

## QUANTIFICATION OF 8-ISO-PROSTAGLANDIN- $F_{2\alpha}$ AND 2,3-DINOR-8-ISO-PROSTAGLANDIN- $F_{2\alpha}$ IN HUMAN URINE USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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**Abstract**—Quantification of 8-iso-prostaglandin  $F_{2\alpha}$  (8-iso-PGF $_{2\alpha}$ ) has been suggested to be a reliable indicator of lipid peroxidation that may be related to in vivo free radical generation, oxidative damage, and antioxidant deficiency. We have developed a LC-MS/MS method to quantify 8-iso-PGF $_{2\alpha}$  and its dinor metabolite, 2,3-dinor-8-iso-prostaglandin  $F_{2\alpha}$  (2,3-dinor-8-iso-PGF $_{2\alpha}$ ), in human urine samples. After an initial purification step using an automated C18 solid phase extraction procedure, the urine sample was injected directly into a liquid chromatography (LC) system and detected with tandem mass spectrometry. The detection limit of the assay was 9 pg for 8-iso-PGF $_{2\alpha}$  and 3 pg for 2,3-dinor-8-iso-PGF $_{2\alpha}$  with both inter- and intraday variations of less than 12%. The inaccuracies were less than 3% for both analytes at three different levels. The urinary excretion rate of 2,3-dinor-8-iso-PGF $_{2\alpha}$  was higher than that of 8-iso-PGF $_{2\alpha}$ , and changed in proportion to the parent compound ( $R = 0.70$ ,  $n = 60$ ). Values obtained with this method showed good linear correlation to duplicate 8-iso-PGF $_{2\alpha}$  measurements performed with GCMS ( $R = 0.97$ ,  $n = 15$ ). The mean excretion rates of 8-iso-PGF $_{2\alpha}$  and 2,3-dinor-8-iso-PGF $_{2\alpha}$  were significantly higher in smokers than in nonsmokers ( $0.53 \pm 0.37$  vs.  $0.25 \pm 0.15$   $\mu\text{g/g}$  creatinine,  $p = 0.002$  for 8-iso-PGF $_{2\alpha}$  and  $8.9 \pm 3.8$  vs.  $4.6 \pm 2.6$   $\mu\text{g/g}$  creatinine,  $p = 0.003$  for 2,3-dinor-8-iso-PGF $_{2\alpha}$ , respectively). The excellent accuracy, reproducibility, and high throughput of this method should permit it to be used in large clinical studies and standard clinical laboratories. © 2003 Elsevier Science Inc.

**Keywords**—Free radicals, Quantification, 8-Iso-prostaglandin  $F_{2\alpha}$ , 2,3-dinor-8-iso-prostaglandin  $F_{2\alpha}$ , LC-MS/MS, Human urine, Oxidative stress

### INTRODUCTION

Oxidative stress is thought to play an important contributory role in the pathogenesis of numerous degenerative or chronic diseases, such as arteriosclerosis [1], cancer [2], tissue injury [3,4], and aging [5–10]. Considerable attention has focused on identifying suitable biomarkers to assess in vivo rates of oxidative damage. Candidate biomarkers can be classified into three major groups: markers of oxidative damage to lipids, proteins, and nucleic acids (DNA and RNA).

The compound, 8-iso-prostaglandin  $F_{2\alpha}$ , is one of a large number of prostanes produced predominantly by

free radical-catalyzed peroxidation of arachidonic acid [11]. Quantification of 8-iso-PGF $_{2\alpha}$  has been suggested to be a reliable indicator of lipid peroxidation that may be related to in vivo free radical generation, oxidative damage, and antioxidant deficiency [11–17]. Elevated levels of 8-iso-PGF $_{2\alpha}$  have been reported in animal models with free radical injury [18,19], in patients with cardiovascular disease [1], in Alzheimer's disease [20,21], in heavy smokers [22,23], and in type 2 diabetics [24,25].

Of the many different prostaglandin oxidation products that can be measured, free 8-iso-PGF $_{2\alpha}$  in urine is particularly useful because sampling is noninvasive, and sample preparation is simpler than for plasma or other lipid-containing tissues or fluids, where artificial formation of isoprostanes during sample processing may occur.

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The measurement of its major metabolites, such as 2,3-dinor-8-iso-PGF<sub>2α</sub> [26] and 2,3-dinor-5,6-dihydro-8-iso-PGF<sub>2α</sub> [27] in urine samples, has also been demonstrated to be a useful tool to evaluate 8-iso-PGF<sub>2α</sub> total body production *in vivo* more accurately. A stronger correlation was observed between 8-iso-PGF<sub>2α</sub> and 2,3-dinor-8-iso-PGF<sub>2α</sub>, than that of 2,3-dinor-5,6-dihydro-8-iso-PGF<sub>2α</sub> [26]. However, since urinary 2,3-dinor-8-iso-PGF<sub>2α</sub> was first described by Chiabrando *et al.* in 1999 [27], no further information has become available regarding its biological role, its relationship to levels of 8-iso-PGF<sub>2α</sub>, or its measurement in human or animal samples.

Various analytical methods have been used to measure 8-iso-PGF<sub>2α</sub>, including Enzyme Immunoassay (EIA) [24,28,29], radioimmunoassay (RIA) [30], gas-chromatography mass spectrometry (GCMS) [22,31–33], and liquid chromatography mass spectrometry (LC-MS) [34].

Analysis of 8-iso-PGF<sub>2α</sub> in urine samples by GCMS has been accepted by most researchers as the “gold standard technique.” However, extensive sample preparation procedures are required for this method, including C18 and silica solid phase extractions (SPE), thin-layer chromatography (TLC) purification, two derivatization steps, and several drying and reconstitution steps before samples can be injected onto the GCMS column. These complex and time-consuming sample preparation procedures make this technique less than ideal to handle large number of samples for determination of 8-iso-PGF<sub>2α</sub> in a clinical laboratory environment.

The EIA methods utilizing a 96 well plate, also called enzyme-linked immunosorbent assay (ELISA), have been developed and made commercially available by at least three different manufacturers with four different formats. Their performance compared to the standard GCMS method has been inconsistent among reported studies [24,28]. Based on experience in our laboratory, three of the commercially available kits require sample preparation steps similar to that of GCMS. We found that, without these sample purification steps, the ELISA assays could generate results, which appeared to be over 1000-fold higher than they should be. Moreover, the extensive sample preparation steps diminish the high throughput nature of ELISA methodology, so that they no longer offer any real advantage over the GCMS method. One of the commercially available ELISA kits was advertised as designed to measure urinary 8-iso-PGF<sub>2α</sub> without a need for sample preparation and purification. However, in our hands, values of replicate determinations frequently differed greatly and the average coefficient of variation was unacceptably high (>40%).

Liquid-chromatography tandem mass spectrometry (LC-MS/MS) following a C18 SPE preparation step has also been used to assess urinary F<sub>2</sub>-isoprostanes, as

described by Li and colleagues with promising results [34]. However, while repeating their procedure, we noticed that the sample throughput was limited not only by a long LC separation time, but also by repeated contamination of the curtain plate, which facilitates transfer of the sample from the atmospheric LC system to the ultra high vacuum chamber of the mass spectrometer. This phenomenon probably occurs because urine matrix could not be entirely removed during the C18 SPE purification step, and results in signal attenuation and reduced sensitivity. As a result, fewer than 20 samples could be consecutively processed before the curtain plate had to be cleaned to restore signal intensity.

