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A New Automated Method to Analyze Urinary 8-Hydroxydeoxyguanosine by a High-performance Liquid Chromatography–Electrochemical Detector System

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Urine/8-hydroxydeoxyguanosine/HPLC-ECD/Oxidative DNA damage.

A new method was developed to analyze urinary 8-hydroxydeoxyguanosine (8-OH-dG) by high-performance liquid chromatography (HPLC) coupled to an electrochemical detector (ECD). This method is unique because (i) urine is first fractionated by anion exchange chromatography (polystyrene-type resin with quaternary ammonium group, sulfate form) before analysis by reverse phase chromatography; and (ii) the 8-OH-dG fraction in the first HPLC is precisely and automatically collected based on the added ribonucleoside 8-hydroxyguanosine marker peak, which elutes 4–5 min earlier. Up to 1,000 human urine samples can be continuously analyzed with high accuracy within a few months. This method will be useful for studies in radiotherapy, molecular epidemiology, risk assessment, and health promotion.

INTRODUCTION

Since 1983, my colleagues and I have been studying oxidative DNA damage in vitro and in vivo[1,2]. A particularly important lesion is 8-hydroxydeoxyguanosine (8-OH-dG, 7,8-dihydro-8-oxodeoxyguanosine, 8-oxodG), produced by the oxidation of deoxyguanosine, because it is formed in cellular DNA by ionizing radiation and many chemical carcinogens[3], and it has biological significance, since it induces mutations and its repair enzymes are ubiquitously present in bacteria and mammalian cells[4–6]. Recently, the effects of reactive oxygen species (ROS) on the pathogenesis of various diseases, including cancer, have been widely studied. However, because ROS themselves have short half-lives, it is difficult to establish a cause-and-effect relationship between ROS and disease. The analysis of urinary 8-OH-dG, a reliable marker of cellular oxidative stress, will provide insight into this problem.

Ames and his collaborators first reported the presence of 8-OH-dG in human, rat, and mouse urine[7]. Since then several groups have reported its analysis by different methods. Urinary 8-OH-dG has been analyzed by at least nine methods, namely, (1) high-performance liquid chromatography (HPLC), using a reverse phase column coupled to an electrochemical detector (ECD) after enrichment on an 8-OH-dG antibody affinity column[8]; (2) the HPLC-ECD method after purification on a reverse phase column[9]; (3) the HPLC-ECD method after purification on reverse phase and cation exchange columns[10]; (4) the HPLC-ECD method after purification on reverse phase and two carbon columns[11]; (5) the HPLC-ECD method after purification on two different cation exchange columns[12]; (6) the HPLC-ECD method after purification on a multi-function column, with the functions of gel filtration, reverse phase, and cation exchange columns[13]; (7) the gas-chromatography mass spectrometry (GC-MS) method[14]; (8) the HPLC electrospray tandem mass spectrometry (LC-MS-MS) method[15]; and (9) the enzyme-linked immunosorbent assay (ELISA) method[16]. However, none of these methods is widely employed for the following reasons. Method 1 uses a commercially unavailable affinity column with an 8-OH-dG antibody, and the recovery is not consistent. In methods 2–6, the determination of the 8-OH-dG fraction collection timing is difficult, because it changes day by day and also depends on the sample injected. In methods 7 and 8, an isotope-labeled internal standard that is not commercially available must be added to the urine for an exact measurement, because of the low recovery. Particularly in the LC-MS-MS method, repeated injections of urine samples reduce the sensitivity. The urinary 8-OH-dG levels measured by the ELISA method were at least two times higher than those obtained by the HPLC-ECD method[17], possibly because the commercial monoclonal antibody may cross-react with other components in urine, and also the reproducibility of the analyses was very low[18].

In this paper I describe a new automated method for urinary 8-OH-dG analysis, using an anion exchange column at the first step followed by separation with a reverse phase column, which allows the detection of 8-OH-dG as a single peak by an EC

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detector. Furthermore, in this system the 8-OH-dG fraction is accurately collected and injected into the second column by monitoring the position of the added ribonucleoside 8-hydroxyguanosine (8-OH-G) marker peak.

