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# Rapid and sensitive quantification of 8-isoprostaglandin $F_{2\alpha}$ in human plasma and urine by liquid chromatography–electrospray ionization mass spectrometry

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**Abstract**

The isoprostane, 8-isoprostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), is produced non-enzymatically by direct oxidation of arachidonic acid on the cell surface by oxygen radicals. We developed a new assay method for 8-iso-$PGF_{2\alpha}$ using $^{2}H_{4}$-8-iso-$PGF_{2\alpha}$ as the internal standard (I.S.) by high-performance liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS). For this assay, we established a very simple and rapid pretreatment method using a membrane filter-type solid-phase extraction column (Empore™ disk cartridge) for human urine extracts or intact plasma. LC–ESI-MS was performed in the selected ion monitoring (SIM) mode using target ions at $m/z$ 353.24 (8-iso-$PGF_{2\alpha}$) and $m/z$ 357.26 (I.S.) with a resolution of 1500. The imprecision for this method was below 13.7%. Mean inaccuracy was 8.7% for added levels of 8-iso-$PGF_{2\alpha}$ up to 5000 pg/ml of urine and 500 pg/ml of plasma. Determination of plasma and urinary 8-iso-$PGF_{2\alpha}$ concentrations in healthy subjects by the present method revealed that its urinary concentration in smokers tends to be higher than that in nonsmokers. 

*Keywords:* 8-Isoprostaglandin $F_{2\alpha}$

## 1. Introduction

Isoprostanes are formed from arachidonic acid in vivo not by involvement of oxidizing enzymes such as cyclooxygenase but by free radical-catalyzed peroxidation. Free $F_2$-isoprostanes are released from the esterified stores on the cell surface by the action of phospholipases. In theory, free $F_2$-isoprostanes can be grouped into four subtypes (regioisomers I, II, III and IV), with regioisomers I and IV being relatively stable [1]. A diastereoisomer of regioisomer IV, 8-iso-prostaglandin (PG) $F_{2\alpha}$, is recently attracting attention as a new oxidative stress marker in vivo, because its plasma level is related to the extent of cell injury or lipid peroxidation [2–4]. Urinary 8-iso-$PGF_{2\alpha}$ and other $F_2$-isoprostanes have been reported to increase with smoking and depend on pathological states including aging [5]. It has been reported that, in the urine of a child patient with a Zellweger syndrome, a β-oxidation deficiency in peroxisomes, 8-iso-$PGF_{2\alpha}$ was excreted in amounts 124-fold higher than that in healthy children [6]. Therefore, urinary or plasma 8-iso-$PGF_{2\alpha}$ is a new marker which reflects not only the oxidative stress in vivo but also the status of various diseases [7] and may be a useful tool for evaluating the pathological status.

As for quantification of 8-iso-$PGF_{2\alpha}$, GC–MS,

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EIA and RIA methods have been reported to date [3,5–9]. These methods, although relatively speedy in the final measurement step, require pretreatments with many laborious and time-consuming processes; i.e., after the concentration and extraction of samples on solid-phase extraction cartridges of $C_{18}$ or silica gel, 8-iso-$PGF_{2\alpha}$ has to be separated by TLC or HPLC and derivatized. Since arachidonyl-containing lipids in biological samples, especially in plasma and tissues, are very unstable and readily undergo autooxidation in vitro even in the presence of antioxidants, biological samples have to be carefully treated.

Recent progress in the field of mass spectrometry has resulted in development of new techniques such as electrospray ionization (ESI) and tandem mass spectrometry (MS–MS) which have markedly improved detection sensitivity for drugs, and quantification in the order of picograms is becoming possible. Therefore, LC–MS–MS has attracted attention as a highly sensitive and specific first-choice method for assaying clinical samples. Recently, Lawson et al. have reported $F_2$-isoprostane analysis using LC–MS–MS [10]. In the present study, we investigated a quantification method for urinary and plasma 8-iso-$PGF_{2\alpha}$ in order to simplify the sample pretreatment and improve the assay sensitivity. We established a highly sensitive and specific assay method for 8-iso-$PGF_{2\alpha}$, combining liquid–liquid extraction and solid-phase extraction for the sample pretreatment, and a quantification method using a sector-type LC–ESI-MS with a high-resolution mode, instead of using LC–MS–MS. Further, plasma and urinary 8-iso-$PGF_{2\alpha}$ concentrations in healthy subjects were determined by the present method.