We now report a new LC-MS/MS method, that is rapid, semiautomated, and provides accurate and precise measurements of 8-iso-PGF<sub>2α</sub> in human urine samples. Both 8-iso-PGF<sub>2α</sub> and its dinor metabolite, 2,3-dinor-8-iso-PGF<sub>2α</sub>, can be measured simultaneously. The sample throughput is increased significantly (over 100 samples can be processed daily) due to employment of rapid SPE and LC-MS/MS procedures, optimal removal of urine matrix, and minimum contamination of the curtain plate. Moreover, by reducing the extent of manual operations, this procedure also reduces the potential for human error.

## MATERIALS AND METHODS

### *Reagents and standards*

All organic solvents were HPLC grade and obtained from Fisher Scientific (Pittsburgh, PA, USA). 8-iso-PGF<sub>2α</sub>, 8-iso-15(R)-prostaglandin F<sub>2α</sub> (8-iso-15(R)-PGF<sub>2α</sub>), prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>), 15(R)-prostaglandin F<sub>2α</sub> (15(R)-PGF<sub>2α</sub>), 11β-prostaglandin F<sub>2α</sub> (9α,11β-PGF<sub>2α</sub>), 9β prostaglandin F<sub>2α</sub> (9β,11α-PGF<sub>2α</sub>), and isotope labeled 8-iso-prostaglandin F<sub>2α</sub>-D4 (8-iso-PGF<sub>2α</sub>-D4), and 2,3-dinor-8-iso-PGF<sub>2α</sub>, were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). All of the PGF<sub>2α</sub> isomers were dissolved or diluted into adequate volumes of ethanol to generate stock solutions, which were aliquoted into small vials and stored at −70°C.

### *Clinical sample collection*

Random urine samples from 41 subjects (26 males and 15 females) were collected. The mean age of the subjects was 50 years with a range of 32–80 years. Twenty-two of the subjects had never smoked or had quit smoking at least 15 years prior (11 men and 11 women), while the remaining subjects were active smokers (15 men and 4 women). The mean age of the smokers was slightly lower than nonsmokers (46 vs. 54 years old, *p* = 0.028). Random urine samples, collected from an additional group of 19 subjects, were included in an analysis

of correlating 8-iso-PGF $_{2\alpha}$  and 2,3-dinor-8-iso-PGF $_{2\alpha}$ . Smoking status and other health-related information were unavailable for these 19 subjects. Urine creatinine was measured using standard clinical methodology on a Synchron Clinical System LX20 (Beckman Coulter, Fullerton, CA, USA).

#### Sample preparation

All of the urine samples were aliquoted and stored at  $-70^{\circ}\text{C}$  until analysis. The freshly thawed urines were mixed for a few seconds on a vortex shaker and centrifuged at  $8000 \times g$  for 3 min to precipitate solids. One milliliter of urine supernatant was transferred into a glass tube, spiked with 10 ng of an internal standard (8-iso-PGF $_{2\alpha}$ -D4), and diluted with 1 ml of deionized (DI) water. After gently mixing, the treated urine was purified through a C18 solid phase extraction (SPE) cartridge on an automated SPE workstation (RapidTrace; Zymark, Hopkinton, MA, USA), as follows: A Varian's Bond Elut C18 cartridge (3CC/500 mg; Varian, Harbor City, CA, USA) was solvated with 5 ml of ethanol and equilibrated with 5 ml of DI water. The sample was loaded and washed sequentially with 5 ml of water, 5 ml of ethanol:water (5:95 v/v), and 1 ml of hexane. The column was then eluted with 2 ml of ethyl acetate. The sample eluent was evaporated to dryness under a stream of nitrogen gas and reconstituted in 50  $\mu\text{l}$  of acetonitrile:water (20:80 v/v) solution. We also tested a commercial 8-isoprostane affinity column (Cayman Chemical Company) for urine purification. After draining off the column buffer solution, 1 ml of urine sample, spiked with 10 ng of 8-iso-PGF $_{2\alpha}$ -D4, was loaded onto the affinity column. The column was washed with 10 ml of Eicosanoid Affinity Column Buffer and 10 ml DI water. The targeted analytes were eluted with 5 ml of Eicosanoid Affinity Column Elution Solution. The sample eluent was evaporated to dryness under a steam of nitrogen gas and reconstituted in 50  $\mu\text{l}$  of acetonitrile:water (20:80 v/v) solution.

#### LC-MS/MS analysis

The HPLC system consisted of two Shimadzu LC-10AD pumps, a Shimadzu degasser (Shimadzu Scientific Instruments, Columbia, MD, USA), and a Perkin Elmer autosampler (Perkin Elmer LLC, Norwalk, CT, USA). Ten microliters of the reconstituted urine sample were injected onto a YMC ODS-AQ column ( $2.0 \times 50$  mm, 3  $\mu\text{m}$  particle size; Waters, Milford, MA, USA) with an identical guard column ( $2.0 \times 10$  mm, 3  $\mu\text{m}$ ). The sample was delivered at a flow rate of 200  $\mu\text{l}/\text{min}$  (min). The mobile phases consisted of methanol:acetonitrile (5:95 v/v) (A) and 2 mM ammonium acetate (B). The HPLC separation was carried out with a solvent gradient

program of: 15% A at time 0, a linear increase to 70% A at 6 min, a linear increase to 100% A at 8 min, than a linear decrease from 100 to 15% A within 1 min. A switch valve was used to inject only the components eluted between 3 and 8 min into the mass spectrometer chamber. A 30 s equilibration time was used for each sample. The total HPLC running time was 9.5 min.

The HPLC system was directly interfaced with a triple stage quadrupole mass spectrometer (API2000; Applied Biosystem, Foster City, CA, USA) equipped with a TurboIonSpray ionization source. Negative electrospray was used as the means of ionization. Instrument control, data acquisition, and data analysis were carried out with Analyst software (Applied Biosystem). Nitrogen was used as the collision gas. The mass spectrometer was optimized in the multiple reaction-monitoring (MRM) mode by diffusing 1  $\mu\text{g}/\text{ml}$  of 8-iso-PGF $_{2\alpha}$ , 8-iso-PGF $_{2\alpha}$ -D4, or 2,3-dinor-8-iso-PGF $_{2\alpha}$  standard solutions. The ion pairs of  $m/z$  353/193,  $m/z$  357/197, and  $m/z$  325/237 were used to monitor 8-iso-PGF $_{2\alpha}$ , 8-iso-PGF $_{2\alpha}$ -D4, and 2,3-dinor-8-iso-PGF $_{2\alpha}$ , respectively. A seven point linear calibration curve was established using both internal and external standards over a range of 0–40 ng/ml. Ten nanograms of internal standard (8-iso-PGF $_{2\alpha}$ -d4) were added into each 1 ml of urine sample or 200  $\mu\text{l}$  of calibration standard. The seven level calibration standards were measured at the beginning and the end of the unknown samples. Two levels of urine based quality control samples, one within the "normal" range (Level 1) and the other one elevated by spiking with 8-iso-PGF $_{2\alpha}$  and 2,3-dinor-8-iso-PGF $_{2\alpha}$  standards (Level 2), were injected at the beginning, the end, and after every 10 samples to monitor the inter- and intraday accuracy and precision.

