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ARTICLE

Determination of F_2-Isoprostanes in Urine by Online Solid Phase Extraction Coupled to Liquid Chromatography with Tandem Mass Spectrometry

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F_2-isoprostanes are a unique class of prostaglandin-like compounds formed in vivo, which have been established as biomarkers of oxidative stress. Accurate analysis has been challenging due to lack of specificity for the isoforms of isoprostanes and lengthy sample preparation procedures to enable trace quantitative analysis. A quantitative analytical method was developed for the determination of F_2-isoprostanes in rat and hamster urine by online solid phase extraction (SPE) coupled with liquid chromatography and tandem mass spectrometry (LC-MS/MS). The online SPE LC-MS/MS procedure has significant advantages over alternative methods with respect to specificity, sensitivity, simplicity, and speed. The assay enables the detection of $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-IV, and $iPF_{2\alpha}$-VI over a linear dynamic range of 0.1−50 ng/mL in rat urine samples. This range covers the basal levels of these F_2-isoprostanes. The limit of quantitation (LOQ) for the standard isoprostanes was about 0.3 ng/mL. The average recoveries ranged from 73 to 115% depending upon the individual F_2-isoprostane isomers in rat urine. Additionally, the method was used to determine increases of endogenous urine $iPF_{2\alpha}$-VI and $iPF_{2\alpha}$-III in hamsters challenged with either low-fat or high-fat diets.

KEYWORDS: Determination of urine isoprostanes; online SPE-LC/MS/MS; mass spectrometry

INTRODUCTION

Oxidative stress plays a major role in the pathogenesis of many human diseases, including cardiovascular diseases, type II diabetes, neurological diseases, and cancers (*1*, *2*). F_2-isoprostanes (F_2-iPs) are a class of prostaglandin-like molecules formed by free radical attack on arachidonic acid, a polyunsaturated fatty acid that is a subclass of the lipid mediator group known as eicosanoids. These isoprostanes are regarded as one of the most reliable and valuable biomarkers of oxidative stress and lipid peroxidation because isoprostanes are generated in the phospholipid domain of cell membranes at the site of free radical attack (*1*−*6*). The isoprostanes are cleaved by phospholipases, circulate in both esterified and unesterified forms, and are excreted in urine. F_2-isoprostanes possess a 1,3-hydroxycyclopentane ring with hydroxyls in the syn configuration. Depending on the methylene hydrogens abstracted by free radical attack, up to 64 isomers in 4 structural classes can be generated (*1*, *3*). In addition, isoprostanes are isomers of enzymatically formed prostaglandins, leukotrienes, and epoxyeicosatrienoic acids (*4*, *5*).

F_2-isoprostanes are chemically stable with little variability in healthy humans and are reportedly present in detectable amounts in all normal biological tissues and fluids analyzed, including plasma (35 ± 6 pg mL^{-1}), urine (1.6 ± 0.6 ng mg^{-1} creatinine), cerebrospinal fluid, and bile (23 ± 1 pg mL^{-1}) (*2*). The basal levels for the isoprostanes allow for an assessment of the effects of diseases and permit the determination of the extent to which various therapeutic interventions affect levels on endogenous oxidant stress. Elevations of F_2-isoprostanes in human body fluids and tissues have been found in a number of human disorders, which include atherosclerosis, hypercholesterolemia, diabetes, obesity, neurodegenerative diseases, and rheumatoid arthritis. Furthermore, treatments for some of these conditions, including antioxidant supplementation, antidiabetic treatments, and weight management, have been shown to decrease production of F_2-isoprostanes. The quantification of F_2-isoprostanes in urine is the most convenient and least invasive. Autoxidation does not readily occur in urine due to low lipid content. Thus, these compounds represent their endogenous production and give a highly precise and accurate index of in vivo oxidative stress (*4*). Isoprostanes exist primarily in free acid form in urine, and the concentrations are reportedly approximately 40 times higher in urine than in plasma (*2*). However, a significant portion of urinary isoprostanes conjugate with glucuronic acid (*7*). Because the extent of glucuronidation varies widely among individuals, ranging from 28 to 80%, specimens require pretreatment with β-glucuronidase

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prior to analysis to provide a more accurate assessment of oxidative stress.

The role of isoprostanes is in many instances still obscure because quantitation of these compounds in urine and plasma is difficult. Many of the previous studies are challenged by extensive sample preparation or inadequate, nonsensitive, and nonspecific in vitro bioassays. Different assay methods have attempted to overcome some of the challenges of quantifying F_2-isoprostanes, which include (a) multiple molecular forms of isoprostanes, (b) isoprostanes conjugated with glucuronic acid, and (c) isomers such as $PGF_{2\alpha}$, a prostaglandin that may potentially interfere with the determination of isoprostanes (*2*).