## 2. Experimental

### 2.1. Materials and reagents

HPLC-grade acetonitrile and acetic acid were obtained from Nacalai Tesque (Kyoto, Japan). 8-Iso-$PGF_{2\alpha}$, [3,3,4,4-$^2H_4$]8-iso-$PGF_{2\alpha}$ for use as the internal standard (I.S.) (Fig. 1), 9β,11α-$PGF_2$, 9α,11β-$PGF_2$, $PGF_{2\alpha}$ and other PG standards were purchased from Cayman (Ann Arbor, MI, USA). 5-Hydroxytempo, butylated hydroxytoluene (BHT)

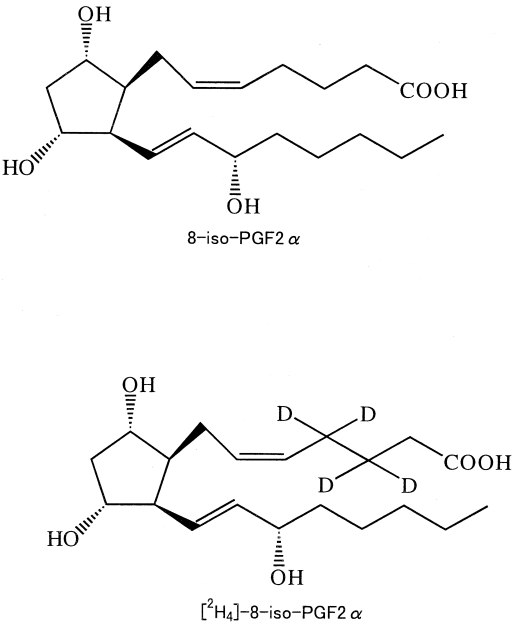


Fig. 1. Chemical structures of 8-iso-$PGF_{2\alpha}$ and I.S.

and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (St. Louis, MO, USA). Empore™ disk cartridges (3 *M*, USA) [11] were purchased from GL Science (Tokyo, Japan). Other regents and solvents used were of the commercial by available analytical grade.

### 2.2. Instrumentation and conditions

An HPLC model HP1100 system (Hewlett, Packard, USA) with a Symmetry $C_8$ column (3.9×150 mm, 5 μm; Waters, USA) was used. As the mobile phases, 0.1% $CH_3COOH$ (pH 3) (A) and acetonitrile (B) were used and isocratic elution was run with an A/B ratio of 7/3, at a flow-rate of 0.35 ml/min. 4-Sector type MStation 700 tandem mass spectrometer (Jeol, Japan) equipped with an electrospray ionization source was used in negative ion (NI) selected ion monitoring (SIM) mode. In ESI-MS, the temperatures of the desolvating plate and orifice 1 were set at 80 and 200°C, respectively. The voltages of the ring lens and orifice 1 were set at 90 and 50 V, respectively. LC–ESI-MS was carried out using nitrogen to assist nebulization. Quasi-molecular ions (deprotonated ions), *m*/*z* 353.24 and *m*/*z* 357.26 for

8-iso-$PFG_{2\alpha}$, and I.S., respectively, were monitored for 500 ms each in the SIM mode at a mass spectral resolution of 1500. The mass calibration was carried out using the cluster ions of trifluoroacetic acid at $m/z$ 248.9599 and 384.9348.