## RESULTS AND DISCUSSION

#### LC-MS/MS

Under negative electrospray ionization mode, the most abundant molecular ions were  $m/z$  353 for 8-iso-PGF $_{2\alpha}$ ,  $m/z$  357 for 8-iso-PGF $_{2\alpha}$ -D4, and  $m/z$  325 for 2,3-dinor-8-iso-PGF $_{2\alpha}$ . The negatively charged molecular ions undergo extensive collision-induced dissociation. The molecular structures and product ion spectra of all three targeted compounds are shown in Fig. 1. The fragmentation patterns were similar between 8-iso-PGF $_{2\alpha}$  and 8-iso-PGF $_{2\alpha}$ -d4, except that the daughter ions of 8-iso-PGF $_{2\alpha}$ -D4 were always four-units higher than those of 8-iso-PGF $_{2\alpha}$ . This indicates that the majority of the stable daughter ions were generated from chain A, which was labeled with four deuterium atoms. The most intensive daughter ions were  $m/z$  193 for 8-iso-PGF $_{2\alpha}$ ,

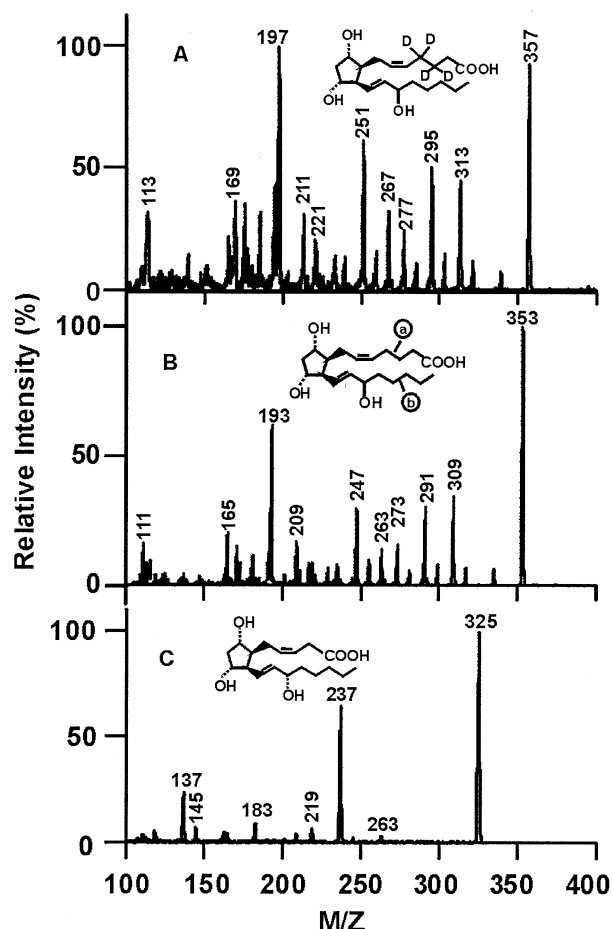


Fig. 1. Product ion spectra of 8-iso-PGF<sub>2α</sub>-d<sub>4</sub> (A), 8-iso-PGF<sub>2α</sub> (B), and 2,3-dinor-8-iso-PGF<sub>2α</sub> (C).

m/z 197 for 8-iso-PGF<sub>2α</sub>-D<sub>4</sub>, and m/z 236 for 2,3-dinor-8-iso-PGF<sub>2α</sub>.

The product ion spectra of the five 8-iso-PGF<sub>2α</sub> isomers: PGF<sub>2α</sub>, 15(R)-PGF<sub>2α</sub>, 15(R)-8-iso-PGF<sub>2α</sub>, 9α,11β-PGF<sub>2α</sub>, and 9β,11α-PGF<sub>2α</sub>, were also determined under the same condition as that of 8-iso-PGF<sub>2α</sub>. All of the PGF<sub>2α</sub> isomers showed the same fragmentation pattern with m/z 193 as the most abundant daughter ion, followed by the daughter ion of m/z 309. Therefore it is very important to develop a good LC program to separate 8-iso-PGF<sub>2α</sub> from all of its isomers, so that a reliable result can be achieved during quantification of 8-iso-PGF<sub>2α</sub> in urine samples.

Liquid chromatographic separation of 8-iso-PGF<sub>2α</sub>, 8-iso-15(R)-PGF<sub>2α</sub>, PGF<sub>2α</sub>, 15(R)-PGF<sub>2α</sub>, 9α,11β-PGF<sub>2α</sub>, and 9β,11α-PGF<sub>2α</sub>, was carried out after diluting each standard stock solution into 20% acetonitrile: water. Fig. 2 showed the LC-MS/MS chromatogram of a mixture of PGF<sub>2α</sub> isomers, containing 10 μg/l of each PGF<sub>2α</sub> like compounds. It can be seen that 8-iso-PGF<sub>2α</sub> was clearly separated from all other PGF<sub>2α</sub> isomers.

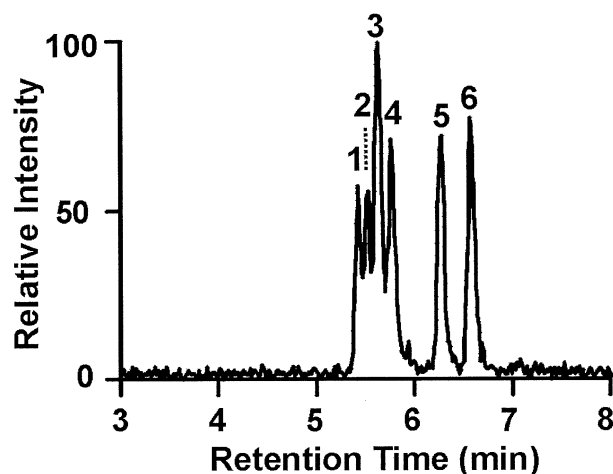


Fig. 2. The LCMSMS chromatograms of a standard solution, containing 10 ng/ml of each PGF<sub>2α</sub> isomers, detected under MRM detection mode for ion pairs of m/z 353/193. Peak (1): 8-iso-15(R)-PGF<sub>2α</sub>; (2): 9β,11α-PGF<sub>2α</sub>; (3) 8-iso-PGF<sub>2α</sub>; (4): 9α,11β-PGF<sub>2α</sub>; (5): 15(R)-PGF<sub>2α</sub>; and (6) PGF<sub>2α</sub>.