Several strategies have been developed in an attempt to quantify F_2-isoprostanes. However, most published methods for F_2-isoprosane measurements are laborious, with insufficient validation procedures and relatively low recovery rates (*8*−*11*). Many of these protocols involve HPLC and thin-layer chromatography (TLC) for purification and capillary gas chromatography (GC) with negative ion electron capture chemical ionization mass spectrometry (MS) or GC-MS/MS for analysis (*12*−*16*). Others utilize solid phase extraction (SPE) and TLC for purification and LC-MS for analysis (*17*). Alternative methods also have been developed to quantify F_2-isoprostanes using immunological approaches (*18*). Several commercially available immunoassay kits have been generated against $iPF_{2\alpha}$-III. Although these approaches are highly sensitive, they have limited specificity. In contrast, LC-MS/MS methods have recently been successfully developed to quantify F_2-isoprostane biomarkers due to their high specificity, sensitivity, and accuracy (*19*−*30*). One advantage of LC-MS/MS methods is that the sample preparation for analysis is simpler than that for GC-MS because it does not require a derivatization step and therefore is less prone to artifacts and loss of material. Ohashi and Yoshikawa (*17*) reported a method for the determination of F_2-isoprostanes in plasma and urine, which involves SPE followed by LC−electrospray ionization (ESI)/MS in selective ion monitoring (SIM) mode. In addition, several groups have also reported the estimation of isoprostanes with LC-MS/MS (*2*, *12*). However, many of these methods still require multiple sample preparation steps and off-line analysis.

In this study, we specifically focused on the detection and quantification of F_2-isoprostanes, $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-IV, and $iPF_{2\alpha}$-VI, which have been specifically linked to oxidative stress in both animal and human studies (*1*, *20*). Because there have been several studies previously reported measuring F_2-isoprostanes in rat urine (*31*−*33*), we developed a quantitative F_2-isoprostane method using rat urine. The method was then applied to measure endogenous F_2-isoprostanes in urine from Syrian Golden hamsters, an important model in the study of fat uptake and digestion. To the best of our knowledge F_2-isoprostanes have not been previously reported from hamster urine. To achieve high specificity and sensitivity for the detection of these F_2-isoprostanes, an online SPE-LC-MS/MS method was developed for rapid analyte identification and quantification. In addition, this method was developed to demonstrate quantitation in the comparison of urine samples from hamsters fed low-fat and high-fat diets. This study describes a significant advancement incorporating online SPE coupled with LC-MS/MS to minimize sample handling, reduce analysis time, and allow sensitive detection and quantitation of F_2-isoprostane isomers with limited sample quantities.

MATERIALS AND METHODS

Materials and Chemicals. Representative isomers from three of the F_2-iP classes were purchased from Cayman Chemicals (Ann Arbor, MI), along with stable isotope-labeled versions for use as internal standards: $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-III-d_4, $iPF_{2\alpha}$-IV, $iPF_{2\alpha}$-IV-d_4, $iPF_{2\alpha}$-VI, and $iPF_{2\alpha}$-VI-d_4. Standards were received in acetonitrile, ethanol, or methyl acetate. Chemical structures of analytes included in this study are shown in **Figure 1**. SPE cartridges, Hysphere C18HD, 10 × 2.0 mm, 7 μm, were purchased from Spark-Holland (Plainsboro, NJ). A Glucuronidase Sample Treatment Kit was obtained from Oxford Biomedical Research (Oxford, MI). HPLC-grade water from EM Science (Gibbstown, NJ) was used in the preparation of all aqueous solutions and mobile phases, HPLC-grade acetonitrile and methanol were purchased from Mallinckrodt-Baker (Paris, KY). Glacial acetic acid and ammonium hydroxide (28% v/v) were obtained from Fisher Scientific (Pittsburgh, PA). Histology-grade ethanol with 5% isopropanol and 5% methanol was purchased from EMD Chemicals (Gibbstown, NJ). A colorimetric microplate assay for creatinine was purchased from Oxford Biomedical Research.

Animal Studies. Twenty male and female rats, 75−99 g, had unlimited access to their assigned diet and distilled water. Diet was provided fresh daily. The rats were housed individually in stainless steel metabolism wire-bottom cages and kept at 22 °C in a humidity- and light-controlled environment (12 h light/dark cycle). Urine was collected in glass jars overnight and frozen at −80 °C until analysis.

Male Syrian Golden hamsters with a starting body weight of approximately 75 g (LVG strain, Charles River, Wilmington, MA) were acclimatized and fed commercial rodent chow (Ralston Purina, St. Louis, MO) and water ad libitum for 7 days. The animals were housed individually in wire-bottom cages in an environmentally controlled room maintained at 20−22 °C and 60% relative humidity. A 12 h alternating light/dark cycle was maintained. All animal experimental protocols were approved by the Western Regional Research Center (WRRC) Animal Care and Use Committee, USDA, Albany, CA.