### 2.3. Sample preparation

Aliquots of urine 1 ml and plasma 1 ml spiked with 5 and 1 ng, respectively, of I.S. were acidified to pH 3 with 1 *M* HCl. The urine solution was extracted with ethyl acetate 3 ml and the resulting organic layer was evaporated under a stream of nitrogen. The residue was dissolved in 5% (v/v) ethanol (pH 3) 2 ml. For solid-phase extraction, the solution of urine extracts or plasma sample was applied to an Empore disk cartridge pre-conditioned with methanol 0.5 ml and 1 m*M* HCl 0.5 ml. The cartridge was washed with 1 m*M* HCl 0.5 ml then heptane 0.5 ml. 8-Iso-$PGF_{2\alpha}$ and I.S. were eluted with ethyl acetate containing 1% (v/v) methanol 1 ml, and the eluate was evaporated under a stream of nitrogen. The residue was dissolved in the mobile phase for HPLC 0.3 ml, then the solution was filtered with a membrane filter (0.45 μm), and 0.1 ml of the filtrate was injected into the LC–ESI-MS system.

### 2.4. Preparation of calibration curves and determination of added 8-iso-$PGF_{2\alpha}$

Human urine 1 ml and plasma lml samples for three male subjects spiked with 8-iso-$PGF_{2\alpha}$ (urine, 100, 200, 500, 1000, 2000, 5000 pg; plasma, 20, 50, 100, 500 pg) and I.S. (urine, 5 ng; plasma, 1 ng) were treated and analyzed by the procedure described above to obtain the calibration curves. The calibration curves were drawn by plotting the peak area ratio (8-iso-$PGF_{2\alpha}$ to I.S.) versus the concentration of 8-iso-$PGF_{2\alpha}$. Determination of the intra- and inter-day assay reproducibility was carried out with three and six injections, respectively.

### 2.5. Stability

The stability in biological samples was assessed by identical analysis of the pooled urine and the pooled plasma from six and four donors, respectively. Concentrations of 8-iso-$PGF_{2\alpha}$ in these samples were determined at the end of three freeze–thaw cycles between −80°C and room temperature, and after storage for 3 or 6 months at −80°C and for 1 (plasma) or 3 (urine) months at −20°C.

### 2.6. Human study

A spot urine sample was collected in a spitz-tube containing 1 m*M* each of 5-hydroxytempo and EDTA (final concentration) from 19 healthy male subjects (age, 27–51 years; eight smokers and 11 nonsmokers) early in the morning. Seven ml each of blood was collected in an evacuated tube containing EDTA-2Na from 17 healthy male subjects (age, 27–47 years; seven smokers and 10 nonsmokers) early in the morning. The blood sample was immediately centrifuged (3500 $g\times5$ min) to give a plasma sample, which was transferred in a spitz-tube containing 0.05% BHT (final concentration). All samples were stored frozen at −80°C in a freezer until analysis. A portion of the urine sample was used for determination of the creatinine concentration at BML (Tokyo, Japan).

To investigate the inter-day variations of 8-iso-$PGF_{2\alpha}$, plasma and urine were collected three times at 2-week intervals from four healthy male subjects (age, 44–47 years; two smokers and two nonsmokers).

## 3. Results and discussion

### 3.1. Conditions for LC–ESI-MS

In the ESI mass spectra of 8-iso-$PGF_{2\alpha}$ and I.S. (Fig. 2), their deprotonated molecular ions $[M-H]^-$ were observed at $m/z$ 353 and $m/z$ 357, respectively, which were monitored in the SIM mode. Effects of the orifice lens voltage (0–150 V) and ring lens voltage (30–150 V) on the sensitivity of analysis in ESI were investigated. The intensities of deprotonated molecular ions were greatest at a ring lens voltage of 90 V and an orifice lens voltage of 50 V. Therefore, these conditions were used to carry out LC–ESI-MS in the SIM mode. The SIM chromatograms of the samples prepared with 8-iso-$PGF_{2\alpha}$, 9β,11α-$PGF_2$, 9α,11β-$PGF_2$, $PGF_{2\alpha}$ [A], I.S. [B],

