Quantitation of isoprostane isomers in human urine from smokers and nonsmokers by LC-MS/MS[1]

Weiying Yan,* Gary D. Byrd,[2,†] and Michael W. Ogden[†]

Department of Physiology and Pharmacology,* Wake Forest University Medical Center, Winston-Salem, NC; and Human Studies Division,[†] R. J. Reynolds Tobacco Company, Winston-Salem, NC

Abstract A simple, rapid liquid chromatography-tandem mass spectrometry method was developed to identify and quantitate in human urine the isoprostanes iPF$_{2\alpha}$-III, 15-epi-iPF$_{2\alpha}$-III, iPF$_{2\alpha}$-VI, and 8,12-iso-iPF$_{2\alpha}$-VI along with the prostaglandin PGF$_{2\alpha}$ and 2,3-dinor-iPF$_{2\alpha}$-III, a metabolite of iPF$_{2\alpha}$-III. Assay specificity, linearity, precision, and accuracy met the required criteria for most analytes. The urine sample storage stability and standard solution stability were also tested. The methodology was applied to analyze 24 h urine samples collected from smokers and nonsmokers on controlled diets. The results for iPF$_{2\alpha}$-III obtained by our method were significantly correlated with results by an ELISA, although an ~2-fold high bias was observed for the ELISA data. For iPF$_{2\alpha}$-III and its metabolite 2,3-dinor-iPF$_{2\alpha}$-III, smokers had significantly higher concentrations than nonsmokers (513 ± 275 vs. 294 ± 104 pg/mg creatinine; 3,030 ± 1,546 vs. 2,046 ± 836 pg/mg creatinine, respectively). The concentration of iPF$_{2\alpha}$-VI tended to be higher in smokers than in nonsmokers; however, the increase was not statistically significant in this sample set. Concentrations of the other three isoprostane isomers showed no trends toward differences between smokers and nonsmokers. Among smokers, the daily output of two type VI isoprostanes showed a weak correlation with the amount of tobacco smoke exposure, as determined by urinary excretion of total nicotine equivalents.—Yan, W., G. D. Byrd, and M. W. Ogden. **Quantitation of isoprostane isomers in human urine from smokers and nonsmokers by LC-MS/MS.** *J. Lipid Res.* **2007.** 48: **1607–1617.**

Supplementary key words liquid chromatography-tandem mass spectrometry • isoprotanes • prostaglandins • quantitation • validation • oxidative stress • cigarette smoking

Isoprostanes are prostaglandin-like compounds produced in vivo by nonenzymatic free radical-induced peroxidation of arachidonic acid (1). Two nomenclatures for isoprostanes have been proposed, by Taber, Morrow, and Roberts (2) and Rokach et al. (3); the Rokach nomenclature was adopted here. Isoprostanes have a wide daily variation in secretion in humans. Both physiological factors, such as age, gender, ethnicity, and female hormones, and pathophysiological factors related to various diseases affect isoprostane levels. In addition, some exogenous factors, such as diet, smoking, alcohol, and exercise, can affect isoprostane levels (4). Quantitation of these compounds has been proposed as a reliable index of lipid peroxidation and oxidative stress in vivo (5). Increased levels of isoprostanes in human body fluid and tissues have been found in an increasing number of diseases, including vascular disease involving atherosclerosis, ischemia-reperfusion injury, and inflammation; Alzheimer's and other neurodegenerative diseases; diabetes; and pulmonary diseases (6, 7).

Recently, Dentchev et al. (8) showed that iPF$_{2\alpha}$-VI increases in a mouse model of retinal degeneration. Measurement of isoprostanes is important in delineating the role of lipid peroxidation in human pathophysiology (9, 10). Furthermore, some of the isoprostanes possess potent biological activity (11–14). An association between cigarette smoking and the risk of pulmonary and cardiovascular disease is well established (15); however, the underlying mechanism for this effect is not fully understood. One hypothesis is that this association is attributable to enhanced oxidation of tissues and/or circulating lipids. The gaseous phase of cigarette smoke has been reported to induce the oxidation of tissue and circulating lipids in vitro (16). Morrow et al. (17) reported that plasma levels of free and esterified F$_2$-isoprostanes were significantly higher in smokers than in nonsmokers and decreased significantly after 2 weeks of smoking abstinence. Significantly higher concentrations of iPF$_{2\alpha}$-III in urine (18, 19), exhaled breath condensate (20), plasma, serum, and leg lymph

Abbreviations: CID, collision-induced dissociation; ESI, electrospray ionization; FDA/CDER, Food and Drug Administration Center for Drug Evaluation and Research; HCl, hydrochloric acid; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LOQ, limit of quantitation; MRM, multiple reaction monitoring; NH_4OH, ammonium hydroxide; QC, quality control; RSD, relative standard deviation.

[1] Part of this work was presented at the 54th Annual Conference of the American Society for Mass Spectrometry (Seattle, WA, June, 2006).

[2] To whom correspondence should be addressed.
e-mail: byrdg@rjrt.com

Manuscript received 22 February 2007 and in revised form 30 March 2007.
Published, JLR Papers in Press, April 24, 2007.
DOI 10.1194/jlr.M700097-JLR200

(21) of smokers than nonsmokers has been reported. Maternal cigarette smoking increased F_2-isoprostanes in umbilical vessels (22). Vitamin C supplementation decreased $iPF_{2\alpha}$-III in plasma from smokers (23) and nonsmokers exposed to environmental tobacco smoke (24). Plasma, serum, and urinary levels of $iPF_{2\alpha}$-III decreased significantly after quitting cigarette smoking (25, 26) and increased after restarting smoking (27).

Reilly et al. (28) also reported that urinary $iPF_{2\alpha}$-III was increased in healthy cigarette smokers, but upon cessation, levels failed to decline to those observed in nonsmokers. They also found vitamin C alone or combined with vitamin E depressed the excretion of $iPF_{2\alpha}$-III in smokers' urine. A recent study investigating biomarkers of harm in smokers and nonsmokers found that nonsmokers had significantly reduced urinary $iPF_{2\alpha}$-III compared with smokers (29). On the other hand, some studies with small, targeted populations have revealed no significant differences in urinary $iPF_{2\alpha}$-III levels in smokers and nonsmokers (30–32). Overall, however, most studies show some relationship, with increased levels of isoprostanes associated with smoking. Recent deliberations by several work groups that focused on tobacco-related health issues considered isoprostanes promising in evaluating potential reduced-exposure tobacco products (33).

All of the research cited above is focused mainly on the extensively studied isomer $iPF_{2\alpha}$-III. Lately, a few studies have focused on other isoprostanes. One study reported $iPF_{2\alpha}$-III along with its metabolite, 2,3-dinor-8-iso-prostaglandin $F_{2\alpha}$ (2,3-dinor-$iPF_{2\alpha}$-III), to be significantly higher in smokers compared with nonsmokers (34). Another isomer, $iPF_{2\alpha}$-VI, was found to be increased in the urine of cigarette smokers along with $iPF_{2\alpha}$-III (35). Much work remains to evaluate the effect of smoking on the generation of individual isoprostane isomers.

Two primary analytical approaches have been adopted for isoprostane measurements: immunological methods and mass spectrometry. Immunological approaches include ELISA (36) and RIA (37, 38), which are relatively inexpensive and easy to perform. These methods are considered to give only a semiquantitative estimation of isoprostane levels, because cross-reactivity is significant (36, 39). GC-MS has a low limit of detection; however, it usually requires at least one extraction step (sorbents used include C18, silica, aminopropyl NH_2, or immunoaffinity) and one purification step (thin-layer chromatography or liquid chromatography). GC-MS also requires derivatization of the analytes, which complicates the analysis by adding more time and potential loss of target compounds (30, 40–52). As such, it is not considered a high-throughput approach.

Liquid chromatography-mass spectrometry (LC-MS) is an attractive alternative to GC-MS, because it has comparable sensitivity and derivatization is not necessary. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI) has been used successfully to separate four classes of F_2-isoprostanes and to quantitate or semiquantitate eight isomers (53). Taylor et al. (54) reported a validated method to separate and determine several type III isoprostanes and arachidonic acid in plasma by LC-MS/MS. Liang et al. (34) quantified $iPF_{2\alpha}$-III and 2,3-dinor-$iPF_{2\alpha}$-III with a total HPLC run time of 9.5 min. The method showed potential for the routine determination of urinary $iPF_{2\alpha}$-III and 2,3-dinor-$iPF_{2\alpha}$-III in clinical samples. Several applications of $iPF_{2\alpha}$-III quantitation by LC-MS were reported to evaluate the role of oxidative stress in different diseases (31, 32, 55–57). Recently, F_2-isoprostane regioisomers and diastereomers formed in vitro were separated and identified by LC-MS (58).

Although isoprostanes occur in various biological fluids, a urine assay is attractive because it is noninvasive, minimizes subject discomfort, requires little expertise to collect the specimen, and thus may be more suitable for large clinical trials. Plus, it circumvents the problem of artifactual generation of isoprostanes compared with plasma or other lipid-containing tissue or fluid (59, 60).

The goal of our work was to develop a simple, accurate, and fast method for the determination of isoprostanes in human urine by LC-MS/MS to permit high-throughput sample analysis. The isomers selected for study were based on commercially available standards and include $iPF_{2\alpha}$-III, 8,12-iso-$iPF_{2\alpha}$-VI, 15-epi-$iPF_{2\alpha}$-III, $PGF_{2\alpha}$, $iPF_{2\alpha}$-VI, and 2,3-dinor-$iPF_{2\alpha}$-III, a major metabolite of $iPF_{2\alpha}$-III (structures shown in **Fig. 1**). These compounds have been identified in human urine, and some have been characterized based on their biological activity (11, 61). The prostaglandin $PGF_{2\alpha}$ is an isomer of $iPF_{2\alpha}$-III and was included in this assay even though it is not an isoprostane. The method described here was validated according to Food and Drug Administration Center for Drug Evaluation and Research (FDA/CDER) guidelines (62). The method was applied in a study of 24 h urine samples from smokers and nonsmokers on controlled diets. Results from smokers and nonsmokers were compared along with the degree of smoking as determined by total nicotine uptake.