Under MRM detection mode, the typical LC-MS/MS chromatograms of a standard solution, containing 2 ng/ml of 8-iso-PGF<sub>2α</sub> and 2 ng/ml of 2,3-dinor-8-iso-PGF<sub>2α</sub>, and a urine sample are shown in Fig. 3. After spiking 10 ng of 8-iso-PGF<sub>2α</sub>-d<sub>4</sub> as internal standard, both the standard solution and urine sample underwent the same SPE purification and analysis with the LC-MS/MS protocols, as described above. From Fig. 3(I), one can see that in all three monitored channels, there were no additional peaks on the chromatograms of the standard solution. In urine samples, however, an unknown peak appeared on the 8-iso-PGF<sub>2α</sub>-D<sub>4</sub> chromatogram [Fig. 3(II) (A), peak (b)]. Due to unknown matrix effects, retention time shift had been observed in some urine samples. All of the analytes were retained in the LC column longer than they used to be. To eliminate any possible interferences or artifacts during identification and quantification of the internal standard, 8-iso-PGF<sub>2α</sub>-D<sub>4</sub>, a urine sample was extracted with and without adding 8-iso-PGF<sub>2α</sub>-D<sub>4</sub>. No signal was detected between 5.0 to 6.0 min for the sample processed without 8-iso-PGF<sub>2α</sub>-D<sub>4</sub>. However, peak (b) was consistently present with the same intensities. This indicates that there is no interference with the measurement of the internal standard, 8-iso-PGF<sub>2α</sub>-4.

Figure 3(II) (B) showed that neither 9α,11β-PGF<sub>2α</sub> nor 9β,11α-PGF<sub>2α</sub> have been detected from urine, and 8-iso-PGF<sub>2α</sub> was baseline separated from all other naturally existed PGF<sub>2α</sub> isomers. Although there are no interferences from the PGF<sub>2α</sub> like isomers to the quantification of 8-iso-PGF<sub>2α</sub>, the interferences from other types of naturally existing prostaglandins might exist. Therefore, a pooled urine extract was monitored under prod-

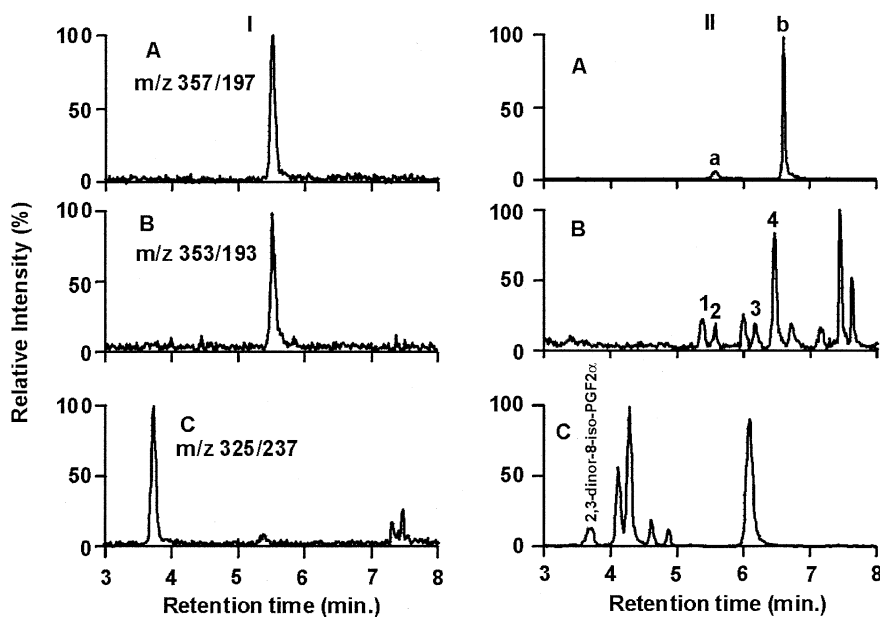


Fig. 3. The LCMSMS chromatograms of standard solution (I), containing 2 ng/ml of 8-iso-PGF<sub>2α</sub> & 2,3-dinor-8-iso-PGF<sub>2α</sub>, and urine sample (II) detected under MRM detection mode. The ion pairs of m/z 357/197, m/z 353/193, m/z 325/237 were used to monitor 8-iso-PGF<sub>2α</sub>-d4 (as internal standard) (A), 8-iso-PGF<sub>2α</sub> (B), and 2,3-dinor-8-iso-PGF<sub>2α</sub> (C), respectively. Peak (a): 8-iso-PGF<sub>2α</sub>-D4 ; (b): unknown component; peak (1): 8-iso-15(R)-PGF<sub>2α</sub>; (2): 8-iso-PGF<sub>2α</sub>; (3): 15(R)-PGF<sub>2α</sub>; and (4) PGF<sub>2α</sub>.

uct-ion scan monitoring (PIM) mode for ion m/z 353. A strong peak was observed at the retention time characteristic of 8-iso-PGF<sub>2α</sub>, which was not proportional to the actual amount of 8-iso-PGF<sub>2α</sub> in the sample. The product ion spectrum of this peak showed that the main daughter ion of the compound is m/z 273, and fragment ion of m/z 193 was not detectable. When 10 μl of 40 ng/ml 8-iso-PGF<sub>2α</sub> standard solution was injected into the LC system and monitored under the same PIM mode for the negative ion of m/z 353, only a single peak was observed on the chromatogram, with a product ion spectrum matching that obtained through direct diffusion of the 8-iso-PGF<sub>2α</sub> standard solution. However, the signal intensity was an order of magnitude smaller than that of urine samples, although the concentration of 8-iso-PGF<sub>2α</sub> in the urine extract was approximately five times less than that of the standard. This indicates that in the urine sample an unknown interference compound co-eluted with 8-iso-PGF<sub>2α</sub>, and the concentration of the co-eluent was much higher than that of 8-iso-PGF<sub>2α</sub>. Because this compound has the same m/z for the [M-H]<sup>-</sup> ion as that of 8-iso-PGF<sub>2α</sub>, it appears to be unfeasible to quantify 8-iso-PGF<sub>2α</sub> under single mass spectrometer detection mode by monitoring the molecular ion only.

To verify the possible interference of this co-eluent compound in the quantification of 8-iso-PGF<sub>2α</sub>, the 8-isoprostane affinity column (Cayman Chemical Company) was used to purify urine samples. After purification with the affinity column, the reconstituted urine

sample was injected into the same LC-MS/MS system, and analyzed with the same methods and instrumental settings as those of the previous tests. Compared to C18 SPE cartridge, the affinity column was more effective in purifying the urine samples. The chromatograms were very simple with only one peak appearing in each 8-iso-PGF<sub>2α</sub> and 8-iso-PGF<sub>2α</sub>-d4 detection channel. None of the PGF<sub>2α</sub> isomers can be detectable. And no signal could be detected in 2,3-dinor-8-iso-PGF<sub>2α</sub> monitoring channel. Under product-ion-scan monitoring mode for negative ion m/z 357 the peak was verified as 8-iso-PGF<sub>2α</sub>-d4. No signal could be detected under product-ion-scan monitoring mode for negative ion m/z 353 after HPLC separation, since the concentration of 8-iso-PGF<sub>2α</sub> was too small to be detected. The same urine sample was tested in triplicate after either C18-SPE cartridge or affinity column purification procedures, similar concentrations of 8-iso-PGF<sub>2α</sub> were measured after either purification method ( $0.39 \pm 0.03$  ng/ml with C18 SPE and  $0.36 \pm 0.03$  ng/ml with affinity column procedures). This finding indicated that the interference of the co-eluted compound was totally eliminated under MRM mode, so that the same quantitative results could be obtained with either C18 SPE or affinity column purification procedures. Because 2,3-dinor-8-iso-PGF<sub>2α</sub> was not recovered from the affinity column, the C18 SPE cartridge remained our first choice for simultaneous determination of both 8-iso-PGF<sub>2α</sub> and 2,3-dinor-8-iso-