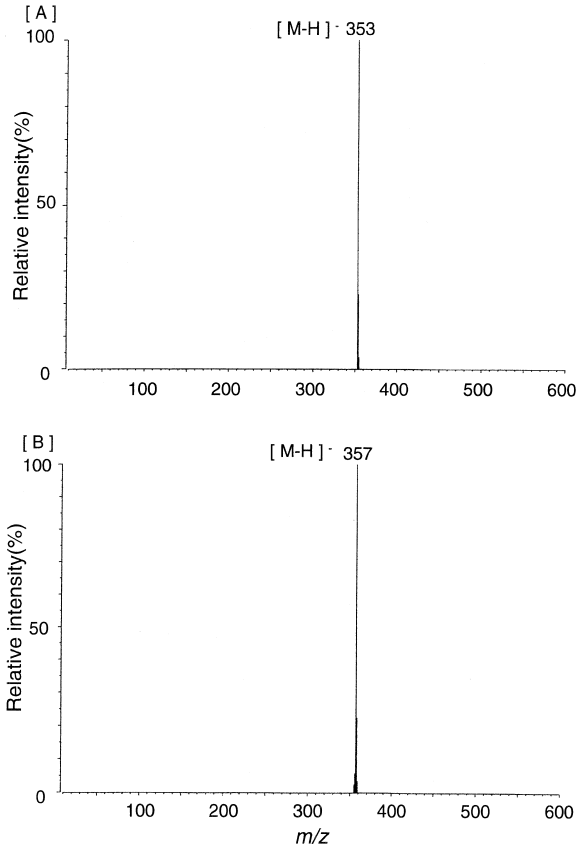


Fig. 2. ESI mass spectra of 8-iso-$PGF_{2\alpha}$ and I.S.

human plasma [C] and human urine [D] are shown in Fig. 3.

### 3.2. Calibration curves

The preparation of plasma and urine samples was very simple. Empore disk cartridges, membrane filter-type solid-phase extraction columns, were used for a small volume of urine extracts or intact plasma samples. This preparation method yielded an almost quantitative recovery (95%). The eluent volume used for this solid-phase extraction was so small that the solvent could be removed very rapidly in a few minutes.

Since 8-iso-$PGF_{2\alpha}$ is a diastereoisomer of regioisomer IV of $F_2$-isoprostanes, the other diastereoisomers available, 9β,11α-$PGF_2$, 9α,11β-$PGF_2$ and $PGF_{2\alpha}$ and other PG standards were used to investigate the separation conditions in HPLC. Under the described conditions, separation of these four diastereoisomers and I.S. on the mass chromatogram was satisfactory and no interference between the peaks was observed (Fig. 3). Retention times obtained from the analysis of 8-iso-$PGF_{2\alpha}$ and other PG standards available were as follows: $PGF_{2\alpha}$ for 35.35 min, 9β,11α-$PGF_2$ for 24.83 min, 9α,11β-$PGF_2$, for 27.75 min, 8-iso-$PGF_{2\alpha}$ for 26.12 min, 8-iso-13,14-dihydro-15-keto-$PGF_{2\alpha}$ for 37.55 min, 13,14-dihydro-15-keto-$PGF_{2\alpha}$ for 38.55 min, 15-R-$PGF_{2\alpha}$ for 34.53 min, *trans*-$PGF_{2\alpha}$ for 33.55 min.