MATERIALS AND METHODS

Materials

$iPF_{2\alpha}$-III, 15-epi-$iPF_{2\alpha}$-III, $iPF_{2\alpha}$-VI, 8,12-iso-$iPF_{2\alpha}$-VI, $PGF_{2\alpha}$, 2,3-dinor-$iPF_{2\alpha}$-III, and $iPF_{2\alpha}$-III-d_4 were obtained from Cayman Chemicals (Ann Arbor, MI). Urea, potassium chloride, hippuric acid, ammonium chloride, anhydrous magnesium sulfate, anhydrous calcium chloride, oxalic acid, anhydrous sodium silicate, creatinine, anhydrous sodium sulfate, and pepsin were from Sigma-Aldrich (St. Louis, MO). Citric acid, anhydrous sodium dihydrogen phosphate, and glucose were from Aldrich (Milwaukee, WI). Lactic acid was obtained from Fluka (Lausanne, Switzerland). High-purity water from a Milli-Q A10 Synthesis purification system (Millipore, Bedford, MA) was used for all solutions. High-purity acetonitrile, methanol, and hexane were obtained from Burdick and Jackson (Muskegon, MI). Ammonium hydroxide (NH_4OH) and sodium chloride were obtained from Acros Organics (Fair Lawn, NJ), and hydrochloric acid (HCl) was obtained from Fisher Scientific (Fair Lawn, NJ). Urine samples from 24 h collections were portioned into 50 ml plastic (modified polystyrene) centrifuge tubes (Corning, Corning, NY) and stored at −80°C until analysis. After thawing, 2 ml was divided into aliquots on deep 96-well plates (Phenomenex, Torrance, CA). Centrifugation of samples was done on an IEC Centra-GP8R

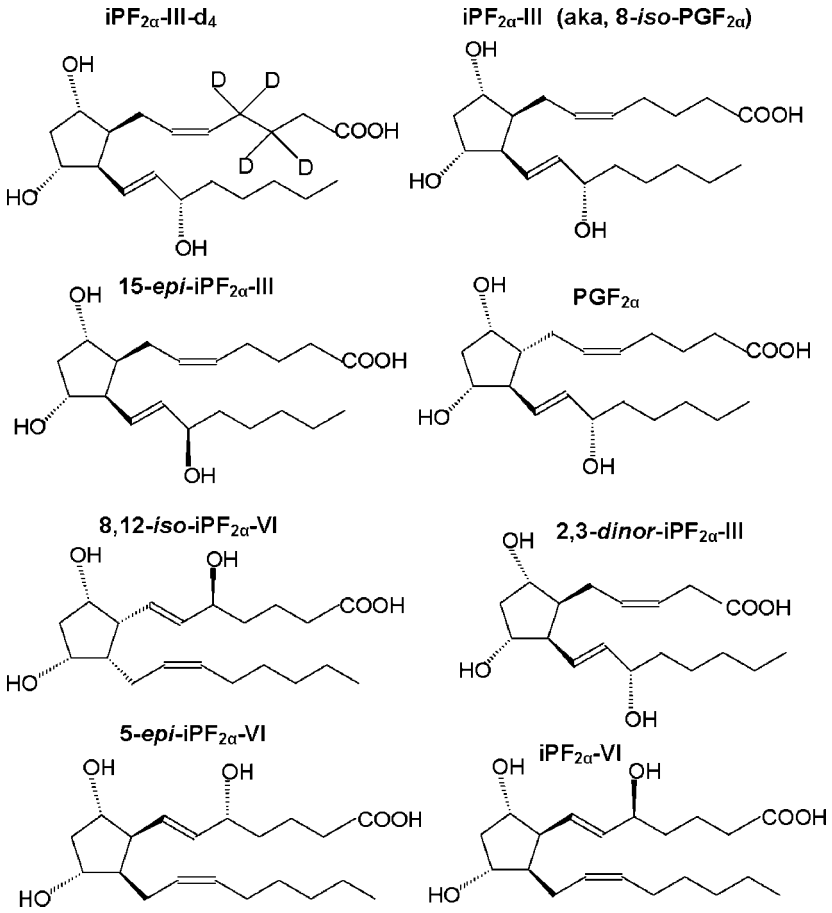

Fig. 1. Structures of analytes and the internal standard $iPF_{2\alpha}$-III-d_4.

centrifuge (Needham Heights, MA). Oasis® HLB and MAX 96-well extraction plates (10 and 30 mg) were from Waters (Milford, MA). Extraction was performed on a Phenomenex 96-well plate manifold with a customized insert to adapt shallow 0.5 ml 96-well plates (Agilent Technologies, Palo Alto, CA) for collection.

Sample preparation

For each sample, 2 ml of urine was fortified with 50 μl of $iPF_{2\alpha}$-III-d_4 internal standard solution (20 ng/ml) to a concentration of 500 pg/ml. It was then acidified to approximately pH 3 by the addition of 40 μl of 1 N HCl and centrifuged (3,500 rpm for 10 min at 4°C) to remove suspended material. The supernatant was loaded into a well on an HLB 96-well extraction plate (10 mg), which had been preconditioned with 1 ml of methanol and equilibrated with 1 ml of water. The plate was washed successively with 0.5 ml of 0.1 N HCl, 0.5 ml of 5:95 methanol-water, and 0.1 ml of hexane. Then, analytes and internal standard were eluted into the sample collection plate with two aliquots of 80 μl of methanol containing 0.5% NH_4OH. After adding 100 μl of water to the eluent, the sample plate was transferred to the autosampler tray.

Standards preparation

Primary standards of individual analytes were prepared separately. Primary standards of $iPF_{2\alpha}$-III in methanol were prepared by accurately weighing ~1 mg of $iPF_{2\alpha}$-III into 100 ml of methanol (10 μg/ml). The other primary standards were prepared by transferring small volumes of stock solution to appropriate volumes of methanol. Resulting concentrations were 10, 25, 25, 25, and 12.5 μg/ml for 15-epi-$iPF_{2\alpha}$-III, $iPF_{2\alpha}$-VI, 8,12-iso-$iPF_{2\alpha}$-VI, 2,3-dinor-$iPF_{2\alpha}$-III, and $PGF_{2\alpha}$, respectively. Primary standards were diluted to four working solutions by 80:20 water-methanol to prepare solutions for fortifying synthetic urine to be used for calibration standards and quality control (QC) samples. A solution of $iPF_{2\alpha}$-III-d_4 was prepared at 20 ng/ml by transferring 10 μl of $iPF_{2\alpha}$-III-d_4 stock (100 μg/ml) solution into 50 ml of 80:20 water-methanol for spiking standards and samples.

Calibration standards were prepared in 2 ml aliquots of synthetic urine. The formula for synthetic urine was adapted from a published reference with minor changes (63). Yellow food color was not added, because the cosmetic appearance was not required. Instead of nitric acid, NH_4OH was added to adjust the synthetic urine to approximately pH 6. It was then divided into aliquots in 50 ml plastic centrifuge tubes and stored at −20°C until use. Six standards were prepared in the range 30–2,000 pg/ml for $iPF_{2\alpha}$-III and 15-epi-$iPF_{2\alpha}$-III, 30–10,000 pg/ml for $iPF_{2\alpha}$-VI, 8,12-iso-$iPF_{2\alpha}$-VI, and 2,3-dinor-$iPF_{2\alpha}$-III, and 30–5,000 pg/ml for $PGF_{2\alpha}$. An additional standard as a blank containing only internal standard was also prepared. QC samples were prepared at the limit of quantitation (LOQ), 50%, and 95% of the calibration range, by spiking the synthetic urine with working solutions independently prepared from those used to make the calibration standards. A 50 μl aliquot of internal standard ($iPF_{2\alpha}$-III-d_4 at 20 ng/ml) was added to each standard and QC sample, yielding a concentration of 500 pg/ml. The standards and QC samples were extracted and prepared the same as the urine samples described above.

LC-MS/MS

Analysis of the prepared samples and standards was accomplished using an Agilent 1100 LC system (Palo Alto, CA) connected to a Micromass Quattro Ultima triple-stage mass spectrometer (Manchester, UK). Both systems were controlled using Micromass MassLynx software. The LC method used a 150 × 3 mm (5 μm) Phenomenex Luna C18(2) column preceded by a 4 × 2 mm guard column. The mobile phase consisted of A (0.15% NH_4OH in water) and B (0.15% NH_4OH in 95:5 acetonitrile-methanol) with a gradient of 6% B at 0 min to 21% B at 17 min (total run time of 20 min). The flow rate was 1 ml/min, with 10% of the column effluent introduced into an electrospray interface operated in the negative ion mode. The interface used nitrogen desolvation gas at 650–700 l/h and 400°C. The instrument was operated in the multiple reaction monitoring (MRM) mode, and the MRM transitions monitored are shown in **Table 1**. The collision cell pressure was 2.25×10^{-3} mBar (±10%). Quantitation was done using Micromass QuanLynx software. For the optimization of cone voltages and collision energies during method development, a solution of each analyte was infused into the ESI source at 5 μl/min using a syringe pump (Pump 11; Harvard Apparatus, Holliston, MA). Product ion (daughter ion) mass spectra were recorded using the continuum averaging mode of operation.

Urine sample collection

Samples from a urine mutagenicity study were analyzed with our method. In that study, 32 smokers and 12 nonsmokers were recruited. There were 16 male smokers, 16 female smokers, 6 male nonsmokers, and 6 female nonsmokers. The mean ages of smokers and nonsmokers were equivalent (44 ± 9 and 44 ± 7 years, respectively). Two consecutive 24 h urine samples were collected at 1 week after subjects were placed on a controlled low-mutagen diet (grilled, fried, or other heavily pyrolyzed foods eliminated, plus other restrictions). The study protocols were approved by an internal human resources review committee, and all subjects gave informed consent after learning the details of the study. Urine creatinine measurements and ELISA determination of iPF2α-III were done by Covance (Harrogate, UK). Nicotine and nine of its metabolites in urine were determined for these samples using a modification of a published method (64).

