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Determination of the oxidative stress biomarker urinary 8-hydroxy-2′-deoxyguanosine by automated on-line in-tube solid-phase microextraction coupled with liquid chromatography–tandem mass spectrometry

Hiroyuki Kataoka*, Keisuke Mizuno, Eri Oda, Akihiro Saito

School of Pharmacy, Shujitsu University, Nishigawara, Okayama 703-8516, Japan

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ABSTRACT

A simple and sensitive method for the determination of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage in human urine, was developed using automated on-line in-tube solid-phase microextraction (SPME) coupled with stable isotope-dilution liquid chromatography–tandem mass spectrometry (LC–MS/MS). Creatinine was also analyzed simultaneously to normalize urine volume by the in-tube SPME LC–MS/MS method, and 8-OHdG and creatinine were separated within 3 min using a Zorbax Eclipse XDB-C8 column. Electrospray MS/MS for these compounds was performed on an API 4000 triple quadruple mass spectrometer in the positive ion mode by multiple reaction monitoring. The optimum in-tube SPME conditions were 20 draw/eject cycles of 40 μL of sample at a flow rate of 200 μL/min using a Carboxen 1006 PLOT capillary column as an extraction device. The extracted compounds were easily desorbed from the capillary by passage of the mobile phase, and no carryover was observed. The calibration curve for 8-OHdG using its stable isotope-labeled internal standard was linear in the range of 0.05–10 ng/mL, and the detection limit was 8.3 pg/mL. The intra-day and inter-day precision (relative standard deviations) were below 3.1% and 9.6% ($n = 5$), respectively. This method was applied successfully to the analysis of urine samples without any other pretreatment and interference peaks, with good recovery rates above 91% in spiked urine samples. The limits of quantification of 8-OHdG and creatinine in 0.1 mL urine samples were about 0.32 and 0.69 ng/mL (S/N = 10), respectively. This method was utilized to assess the effects of smoking, green tea drinking and alcohol drinking on the urinary excretion of 8-OHdG.

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1. Introduction

Oxidative stress due to an excess of reactive oxygen species (ROS) may play a role in the development and progression of many acute and chronic diseases [1,2]. ROS, such as superoxide radicals, singlet oxygen, hydrogen peroxide, and hydroxyl radicals, can be generated endogenously during physiological processes and exogenously during exposure to ultraviolet rays, radiation, air pollutants, cigarette smoking, and toxic chemicals, including asbestos fibers, heavy metals, and polycyclic aromatic hydrocarbons [3–5]. Free radicals and ROS attack guanine bases in nuclear and mitochondrial DNA, resulting in DNA mutations and cell death. Oxidative DNA damage is highly associated with the pathogenesis of various diseases, such as cancer [6,7], atherosclerosis [6], diabetes [6,8,9], gastrointestinal diseases [10] and Huntington's disease [11]. Therefore, early diagnosis of these diseases is crucial in maintaining human health and for successful therapy, indicating a need to reliably and quantitatively measure biomarkers of oxidative stress. 8-Hydroxy-2′-deoxyguanosine (8-OHdG), one of the major stable end products of non-enzymatic DNA oxidation, is regarded as an important biomarker of oxidative stress, and has been utilized not only as an indicator of endogenous oxidative DNA damage but as a risk factor for cancer and many degenerative diseases [3,4,6,7,12]. For example, elevated levels of urinary 8-OHdG have been detected in smokers [13–15], workers exposed to carcinogens [16–19], patients with various types of cancer [20], glaucoma [21], hypertension [22,23], diabetes [24,25], Alzheimer's disease [26], and major depression and myalgic encephalomyelitis/chronic fatigue syndrome [27]. However, measurements of 8-OHdG in bio-

* Corresponding author. Fax: +81 86 271 8342.
E-mail address: hkataoka@shujitsu.ac.jp (H. Kataoka).

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logical samples are challenging because of the trace amounts of 8-OHdG in complex matrices.

Urinary 8-OHdG is widely used as a biomarker of oxidative stress, because urine collection is easy and non-invasive. 8-OHdG concentrations in human urine samples are usually measured by enzyme-linked immunosorbent assay (ELISA) [28–31], capillary electrophoresis (CE) [32–35], high-performance liquid chromatography (HPLC) [36–38], or liquid chromatography–tandem mass spectrometry (LC–MS/MS) [29,30,39–44]. Commercially available ELISA kits are frequently used to quantify 8-OHdG because of their reduced instrumentation, lower costs and ease of use. However, these assays show variable performance, and their results correlate poorly with mass spectrometric techniques due to the cross-reactivity of 8-OHdG with urea [6,7,30,31]. CE and HPLC with UV or electrochemical detection (ECD) methods are not very sensitive (detection limit: 0.1–1.0 ng/mL), ECD has poor stability. In contrast, LC–MS/MS methods can sensitively and selectively measure 8-OHdG concentrations (detection limit: 7–160 pg/mL). Most of these assay methods, however, require time-consuming sample preparation procedures, such as chemical derivatization or solid-phase extraction (SPE) to remove coexisting substances in urine samples prior to analysis. Furthermore, urinary creatinine concentrations must be measured separately using colorimetric methods to normalize urine volume, because the urinary excretion rate of creatinine is relatively constant over time and the level of urinary 8-OHdG is expressed relative to the amount of creatinine. Therefore, simultaneous determination of urinary 8-OHdG and creatinine in single sample by LC–MS/MS can save time and trouble. Although simultaneous determination of 8-OHdG and creatinine has been reported [42], this method requires time-consuming sample preparation procedures to remove coexisting substances in urine samples prior to analysis.