Calibration curves for urinary and plasma 8-iso-$PGF_{2\alpha}$ were prepared using spiked samples for three male subjects (urine, 100–5000 pg/urine 1 ml of 8-iso-$PGF_{2\alpha}$ 5 ng/urine 1 ml of I.S.; plasma, 20–500 pg/plasma 1 ml of 8-iso-$PGF_{2\alpha}$ and 1 ng/plasma 1 ml of I.S.). Each calibration curve shows good linearity throughout the range with a regression coefficient of 0.999. Each calibration curve obtained from SIM analysis for the three subjects were as follows: plasma sample, $y = 0.053 + 1.001x$, $y = 0.040 + 1.003x$, $y = 0.038 + 1.001x$, for urine sample; $y = 0.787 + 1.005x$, $y = 0.778 + 0.997x$, $y = 0.756 + 1.008x$. Data for inaccuracy and imprecision (Tables 1 and 2) show that this assay method for plasma and urine sample is quite consistent and reliable with low relative errors (RE$<$8.7%) and relative standard deviations (RSD$<$13.7%). The lower limits of quantitation (LOQ) of plasma and urinary 8-iso-$PGF_{2\alpha}$, were 20 pg and 100 pg/ml, respectively. The concentrations of intrinsic 8-iso-$PGF_{2\alpha}$ (measured concentration minus added concentration) were almost the same: 52, 44 and 47 pg/ml plasma and 790, 770 and 760 pg/ml urine for three male subjects in this experiment. Therefore, the added amounts of 8-iso-$PGF_{2\alpha}$ were considered to be recovered quantitatively. Instrumental accuracy and precision of inter- and intra-day assays show that this assay method is quite consistent and reliable with low relative errors (RE$<$10.0%) and relative standard deviations (RSD$<$6.1%). The limits of detection (LOD) of the method were determined to be about 2 pg/ml of plasma 1 ml and urine 1 ml for I.S.

### 3.3. Stability

The stability of 8-iso-$PGF_{2\alpha}$ was studied with pooled plasma and urine samples from four and six