Statistical analysis

Statistical analysis of the data was performed using log-transformed two-way ANOVA on smokers versus nonsmokers and female versus male subjects. Linear regression analysis with correlation coefficients (r) and P values for significance of slope were used to assess the effects of total nicotine uptake on each analyte. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Mass spectrometry of analytes

Weakly acidic isoprostane isomers ($Pk_a \sim 5$) can be deprotonated to form $(M\text{-}H)^-$ precursor ions in the ESI source and detected in the negative ion mode. MS/MS can be used to fragment the precursor ions and form specific product ions. One analyte from each class of isomers was selected for optimization of mass spectrometry parameters such as cone voltage and collision energy. $iPF_{2\alpha}$-III, 8,12-iso-$iPF_{2\alpha}$-VI, and 2,3-dinor-$iPF_{2\alpha}$-III were chosen as representative analytes for class III, VI, and the dinor metabolite, respectively. Optimum cone voltages were chosen from the apex of the plot of $(M\text{-}H)^-$ intensity against the cone voltage. For example, the $(M\text{-}H)^-$ intensity of $iPF_{2\alpha}$-III maximized at a capillary voltage of 2.9 kV. The collision-induced dissociation (CID) mass spectra of $(M\text{-}H)^-$ from the representative analytes are shown in **Fig. 2**. A comparison of mass spectra for $iPF_{2\alpha}$-III and 8,12-iso-$iPF_{2\alpha}$-VI shows that, although their $(M\text{-}H)^-$ ions are isobaric (m/z 353), class VI analytes have a very specific fragment at m/z 115, whereas class III analytes have an abundant fragment at m/z 193; this is consistent with other literature reports (53, 65). Class VI analytes also have the m/z 193 fragment, but only at 10% of m/z 115 intensity in the spectra. To avoid interference, it is important that no class VI analytes coelute with class III analytes in the chromatogram. MRM was used to monitor the different classes of isoprostanes to improve specificity. The collision energy for the CID was chosen where the selected product ion had a maximum intensity. The optimized MS conditions and MRM transitions are summarized in Table 1. The CID transition m/z 357 → 197 was selected for the internal standard $iPF_{2\alpha}$-III-d_4.

Chromatography of isoprostanes

Buffer additives in the mobile phase affect not only the separation of analytes but also the sensitivity of detection. The ESI process is favored when analytes are highly ionized by the time they reach the interface. By infusing 1 μg/ml $iPF_{2\alpha}$-III in different buffer solutions, we found that the intensity of ESI response was higher in NH_4OH buffer solutions than in ammonium acetate (2–20 mM). At a concentration of 0.15% NH_4OH, no ion suppression was observed (tested by infusing $iPF_{2\alpha}$-III with 0.05, 0.1,

TABLE 1. MRM transitions and analyte-specific mass spectrometry parameters

Compounds Monitored	Transition	MRM Transition	Dwell	Cone	Collision Energy
			s	*V*	*eV*
2,3-Dinor-$iPF_{2\alpha}$-III	Primary	m/z 325 → 237	0.25	39	11
	Secondary[a]	m/z 325 → 137	0.25	39	15[b]
8,12-Iso-$iPF_{2\alpha}$-VI, $iPF_{2\alpha}$-VI	Primary	m/z 353 → 115	0.25	45	21
	Secondary	m/z 353 → 201	0.25	45	21
$iPF_{2\alpha}$-III, 15-epi-$iPF_{2\alpha}$-III, $PGF_{2\alpha}$	Primary	m/z 353 → 193	0.25	43	23
	Secondary	m/z 353 → 165	0.25	43	24[b]

MRM, multiple reaction monitoring.
[a] Secondary transitions were monitored during specificity evaluation.
[b] Different from the primary MRM transition, adjusted for higher sensitivity.

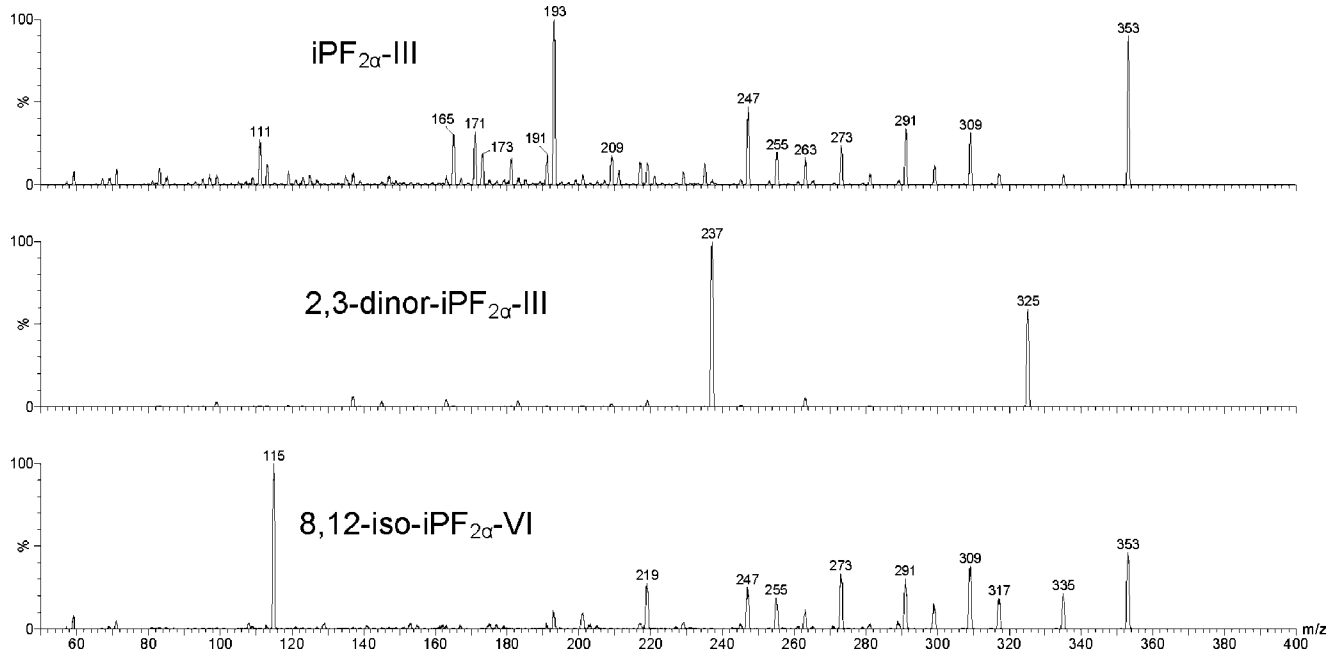

Fig. 2. Representative collision-induced dissociation (CID) mass spectra of selected $(M\text{-}H)^-$ ions. Experimental conditions for $iPF_{2\alpha}$-III were as follows: electrospray ionization (ESI) negative mode; capillary voltage, 3.0 kV; cone voltage, 43 V; collision energy, 23 eV; desolvation gas at 600 l/h and 280°C; 1 μg/ml infused to mass spectrometer with a flow rate of 5 μl/min. Experimental conditions for 2,3-dinor-$iPF_{2\alpha}$-III were as follows: ESI negative mode; capillary voltage, 2.9 kV; cone voltage, 39 V; collision energy, 12 eV; desolvation gas at 700 l/h and 400°C; 2 μg/ml infused to mass spectrometer with a flow rate of 5 μl/min. Experimental conditions for 8,12-iso-$iPF_{2\alpha}$-VI were as follows: cone voltage, 45 V; collision energy, 22 eV; others were as for 2,3-dinor-$iPF_{2\alpha}$-III.

0.2. 0.4, and 0.5% NH_4OH in the solution), and the pH was 9.5, which is in the specified pH range of the particular column used [150 × 3 mm Phenomenex Luna C18(2)]. Good resolution of the isoprostanes and high efficiency were achieved when using 0.15% NH_4OH in the mobile phase with a gradient starting with a low percentage of organic solvent (6%). By fortifying urine samples with authentic standards, $iPF_{2\alpha}$-III, 15-epi-$iPF_{2\alpha}$-III, $iPF_{2\alpha}$-VI, 8,12-iso-$iPF_{2\alpha}$-VI, $PGF_{2\alpha}$, and the metabolite 2,3-dinor-$iPF_{2\alpha}$-III were identified (**Fig. 3**). The MRM transition *m/z* 353 → 151 for class V isomers was recorded according to a literature report (53), although no identification of the class V isomers was done in that study because of lack of standards.

A porous graphite carbon column was also tried for the separation based on one literature report (57). However, in our experience, the column efficiency degraded after <10 injections of urine sample extracts. This column is such a strong reverse stationary phase that it retains any hydrophobic compounds passing through the column. Some compounds did not elute easily (or at all), and the column required regeneration with tetrahydrofuran with strong acid (trifluoroacetic acid) or base (triethyl amine). This column was abandoned, because it was not suitable for our purpose (high sample throughput).

Sample preparation and extraction

A 96-well solid phase extraction plate with 10 mg of sorbent per well was used for the extraction of analytes from human urine. This plate enabled the preparation of 84 samples simultaneously along with the calibration standards and QC samples. This format also permitted the automation of sample preparation by liquid-handling instruments. Both Waters Oasis® HLB (10 mg) and MAX (10 mg) sorbents were investigated. In the extraction procedure using the MAX plate, 2 ml of urine was basified by adding 40 μl of 10% NH_4OH and then loaded into the plate well. Each well was washed with 0.5% NH_4OH in water and either plain methanol or methanol with 0.5% NH_4OH. The analytes were eluted with 2% formic acid in methanol. Although MAX is supposedly more selective for acidic compounds, the recovery for $iPF_{2\alpha}$-III was only about half that of the HLB plate, and this approach (MAX) was abandoned. The third wash step of HLB sorbent extraction procedure used 0.1 ml of hexane, as reported in other C18 sorbent methods (31, 34, 35, 49). In our study, this washing step did not result in significant loss of analytes, and considering that it might help to clean up the sample, this step was retained in the extraction procedure.