MATERIALS AND METHODS

Material

The anion exchange resin, MCI GEL CA08F (7 μm, Cl^- form) was purchased from Mitsubishi Chemical Corp., Japan. A 5 g portion of it was washed with 100 ml of 1 N NaOH, then with deionized water until a neutral pH was achieved. The resin was then washed with 100 ml of 1 N H_2SO_4, followed by washing with deionized water to a neutral pH. The resin was manually packed within guard (1.5 × 50 mm) and main (1.5 × 150 mm) columns for HPLC-1. The ribonucleoside 8-OH-G was prepared according to the method of Ikehara *et al.*[19], and was further purified by preparative HPLC with the use of a reverse-phase column with solvent B (see below). The antiseptic, Reagent MB, was purchased from MC Medical Inc., Japan. HPLC grades of methanol and acetonitrile were purchased from Wako Pure Chemical Industries, Ltd., and Kanto Chemical Co., Inc., Japan, respectively.

Urine samples

Frozen urine samples were defrosted and mixed completely to form homogeneous suspensions; 50 μl of each was then mixed with the same volume of a dilution solution containing the ribonucleoside marker 8-hydroxyguanosine (120 μg/ml) and 4% acetonitrile in a solution of 130 mM NaOAc (pH 4.5) and 0.6 mM H_2SO_4. The pH of the diluted urine solution should be below 7 to obtain good separation. The urine solutions were stored in a refrigerator at 5°C for 2-3 hours, then centrifuged at 13,000 rpm for 5 min. A 70 μl aliquot of each supernatant was transferred to a vial tube for analysis in the apparatus.

Apparatus and 8-OH-dG analysis

The urinary 8-OH-dG level was determined by using the apparatus shown in Fig. 1, in which pump 1 (Shiseido Nanospace SI-2), the sampling injector (Gilson 231XL), the guard column for HPLC-1(valve 1, pump 3), the HPLC-1 column, the UV detector (Toso UV-8020, micro cell), (valve 2, loop, pump 2), the HPLC-2 column, and the EC detector (ESA Coulochem II) were connected. The guard and main columns for HPLC-1 were set in a column oven at 65°C, and the HPLC-2 column was set in a column oven at 40°C. A 20 μl aliquot of the diluted urine sample was injected into HPLC-1 (MCI GEL CA08F, 7 μm, 1.5 × 150 mm, solvent A, 37 μl/min) from the sampling injector, via the guard column (the same as above, except that the column length is 50 mm), and the chromatogram was recorded by a UV detector (254 nm). In this method, the 8-OH-dG fraction was unequivocally and precisely collected, depending on the relative elution position from the peak of the added marker, 8-OH-G, and automatically injected into the HPLC-2 column (Shiseido, Capcell

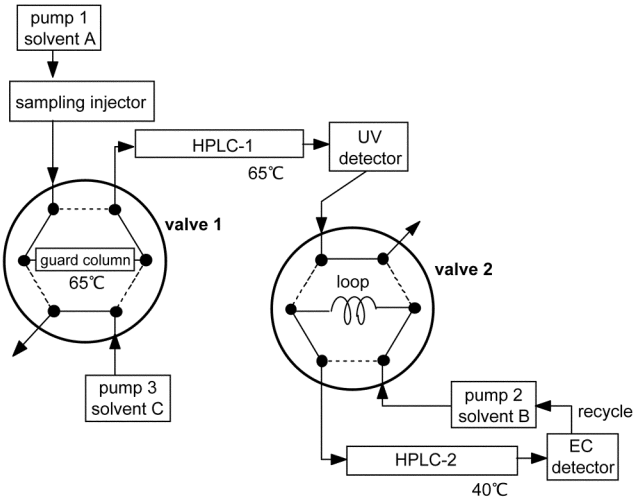

Fig. 1. Outline of the apparatus for urinary 8-OH-dG analysis.