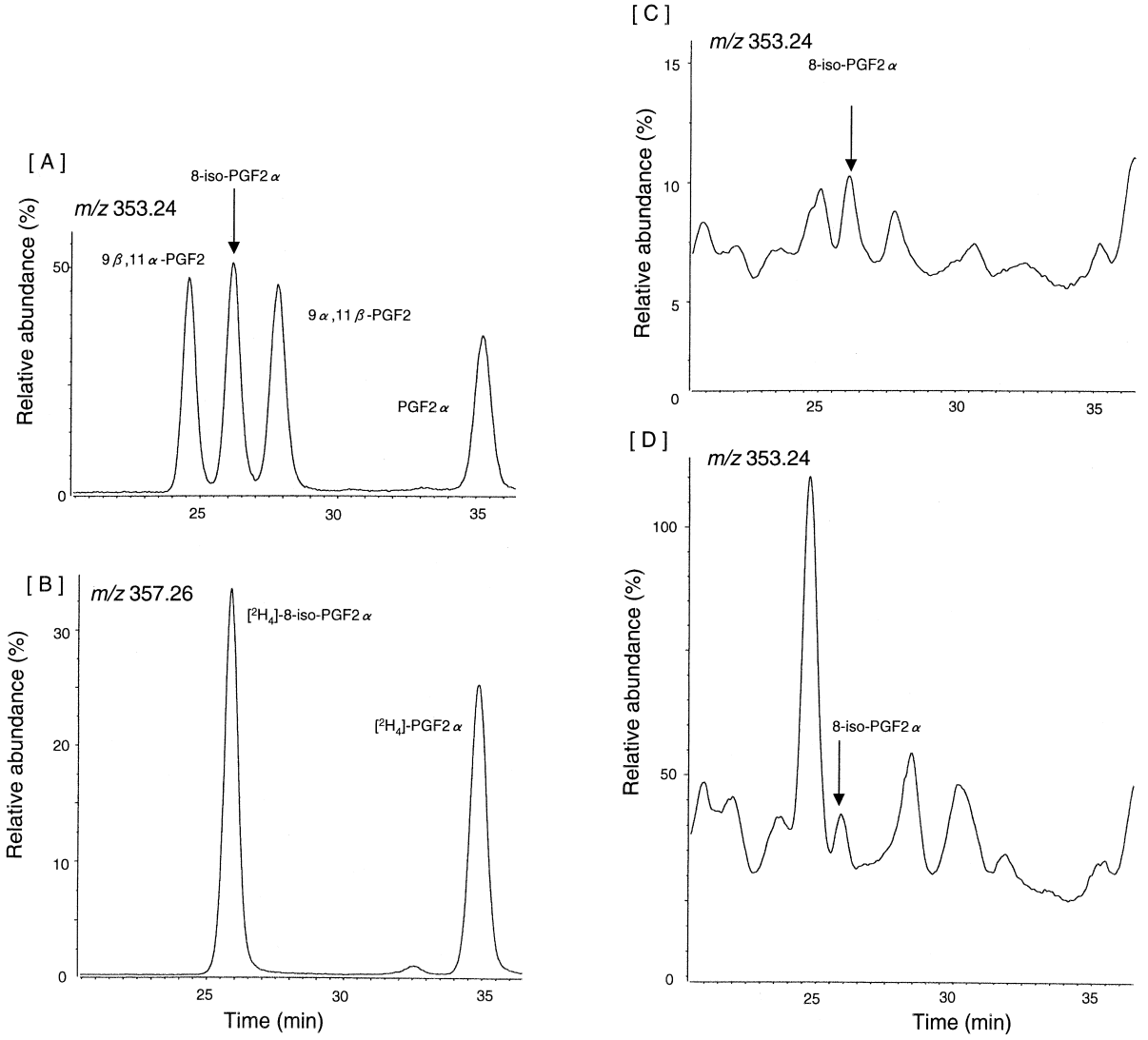


Fig. 3. ESI mass chromatograms of 8-iso-$PGF_{2\alpha}$, I.S. and three diastereoisomers.

Table 1
Imprecision and inaccuracy of SIM analysis of 8-iso-$PGF_{2\alpha}$ in human plasma

| 8-Iso-$PGF_{2\alpha}$ spiked (pg/ml) | 8-Iso-$PGF_{2\alpha}$ measured minus the basal level[a] (pg/ml) for subject: | | | Amount measured (mean±SD) (pg/ml) | Precision (RSD) (%) | Accuracy (RE) (%) |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | | | |
| 20 | 21 | 18 | 16 | 18±2.5 | 13.7 | −8.3 |
| 50 | 56 | 48 | 45 | 50±5.7 | 11.4 | −0.7 |
| 100 | 114 | 117 | 95 | 109±11.9 | 11.0 | 8.7 |
| 500 | 516 | 508 | 496 | 507±10.1 | 2.0 | 1.3 |

[a] Mean basal levels were 52, 44 and 47 pg/ml, respectively.

Table 2
Imprecision and inaccuracy of SIM analysis of 8-iso-$PGF_{2\alpha}$ in human urine

| 8-Iso-$PGF_{2\alpha}$ spiked (pg/ml) | 8-Iso-$PGF_{2\alpha}$ measured minus the basal level[a] (pg/ml) for subject: | | | Amount measured (mean±SD) (pg/ml) | Precision (RSD) (%) | Accuracy (RE) (%) |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | | | |
| 100 | 98 | 87 | 105 | 97±9.1 | 9.4 | −3.3 |
| 200 | 187 | 196 | 213 | 199±13.2 | 6.6 | −0.7 |
| 500 | 486 | 492 | 521 | 500±18.7 | 3.7 | −0.1 |
| 1000 | 1021 | 995 | 1025 | 1014±16.3 | 1.6 | 1.4 |
| 2000 | 2013 | 1986 | 2018 | 2006±17.2 | 0.9 | 0.3 |
| 5000 | 5014 | 5034 | 4998 | 5015±18.0 | 0.4 | 0.3 |

[a] Mean basal levels were 790, 770 and 760 pg/ml, respectively.

donors, respectively (Tables 3 and 4). In the pooled plasma (Table 3), the 8-iso-$PGF_{2\alpha}$ concentration at the beginning of the stability test was unchanged after 3 and 6 months of storage at −80°C. On the other hand, when the sample was stored at −20°C for 1 month, the initial 8-iso-$PGF_{2\alpha}$ increased somewhat (120.8%). Furthermore, an approximately 1.4-fold increase in the concentration (137.7%) was observed after three freeze–thaw cycles between −80°C and room temperature. These increases were considered to show further production of intrinsic 8-iso-$PGF_{2\alpha}$ in the plasma by possible autooxidation under these conditions.