Validation results

The internal standard was monitored by the transition *m/z* 357 → 197, and no coeluting interferences were observed from the synthetic urine or authentic urines. In addition to the primary MRM transition monitored, a secondary MRM transition was monitored for each analyte (Table 1). Ratios of the two transitions for each analyte in three standards of spiked synthetic urine, three standards of spiked aqueous mobile phase samples, and six authentic

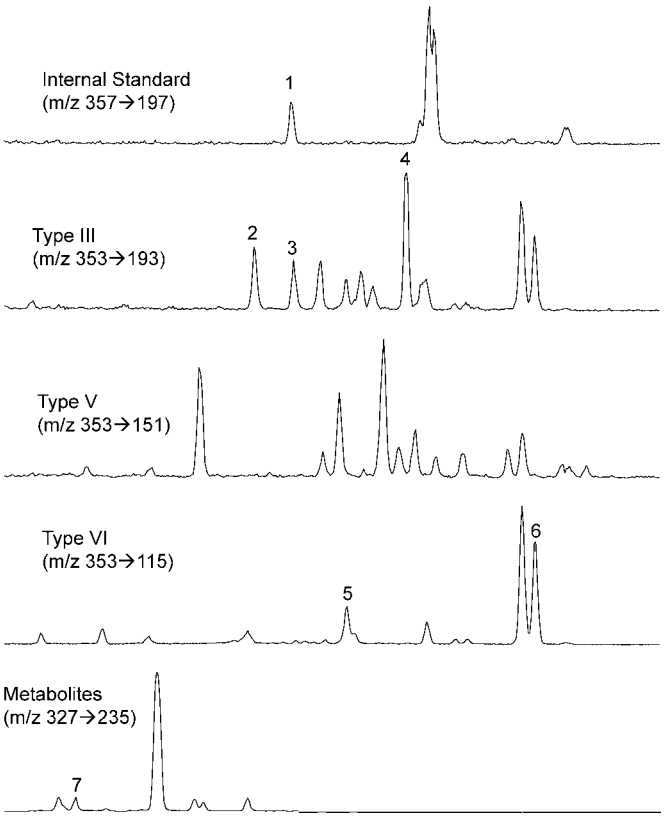

Fig. 3. Multiple reaction monitoring (MRM) chromatograms of urine extract. Peak 1, $iPF_{2\alpha}$-III-d_4 [retention time (RT), 11.00 min]; peak 2, 15-epi-$iPF_{2\alpha}$-III (RT, 10.32 min); peak 3, $iPF_{2\alpha}$-III (RT, 11.06 min); peak 4, $PGF_{2\alpha}$ (RT, 13.15 min); peak 5, $iPF_{2\alpha}$-VI (RT, 12.05 min); peak 6, 8,12-iso-$iPF_{2\alpha}$-VI (RT, 15.56 min); peak 7, 2,3-dinor-$iPF_{2\alpha}$-III (RT, 7.01 min). Experimental conditions for liquid chromatography were as follows: column, 150 × 3 mm (5 μm) Phenomenex Luna C18(2); room temperature; mobile phase, A = 0.15% ammonium hydroxide (NH_4OH) in water and B = 0.15% NH_4OH in 95:5 acetonitrile-methanol; gradient, 6% B at 0 min to 21% B at 17 min; total run time, 20 min; flow rate, 1 ml/min with 100 μl/min introduced to the mass spectrometer; injection volume, 40 μl. Mass spectrometric parameters were as follows: ESI in negative mode; capillary voltage, 2.9 kV; desolvation gas, 700 l/h at 400°C; collision cell pressure, 2.25×10^{-3} mBar (±10%); for CID transitions, cone voltage, and collision energy, see Table 1.

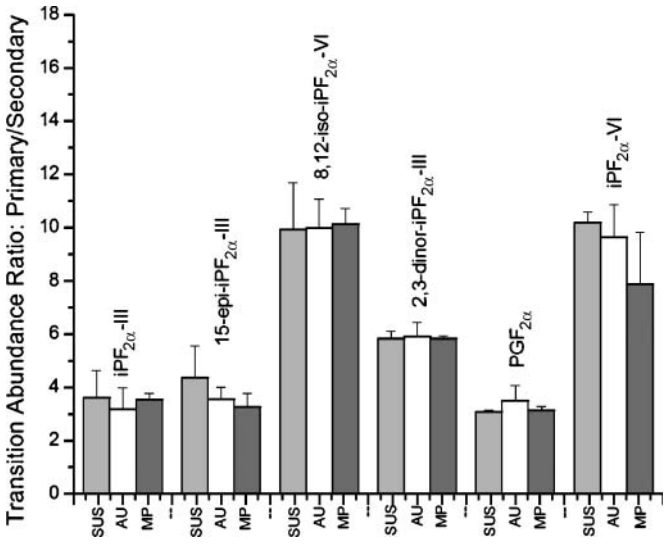

Fig. 4. MRM transition ratios in different matrices. SUS, three synthetic urine samples spiked at low-, medium-, and high-level standards; AU, six authentic urine samples from six subjects; MP, three mobile phase samples spiked at low-, medium-, and high-level standards. Error bars represent one standard deviation unit.

urine samples were monitored. **Figure 4** shows the mean transition ratio for each analyte in different matrices. A Student's *t*-test for significance (66) showed that the mean transition ratio was not significantly different in either synthetic or authentic urine from that in the mobile phase for any of the analytes. Thus, the assay was specific for all six analytes in urine by this measure; however, the $iPF_{2\alpha}$-VI isomer was not supplied in sufficient purity to enable adequate quantitation. The $iPF_{2\alpha}$-VI standard was actually a mixture of $iPF_{2\alpha}$-VI and another isomer (likely 5-epi-$iPF_{2\alpha}$-VI), with two peaks observed in the chromatogram. Thus, complete validation was not extended to $iPF_{2\alpha}$-VI. Therefore, the method is not considered valid for that analyte, although quantitative data are supplied for $iPF_{2\alpha}$-VI in this report.

The linearity of the assay was assessed using a series of six calibration standards independently prepared as described above. The calibration range covers 30 to 2,000 pg/ml for $iPF_{2\alpha}$-III and 15-epi-$iPF_{2\alpha}$-III, 30 to 10,000 pg/ml for 2,3-dinor-$iPF_{2\alpha}$-III and 8,12-iso-$iPF_{2\alpha}$-VI, and 30 to 5,000 pg/ml for $PGF_{2\alpha}$. All calibration plots were linear with 1/X weighting for the regression. At least five of six nonzero standards met the criteria that the calculated concentration for each standard must be ±15% of the prepared concentration (±20% at the LOQ) and the relative standard deviation (RSD) must be <15% (<20% at the LOQ). Correlation coefficients were >0.99. LOQ for the method was set at 30 pg/ml for all six analytes and tested. Accuracy at LOQ was ±20% of the predicted concentration with RSD < 20% (n = 6). The raw peak-to-peak signal-to-noise ratio ranged from 10 to 40 for the six analytes in synthetic urine-spiked samples at LOQ. LOQ was the lowest standard on the calibration curve.

Intrabatch accuracy and precision were assessed on three separate batches of synthetic urine spiked with the analytes at LOQ, 50%, and 95% of the calibrated range. For the mean of six replicates at each level, agreement of ±15% of the prepared and measured concentrations was obtained, with RSD < 15% for the five analytes ($iPF_{2\alpha}$-III, 15-epi-$iPF_{2\alpha}$-III, 8,12-iso-$iPF_{2\alpha}$-VI, $PGF_{2\alpha}$, and 2,3-dinor-$iPF_{2\alpha}$-III) in each batch. Interbatch accuracy and precision were assessed on the mean concentrations from three batches as summarized in **Table 2**. The means at each concentration were ±15% of the prepared concentration, with RSD < 15% for the three batches.

Stability was tested on two sets of triplicate samples: *1*) synthetic urine samples spiked at LOQ, 50%, and 95% of the calibration range; and *2*) authentic urine samples from two subjects, one with endogenous analytes at relatively high levels and one with endogenous analytes at relatively low levels. For freeze-thaw stability, triplicate sam-

TABLE 2. Summary of data for interbatch accuracy and precision

Sample	Preparation	Measured (mean ± SD)	% Delta	RSD
	pg/ml			%
$iPF_{2\alpha}$-III				
LOQ	30	30.1 ± 3.6	0.5	11.9
50%M	1,000	1,036.4 ± 57.2	3.6	5.5
95%M	1,900	1,948.9 ± 74.9	2.6	3.8
15-Epi-$iPF_{2\alpha}$-III				
LOQ	30	30.5 ± 3.3	1.6	10.9
50%M	1,000	977.1 ± 44.2	−2.3	4.5
95%M	1,900	1,814.7 ± 72.1	−4.5	4.0
8,12-Iso-$iPF_{2\alpha}$-VI				
LOQ	30	34.4 ± 2.3	14.8	6.6
50%M	5,000	5,088.0 ± 255.8	1.8	5.0
95%M	9,500	9,526.9 ± 383.4	0.3	4.0
2,3-Dinor-$iPF_{2\alpha}$-III				
LOQ	30	31.1 ± 2.9	3.5	9.3
50%M	5,000	5,097.8 ± 234.8	2.0	4.6
95%M	9,500	9,452.6 ± 349.1	−0.5	3.7
$PGF_{2\alpha}$				
LOQ	30	29.4 ± 3.1	−1.9	10.4
50%M	2,500	2,578.2 ± 161.9	3.1	6.3
95%M	4,750	4,789.6 ± 203.7	0.8	4.3

LOQ, limit of quantitation; RSD, relative standard deviation. % Delta represents the percentage difference of the measured concentration and the prepared concentration. 50%M and 95%M represent 50% and 95% of the maximum calibration level, respectively.

ples at each concentration for synthetic urine samples and authentic urine samples were subjected to three complete freeze-thaw cycles with freezing at −20°C and thawing at room temperature. Short-term stability was tested by freezing and thawing at room temperature and allowing the samples to remain at room temperature for 24 h. Long-term stability was tested on triplicate samples at each concentration for synthetic urine samples and authentic urines samples by freezing them for 6 weeks before thawing and analyzing the samples. The spiked synthetic urine samples were ±15% of the prepared concentration (±20% at LOQ). The endogenous analytes in authentic urine samples were ±15% of the fresh concentration after freeze-thaw, short-term, and long-term storage. Autosampler storage stability was assessed on the two sets of samples mentioned above with three duplicates by reanalyzing them after remaining in the autosampler tray for 2 days at room temperature. Variation was <15% for all spiked synthetic urine samples and unspiked authentic urine samples. Primary standards were found to be stable for up to 6 weeks at 4°C. Diluted solutions (working solutions) were found to be stable for up to 6 weeks in the dark at room temperature. In addition, freshly prepared and 7 week old $iPF_{2\alpha}$-III-d_4 spiking solutions (10 ng/ml) were monitored in the same batch of runs. Comparison of peak areas showed no significant decomposition to d_3, d_2, d_1, and d_0 through hydrogen/deuterium exchange after 7 weeks.