Table 1. Percent Recovery of 8-iso-PGF<sub>2α</sub> and 2,3-dinor-8-iso-PGF<sub>2α</sub> from Urine Samples

	Urine Sample <sup>a</sup> (ng/ml)	Spiked Amount (ng/ml)	Urine + Spike <sup>a</sup> (ng/ml)	Recovery (%)
8-iso-PGF <sub>2α</sub>	0.34 ± 0.02	0.40	0.73 ± 0.04	98
	0.53 ± 0.02	1.0	1.51 ± 0.02	97
	0.24 ± 0.02	2.0	2.25 ± 0.06	104
2,3-dinor-9-iso-PGF <sub>2α</sub>	2.81 ± 0.31	3.0	5.75 ± 0.42	98
	6.98 ± 0.40	10.0	16.85 ± 1.05	99

<sup>a</sup> Results presented as mean ± SD, *n* = 6.

PGF<sub>2α</sub>. The latter purification approach was used in all subsequent studies.

The existence of 2,3-dinor-8-iso-PGF<sub>2α</sub> was confirmed by monitoring the same urine extract under the PIMS mode for negative ion *m/z* 326. Although the signal intensities were weak, the product ion spectra consistently matched that obtained from the 2,3-dinor-8-iso-PGF<sub>2α</sub> standard solution.

#### Method validation

Seven standard solutions, containing 0.2–10 ng/ml of each target analyte, were used to evaluate the method sensitivity. Detection limits of 9 pg for 8-iso-PGF<sub>2α</sub> and 3 pg for 2,3-dinor-8-iso-PGF<sub>2α</sub> (total injection mass) were determined at the signal-to-noise ratio (S/N) of three. The assay remained linear over a range of 10 pg to 1 ng of 8-iso-PGF<sub>2α</sub> or 2,3-dinor-8-iso-PGF<sub>2α</sub> injected onto the LC column. The accuracy of the procedure was demonstrated by spiking urine samples with three levels of 8-iso-PGF<sub>2α</sub> standard, 0.4, 1.0 and 2.0 ng/ml, and two levels of 2,3-dinor-8-iso-PGF<sub>2α</sub>, 3.0 and 10.0 ng/ml. Six independent extractions were performed on each spiked and unspiked urine sample on three different days. The results are provided in Table 1. Two pooled urine samples, Level 1 and Level 2, were used to evaluate inter- and intraassay precision and as quality control samples to monitor the day to day performance of the assay. Both intra- (*n* = 6) and inter- (*n* = 20 for over 3 months) assay variation were <12% for level 1 and <9% for level 2 (data not shown). More than 50 urine samples have been assayed continuously without significant contamination of the curtain plate. The variation of the signal intensities for the same standard, tested at the beginning and after 50 urine samples, was less than 5%. This finding suggests that the majority of the urine matrix is removed by the C18 SPE step and the column-switch technique. Thus, the combination of C18 SPE sample preparation and column switch technique reduced the contamination of the mass spectrometer interface, and makes this method more robust. A comparison study of our method with the standard GCMS method was performed on 15 urine samples kindly provided by Dr. L. J. Roberts (Vanderbilt University, Nashville, TN, USA), which had

been previously assayed in his laboratory [33]. The correlation of results obtained using these two methods was excellent (*R* = 0.97, *n* = 15, Fig. 4). However, a systematic negative bias of approximately 50% was observed for our results compared with those obtained with GCMS method. This difference is probably due to the fact that derivatization procedures used for the GCMS method combine three prostaglandin F<sub>2</sub> isomers, 8-iso-PGF<sub>2α</sub>, 9β, 11α-PGF<sub>2α</sub>, and 8-iso-15(R)-PGF<sub>2α</sub>, to form a single peak, as detected by GCMS. Therefore, the estimates obtained by GCMS represent the sum of all three isomers [35]. No derivatization procedure was involved in the LC-MS/MS procedure. Meanwhile, 8-iso-PGF<sub>2α</sub> was clearly separated from all above-noted isomers during LC procedure (Fig. 2). So that 8-iso-PGF<sub>2α</sub> is the only isomer quantified in this assay.

Both 8-iso-PGF<sub>2α</sub> and 2,3-dinor-8-iso-PGF<sub>2α</sub> were determined simultaneously on 60 human urine samples. In this group of samples, the mean excretion rate of 2,3-dinor-8-iso-PGF<sub>2α</sub> (6.2 ± 3.6 μg/g creatinine) was over an order of magnitude higher than that of 8-iso-PGF<sub>2α</sub> (0.29 ± 0.22 μg/g creatinine). Nonetheless, a significant correlation was observed between 8-iso-PGF<sub>2α</sub> and 2,3-dinor-8-iso-PGF<sub>2α</sub> (*R* = 0.70, *n* = 60) (Fig. 5). This observation suggests that 2,3-dinor- 8-iso-

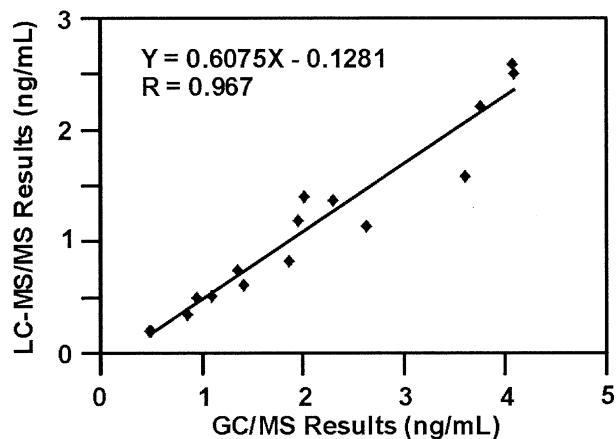


Fig. 4. Linear regression of the 8-iso-PGF<sub>2α</sub> values measured by the LC-MS/MS method and the standard GCMS method.

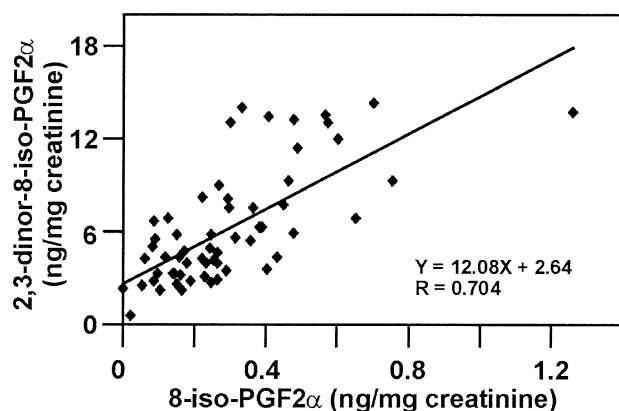


Fig. 5. Linear regression of 8-iso-PGF<sub>2α</sub> and 2,3-dinor-8-iso-PGF<sub>2α</sub>. Concentrations measured in the same urine samples.