After the acclimatization, hamsters were weighed and randomized into two diet groups, namely, the low-fat diet (n = 10) and high-fat diet (n = 10) groups. Hamsters were fed either a 10% kcal low-fat diet or a 45% kcal high-fat diet. Hamsters were fed for 12 weeks with water available ad libitum. After 12 weeks, the hamsters were sacrificed, and urine was collected by puncturing the bladder with a needle-equipped syringe. Approximately 2 mL of urine was collected from each animal. Urine samples were promptly frozen and stored at −80 °C prior to analysis.

Sample Preparation. Stock solutions of individual analytes were prepared at a concentration of 10.0 μg/mL, purged with nitrogen, and stored at −20 °C. Approximately 200 μL of each 10.0 μg/mL individual stock solution was combined and then diluted with methanol to prepare five spiking solutions with concentrations of 0.5, 1, 5, 10, and 50 ng/mL. Calibration solutions were prepared fresh daily by diluting each spiked solution 10-fold with water. Concentrations were 0.05, 0.1, 0.5, 1, 5, 10, 20, 35, and 50 ng/mL in 10:90 methanol/water. These calibration standards were analyzed with sample sets for both rat and hamster urine.

To facilitate LC-MS/MS, stable isotope-labeled (SIL) $iPF_{2\alpha}$-III-d_4 ($C_{20}H_{30}D_4O_5$), $iPF_{2\alpha}$-IV-d_4 ($C_{20}H_{30}D_4O_5$), and $iPF_{2\alpha}$-VI-d_4 ($C_{20}H_{30}D_4O_5$) were used. The presence of four deuterium atoms for each F_2-isoprostane ensured that identical chromatographic retention and matrix ionization efficiency effects were achieved for both the internal standard and analyte, thereby minimizing the quantitation variability (*34*). The 0.1 μg/mL SIL solution was prepared by combining 20 μL of each of the deuterated SIL stock solutions with 1.94 mL of water to obtain 2 mL of mixed SIL spiking solution for each standard. All samples were spiked with SIL standard to have an identical final known concentration of 10 ng/mL. Validation samples were prepared to evaluate accuracy and precision at concentrations of 0.5, 1, 5, 10, and 50 ng/mL applying the same procedure used to prepare the standard samples. Blank samples were analyzed with each set of standard samples to verify the assay selectivity in each of the multiple reaction monitoring (MRM) transitions.

Fortified rat urine samples were prepared by combining 50 μL of spiking solution with 450 μL of rat urine and gently mixed by vortexing. In addition, approximately 5 μL of β-glucuronidase solution (125 U/μL) was incubated at 37 °C for 2 h. Following incubation, the solution was cooled to room temperature and centrifuged at 20800g for approximately 3 min. Concentrations of the analysis solutions were equivalent to the calibration standard solutions: 0.05, 0.1, 0.5, 1, 5, 10, and 50 ng/mL.

Similarly, approximately 100 μL of hamster urine was combined with 5 μL of β-glucuronidase solution and incubated at 37 °C for 2 h. Following incubation, the solution was cooled to room temperature and diluted with 10 μL of methanol. The solution was centrifuged for 3 min at 20800g.