To test the dilution effect, seven synthetic urine samples were spiked at three times the upper LOQ, which is 6,000 pg/ml for $iPF_{2\alpha}$-III and 15-epi-$iPF_{2\alpha}$-III, 30,000 pg/ml for 8,12-iso-$iPF_{2\alpha}$-VI and 2,3-dinor-$iPF_{2\alpha}$-III, and 15,000 pg/ml for $PGF_{2\alpha}$. The samples were then diluted 4-fold with distilled deionized water and prepared and analyzed as the other samples. The measured amounts multiplied by 4 were ±15% of the prepared amounts, with RSD ⩽ 8% for all analytes, showing no significant effect of 4-fold dilution.

Most reported methods for isoprostane quantitation have used calibration curves generated from standards prepared in mobile phase (or aqueous buffer). Although this avoids interference of the endogenous analytes present in the authentic urine, which results in a high constant (y intercept) in the regression equation, the FDA/CDER guidelines recommend that calibration standards be prepared in the same matrix as the samples. While using authentic urine to prepare calibration standards, the concentration of endogenous analytes can be calculated from the regression equation of the calibration curve (32, 39). In our method, synthetic urine was selected for the preparation of standards because it is easily manufactured in the laboratory from commercially available chemicals, is readily prepared as needed, and also avoids interferences from endogenous analytes. In addition, synthetic urine composition is constant compared with that of authentic urine, which is more variable from one subject to another. A recovery experiment was carried out to test the suitability of the synthetic urine matrix for standard preparation. Two milliliter aliquots of authentic urine were spiked at 0, low (2–10× LOQ), 25%, and 50% of calibration range levels and measured against standards prepared in synthetic urine. Basal concentrations in the authentic urine samples were determined from the unspiked samples. The acceptance criterion is that the percentage recovery of the spiked concentration should be within 85–115% (80–120% at LOQ). Based on the recovery results (**Table 3**), synthetic urine was accepted as a suitable matrix for $iPF_{2\alpha}$-III, 15-epi-$iPF_{2\alpha}$-III, 8,12-iso-$iPF_{2\alpha}$-VI, and $PGF_{2\alpha}$ but was borderline for 2,3-dinor-$iPF_{2\alpha}$-III. For the latter analyte, <80% was recovered when spiked into authentic urine. However, by

TABLE 3. Recovery results for the suitability of synthetic urine matrix for calibration standard preparation

Sample	Spiked	Measured (mean ± SD)	Recovery	RSD
	pg/ml		%	
$iPF_{2\alpha}$-III				
LOQ × 2	60	56.1 ± 10.8	93.5	19.2
25%M	500	507.9 ± 42.2	101.6	8.3
50%M	1,000	940.0 ± 59.5	94.0	6.3
15-Epi-$iPF_{2\alpha}$-III				
LOQ × 2	60	49.1 ± 4.0	81.9	8.1
25%M	500	424.6 ± 32.2	84.9	7.6
50%M	1,000	8,63.0 ± 91.9	86.3	10.7
8,12-Iso-$iPF_{2\alpha}$-VI				
LOQ × 10	300	294.3 ± 62.7	98.1	21.3
25%M	2,500	2,199.4 ± 233.7	88.0	10.6
50%M	5,000	4,377.4 ± 635.7	87.5	14.5
$PGF_{2\alpha}$				
LOQ × 5	150	135.6 ± 10.8	90.4	7.9
25%M	1,250	1,235.7 ± 63.5	98.9	5.1
50%M	2,500	2,333.7 ± 123.1	93.3	5.3
2,3-Dinor-$iPF_{2\alpha}$-III				
LOQ × 10	300	245.2 ± 30.4	81.7	12.4
25%M	2,500	1,989.0 ± 246.1	79.6	12.4
50%M	5,000	3,765.0 ± 375.7	75.3	10.0

Spiked concentration of analytes in authentic urine calculated based on a calibration curve prepared in synthetic urine. 25%M and 50%M represent 25% and 50% of the maximum calibration level, respectively.

simply diluting the urine sample 6-fold with water or increasing HLB extraction sorbent from 10 to 30 mg without changing any other sample preparation procedure, the recovery for 2,3-dinor-iPF$_{2\alpha}$-III was improved to an acceptable range. The likely reason for the low recovery of 2,3-dinor-iPF$_{2\alpha}$-III was overloading of the 10 mg solid phase extraction well. Ion suppression variation may also play a role in the reduced recovery for 2,3-dinor-iPF$_{2\alpha}$-III, because it elutes several minutes before the internal standard.

Comparison of LC-MS/MS and ELISA

Results for iPF$_{2\alpha}$-III obtained by this LC-MS/MS method were compared with results from an ELISA determination and found to be significantly correlated ($r = 0.890$, $P < 0.0001$); however, an ~2-fold higher concentration bias was observed for the ELISA data (**Fig. 5**). We hypothesize that the difference was attributable to cross-reactivity in ELISA in which more than one isoprostane isomer responded. The LC-MS/MS method described here demonstrates specificity for the single isomer iPF$_{2\alpha}$-III and is likely more representative of the true concentration.

Results for human urine

Our method was applied to a set of 24 h urine samples. The six analytes were determined in two 24 h urine samples from 32 smokers and 12 age- and sex-matched nonsmokers using the LC-MS/MS method. From the measured concentration of each analyte, adjusted measurements were calculated, including mass per milligram of creatinine and total mass per day (**Table 4**). The latter units are possible because 24 h urine samples were collected; moreover, they may be more indicative of total oxidative stress. The mean values of iPF$_{2\alpha}$-III were 513 ± 275 and 294 ± 104 pg/mg creatinine in smokers and nonsmokers, respectively. These results are consistent with results in the literature using an LC-MS/MS method with a reverse phase column (34). The mean concentrations of 2,3-dinor-iPF$_{2\alpha}$-III (3,030 ± 1,546 and 2,046 ± 836 pg/mg creatinine in smokers and nonsmokers, respectively) were slightly lower than the results in the same study.

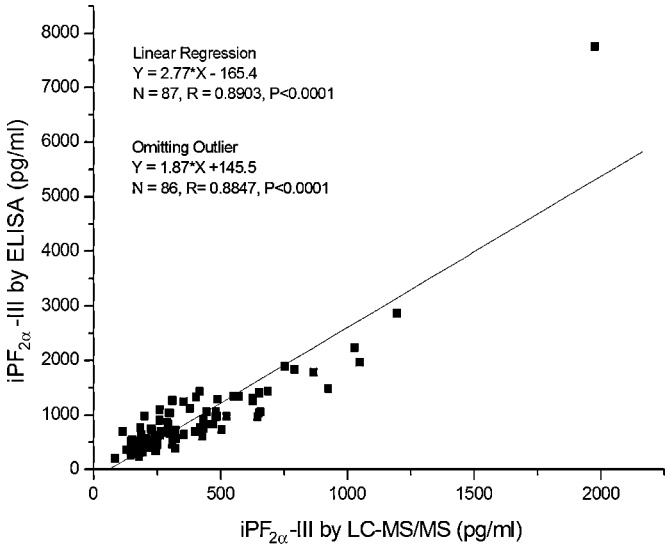

Fig. 5. Correlation of iPF$_{2\alpha}$-III measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and ELISA.

As shown in Table 4, excretion of iPF$_{2\alpha}$-III and 2,3-dinor-iPF$_{2\alpha}$-III was significantly higher (except for output per day of 2,3-dinor-iPF$_{2\alpha}$-III, which was marginally significant at $P = 0.06$) in smokers than in nonsmokers in units of both pg/mg creatinine and μg/day. The same trend was observed when male subjects and female subjects were compared separately in units of pg/mg creatinine. The increase of the total output per day was not statistically significant for iPF$_{2\alpha}$-III and 2,3-dinor-iPF$_{2\alpha}$-III (except for iPF$_{2\alpha}$-III in the female group) when the male and female groups were compared separately. The concentration of iPF$_{2\alpha}$-VI in units of μg/mg creatinine and μg/day tended to be higher in smokers than in nonsmokers but did not reach statistical significance. The average excretion of 15-epi-iPF$_{2\alpha}$-III, 8,12-iso-iPF$_{2\alpha}$-VI, and PGF$_{2\alpha}$ was not different between smokers and nonsmokers in units of μg/mg creatinine or μg/day. The mean level of all isoprostanes analyzed (iPF$_{2\alpha}$-III, 2,3-dinor-iPF$_{2\alpha}$-III, 15-epi-iPF$_{2\alpha}$-III, 8,12-iso-iPF$_{2\alpha}$-VI, and iPF$_{2\alpha}$-VI) was significantly higher in women than in men in units of pg/mg creatinine but not in units of μg/day. The same difference was observed in both smokers and nonsmokers, and these results are consistent with iPF$_{2\alpha}$-III and 2,3-dinor-iPF$_{2\alpha}$-III values (pg/mg creatinine) reported in the literature (55, 67). iPF$_{2\alpha}$-III in plasma was also reported to be significantly higher in women than in men (24). The reason for the higher level of isoprostanes in women was not clear, although greater susceptibility to lipid oxidation in female smokers is a possibility (55). Interestingly, in our study, the total excretion per day of analytes was not different by gender. Investigation of the clinical conditions associated with systemic oxidative stress showed that smoking, gender, age, body mass index, glucose, total cholesterol, and history of cardiovascular disease are all factors that can affect the excretion of iPF$_{2\alpha}$-III (67). Note that the sample size in our study was limited and only gender and smoking status were considered. It is possible that other factors not considered here may have affected our results. Also, a limitation of this study is that the number of nonsmokers included was far less than the number of smokers.