In-tube solid-phase microextraction (SPME), using an open tubular fused-silica capillary with an inner surface coating as the SPME device, is simple and can be coupled easily online with HPLC and LC–MS. In-tube SPME allows convenient automation of the extraction process, which not only reduces analysis time, but provides greater precision and sensitivity than manual off-line techniques. We recently developed an in-tube SPME method, coupled with LC–MS and LC–MS/MS, to determine urinary concentrations of nicotine and cotinine [45], doping agents [46], and heterocyclic amines [47]. The details of the in-tube SPME technique and its applications have been summarized in several reviews [48–50]. Here we describe an automated on-line in-tube SPME LC–MS/MS method for simultaneous determination of 8-OHdG and creatinine in urine samples. Using this method, we assessed the effects of oxidative stress by smoking and antioxidant intake by green tea or alcohol drinking on the urinary excretion of 8-OHdG.

2. Experimental

2.1. Materials

8-OHdG and creatinine were purchased from Wako Pure Chemicals (Osaka, Japan), and their stable isotope-labeled compounds, 8-OHdG-$^{15}N_5$ (chemical purity 99.1%, isotopic enrichment 99.0%) and creatinine-d_3 (chemical purity 100%, isotopic enrichment 99.9%), used as internal standards (IS), were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA) and Toronto Research Chemicals Inc. (Toronto, Canada), respectively (Fig. S1). 8-OHdG, creatinine, 8-OHdG-$^{15}N_5$ and creatinine-d_3 were dissolved in methanol to concentrations of 1.0 mg/mL. These standard solutions were stored at 4 °C and diluted in pure water to the required concentrations prior to use. LC–MS grade acetonitrile and water used as mobile phases were purchased from Kanto Chemical (Tokyo, Japan). All other chemicals were of analytical grade.

2.2. Instruments and analytical conditions

The LC system was a Model 1100 series (Agilent Technologies, Böblingen Germany), consisting of a binary pump, an online-degasser, an autosampler, a column compartment, a diode array detector, and an HP ChemStation. A Zorbax Eclipse XDB-8 column (150 mm × 4.6 mm, particle size 5 μm; Agilent Technologies) was used for LC separation at a column temperature of 30 °C, with a mobile phase consisting of 5 mM ammonium formate/acetonitrile (90/10, v/v) at a flow rate of 1.0 mL/min.

Electrospray MS/MS for 8-OHdG, creatinine and their stable isotope-labeled compounds was performed on an API 4000 triple quadruple mass spectrometer (Applied Biosystems, Foster City, CA, USA), equipped with a turbo ion spray interface operated in positive ion mode at 5500 V and 600 °C. Nitrogen, which acted as a nebulizing and drying gas, was generated using a Kaken N_2 generator (System Instruments Co., Ltd., Tokyo, Japan). The ion sources for gases 1 (GS1) and 2 (GS2) flow rates were set at 40 and 60 L/min, respectively. The curtain gas (CUR) flow rate was set at 20 L/min and the collision gas (CAD) flow rate at 4.0 L/min. Other setting parameters, including dwell time, declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP), are shown in Table 1. Multiple reaction monitoring (MRM) was used for quantification of the protonated precursor molecular ions $[M+H]^+$ and the related product ions for each compound. Quadrupoles Q1 and Q3 were set at unit resolution (Table 1). LC–MS/MS data were processed using Analyst Software 1.3.1 (Applied Biosystems).

2.3. In-tube solid-phase microextraction

The in-tube SPME device consisted of a GC capillary column (60 cm × 0.32 mm i.d.; 48 μL internal volume) placed between the injection loop and the injection needle of the autosampler. The injection loop was retained in the system to avoid fouling of the metering pump. A 2.5-cm sleeve of 1/16-in. polyetheretherketone (PEEK) tubing, with an internal diameter of 330 μm, was placed at each end of the capillary. Standard 1/16-in. stainless steel nuts, ferrules, and connectors were used to complete the connections. The extraction efficiencies of non-coated fused silica, CP-Sil 5CB (100% polydimethylsiloxane, film thickness 5 μm), CP-Sil 19CB (14% cyanopropyl phenyl methylsilicone, film thickness 1.2 μm), CP-Wax 52CB (polyethylene glycol, film thickness 1.2 μm) (Varian Inc., Lake Forest, CA), Supel-Q PLOT (divinylbenzene polymer, film thickness 17 μm), and Carboxen 1006 PLOT (Carboxen molecular-sives, film thickness 17 μm) (Supelco, Bellefonte, PA, USA) were compared. The autosampler software was programmed to control the in-tube SPME extraction, desorption, and injection. For sampling and extraction, 2-mL screw-cap autosampler vials equipped with silicon/PTFE septa were each filled with 1 mL of sample for extraction and set onto the sample tray in the autosampler programmed to control the SPME extraction, desorption and injection steps. In addition, two 2-mL autosampler vials with septa, one containing 1.5 mL of methanol and the other containing 1.5 mL of water, were set into the autosampler. The capillary column was washed and conditioned by two repeated draw/eject cycles (40 μL each) of these solvents, and a 50-μL air plug was drawn prior to the extraction step. This air gap was necessary not only to avoid sample mixing but to desorb the analyte from the capillary coating by the mobile phase during the ejection step. 8-OHdG, creatinine and the IS compounds were extracted onto the capillary coating by 20 repeated draw/eject cycles of 40 μL of sample at a flow rate of 200 μL/min with the six-port valve in the LOAD position. After

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