Pak C18, 5 μm, 4.6 × 250 mm, solvent B, 1 ml/min). For this purpose, new program software was used for the Gilson 231XL sampling injector. Namely, after waiting for a defined time (T1), the sampling injector started to monitor a defined level of the UV absorbance signal, which enabled the recognition of the 8-OH-G marker peak (Fig. 2). After a defined time interval (T2), the collection of the 8-OH-dG-containing fraction started in the loop connected to valve 2, and after a defined interval (T3), the 8-OH-dG fraction in the loop was injected into the HPLC-2 column by valve switching. The 8-OH-dG was detected by a Coulochem II EC detector with a guard cell (5020) and an analytical cell (5011) (applied voltage: guard cell, 400 mV; E1, 280 mV; E2, 350 mV). The applied voltages (E1, E2) for the maximum sensitivity and selectivity for 8-OH-dG detection were different, depending on each analytical cell (5011). About 13 min after each urine injection, the guard column back flash (solvent C, 37 μl/min) was started and continued for 32 min; the solution was then changed to the starting buffer (solvent A) by valve switching. The solvents used were: solvent A, 2% acetonitrile in 0.3 mM sulfuric acid; solvent B, 10 mM sodium phosphate buffer (pH 6.7), 5% methanol, plus an antiseptic Reagent MB (100 μl/liter); and solvent C, 0.5 M ammonium sulfate: acetonitrile (7:3, v/v). Solvent B (1 liter) could be recycled from the ECD to pump 2 for one week during the analysis of 80–100 urine samples. Under these standard conditions, one sample was analyzed per hour. Usually 16–20 urine samples and four randomly inserted 8-OH-dG standard samples were analyzed per day.

When a thicker column (2–4.6 mm diameter) was used for HPLC-1, the Gilson 233XL sampling injector was used instead of the 231XL model. It has the functions of injection of the urine sample, collection of the 8-OH-dG fraction, mixing, and injection into HPLC-2. In this system, the 8-OH-dG fraction was automatically collected based on the relative elution position from the 8-OH-G peak. For this purpose, new program software was used with the Gilson 233XL sampling injector. Namely,

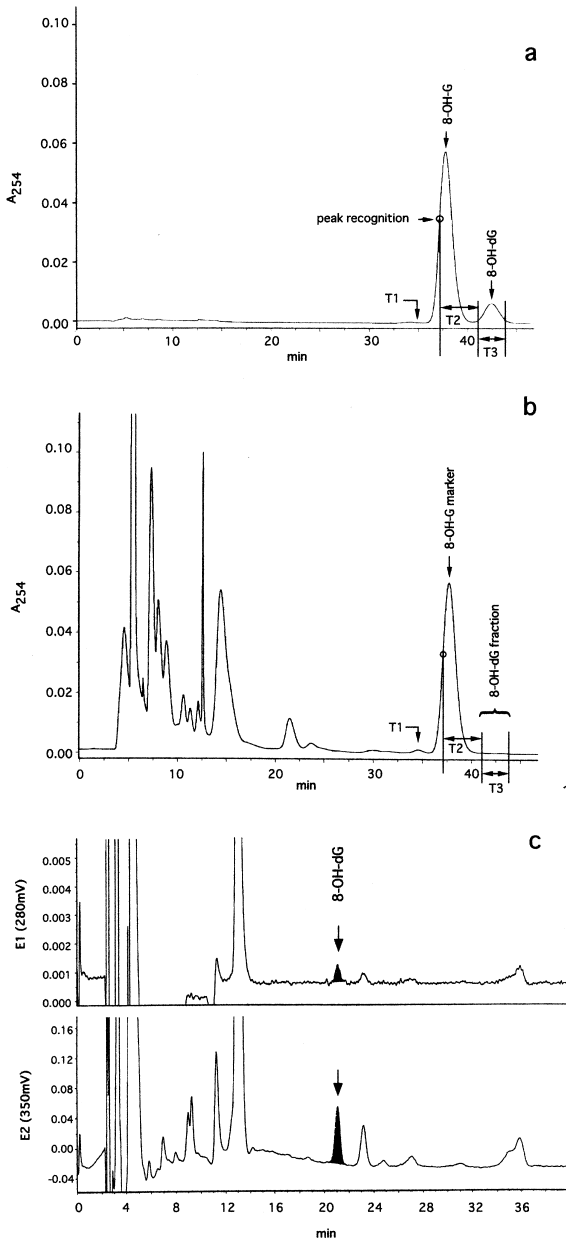

Fig. 2. HPLC-1 chromatograms of (a) 8-OH-G and 8-OH-dG standards and (b) human urine sample (male nonsmoker, age 56) containing the 8-OH-G marker; (c) HPLC-2 chromatogram of the 8-OH-dG fraction obtained by HPLC-1. Urinary 8-OH-dG concentration, 4.79 ng/ml. The huge peak around 13 min is the ribonucleoside 8-OH-G contaminating the 8-OH-dG fraction.