One potential mechanism of increased oxidative stress associated with cigarette smoking is the direct actions of various oxidants and preoxidants present in tobacco smoke (68). Obata et al. (19) found that the duration of smoking and the number of cigarettes per day were not correlated with urine isoprostane concentration. Reilly et al. (28) found that urinary cotinine was higher in heavy smokers and tended to correlate with urinary iPF$_{2\alpha}$-III, but this difference did not attain statistical significance. Tsikas et al. (30) showed a weak linear correlation between creatinine-corrected urinary iPF$_{2\alpha}$-III level and number of cigarettes reportedly smoked. To investigate the relationship between isoprostane excretion and cigarette smoke exposure, concentrations of urinary nicotine and nine nicotine metabolites (cotinine, cotinine-*N*-glucuronide, nicotine-*N*-glucuronide, *trans*-3′-hydroxycotinine, *trans*-3′-

TABLE 4. Summary of measurements (means ± SD) of urine samples from smokers and nonsmokers

Analyte	Group	pg/ml		pg/mg creatinine			μg/day		
		Smoker	*Nonsmoker*	*Smoker*	*Nonsmoker*	P^a	*Smoker*	*Nonsmoker*	P^a
$iPF_{2\alpha}$-III (ELISA)	Total	1,051 ± 991	630 ± 342	1,152 ± 431	604 ± 196	0.0000	1.396 ± 0.466	0.829 ± 0.269	0.0000
	Male	1,229 ± 1,306	568 ± 398	976 ± 367	521 ± 181	0.0001	1.528 ± 0.422	0.955 ± 0.321	0.0010
	Female	877 ± 500	693 ± 287	1,322 ± 407	688 ± 181	0.0001	1.268 ± 0.477	0.703 ± 0.117	0.0004
$iPF_{2\alpha}$-III	Total	437 ± 312	293 ± 129	513 ± 275	294 ± 104	0.0015	0.592 ± 0.246	0.399 ± 0.109	0.0075
	Male	453 ± 362	231 ± 86	380 ± 204	231 ± 54	0.0494	0.579 ± 0.237	0.426 ± 0.108	0.1500
	Female	421 ± 258	355 ± 138	647 ± 274	356 ± 105	0.0080	0.606 ± 0.257	0.373 ± 0.108	0.0162
15-Epi-$iPF_{2\alpha}$-III	Total	383 ± 244	377 ± 174	450 ± 251	387 ± 164	0.52	0.532 ± 0.250	0.518 ± 0.153	0.84
	Male	410 ± 280	286 ± 108	341 ± 195	288 ± 69	0.70	0.532 ± 0.253	0.533 ± 0.151	0.77
	Female	356 ± 203	469 ± 183	559 ± 255	486 ± 172	0.61	0.532 ± 0.252	0.503 ± 0.161	1.00
8,12-Iso-$iPF_{2\alpha}$-VI	Total	2,063 ± 1,096	2,208 ± 1,092	2,485 ± 1,169	2,293 ± 1,124	0.61	2.928 ± 1.064	3.078 ± 1.093	0.70
	Male	2,185 ± 1,184	1,625 ± 561	1,918 ± 908	1,653 ± 479	0.67	2.961 ± 1.077	3.068 ± 0.999	0.77
	Female	1,941 ± 1,004	2,791 ± 1,198	3,052 ± 1,136	2,933 ± 1,232	0.76	2.894 ± 1.067	3.088 ± 1.226	0.80
2,3-Dinor-$iPF_{2\alpha}$-III	Total	2,380 ± 1,023	2,083 ± 1,183	3,030 ± 1,546	2,046 ± 836	0.0023	3.537 ± 1.346	2.723 ± 0.768	0.06
	Male	2,331 ± 964	1,365 ± 501	2,074 ± 706	1,354 ± 244	0.0164	3.275 ± 1.016	2.497 ± 0.544	0.16
	Female	2,429 ± 1,091	2,801 ± 1,245	3,986 ± 1,572	2,738 ± 598	0.0420	3.799 ± 1.583	2.949 ± 0.909	0.21
$PGF_{2\alpha}$	Total	1,031 ± 1,428	979 ± 471	1,059 ± 565	986 ± 414	0.97	1.242 ± 0.565	1.34 ± 0.423	0.39
	Male	1,240 ± 1,958	789 ± 367	853 ± 545	768 ± 175	0.82	1.302 ± 0.655	1.42 ± 0.381	0.34
	Female	828 ± 530	1,168 ± 501	1,259 ± 516	1,204 ± 473	0.77	1.184 ± 0.465	1.26 ± 0.464	0.79
$iPF_{2\alpha}$-VI[b]	Total	3,675 ± 2,054	3,453 ± 1,641	4,307 ± 1,980	3,484 ± 1,423	0.14	5.169 ± 1.969	4.684 ± 1.389	0.63
	Male	3,982 ± 2,256	2,597 ± 1,111	3,386 ± 370	2,583 ± 720	0.21	5.256 ± 1.702	4.762 ± 1.416	0.66
	Female	3,368 ± 1,813	4,308 ± 1,674	5,229 ± 2,083	4,384 ± 1,396	0.39	5.083 ± 2.229	4.606 ± 1.421	0.81
Creatinine	Total	98 ± 65[c]	105 ± 45[c]				1.325 ± 0.542[d]	1.459 ± 0.470[d]	
	Male	129 ± 75[c]	103 ± 37[c]				1.671 ± 0.525[d]	1.845 ± 0.264[d]	
	Female	67 ± 33[c]	107 ± 54[c]				0.980 ± 0.272[d]	1.074 ± 0.261[d]	

[a] Comparisons of smokers and nonsmokers in total and by gender using log-transformed ANOVA.
[b] Integration of two peaks (mixture of $iPF_{2\alpha}$-VI and 5-epi-$iPF_{2\alpha}$-VI).
[c] In units of mg/dl.
[d] In units of g/day.

hydroxycotinine-*O*-glucuronide, cotinine-*N*-oxide, nicotine-*N*-oxide, norcotinine, and nornicotine) were measured by LC-MS/MS. The results are presented as nicotine equivalents, which is the molar sum of nicotine and each metabolite. Determination of total nicotine equivalents is considered a good estimate of total exposure to cigarette smoke (69–71). The linear regression of isoprostane excretion against the total nicotine equivalent excretion in the smokers' group revealed that only $iPF_{2\alpha}$-VI and 8,12-iso-$iPF_{2\alpha}$-VI had significant correlations (although very weak) with nicotine uptake (**Table 5**). In all cases for daily excretion, the linear correlation between isoprostanes and nicotine uptake was weak, with $r \leqslant 0.18$–0.40, indicating that factors other than nicotine uptake affect isoprostane excretion.

In this set of samples, the mean excretion of 2,3-dinor-$iPF_{2\alpha}$-III was approximately six times higher than that of $iPF_{2\alpha}$-III. The correlation between 2,3-dinor-$iPF_{2\alpha}$-III and its parent compound $iPF_{2\alpha}$-III was significant ($r = 0.7619$, $P < 0.0001$). This observation is consistent with the literature findings that $iPF_{2\alpha}$-III together with its metabolite 2,3-dinor-$iPF_{2\alpha}$-III might provide an integrated index of $iPF_{2\alpha}$-III formation and lipid oxidation in vivo (34, 72).

Although the number of subjects in this study was limited, it is interesting that some isoprostanes seem to be more increased than others in smokers. Metabolic stability may play a role in the observed levels of isoprostanes. For example, perhaps all isoprostanes are increased equally by any source of oxidative stress (e.g., smoking) but some are more efficiently metabolized, so that their determined concentrations appear less affected by variations in oxidant levels. Such an event would make highly metabolized isoprostanes appear to be less correlated with smoking than less metabolized isoprostanes. Another possibility is that exposure to different types of oxidants may affect the mechanisms that create isoprostanes and thereby affect their distribution. Perhaps further research will provide clues to what is occurring with exposure to smoke.

In conclusion, an LC-MS/MS method was developed and validated to quantitate $iPF_{2\alpha}$-III, 15-epi-$iPF_{2\alpha}$-III, $iPF_{2\alpha}$-VI, and 8,12-iso-$iPF_{2\alpha}$-VI along with prostaglandin $PGF_{2\alpha}$ and the metabolite 2,3-dinor-$iPF_{2\alpha}$-III in human urine. This method can be helpful for high-throughput measurement of isoprostanes for investigation of the pathophysiological role of lipid peroxidation in associated human diseases. The data reported here support literature reports that $iPF_{2\alpha}$-III and its metabolite 2,3-dinor-$iPF_{2\alpha}$-III are increased slightly in smokers. Among smokers, the total output of isoprostanes $iPF_{2\alpha}$-VI and 8,12-iso-$iPF_{2\alpha}$-VI shows a

TABLE 5. Summary of linear regression results of excretion of each analyte against the daily output of nicotine equivalents in the same urine samples from each smoker

Analyte	*r*	*P*
$iPF_{2\alpha}$-III	0.2899	0.1075
15-Epi-$iPF_{2\alpha}$-III	0.1896	0.2986
8,12-Iso-$iPF_{2\alpha}$-VI	0.4010	0.0229
2,3-Dinor-$iPF_{2\alpha}$-III	0.2052	0.2597
$iPF_{2\alpha}$-VI[a]	0.3723	0.0359
$PGF_{2\alpha}$	0.1855	0.3095

[a] Combination of two peaks (mixture of $iPF_{2\alpha}$-VI and 5-epi-$iPF_{2\alpha}$-VI).

slight positive correlation with the amount of exposure to tobacco smoke (nicotine uptake). As there are numerous isoprostane isomers formed in the body, it may be possible that others not determined in this study are more correlated with cigarette smoking and will provide a useful tool for monitoring this form of oxidative stress.

The authors thank Walt Morgan and Tom Steichen for their assistance with statistical analyses in this work and David Heavner for the measurement of nicotine and its metabolites.