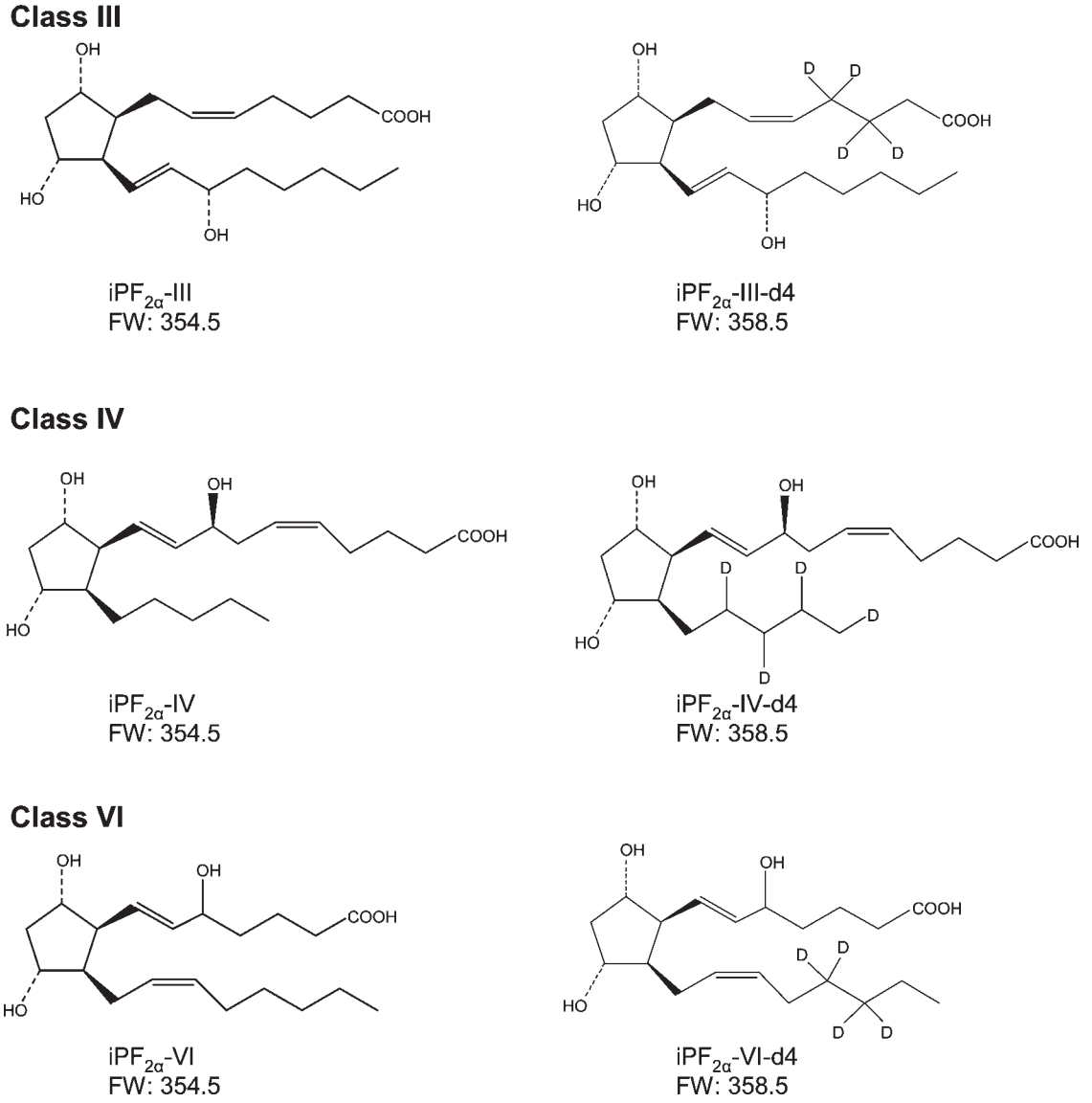

Figure 1. Chemical structures of three F_2-isoprostane isomers from classes III, IV, and VI and corresponding stable isotope versions.

A 40 μL aliquot of the treated sample was diluted with 50 μL of water and spiked with 10 μL of mixed SIL spiked solution. Calibration solutions were also diluted for analysis by combining 40 μL calibration solutions with 50 μL of water and 10 μL of mixed SIL spiked solutions. The injection volume for these samples was 20 μL.

Creatinine Content. Determination of oxidant status using urinary isoprostane measurements requires normalization of the isoprostane levels. The creatinine content in urine was determined by a colorimetric assay. The assay is based on the reaction of urinary creatinine metabolite with picric acid under alkaline conditions to produce an orange color, which was quantified by absorption spectroscopy at 490 nm on a Biotek Synergy HT Multi-Detection Microplate Reader (Winooski, VT). Urine samples were diluted 4-fold with water prior to analysis as described by the manufacturers.

Determination and Quantitation of Isoprostanes. SPE was performed on a Symbiosis Pharma system (Spark Holland, Emmen, The Netherlands). A C18 HD SPE cartridge was solvated using 1 mL of acetonitrile at 5 mL/min and equilibrated with 1 mL of 0.5% NH_4OH in water at 5 mL/min. The sample containing isoprostanes was extracted with 0.5% NH_4OH in water (1 mL at 2 mL/min), followed by three consecutive wash solutions (1 mL each at 2 mL/min): Wash 1 was acetonitrile/water 5:95 with 0.5% NH_4OH; wash 2 was water; wash 3 was methanol/water 20:80. The isoprostanes were eluted onto a Gemini C18 reverse-phase column (5 μm particle size, 50 × 2.0 mm) from Phenomenex (Torrance, CA) with a Gemini C18 precolumn (4 × 2.0 mm) containing C18 reversed-phase resin using the initial HPLC column conditions and an 8 min elution time. The initial LC column conditions were 85% mobile phase A (water with 0.02% acetic acid) and 15% mobile phase B (acetonitrile with 0.02% acetic acid). This condition was maintained for 3 min followed by a gradient separation at a flow rate of 0.3 mL/min. The gradient regimen included a linear gradient over 2 min ranging from 15 to 29% mobile phase B, followed by a linear gradient over 2 min from 29 to 40% mobile phase B, and a final isocratic elution at 100% mobile phase B at 10.25 min, holding for 2 min. The column was re-equilibrated before the next injection as described above. The run time was 12.5 min with a cycle time of 16 min. The injection volume was 10 μL. A switching valve (Valco Instruments Co. Inc., Houston, TX) was used to divert the solvent flow to waste outside the retention time of the analytes. During the HPLC run, the SPE clamp was flushed with 0.02% acetic acid in water for 1 min at 5 mL/min, then solvated, and equilibrated for subsequent injections.

