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Analysis of urinary 8-isoprostane as an oxidative stress biomarker by stable isotope dilution using automated online in-tube solid-phase microextraction coupled with liquid chromatography–tandem mass spectrometry

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ABSTRACT

We have developed a simple and sensitive method for the determination of the oxidative stress biomarker 8-isoprostane (8-IP) in human urine by automated online in-tube solid-phase microextraction (SPME) coupled with liquid chromatography–tandem mass spectrometry (LC–MS/MS) using a Zorbax Eclipse XDB-8 column and 0.1% formic acid/methanol (25/75, v/v) as a mobile phase. Electrospray MS/MS for 8-IP was performed on an API 4000 triple quadruple mass spectrometer in negative ion mode. The optimum in-tube SPME conditions were 20 draw/eject cycles with a sample size of 40 μL using a Carboxen 1006 PLOT capillary column for the extraction. The extracted compounds were easily desorbed from the capillary by passage of the mobile phase, and no carryover was observed. Total analysis time of this method including online extraction and analysis was about 30 min for each sample. The in-tube SPME LC–MS/MS method showed good linearity in the concentration range of 20–1000 pg/mL with a correlation coefficient $r = 0.9999$ for 8-IP using a stable isotope-labeled internal standard, 8-IP-d_4. The detection limit of 8-IP was 3.3 pg/mL and the proposed method showed 42-fold higher sensitivity than the direct injection method. The intra-day and inter-day precisions (relative standard deviations) were below 5.0% and 8.5% ($n = 5$), respectively. This method was applied successfully to the analysis of urine samples without pretreatment or interference peaks. The recovery rates of 8-IP spiked into urine samples were above 92%. This method is useful for assessing the effects of oxidative stress and antioxidant intake.

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1. Introduction

Oxidative stress, an unfavorable balance between free radical generation and level of antioxidants, has been implicated as one of the major mechanisms underlying many acute and chronic diseases, including cancer, cardiovascular diseases, Alzheimer's disease, and inflammation [1–4]. Reactive oxygen species (ROS), such as superoxide radicals, singlet oxygen, hydrogen peroxide, and hydroxyl radicals, can be generated endogenously during physiological process and exogenously during exposure to ultraviolet rays, radiation, air pollutants, cigarette smoking, and toxic chemicals [5,6]. Free radicals and ROS can damage cellular membrane lipids, proteins, enzymes and DNA [5–7]. These oxidative changes are highly associated with the pathogenesis of cancers [1,3], fibrosis [8,9], affective diseases [10], atherosclerosis [11], essential hypertension [12], systemic lupus erythematosus [13], and diabetes [14]. 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.

Since free radicals and ROS have extremely short half-lives and are difficult to measure directly, attention has focused on biomolecules induced by oxidative stress, both to determine pathological mechanisms of associated diseases and for practical clinical applications as biomarkers [7,15]. The level or activity of primary enzymatic and non-enzymatic oxidant scavengers, as well as the levels of markers associated with the peroxidation of and damage to lipids, protein and DNA, peroxidation/damage markers, may indicate the intensity of oxidative stress. In addition, the activity of enzymes generating ROS and those related to ROS generation can be measured. For example, the mechanisms and dynamics of lipid peroxidation have been intensively analyzed, as have products of these reactions, their involvement in diseases, the effects of inhibition of lipid peroxidation, and the roles of lipid peroxidation in biological signaling [15]. 8-Isoprostane (8-*iso* prostaglandin $F_{2\alpha}$, 8-IP) (Fig. 1) is a stable product of oxidative stress and one of the most reliable biomarkers of lipid peroxidation in the human body [7,14,15].

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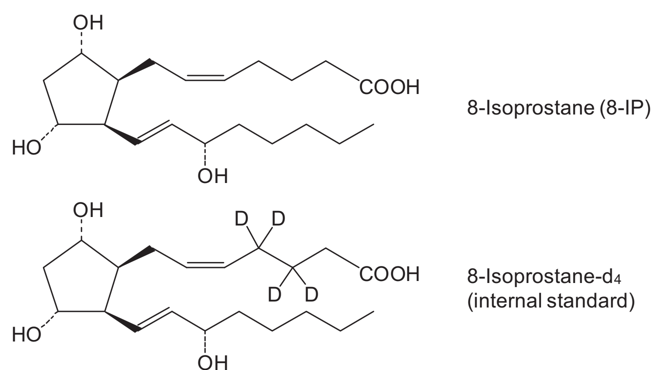

Fig. 1. Structures of 8-isoprostane and 8-isoprostane-d_4, used as an internal standard.