REFERENCES

1. Morrow, J. D., K. E. Hill, R. F. Burk, T. M. Nammour, K. F. Badr, and L. J. Roberts. 1990. A series of prostaglandin-F_2-like compounds are produced *in vivo* in humans by a noncyclooxygenase, free radical-catalyzed mechanism. *Proc. Natl. Acad. Sci. USA.* **87:** 9383–9387.
2. Taber, D. F., J. D. Morrow, and L. J. Roberts. 1997. A nomenclature system for the isoprostanes. *Prostaglandins.* **53:** 63–67.
3. Rokach, J., S. P. Khanapure, S. W. Hwang, M. Adiyaman, J. A. Lawson, and G. A. FitzGerald. 1997. Nomenclature of isoprostanes: a proposal. *Prostaglandins.* **54:** 853–873.
4. Basu, S., and J. Helmersson. 2005. Factors regulating isoprostane formation *in vivo*. *Antioxid. Redox Signal.* **7:** 221–235.
5. Milne, G. L., E. S. Musiek, and J. D. Morrow. 2005. F_2-isoprostanes as markers of oxidative stress in vivo: an overview. *Biomarkers.* **10 (Suppl.):** 10–23.
6. Cracowski, J. L., T. Durand, and G. Bessard. 2002. Isoprostanes as a biomarker of lipid peroxidation in humans: physiology, pharmacology and clinical implications. *Trends Pharmacol. Sci.* **23:** 360–366.
7. Morrow, J. D. 2005. Quantification of isoprostanes as indices of oxidant stress and the risk of atherosclerosis in humans. *Arterioscler. Thromb. Vasc. Biol.* **25:** 279–286.
8. Dentchev, T., Y. Yao, D. Pratico, and J. L. Dunaief. 2007. Isoprostane $F_{2\alpha}$-VI, a new marker of oxidative stress, increases following light damage to the mouse retina. *Mol. Vis.* **13:** 190–195.
9. Montine, T. J., K. S. Montine, W. McMahan, W. R. Markesbery, J. F. Quinn, and J. D. Morrow. 2005. F_2-isoprostanes in Alzheimer and other neurodegenerative diseases. *Antioxid. Redox Signal.* **7:** 269–275.
10. Davi, G., A. Falco, and C. Patrono. 2005. Lipid peroxidation in diabetes mellitus. *Antioxid. Redox Signal.* **7:** 256–268.
11. Cracowski, J. L. 2004. Isoprostanes: an emerging role in vascular physiology and disease? *Chem. Phys. Lipids.* **128:** 75–83.
12. Morrow, J. D. 2006. The isoprostanes—unique products of arachidonate peroxidation: their role as mediators of oxidant stress. *Curr. Pharm. Des.* **12:** 895–902.
13. Janssen, L. J., A. Catalli, and P. Helli. 2005. The pulmonary biology of isoprostaes. *Antioxid. Redox Signal.* **7:** 244–255.
14. Badr, K. F., and T. E. Abi-Antoun. 2005. Isoprostanes and the kidney. *Antioxid. Redox Signal.* **7:** 236–243.
15. Kannel, W. B. 1981. Update on the role of cigarette smoking in coronary artery disease. *Am. Heart J.* **101:** 319–328.
16. Frei, B., T. M. Forte, B. N. Ames, and C. E. Cross. 1991. Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipoprotein properties in human blood plasma. Protective effects of ascorbic acid. *Biochem. J.* **277:** 133–138.
17. Morrow, J. D., B. Frei, A. W. Longmire, J. M. Gaziano, S. M. Lynch, Y. Shyr, W. E. Strauss, J. A. Oates, and L. J. Roberts. 1995. Increase in circulating products of lipid peroxidation (F_2-isoprostanes) in smokers—smoking as a cause of oxidative damage. *N. Engl. J. Med.* **332:** 1198–1203.
18. Bachi, A., E. Zuccato, M. Baraldi, R. Fanelli, and C. Chiabrando. 1996. Measurement of urinary 8-*epi*-prostaglandin $F_{2\alpha}$, 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.
19. Obata, T., K. Tomaru, T. Nagakura, Y. Izumi, and T. Kawamoto. 2000. Smoking and oxidant stress: assay of isoprostane in human urine by gas chromatography-mass spectrometry. *J. Chromatogr. B.* **746:** 11–15.
20. Montuschi, P., J. V. Collins, G. Ciabattoni, N. Lazzeri, M. Corradi, S. A. Kharitonov, and P. J. Barnes. 2000. Exhaled 8-isoprostane as an *in vivo* biomarker of lung oxidative stress in patients with COPD and healthy smokers. *Am. J. Respir. Crit. Care Med.* **162:** 1175–1177.
21. Sinzinger, H., J. Kaliman, and A. Oguogho. 2000. Eicosanoid production and lymphatic responsiveness in human cigarette smokers compared with non-smokers. *Lymphology.* **33:** 24–31.
22. Obwegeser, R., A. Oguogho, M. Ulm, P. Berghammer, and H. Sinzinger. 1999. Maternal cigarette smoking increases F_2-isoprostanes and reduces prostacyclin and nitric oxide in umbilical vessels. *Prostaglandins Other Lipid Mediat.* **57:** 269–279.
23. Dietrich, M., G. Block, M. Hudes, J. D. Morrow, E. P. Norkus, M. G. Traber, C. E. Cross, and L. Packer. 2002. Antioxidant supplementation decreases lipid peroxidation biomarker F_2-isoprostanes in plasma of smokers. *Cancer Epidemiol. Biomarkers Prevent.* **11:** 7–13.
24. Dietrich, M., G. Block, N. L. Benowitz, J. A. Morrow, M. Hudes, P. Jacob, E. P. Norkus, and L. Packer. 2003. Vitamin C supplementation decreases oxidative stress biomarker F_2-isoprostanes in plasma of nonsmokers exposed to environmental tobacco smoke. *Nutr. Cancer.* **45:** 176–184.
25. Pilz, H., A. Oguogho, F. Chehne, G. Lupattelli, B. Palumbo, and H. Sinzinger. 2000. Quitting cigarette smoking results in a fast improvement of *in vivo* oxidation injury (determined via plasma, serum and urinary isoprostane). *Thromb. Res.* **99:** 209–221.
26. Oguogho, A., G. Lupattelli, B. Palumbo, and H. Sinzinger. 2000. Isoprostanes quickly normalize after quitting cigarette smoking in healthy adults. *Vasa.* **29:** 103–105.
27. Chehne, F., A. Oguogho, G. Lupattelli, A. C. Budinsky, B. Palumbo, and H. Sinzinger. 2001. Increase of isoprostane 8-*epi*-$PGF_{2\alpha}$ after restarting smoking. *Prostaglandins Leukot. Essent. Fatty Acids.* **64:** 307–310.
28. Reilly, M., N. Delanty, J. A. Lawson, and G. A. FitzGerald. 1996. Modulation of oxidant stress *in vivo* in chronic cigarette smokers. *Circulation.* **94:** 19–25.
29. Zedler, B. K., R. Kinser, J. Oey, B. Nelson, H. J. Roethig, R. A. Walk, P. Kuhl, K. Rustemeier, G. Schepers, K. Von Holt, et al. 2006. Biomarkers of exposure and potential harm in adult smokers of 3–7 mg tar yield (Federal Trade Commission) cigarettes and in adult non-smokers. *Biomarkers.* **11:** 201–220.
30. Tsikas, D., E. Schwedhelm, M. T. Suchy, J. Niemann, F. M. Gutzki, V. J. Erpenbeck, J. M. Hohfeld, A. Surdacki, and J. C. Frolich. 2003. Divergence in urinary 8-*iso*-$PGF_{2\alpha}$ ($iPF_{2\alpha}$-III, 15-F_{2t}-IsoP) levels from gas chromatography tandem mass spectrometry quantification after thin-layer chromatography and immunoaffinity column chromatography reveals heterogeneity of 8-*iso*-$PGF_{2\alpha}$—possible methodological, mechanistic and clinical implications. *J. Chromatogr. B.* **794:** 237–255.
31. Murai, Y., T. Hishinuma, N. Suzuki, J. Satoh, T. Toyota, and M. Mizugaki. 2000. Determination of urinary 8-*epi*-prostaglandin $F_{2\alpha}$ using liquid chromatography-tandem mass spectrometry: increased excretion in diabetics. *Prostaglandins Other Lipid Mediat.* **62:** 173–181.
32. Ohashi, N., and M. Yoshikawa. 2000. Rapid and sensitive quantification of 8-*iso*- prostaglandin $F_{2\alpha}$ in human plasma and urine by liquid chromatography-electrospray ionization mass spectrometry. *J. Chromatogr. B.* **746:** 17–24.
33. Hatsukami, D. K., N. L. Benowitz, S. Rennard, C. Oncken, and S. S. Hecht. 2006. Biomarkers to assess the utility of potential reduced exposure tobacco products. *Nicotine Tob. Res.* **8:** 600–622.
34. Liang, Y. L., P. Wei, R. W. Duke, P. D. Reaven, S. M. Harman, R. G. Cutler, and C. B. Heward. 2003. 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. *Free Radic. Biol. Med.* **34:** 409–418.
35. Pratico, D., O. P. Barry, J. A. Lawson, M. Adiyaman, S. W. Hwang, S. P. Khanapure, L. Iuliano, J. Rokach, and G. A. FitzGerald. 1998. $IPF_{2\alpha}$-I: an index of lipid peroxidation in humans. *Proc. Natl. Acad. Sci. USA.* **95:** 3449–3454.
36. Proudfoot, J., A. Barden, T. A. Mori, V. Burke, K. D. Croft, L. J. Beilin, and I. B. Puddey. 1999. Measurement of urinary F_2-isoprostanes as markers of in vivo lipid peroxidation—a comparison of enzyme immunoassay with gas chromatography/mass spectrometry. *Anal. Biochem.* **272:** 209–215.
37. Basu, S. 1998. Radioimmunoassay of 8-*iso*-prostaglandin $F_{2\alpha}$: an index for oxidative injury via free radical catalysed lipid peroxidation. *Prostaglandins Leukot. Essent. Fatty Acids.* **58:** 319–325.
38. Helmersson, J., A. Larsson, B. Vessby, and S. Basu. 2005. Active