Negative-ion electrospray ionization (ESI) was performed on an MDS/Sciex API 5000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with a TurboIonSpray source. Analyst version 1.4.2 (Applied Biosystems) was used as the mass spectrometric data system for all analyses. The resolutions of the Q1 and Q3 quadrupoles were set at unit and low, respectively, to enhance sensitivity and enable effective quantitation at the analyte concentrations of interest. MRM transitions were observed at Q1/Q3 for all analytes $[M - H]^-$. The following abundant product ions were chosen: $iPF_{2\alpha}$-III (353.2 → 193.1), $iPF_{2\alpha}$-III-d_4 (357.3 → 197.1), $iPF_{2\alpha}$-IV (353.2 → 127.1), $iPF_{2\alpha}$-IV-d_4 (357.2 → 127.1), $iPF_{2\alpha}$-VI (353.2 → 115.0), $iPF_{2\alpha}$-VI-d_4 (357.2 → 115.0), and $PGF_{2\alpha}$ (353.2 → 193.1).

Isomers were identified on the basis of a combination of Q1 and Q3 ions and chromatographic retention times for given standards. Instrumental parameters for mass spectral acquisition were as follows: curtain gas (CUR) was set at 15 psi, ion source gas 1 (GS1) was 50 psi, ion source gas 2 (GS2) was 55 psi, ion transfer voltage was −4500 V, temperature (TEM) was 600 °C, interface heater (ihe) was on, nitrogen as the collision gas (CAD) was 5, and collision energies were 18−36 V. The collision energy was optimized for each isoprostane to obtain optimum sensitivity using nitrogen as collision gas ($iPF_{2\alpha}$-III, 36 V; $iPF_{2\alpha}$-III-d_4, 36 V; $iPF_{2\alpha}$-IV, 34 V; $iPF_{2\alpha}$-IV-d_4, 31 V; $iPF_{2\alpha}$-VI, 29 V; $iPF_{2\alpha}$-VI-d_4, 29 V; $PGF_{2\alpha}$, 36 V). The settings were optimized for each analyte by infusion of individual standards.

The accuracy and precision of the assay to measure isoprostanes were evaluated on each of two days by analyzing three replicates of control rat urine samples fortified at eight concentrations (0.05, 0.1, 0.5, 1, 2, 5, 10, and 50 ng/mL) using external standard quantitation. Each set was prepared in triplicate and contained a reagent blank 10:90 methanol/water, six control rat urine samples, and rat urine samples fortified at concentrations of 0.05, 0.1, 0.5, 1, 2, 5, 10, and 50 ng/mL. In addition, each quantitation sample set was bracketed by duplicate standard sets, which spanned the dynamic range of 0.5−50 ng/mL. The recovery for each $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-IV, and $iPF_{2\alpha}$-VI was calculated from the area of the signal obtained by analyzing rat urine fortified with a known amount of isoprostane. The concentration was estimated by using an external calibration curve built from eight standard solutions at 0.05, 1, 0.5, 1, 2, 5, 10, and 50 ng/mL.

A series of calibration standards (SIL) were analyzed with each sample set, and peak areas for analytes and internal standards were determined. Calibration curves were obtained by plotting the concentration of analyte on the *x*-axis and the quantitation ratio (peak area of quantitation ion/peak area of SIL ion) on the *y*-axis. A linear fit was applied to the calibration data, and analyte concentrations (ng/mL) were calculated using the linear equation. Isoprostane concentrations were divided by the corresponding creatinine concentration (mg/dL) from the same sample, and the results were reported as nanograms per milligram of creatinine.

RESULTS AND DISCUSSION

Method Development. Multiple molecular forms and isomers of isoprostanes have been observed at basal levels in many different tissues and organs; as a result, analysis requires high sensitivity and selectivity because of the low concentrations and structural similarities of these metabolites. This led to the development of a novel method to minimize sample handling, reduce analysis time, and allow both sensitive detection and quantitation of F_2-isoprostanes in urine by incorporating online SPE coupled with LC-MS/MS. Electrospray ionization (ESI) has been widely applied in the analysis of all classes of eicosanoids. Because isoprostanes have free carboxylic acid groups, ESI results in an abundant $[M - H]^-$ carboxylate ions that maximizes the detection of relatively low concentrations of isoprostanes in negative-ion mode. MRM assays allow for the further improvement of both specificity and quantitation limits of LC-MS/MS assays.