Table 3
Stability of 8-iso-$PGF_{2\alpha}$ in human plasma

| Storage conditions | Recovery (mean±SD, $n=3$) (%) |
|---|---|
| *At −80°C:* | |
| 0 Day | 100.0±6.2 |
| 3 Months | 100.8±3.8 |
| 6 Months | 101.6±2.5 |
| Freeze–thaw (3×) | 137.7±11.3 |
| *At −20°C:* | |
| 1 Month | 120.8±9.5 |

Table 4
Stability of 8-iso-$PGF_{2\alpha}$ in human urine

| Storage conditions | Recovery (mean±SD, $n=3$) (%) |
|---|---|
| *At −80°C:* | |
| 0 Day | 100.0±0.9 |
| 3 Months | 100.2±1.0 |
| 6 Months | 99.5±1.2 |
| Freeze–thaw (3×) | 99.6±2.2 |
| *At −20°C:* | |
| 3 Months | 99.4±1.5 |

In the pooled urine (Table 4), the initial 8-iso-$PGF_{2\alpha}$ concentration was practically unchanged when the sample was stored 3 or 6 months at −80°C or 3 months at −20°C, or after three freeze–thaw cycles between −80°C and room temperature.

### *3.4. Concentrations in human urine and plasma*

The inter-day variations of free 8-iso-$PGF_{2\alpha}$ concentration in plasma and urine from four male subjects are shown Fig. 4. The variations (SEM) observed in urine were relatively large (41–137%), but those in the plasma were small (0.3–4%). A correlation was observed between the plasma and urinary concentration of 8-iso-$PGF_{2\alpha}$ ($r=0.674$) in the inter-day assay. The mean urinary concentration of free 8-iso-$PGF_{2\alpha}$ in smokers (665±137 pg/mg creatinine, $n=8$) tended to be higher than that in non-smokers (426±56 pg/mg creatinine, $n=11$), while those in plasma of smokers and nonsmokers were practically of the same levels, i.e., 46±2 and 42±2 pg/ml, respectively (Fig. 5). The difference in the urinary concentration of 8-iso-$PGF_{2\alpha}$ between smokers and nonsmokers was statistically insignificant assessed with the *t*-test (data not shown).

## 4. Conclusions

In the present study, we established an assay method for 8-iso-$PGF_{2\alpha}$, an oxidative stress marker, in human plasma and urine by LC–ESI-MS. In order