smoking and a history of smoking are associated with enhanced prostaglandin $F_{2\alpha}$, interleukin-6 and F_2-isoprostane formation in elderly men. *Atherosclerosis.* **181:** 201–207.
39. Bessard, J., J. L. Cracowski, F. Stanke-Labesque, and G. Bessard. 2001. Determination of isoprostaglandin $F_{2\alpha}$ type III in human urine by gas chromatography-electronic impact mass spectrometry. Comparison with enzyme immunoassay. *J. Chromatogr. B.* **754:** 333–343.
40. Nourooz-Zadeh, J. 1999. Gas chromatography mass spectrometry assay for measurement of plasma isoprostanes. *Methods Enzymol.* **300:** 13–17.
41. Nourooz-Zadeh, J., M. B. Cooper, D. Ziegler, and D. J. Betteridge. 2005. Urinary 8-*epi*-$PGF_{2\alpha}$ and its endogenous β-oxidation products (2,3-dinor and 2,3-dinor-5,6-dihydro) as biomarkers of total body oxidative stress. *Biochem. Biophys. Res. Commun.* **330:** 731–736.
42. Lee, C. Y. J., A. M. Jenner, and B. Halliwell. 2004. Rapid preparation of human urine and plasma samples for analysis of F_2-isoprostanes by gas chromatography-mass spectrometry. *Biochem. Biophys. Res. Commun.* **320:** 696–702.
43. Zhao, Z., N. M. Hjelm, C. W. K. Lam, and C. S. Ho. 2001. One-step solid-phase extraction procedure for F_2-isoprostanes. *Clin. Chem.* **47:** 1306–1308.
44. Schwedhelm, E., D. Tsikas, T. Durand, F. M. Gutzki, A. Guy, J. C. Rossi, and J. C. Frolich. 2000. Tandem mass spectrometric quantification of 8-iso-prostaglandin $F_{2\alpha}$ and its metabolite 2,3-dinor-5,6-dihydro-8-iso-prostaglandin $F_{2\alpha}$ in human urine. *J. Chromatogr. B.* **744:** 99–112.
45. Tsikas, D., E. Schwedhelm, J. Fauler, F. M. Gutzki, E. Mayatepek, and J. C. Frolich. 1998. Specific and rapid quantification of 8-iso-prostaglandin $F_{2\alpha}$ in urine of healthy humans and patients with Zellweger syndrome by gas chromatography tandem mass spectrometry. *J. Chromatogr. B.* **716:** 7–17.
46. Ferretti, A., and V. P. Flanagan. 1997. Isolation and measurement of urinary 8-iso-prostaglandin $F_{2\alpha}$ by high-performance liquid chromatography and gas chromatography mass spectrometry. *J. Chromatogr. B.* **694:** 271–276.
47. Tsukamoto, H., T. Hishinuma, T. Mikkaichi, H. Nakamura, T. Yamazaki, Y. Tomioka, and M. Mizugaki. 2002. Simultaneous quantification of prostaglandins, isoprostane and thromboxane in cell-cultured medium using gas chromatography mass spectrometry. *J. Chromatogr. B.* **774:** 205–214.
48. Morrow, J. D., W. E. Zackert, J. P. Yang, E. H. Kurhts, D. Callewaert, R. Dworski, K. Kanai, D. Taber, K. Moore, J. A. Oates, et al. 1999. Quantification of the major urinary metabolite of 15-F_{2t}-isoprostane (8-iso-$PGF_{2\alpha}$) by a stable isotope dilution mass spectrometric assay. *Anal. Biochem.* **269:** 326–331.
49. Adiyaman, M., J. A. Lawson, S. P. Khanapure, G. A. FitzGerald, and J. Rokach. 1998. Total synthesis of 17,17,18,18-d_4-$iPF_{2\alpha}$-VI and quantification of iPF(2alpha)-VI in human urine by gas chromatography mass spectrometry. *Anal. Biochem.* **262:** 45–56.
50. Mori, T. A., K. D. Croft, I. B. Puddey, and L. J. Beilin. 1999. An improved method for the measurement of urinary and plasma F_2-isoprostanes using gas chromatography mass spectrometry. *Anal. Biochem.* **268:** 117–125.
51. Walter, M. F., J. B. Blumberg, G. G. Dolnikowski, and G. J. Handelman. 2000. Streamlined F_2-isoprostane analysis in plasma and urine with high-performance liquid chromatography and gas chromatography/mass spectroscopy. *Anal. Biochem.* **280:** 73–79.
52. Schweer, H., B. Watzer, H. W. Seyberth, and R. M. Nusing. 1997. Improved quantification of 8-*epi*-prostaglandin $F_{2\alpha}$ and F_2-isoprostanes by gas chromatography triple-stage quadrupole mass spectrometry: partial cyclooxygenase-dependent formation of 8-*epi*-prostaglandin $F_{2\alpha}$ in humans. *J. Mass Spectrom.* **32:** 1362–1370.
53. Li, H. W., J. A. Lawson, M. Reilly, M. Adiyaman, S. W. Hwang, J. Rokach, and G. A. FitzGerald. 1999. Quantitative high performance liquid chromatography tandem mass spectrometric analysis of the four classes of F_2-isoprostanes in human urine. *Proc. Natl. Acad. Sci. USA.* **96:** 13381–13386.
54. Taylor, A. W., R. S. Bruno, B. Frei, and M. G. Traber. 2006. Benefits of prolonged gradient separation for high-performance liquid chromatography-tandem mass spectrometry quantitation of plasma total 15-series F_2-isoprostanes. *Anal. Biochem.* **350:** 41–51.
55. Harman, S. M., L. Liang, P. D. Tsitouras, F. Gucciardo, C. B. Heward, P. D. Reaven, W. Ping, A. Ahmed, and R. G. Cutler. 2003. Urinary excretion of three nucleic acid oxidation adducts and isoprostane $F_{2\alpha}$ measured by liquid chromatography mass spectrometry in smokers, ex-smokers, and nonsmokers. *Free Radic. Biol. Med.* **35:** 1301–1309.
56. Flores, L., S. Rodela, J. Abian, J. Claria, and E. Esmatjes. 2004. F_2 isoprostane is already increased at the onset of type 1 diabetes mellitus: effect of glycemic control. *Metabolism.* **53:** 1118–1120.
57. Bohnstedt, K. C., B. Karlberg, L. O. Wahlund, M. E. Jonhagen, H. Basun, and S. Schmidt. 2003. Determination of isoprostanes in urine samples from Alzheimer patients using porous graphitic carbon liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B.* **796:** 11–19.
58. Yin, H. Y., N. A. Porter, and J. D. Morrow. 2005. Separation and identification of F_2-isoprostane regioisomers and diastereomers by novel liquid chromatographic/mass spectrometric methods. *J. Chromatogr. B.* **827:** 157–164.
59. Roberts, L. J., and J. D. Morrow. 2000. Measurement of F_2-isoprostanes as an index of oxidative stress *in vivo*. *Free Radic. Biol. Med.* **28:** 505–513.
60. Morrow, J. D., and L. J. Roberts. 1999. Mass spectrometric quantitation of F_2-isoprostanes in biological fluids and tissues as measurement of oxidant stress. *Methods Enzymol.* **300:** 3–12.
61. Marliere, S., J. L. Cracowski, T. Durand, O. Chavanon, J. Bessard, A. Guy, F. Stanke-Labesque, J. C. Rossi, and G. Bessard. 2002. The 5-series F_2-isoprostanes possess no vasomotor effects in the rat thoracic aorta, the human internal mammary artery and the human saphenous vein. *Br. J. Pharmacol.* **135:** 1276–1280.
62. FDA/CDER. 2001. Guidance for Industry on Bioanalytical Methods Validation. FDA/CDER, Rockville, MD.
63. MacLellan, J. A., R. J. Traub, and D. R. Fisher. 1988. Performance Testing of Radiobioassay Laboratories: In Vitro Measurements (Urinalysis). Final Report. National Technical Information Service, Springfield, VA. 8–9.
64. Meger, M., I. Meger-Kossien, A. Schuler-Metz, D. Janket, and G. Scherer. 2002. Simultaneous determination of nicotine and eight nicotine metabolites in urine of smokers using liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B.* **778:** 251–261.
65. Waugh, R. J., J. D. Morrow, L. J. Roberts, and R. C. Murphy. 1997. Identification and relative quantitation of F_2-isoprostane regioisomers formed in vivo in the rat. *Free Radic. Biol. Med.* **23:** 943–954.
66. Miller, J. C., and J. N. Miller. 1988. Statistics for Analytical Chemistry. 2nd edition. Ellis Horwood Limited, Chichester, England. 52–57.
67. Keaney, J. F., M. G. Larson, R. S. Vasan, P. W. F. Wilson, I. Lipinska, D. Corey, J. M. Massaro, P. Sutherland, J. A. Vita, and E. J. Benjamin. 2003. Obesity and systemic oxidative stress—clinical correlates of oxidative stress in the Framingham Study. *Arterioscler. Thromb. Vasc. Biol.* **23:** 434–439.
68. Church, D. F., and W. A. Pryor. 1985. Free-radical chemistry of cigarette-smoke and its toxicological implications. *Environ. Health Perspect.* **64:** 111–126.
69. Byrd, G. D., R. A. Davis, W. S. Caldwell, J. H. Robinson, and J. D. deBethizy. 1998. A further study of FTC yield and nicotine absorption in smokers. *Psychopharmacology (Berl.)* **139:** 291–299.
70. Boswell, C., M. Curvall, R. K. Elswick, and D. Leyden. 2000. Modelling nicotine intake in smokers and snuff users using biological fluid nicotine metabolites. *Biomarkers.* **5:** 341–354.
71. Benowitz, N. L., P. Jacob, I. Fong, and S. Gupta. 1994. Nicotine metabolic profile in man—comparison of cigarette-smoking and transdermal nicotine. *J. Pharmacol. Exp. Ther.* **268:** 296–303.
72. Chiabrando, C., A. Valagussa, C. Rivalta, T. Durand, A. Guy, E. Zuccato, P. Villa, J. C. Rossi, and R. Fanelli. 1999. Identification and measurement of endogenous β-oxidation metabolites of 8-*epi*-prostaglandin $F_{2\alpha}$. *J. Biol. Chem.* **274:** 1313–1319.