To develop the LC-MS/MS assay, product ion scanning experiments were conducted using nitrogen as collision gas, and the collision energy was optimized for each compound to generate the most abundant product ions. These product ion spectra were then used to select the precursor−product ion pairs for the development of MRM assays. The negatively charged ions of the different classes of F_2-isoprostanes undergo extensive collision-induced fragmentation. **Figure 2** shows the product ion spectra of $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-IV, and $iPF_{2\alpha}$-VI. The most abundant molecular ion generated under the ESI mode was m/z 353 for each of these F_2-isoprostanes. The selection of the transitions to be used in the quantitative determinations was based both on the relative intensity of the fragment ions and on the specificity of the examined transitions. The choice of product ions for MRM was consistent with those previously reported in the literature; $iPF_{2\alpha}$-III m/z 353 → 193, $iPF_{2\alpha}$-IV m/z 353 → 127, and $iPF_{2\alpha}$-VI m/z 353 → 115 (*19*, *21*, *23*). In

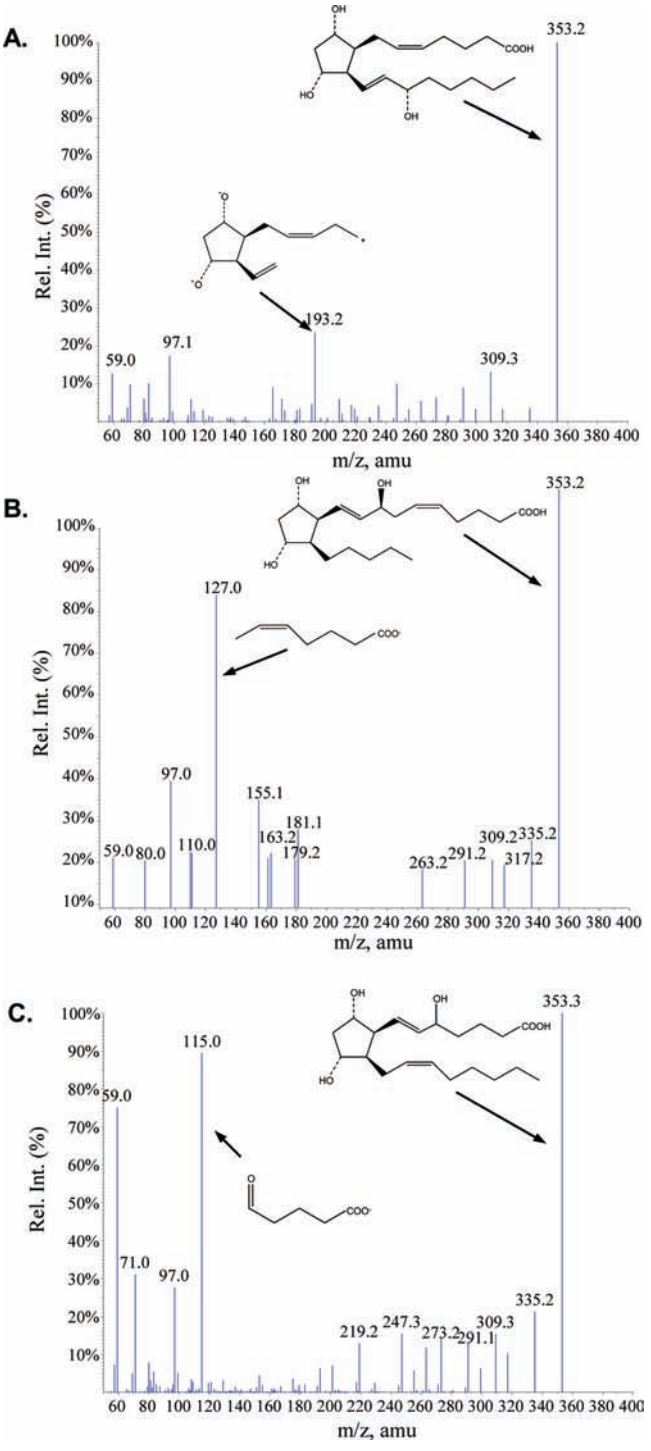

Figure 2. Product ion spectra of F_2-isoprostanes (**A**) $iPF_{2\alpha}$-III, (**B**) $iPF_{2\alpha}$-IV, and (**C**) $iPF_{2\alpha}$-VI.

all cases, the most abundant transition was selected and no significant isotopic crossover was observed.

Because there is precedence that isoprostanes in urine may be excreted as glucuronic acid conjugates, all urine samples were enzymatically pretreated with β-glucuronidase. Pretreatment with β-glucuronidase negates individual differences in the percent of isoprostane glucuronidation, allowing for more accurate assessment of oxidative stress. Following treatment with β-glucuronidase, a selective SPE procedure was developed. A crucial step in the development of the method was sample cleanup, because urine samples have several interferences, primarily due to the presence of other lipids, protein, and other intrinsic components.