PGF<sub>2α</sub> might be valuable as an additional marker to evaluate overall formation of 8-iso-PGF<sub>2</sub> and lipid peroxidation *in vivo*.

#### Clinical studies

We measured 8-iso-PGF<sub>2α</sub> and 2,3-dinor-8-iso-PGF<sub>2α</sub> in random urine samples from 19 smokers and 22 nonsmokers. The results are shown in Table 2. The mean excretion rate of 8-iso-PGF<sub>2α</sub> by smokers ( $0.53 \pm 0.37 \mu\text{g/g creatinine}$ ) was significantly ( $p = 0.002$ ) higher than that of nonsmokers ( $0.25 \pm 0.15 \mu\text{g/g creatinine}$ ). This result is consistent with previous publications showing that smoking is associated with higher rates of oxidative stress [22,23,35], although the elevated level we observed was somewhat less than those previously reported. Higher ( $p = 0.003$ ) 2,3-dinor-PGF<sub>2α</sub> levels were also detected in smoker ( $8.9 \pm 3.8 \mu\text{g/g creatinine}$ ) than in non-smokers ( $4.6 \pm 1.6 \mu\text{g/g creatinine}$ ). This pattern was present in both genders. Mean 8-iso-PGF<sub>2α</sub> levels were at least 2-fold higher in both male ( $0.16 \pm 0.07$  vs.  $0.50 \pm 0.37 \mu\text{g/g creatinine}$ ,  $p < .001$ ) and female smokers ( $0.33 \pm 0.16$  vs.  $0.62 \pm 0.43 \mu\text{g/g creatinine}$ ,  $p$

$= .049$ ). The levels of 2,3-dinor-8-iso-PGF<sub>2α</sub> were also higher in male smokers vs. nonsmokers. ( $7.6 \pm 3.2$  vs.  $3.7 \pm 1.0 \mu\text{g/g creatinine}$ ;  $p = .001$ ), and in female smokers vs. nonsmokers ( $13.7 \pm 0.4$  vs.  $5.5 \pm 1.6 \mu\text{g/g creatinine}$ ;  $p = .002$ ). The corresponding urine creatinine levels, however, did not differ significantly between genders ( $p = .485$ ) or smoking status ( $p = 0.145$ ). The slightly elevated creatinine concentration in smoker's urines could be due to the differences in the timing of sample collections. Samples from nonsmoking subjects were random urines, whereas most of the smoker's urine samples were first or second morning collections, which may have been more concentrated. However, after normalization of all values to creatinine excretion, neither 8-iso-PGF<sub>2α</sub> nor 2,3-dinor-8-iso-PGF<sub>2α</sub> concentrations were related to urinary creatinine levels (Fig. 6). Linear regression between creatinine and normalized 8-iso-PGF<sub>2α</sub> concentrations showed a line with a slope equal to  $-0.01$  and  $R = 0.024$ , indicating that the extent of urine concentration did not appear to influence the measurement of 8-iso-PGF<sub>2α</sub> and 2,3-dinor-8-iso-PGF<sub>2α</sub>.

These experiments demonstrate that both 8-iso-PGF<sub>2α</sub> and 2,3-dinor-8-iso-PGF<sub>2α</sub> can be measured reliably and conveniently in human urine samples using LC/MS-MS. Moreover, these studies suggest that increases of both urinary 8-iso-PGF<sub>2α</sub> and 2,3-dinor-8-iso-PGF<sub>2α</sub> levels can be detected in samples from active smokers. Our data also demonstrate that 2,3-dinor-8-iso-PGF<sub>2α</sub>, a degradation products of 8-iso-PGF<sub>2α</sub>, may be a valuable marker to assess the total endogenous formation of 8-iso-PGF<sub>2α</sub> *in vivo*. Further study is needed to confirm the above results and to further explore the clinical significance of 2,3-dinor-8-iso-PGF<sub>2α</sub> as an oxidative stress marker.

#### CONCLUSION

We have described the development of a new method for analysis of 8-iso-PGF<sub>2α</sub> and one of its major metabolites, 2,3-dinor-8-iso-PGF<sub>2α</sub> in human urine samples as

Table 2. Urinary 8-iso-PGF<sub>2α</sub> and 2,3-dinor-8-iso-PGF<sub>2α</sub> Levels in Smokers and Nonsmokers

	Gender	Nonsmoker <sup>a</sup>	Smoker <sup>b</sup>	<i>p</i> Value
8-iso-PGF <sub>2α</sub> <sup>c</sup> (ng/mg creatinine)	Men	$0.16 \pm 0.07$	$0.50 \pm 0.37$	<.001
	Women	$0.33 \pm 0.16$	$0.62 \pm 0.43$	.049
	Total	$0.25 \pm 0.15$	$0.53 \pm 0.37$	.002
2,3-dinor-8-iso-PGF <sub>2α</sub> <sup>c</sup> (ng/mg creatinine)	Men	$3.7 \pm 1.0$	$7.6 \pm 3.2$	.001
	Women	$5.5 \pm 1.6$	$13.7 \pm 0.4$	.002
	Total	$4.6 \pm 1.6$	$8.9 \pm 3.8$	.003
Creatinine <sup>c</sup> (mg/dl)	Men	$117 \pm 41$	$137 \pm 92$	.352
	Women	$100 \pm 55$	$161 \pm 55$	.362
	Total	$104 \pm 48$	$136 \pm 87$	.145

<sup>a</sup> In nonsmoker group, there were 11 men ( $60 \pm 10$  years) and 11 women ( $48 \pm 9$  years).

<sup>b</sup> In smoker group, there were 15 men ( $45 \pm 10$  years) and 4 women ( $50 \pm 10$  years).

<sup>c</sup> Results presented as mean  $\pm$  SD.

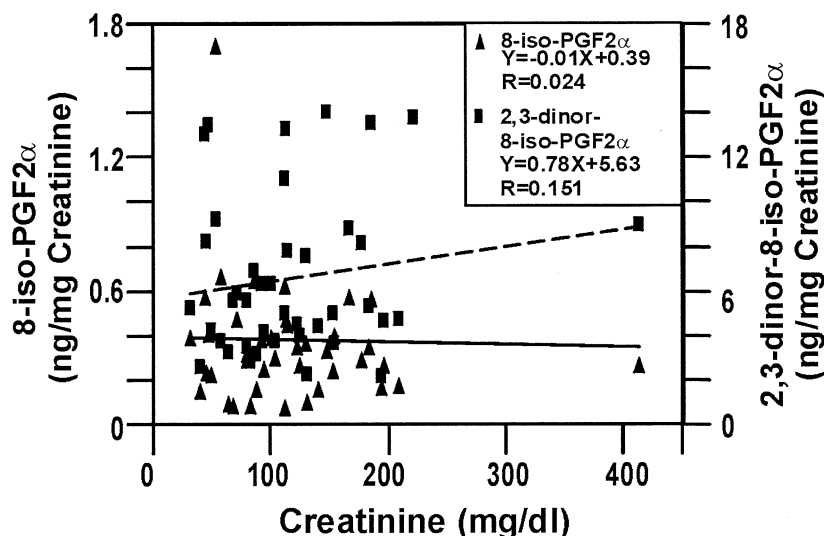


Fig. 6. Linear regression of 8-iso-PGF<sub>2α</sub> (▲) and 2,3-dinor-8-iso-PGF<sub>2α</sub> (■) vs. the corresponding urine creatinine concentrations.