8-IP is a prostaglandin-like compound generated during the free radical-mediated, non-enzymatic peroxidation of arachidonic acid in phospholipids of cell membranes [14,16]. Moreover, the levels of 8-IP are elevated in many human diseases, including systemic lupus erythematosus [17], hepatic fibrosis [18], cancers [19,20], type 2 diabetes [14] and atherosclerosis [21], suggesting the involvement of 8-IP in these diseases. Furthermore, smoking, alcohol intake, exercise, and drug treatment have been reported to increased 8-IP levels [22,23], whereas supplementation with dietary antioxidants and intake of fruits and vegetables were reported to reduce 8-IP concentrations [24].

Although 8-IP can be measured in various biological fluids, including serum/plasma, urine, saliva, and exhaled breath condensate, as an indirect index of oxidative stress and antioxidant deficiency, most clinical studies assay 8-IP in urine, because urine collection is easy and non-invasive. In addition, the levels of 8-IP in urine samples are not influenced by lipid diets and have been shown to correlate with *in vivo* oxidative stress in several animal and human studies [14,17,18,25]. 8-IP concentrations in human urine samples are usually measured by immunoassay [19,25], enzyme-linked immunosorbent assay (ELISA) [22,26,27], gas chromatography–negative ion chemical ionization mass spectrometry (GC–NICI/MS) [28], and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [22,26,27,29–33]. Commercially available immunoassay and ELISA kits are frequently used to quantify 8-IP because of their benefits of reduced instrumentation, lower cost and ease of use. However, these assays show variable performance, and their results correlate poorly with mass spectrometric techniques due to their cross-reactivity with similar compounds. Although GC–NICI/MS methods are robust and sensitive, they require laborious derivatization steps, reducing sample throughput and lengthening turn-around times. In contrast, LC–MS/MS methods can sensitively and selectively measure 8-IP concentrations. However, most LC–MS/MS methods require time-consuming sample preparation procedures, such as liquid–liquid extraction or solid-phase extraction (SPE), 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 to 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 offline techniques. We recently developed an in-tube SPME method, coupled with LC–MS and LC–MS/MS methods, for determination of urinary nicotine and cotinine [34], doping agents [35], and heterocyclic amines [36]. The details of the in-tube SPME technique and its applications have been summarized in several reviews [37,38]. Here we describe an automated online in-tube SPME LC–MS/MS method for determination of 8-IP in urine samples. Using this method, we analyzed the effects of oxidative stress and antioxidant intake.

2. Experimental

2.1. Materials

8-IP and 8-IP-d_4, its stable isotope-labeled internal standard, were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). The structures of these compounds are shown in Fig. 1. Each compound was dissolved in methanol to make stock solutions of 5 μg/mL each, with each solution stored at 4 °C. Prior to use, stock solutions were diluted in pure water to the required concentrations. LC–MS grade methanol and distilled water used as mobile phases were purchased from Kanto Chemical (Tokyo, Japan). All other chemicals were of analytical grade.

2.2. Instrument 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 (250 mm × 2.0 mm, particle size 4 μm; Agilent Technologies), maintained at a temperature of 30 °C, was used for LC separation, with the mobile phase consisting of 0.1% formic acid/methanol (25/75, v/v) at a flow rate of 0.2 mL/min. Electrospray ionization (ESI) MS/MS for 8-IP and 8-IP-d_4 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 negative ion mode at −4500 V and 500 °C. A Kaken N_2 generator (System Instruments Co., Ltd., Tokyo, Japan) was used to generate nitrogen, which acted as a nebulizing and drying gas. The ion sources for gases 1 (GS1) and 2 (GS2) were set at 50 and 65 L/min, respectively. The curtain gas (CUR) flow was set at 10 L/min and the collision gas (CAD) flow at 4.0 L/min. Other set-up parameters included a declustering potential (DP) of −80 V; an entrance potential (EP) of −10 V; a collision energy (CE) of −35 eV; and a collision cell exit potential (CXP) of −15 V. Results were quantified by multiple reaction monitoring (MRM) of the deprotonated precursor molecular ions $[M-H]^-$ and the related product ions for each compound. Quadrupoles Q1 mass (precursor ion) and Q3 mass (product ion) were set at unit resolution. MRM in the negative ionization mode was performed using a dwell time of 500 ms per transition to detect ion pairs m/z 353.30 → 193.10 for 8-IP and m/z 357.30 → 197.10 for 8-IP-d_4. 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.) placed between the injection loop and the injection needle of the autosampler, with the injection loop retained in the system to avoid fouling the metering pump. Capillary connections were facilitated using a 2.5-cm sleeve of 1/16-in polyetheretherketone (PEEK) tubing at each end of the capillary (1 in ≈2.54 cm), with 330 μm i.d. PEEK tubing found to be suitable to accommodate the capillaries used. The connections were completed using normal 1/16-in stainless steel nuts, ferrules, and connectors. 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

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