Table 1. Linearity, Limits of Detection (LOD), and Limits of Quantitation (LOQ) of the SPE-LC-MS/MS Assay for Isoprostanes

compound	av (ng/mL)	SD (*s*)	calcd LOD (3 × S/N) (ng/mL)	calcd LOQ (10 × S/N) (ng/mL)	equation	R^2	no. of samples (*n*)
$iPF_{2\alpha}$-III	0.491	0.035	0.104	0.348	$5.5 \times 10^6 x - 945$	0.999	6
$iPF_{2\alpha}$-IV	0.466	0.029	0.088	0.295	$8.3 \times 10^6 x - 466$	0.999	6
$iPF_{2\alpha}$-VI	0.407	0.029	0.087	0.292	$9.2 \times 10^6 x - 537$	0.998	5

To facilitate sample cleanup, Spark Holland C18 cartridges were selected because they provided good capacity and retention of F_2-isoprostanes, allowing the use of multiple, orthogonal wash steps. F_2-isoprostanes are weakly acidic and hydrophobic compounds. Therefore, a selective SPE wash procedure was developed by taking advantage of the different elution profiles of F_2-isoprostanes and urine matrix interference as a function of both the concentration of acetonitrile and pH to 7.2. The first wash step, using acetonitrile/water 5:95 v/v with 0.5% NH_4OH, removed acidic, moderately polar, hydrophobic matrix components including the elution of yellow interference color bodies in urine. The second wash step, using water, lowered the pH. The final wash step, using methanol/water 20:80 v/v, eluted additional hydrophobic urine components. The isoprostanes were then directly eluted onto an online LC-MS/MS system.

The F_2-isoprostanes were chromatographically resolved on a C18 reverse-phase column. The run time was 12.5 min with a cycle time of 16 min including all of the steps for sample cleanup with SPE. The overall run time to our knowledge is the shortest for a SPE-LC-MS/MS based method described for an eicosanoids analysis and provides the basis for a rapid assay. Because F_2-isoprostanes are isomers of F_2-prostagladin, separation of these isomers is important for the specific analysis of individual F_2-isoprostanes. A good resolution of $iPF_{2\alpha}$-IV (*m*/*z* 353 → 127; rt ∼ 5.35), $iPF_{2\alpha}$-III (*m*/*z* 353 → 193; rt ∼ 5.72), and $iPF_{2\alpha}$-VI (*m*/*z* 353 → 115; rt ∼ 6.38) was observed for each of these isoprostanes. In addition, the MRM product ion for $PGF_{2\alpha}$, a potential interference, was found to be *m*/*z* 353 → 193, which was the same as $iPF_{2\alpha}$-III (data not shown). The two isobaric compounds $iPF_{2\alpha}$-III and $PGF_{2\alpha}$ were chromatographically resolved, rt ∼ 5.72 and rt ∼ 6.64, respectively, which have the same MRM transition, *m*/*z* 353 → 193 (data not shown). Prostaglandin $PGF_{2\alpha}$ is not a biomarker for oxidative stress, but rather a member of a group of lipid compounds that are derived enzymatically from fatty acids. The chromatographic conditions were developed to separate $PGF_{2\alpha}$ from the available F_2-isoprostane isomers.

The recovery of F_2-isoprostanes was affected by several factors including pretreatment of the urine with β-glucuronidase, selective SPE, chromatography, and ionization. However, through the use of stable isotope labels as internal standards spiked in urine before β-glucuronidase treatment, differences originating from each of the above factors were normalized. The technique of isotope dilution represents an accurate method for quantitative chemical analysis in complex matrices and improves precision and accuracy by reducing the problems arising from calibration procedure, sample preparation, and matrix effects.

The calibration curves for external standard quantitation were generated by sampling, three times, each of the eight solutions at different concentrations ranging from 0.05 to 50 ng/mL (0.05, 0.1, 0.5, 1, 2, 5, 10, and 50 ng/mL) (**Table 1**). Representative MRM chromatograms obtained from the analysis of each standard sample of iPF_{2a}-III, iPF_{2a}-IV, and iPF_{2a}-VI in fortified rat urine are presented in **Figure 3**. For each analyte, a standard curve was prepared by plotting the concentration of analyte on the *x*-axis and the peak area on the *y*-axis. A linear fit was applied to the calibration data, and analyte concentrations (ng/mL) were calculated using the linear equation. In all cases, excellent correlation (R^2) coefficients were achieved for the calibration plots (**Table 1**). The limits of quantitation (LOQ) and detection (LOD) were calculated on the basis of the standard deviation of the response and the slope obtained from the linearity plot of each F_2-isprostane. The LODs