indicators of oxidative stress. Both target compounds can be measured simultaneously with a 10 min assay. This study demonstrated that using the isotope labeled 8-iso-prostaglandin as an internal standard is crucial in this method to allow unequivocal identification of 8-iso-PGF<sub>2α</sub> and 2,3-dinor-8-iso-PGF<sub>2α</sub> peaks, despite the retention time shifts in some urine samples. We found no interference from any other naturally existing 8-iso-PGF<sub>2α</sub> isomers. An interfering compound co-eluting with 8-iso-PGF<sub>2α</sub> was detected, which could not be removed by C18 SPE purification procedures. However, the interference of this coeluted compound could be removed entirely either by setting the mass spectrometer to the multiple reaction monitoring mode, or by using an 8-isoprostane affinity column to purify urine samples. When 8-iso-PGF<sub>2α</sub> is the only analyte of interest, better purification performance could be achieved by using the 8-iso-PGF<sub>2α</sub> affinity columns, instead of C18 SPE cartridges. However, using affinity columns precludes the determination of the, 2,3-dinor-8-iso-PGF<sub>2α</sub>, metabolite and possibly other PGF<sub>2α</sub> isomers of potential interest. In addition, due to the column design constraints, the purification procedure with the affinity column is not easily automated, and the cost of each 8-iso-PGF<sub>2α</sub> affinity column is more than 20 times that of a C18 SPE cartridge. Although it has been claimed by the manufacturer that the affinity column can be regenerated at least five times, in our hands the 8-iso-PGF<sub>2α</sub> recovery rate dropped significantly after each regeneration process. In our opinion, the simultaneous determination of both 8-iso-PGF<sub>2α</sub> and its dinor metabolite, plus the low cost and automated cleanup procedures, make the C18 SPE

procedure the better choice for routine tests in a clinical laboratory environment.

We have demonstrated in this study that LC-MS/MS has potential for the routine analysis of urinary 8-iso-PGF<sub>2α</sub> and 2,3-dinor-8-iso-PGF<sub>2α</sub>, in a clinical laboratory environment. This method represents significant advancement in terms of rapidity and simplicity over methods previously reported for measurement of 8-iso-PGF<sub>2α</sub> and its dinor metabolite. The high sensitivity of this technique provides a reliable tool for the determination of the urinary excretion of 8-iso-PGF<sub>2α</sub> even at very low levels. The minimum amount of manual manipulation also reduces the likelihood of human error. The sample cleanup with three different solvent systems and the use of the column-switching technique allows substantial removal of urine matrices, minimizing the contamination and clogging of the mass spectrometer interface, thus greatly increasing sample throughput.

This method could potentially be further simplified by using a newer model of tandem mass spectrometry, which has been claimed by the manufacturer to be 60–100 times more sensitive than the one used in this study. Presumably, at this higher level of sensitivity, samples representing much smaller volumes of urine (and hence matrix) could be injected directly onto the LC column without any sample purification step. The targeted compounds could be selectively determined by the separation power of both HPLC and MS/MS.

We expect that the technique reported herein will make it easier to monitor the effects of antioxidants, drugs, or dietary manipulations on *in vivo* formation and metabolism of 8-iso-PGF<sub>2α</sub>. In view of its specificity,

this analytical procedure may also help better define the validity and limitations of 8-iso-PGF<sub>2α</sub> and its dinor metabolite, 2,3-dinor-8-iso-PGF<sub>2α</sub>, as markers of in vivo oxidative stress in experimental and clinical settings.