after waiting for a defined time (T1), the sampling injector started to monitor a defined level of the UV absorbance signal, which enabled the recognition of the 8-OH-G marker peak. After a defined time interval (T2), the 8-OH-dG-containing fraction began to be collected in a vial tube in the sampling injector, and after a defined interval (T3) the collection was terminated, the 8-

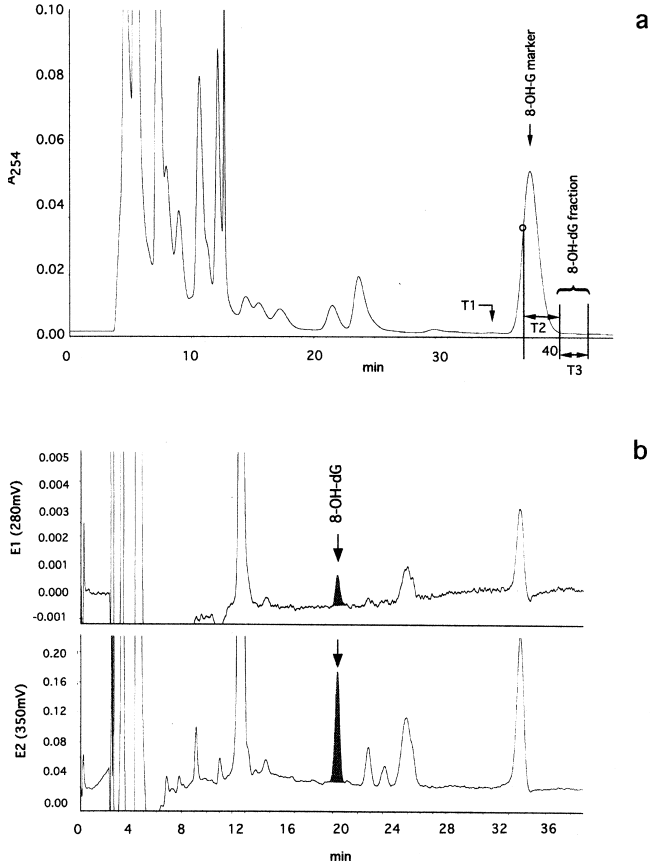

Fig. 3. (a) HPLC-1 and (b) HPLC-2 chromatograms of human urine sample (male smoker, age 43). Urinary 8-OH-dG concentration, 8.55 ng/ml.

OH-dG fraction (200–250 µl) in the vial tube was mixed completely, and 100 µl of it was injected into the HPLC-2 column.

RESULTS

In this new method, the urine was first fractionated on an anion exchange column (polystyrene-type resin with quaternary ammonium group, sulfate form). The 8-OH-dG molecule has an electrically negative character[20], and it may easily change to an anionic form[21] in the anion exchange column and strongly bind to it, though most of the other urinary components elute earlier, particularly at high temperature (65°C). For an accurate collection of the 8-OH-dG fraction, the ribonucleoside 8-OH-G, which elutes 4–5 min earlier than 8-OH-dG does, was added to the urine. To determine the waiting times, T1 and T2, the collection time T3, and the back-flash timing, as well as the 8-OH-G peak recognition level, the 8-OH-dG marker plus the ribonucleoside marker 8-OH-G were injected into HPLC-1 (Fig. 2a). When the urine sample with the 8-OH-G marker was analyzed under the same conditions, most of the urinary UV-absorbing components were eluted from the column during about 35 min (T1) of wait-

