the commonly used method.

Previously, a urine sample collected from a subject was separated into at least two tubes before freezing, one for Cre measurement, the other for 8-OH-dG analysis, which were carried out on different apparatuses, and finally the 8-OH-dG / Cre value was calculated. The successful simultaneous determination of 8-OH-dG and Cre in one apparatus will facilitate the efficient analysis of large numbers of urine samples. It also has the merit that even a dried urine sample on a filter paper can be analyzed, since the 8-OH-dG/Cre value is easily obtained after extraction and injection into this apparatus. This convenient method for the simultaneous measurement of 8-OH-dG and Cre from the same urine sample may be widely used in the future for the risk assessment of environmental agents, and the prediction of oxygen radical-related diseases.

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