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## REFERENCES

- [1] Aviram, M. Review of human studies on oxidative damage and antioxidant protection related to cardiovascular diseases. *Free Radic. Res.* **33**:S85–S97; 2000.
- [2] Brown, N. S.; Bicknell, R. Hypoxia and oxidative stress in breast cancer. Oxidative stress: its effects on the growth, metastatic potential and response to therapy of breast cancer. *Breast Cancer Res.* **3**:323–327; 2001.
- [3] Touyz, R. M. Oxidative stress and vascular damage in hypertension. *Curr. Hypertens. Rep.* **2**:98–105; 2000.
- [4] Dogra, G.; Ward, N.; Croft, K. D.; Mori, T. A.; Barrett, P. H.; Herrmann, S. E.; Irish, A. B.; Watts, G. F. Oxidant stress in nephrotic syndrome: comparison of F(2)-isoprostanes and plasma antioxidant potential. *Nephrol. Dial. Transplant.* **16**:1626–1630; 2001.
- [5] Roberts, L. J. II; Reckelhoff, J. F. Measurement of F(2)-isoprostanes unveils profound oxidative stress in aged rats. *Biochem. Biophys. Res. Commun.* **287**:254–256; 2001.
- [6] Reckelhoff, J. F.; Kanji, V.; Racusen, L. C.; Schmidt, A. M.; Yan, S. D.; Morrow, J.; Roberts, L. J. II; Salahudeen, A. K. Vitamin E ameliorates enhanced renal lipid peroxidation and accumulation of F2-isoprostanes in aging kidneys. *Am. J. Physiol.* **274**:R767–R774; 1998.
- [7] Mecocci, P.; Fano, G.; Fulle, S.; MacGarvey, U.; Shinobu, L.; Polidori, M. C.; Cherubini, A.; Vecchiet, J.; Senin, U.; Beal, M. F. Age-dependent increases in oxidative damage to DNA, lipids, and proteins in human skeletal muscle. *Free Radic. Biol. Med.* **26**:303–308; 1999.
- [8] Leibovitz, B. E.; Siegel, B. V. Aspects of free radical reactions in biological systems: aging. *J. Gerontol.* **35**:45–56; 1980.
- [9] Levi, M.; Rowe, J. W. Renal function and dysfunction in aging. In: Seldin, D. W.; Giebisch, G., eds. *The kidney: physiology and pathophysiology*. New York: Raven Press; 1992:3433–3456.
- [10] Lindemann, R. D.; Goldman, R. Anatomic and physiologic age changes in the kidney. *Exp. Gerontol.* **21**:379–386; 1986.
- [11] Morrow, J. D.; Hill, K. E.; Burk, R. F.; Nammour, T. M.; Badr, K. F.; Roberts, L. J. II. A series of prostaglandin F<sub>2</sub>-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc. Natl. Acad. Sci. USA* **87**:9383–9387; 1990.
- [12] McCall, M. R.; Frei, B. Can antioxidant vitamins materially reduce oxidative damage in humans? *Free Radic. Biol. Med.* **26**:1034–1053; 1999.
- [13] Morrow, J. D.; Roberts, L. J. II. The isoprostanes. Current knowledge and directions for future research. *Biochem. Pharmacol.* **51**:1–9; 1996.
- [14] Patrono, C.; FitzGerald, G. A. Isoprostanes—potential markers of oxidant stress in atherothrombotic disease. *Arterioscler. Thromb. Vasc. Biol.* **17**:2309–2315; 1997.
- [15] Pratico, D. F2-Isoprostanes: sensitive and specific non-invasive indices of lipid peroxidation in vivo. *Atherosclerosis* **147**:1–10; 1999.
- [16] Roberts, L. J. II; Morrow, J. The generation and actions of isoprostanes. *Biochim. Biophys. Acta* **1345**:121–135; 1997.
- [17] Morrow, J. D. The isoprostanes: their quantification as an index of oxidant stress status in vivo. *Drug Metab. Rev.* **32**:377–385; 2000.
- [18] Morrow, J. D.; Awad, J. A.; Kato, T.; Takahashi, K.; Badr, K. F.; Roberts, L. J. II; Burk, R. F. Formation of novel non-cyclooxygenase-derived prostanoids (F2-isoprostanes) in carbon tetrachloride hepatotoxicity. An animal model of lipid peroxidation. *J. Clin. Invest.* **90**:2502–2507; 1992.
- [19] Laight, D. W.; Desai, K. M.; Gopaul, N. K.; Anggard, E. E.; Carrier, M. J. F2-isoprostane evidence of oxidant stress in the insulin resistant, obese Zucker rat: effects of vitamin. *Eur. J. Pharmacol.* **377**:89–92; 1999.
- [20] Pratico, D.; Lee, V.; Trojanowski, J.; Rokach, J.; FitzGerald, G. A. Increased F2-isoprostanes in Alzheimer's disease: evidence for enhanced lipid peroxidation in vivo. *FASEB J.* **12**:1777–1783; 1998.
- [21] Greco, A.; Minghetti, L.; Levi, G. Isoprostanes, novel markers of oxidative injury, help understanding the pathogenesis of neurodegenerative diseases. *Neurochem. Res.* **25**:1357–1364; 2000.
- [22] Bachi, A.; Zuccato, E.; Baraldi, M.; Fanelli, R.; Chiabrando, C. Measurement of urinary 8-Epi-prostaglandin F<sub>2α</sub>, a novel index of lipid peroxidation in vivo, by immunoaffinity extraction/gas chromatography-mass spectrometry. Basal levels in smokers and nonsmokers. *Free Radic. Biol. Med.* **20**:619–624; 1996.
- [23] Morrow, J. D.; Frei, B.; Longmire, A. W.; Gaziano, J. M.; Lynch, S. M.; Shyr, Y.; Strauss, W. E.; Oates, J. A.; Roberts, L. J. II. Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers. Smoking as a cause of oxidative damage. *N. Engl. J. Med.* **332**:1198–1203; 1995.
- [24] Devaraj, S.; Hirany, S. V.; Burk, R. F.; Jialal, I. Divergence between LDL oxidative susceptibility and urinary F(2)-isoprostanes as measures of oxidative stress in type 2 diabetes. *Clin. Chem.* **47**:1974–1979; 2001.
- [25] Mezzetti, A.; Cipollone, F.; Cucurullo, F. Oxidative stress and cardiovascular complications in diabetes: isoprostanes as new markers on an old paradigm. *Cardiovasc. Res.* **47**:475–488; 2000.
- [26] Roberts, L. J.; Moores, K. P.; Zacker, W. E.; Ostes, J. A.; Morrow, J. D. Identification of the major urinary metabolite of the F2-isoprostane 8-iso-prostaglandin-F<sub>2α</sub> in humans. *J. Biol. Chem.* **271**:20617–20620; 1996.
- [27] Chiabrando, C.; Valagussa, A.; Rivalta, C.; Durand, T.; Guy, A.; Zuccato, E.; Villa, P.; Rossi, J.-C.; Fanelli, R. Identification and measurement of endogenous b-oxidation metabolites of 8-iso-prostaglandin-F<sub>2α</sub>. *J. Biol. Chem.* **274**:1313–1319; 1999.
- [28] Bessard, J.; Cracowski, J. L.; Stanke-Labesque, F.; Bessard, G. Determination of isoprostaglandin F<sub>2α</sub> type III in human urine by gas chromatography-electronic impact mass spectrometry. Comparison with enzyme immunoassay. *J. Chromatogr. B Biomed. Sci.* **754**:333–343; 2001.
- [29] Wang, Z.; Ciabattini, G.; Creminon, C.; Lawson, J. A.; FitzGerald, G. A.; Patrono, C.; Maclouf, J. Immunological characterization of urinary 8-epi-prostaglandin F<sub>2α</sub> excretion in man. *J. Pharmacol. Exp. Ther.* **275**:94–100; 1995.
- [30] Helmersson, J.; Basu, S. F2-isoprostane excretion rate and diurnal variation in human urine. *Prostaglandins Leukot. Essent. Fatty Acids* **61**:203–205; 1999.
- [31] Rimbach, G.; Hohler, D.; Fischer, A.; Roy, S.; Virgili, F.; Pallauf, J.; Packer, L. Methods to assess free radicals and oxidative stress in biological systems. *Arch. Anim. Nutr.* **52**:203–222; 1999.
- [32] Pratico, D.; Barry, O. P.; Lawson, J. A.; Adiyaman, M.; Hwang, S. W.; Khanapure, S. P.; Iuliano, L.; Rokach, J.; FitzGerald, G. A. IPF2alpha-I: an index of lipid peroxidation in humans. *Proc. Natl. Acad. Sci. USA* **95**:3449–3454; 1998.
- [33] Morrow, J. D.; Roberts, L. J. II. Mass spectrometric quantification of F2-isoprostanes in biological fluids and tissues as measure of oxidant stress. *Methods Enzymol.* **300**:3–12; 1999.
- [34] Li, H.; Lawson, J. A.; Reilly, M.; Adiyaman, M.; Hwang, S. W.; Rokach, J.; FitzGerald, G. A. Quantitative high performance liquid chromatography/tandem mass spectrometric analysis of the four classes of F2-isoprostanes in human urine. *Proc. Natl. Acad. Sci. USA* **96**:13381–13386; 1999.
- [35] Mori, T. A.; Croft, K. D.; Puddey, I. B.; Beilin, L. J. An improved

method for the measurement of urinary and plasma F<sub>2</sub>-isoprostanes using gas chromatography-mass spectrometry. *Anal. Biochem.* **268**:117–125; 1999.

#### ABBREVIATIONS

DI—deionized

2,3-dinor-8-iso-PGF<sub>2α</sub>—2,3-dinor-8-iso-prostaglandin F<sub>2α</sub>

EIA—enzyme immunoassay

ELISA—enzyme-linked immunosorbent assay

GCMS—gas-chromatography mass spectrometry

HPLC—High performance liquid chromatography

8-iso-PGF<sub>2α</sub>—8-iso-prostaglandin F<sub>2α</sub>

8-iso-PGF<sub>2α</sub>-d<sub>4</sub>—isotope labeled 8-iso-prostaglandin F<sub>2α</sub>-d<sub>4</sub>

LC—liquid chromatography

LC-MS/MS—Liquid-chromatography tandem mass spectrometry

MRM—multiple reaction-monitoring

PIM—product ion-scan monitoring

RIA—radioimmunoassay

S/N—signal/noise ratio

SPE—solid phase extractions

TLC—thin-layer chromatography