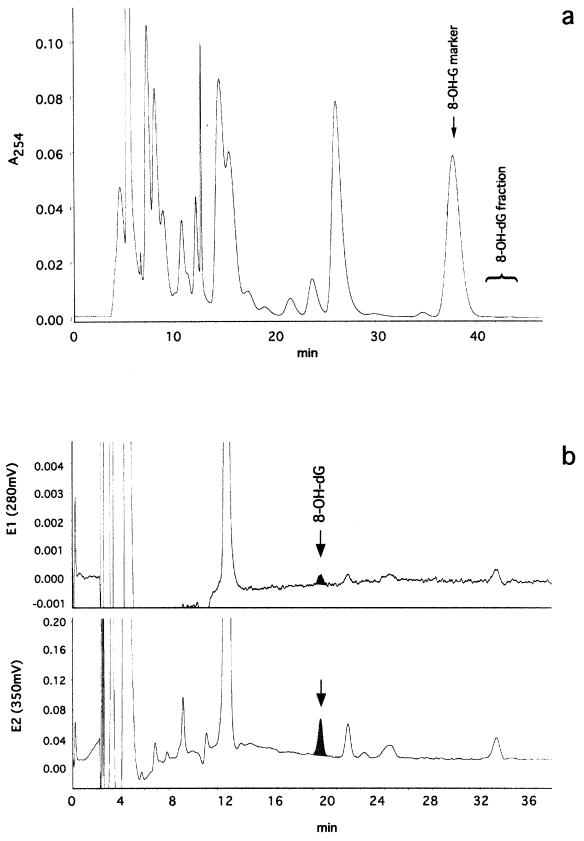

Fig. 4. (a) HPLC-1 and (b) HPLC-2 chromatograms of human urine sample (male nonsmoker, age 22). Urinary 8-OH-dG concentration, 3.07 ng/ml.

ing time (Fig. 2b). Then the 8-OH-G peak was recognized, and after the T2 waiting period, the 8-OH-dG fraction was collected for the T3 time and injected into HPLC-2. The examples of HPLC-1 and -2 profiles in the analyses of three urine samples are shown in Figs. 2b, 2c, 3a, 3b, 4a, and 4b. The 8-OH-dG eluted as a single peak in HPLC-2. Under monitoring with two ECD channels, E1 and E2, with different applied voltages, the 8-OH-dG appeared as a specific ratio of peak heights (see Figs. 2c, 3b, and 4b). Therefore it is easily distinguished from other compounds with the same retention time. The analysis of about 1,500 different human urine samples revealed that only a few showed a partial overlapping of a collateral peak with the 8-OH-dG peak.

DISCUSSION

The source of urinary 8-OH-dG may be the hydrolysis of 8-OH-dGTP by the nucleotide sanitization enzyme MTH1, the nucleotide excision repair of DNA, and the apoptosis of oxidatively damaged cells[22]. Although the mechanisms of 8-OH-dG release into urine are not clear, it could be a useful marker of oxidative stress in vivo. For example, after whole body irradiation of leukemia patients with a dose of 12 Gy, the 8-OH-dG level increased to 8–25 times over normal levels, though one patient showed no increase[23]. Urinary 8-OH-dG measurements will be useful for studying the effects of radiotherapy at the molecular level. Urinary 8-OH-dG may reflect the action of ionizing radiation on radiosensitive tissues. In regard to lifestyle, cigarette smoking increases urinary 8-OH-dG levels[10,13], and moderate exercise reduces its levels[13]. More than 100 papers have been published on urinary 8-OH-dG, but a standard methodology has not been established. The analyses are complicated and not always reproducible. Particularly with the previous HPLC-ECD methods, the determination of the 8-OH-dG collection timing during the first HPLC is difficult, because the elution position changes slightly day by day, as a result of minor changes in the solvent composition, the temperature, and the column- and pump- conditions. Even the urine pH and salt concentration affect the elution position. In the present method, I added the ribonucleoside 8-OH-G standard to each urine sample, and depending on its elution position, 8-OH-dG fraction was precisely collected in the loop for further analysis by HPLC-2. Therefore once the exact conditions are set up by the injection of 8-OH-G and 8-OH-dG (Fig. 2a), one can safely continue to analyze up to 1,000 samples during a few months. Furthermore, the addition of 8-OH-G to the urine may enhance the recovery of 8-OH-dG, particularly when new HPLC-1 and -2 columns are used, by inhibiting the irreversible adsorption of 8-OH-dG to the columns. I hope that this method will be used widely in the future for studies in radiotherapy, molecular epidemiology, risk assessment, and health promotion.

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