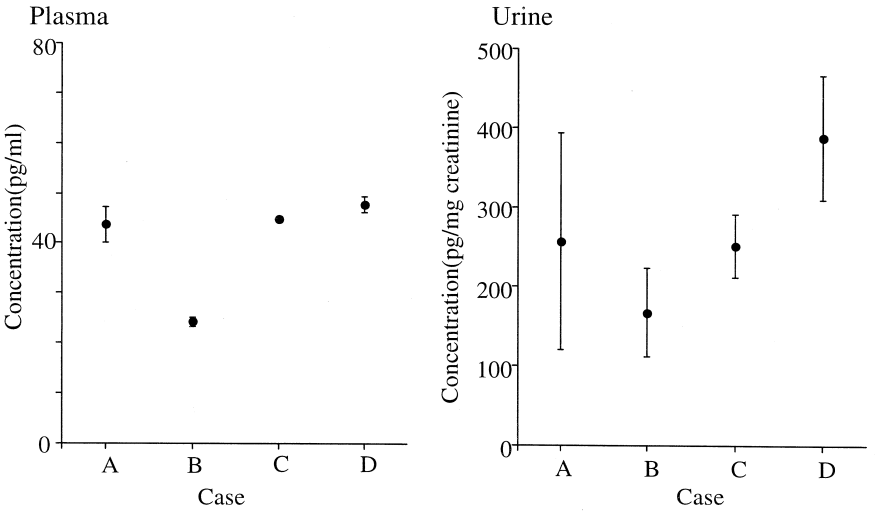


Fig. 4. Inter-day variations of 8-iso-$PGF_{2\alpha}$ in human plasma and urine (mean±SEM, $n=3$).

to circumvent various problems in the conventional assay methods such as laborious and time-consuming pretreatments in GC–MS and possible cross-reactions of the target compound with isomers or analogues in ETA, we used membrane filter-type Empore disk cartridges, which allowed reduction of the eluent volume to less than a few ml, thus reducing the time for solvent removal. Furthermore, LC–MS enables one to analyze the target compounds without derivatization. In the present assay method, a sector-type LC–MS system with high-resolution and high-performance was used, by which a very selective and accurate assay method could be established. Obata et al. have reported the concentrations of $F_2$-isoprostanes in human urine samples using GC–MS method [12]. The results from the investigation with our new LC–MS method were compared with those from the GC–MS method by Obata et al. [13]. It was confirmed that the concentrations obtained from these two experiments were substantially equal.

Plasma and urinary 8-iso-$PGF_{2\alpha}$ in healthy subjects were determined by the present method to assess the effects of smoking on these concentra-

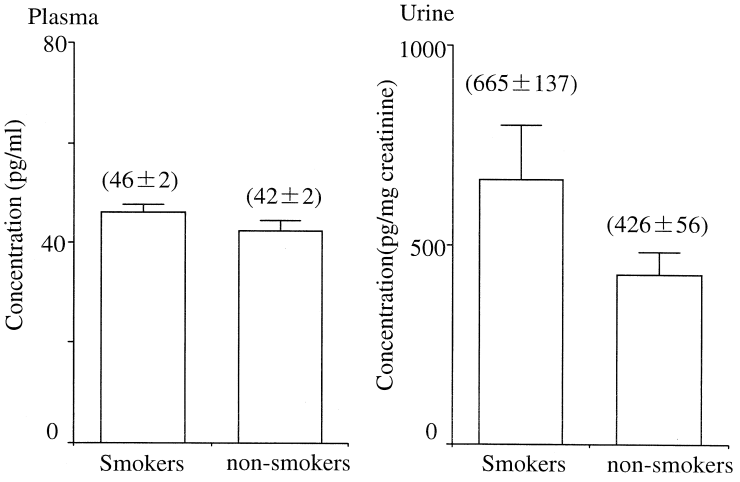


Fig. 5. Concentrations 8-iso-$PGF_{2\alpha}$ in plasma and urine samples from smokers and nonsmokers (mean±SEM).

tions. It was observed that the urinary 8-iso-$PGF_{2\alpha}$ concentration in smokers tended to be higher than that in nonsmokers. It has been reported by Reilly et al. [14] and Morrow et al. [15] that urinary 8-iso-$PGF_{2\alpha}$ levels in smokers were about two times higher than those in nonsmokers. However, in our study, the daily intake of cigarettes ranged from five to 40 in the smokers, and the urinary cotinine levels, which indicate the nicotine content in cigarettes, were not investigated. For these reasons, we think that, though the urinary 8-iso-$PGF_{2\alpha}$ levels in smokers tended to be higher than those in nonsmokers, no significant difference was observed in these levels between the two groups. Thus, the determination of human urinary and plasma 8-iso-$PGF_{2\alpha}$ concentrations may be a convenient diagnostic tool to be able to assess the extent of oxidative stress in vivo not only by smoking but also in other disease states.

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