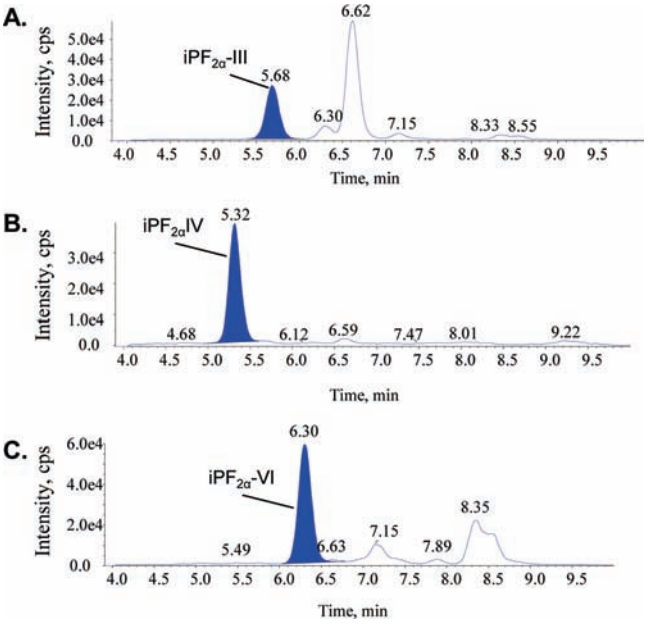

Figure 3. SPE-LC-MS/MS chromatogram results of the rat urine spiked standard solutions containing F_2-isoprostanes detected under multiple reaction monitoring (MRM) mode: (**A**) $iPF_{2\alpha}$-III monitored at MRM transition *m*/*z* 353 → 193; (**B**) $iPF_{2\alpha}$-IV monitored at MRM transition *m*/*z* 353 → 127; (**C**) $iPF_{2\alpha}$-VI monitored at MRM transition *m*/*z* 353 → 115.

Table 2. Recovery and Precision of Isoprostanes from Spiked Rat Urine

analyte	fortification (ng/mL)	av recovery (%)	*n*	precision %RSD	pooled precision %RSD
$iPF_{2\alpha}$-III	0.5	98	6	7.1	
	1	114	6	6.8	
	5	115	6	9.1	
	10	102	6	12.4	
	50	107	6	13.2	
					10.1
$iPF_{2\alpha}$-IV	0.5	93	6	6.3	
	1	107	6	7.5	
	5	106	6	8.4	
	10	94	6	13.2	
	50	98	6	12.7	
					10.0
$iPF_{2\alpha}$-VI	0.5	73	5	27.8	
	1	96	6	8.9	
	5	99	6	9.7	
	10	89	6	11.7	
	50	96	6	12.9	
					15.8

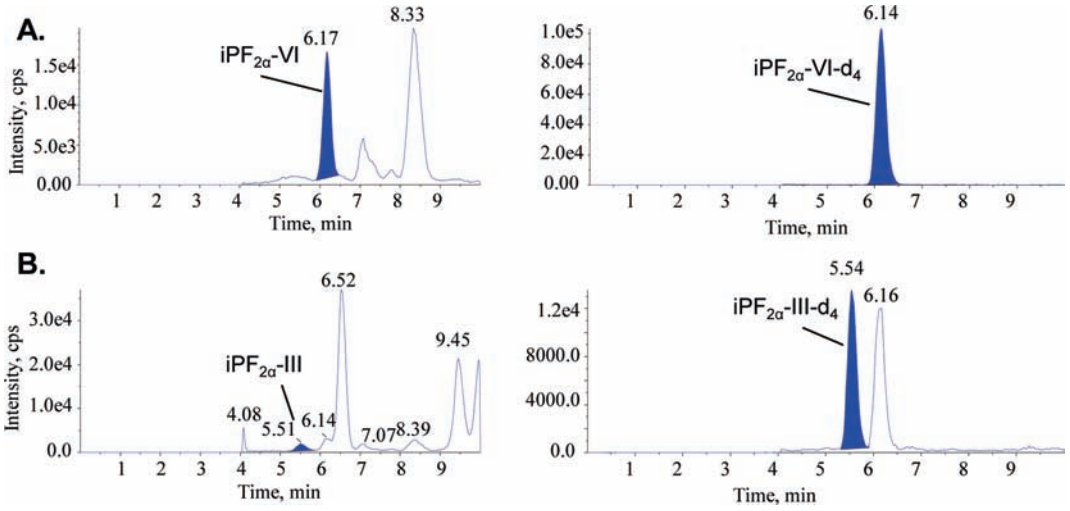

Figure 4. Representative MRM chromatograms obtained the analysis of hamster urine samples: (**A**) left, $iPF_{2\alpha}$-VI (*m*/*z* 353 → 115); right, $iPF_{2\alpha}$-VI-d_4 SIL (*m*/*z* 357 → 115); (**B**) left, $iPF_{2\alpha}$-III (*m*/*z* 353 → 193); right, $iPF_{2\alpha}$-III-d_4 (*m*/*z* 357 → 197).

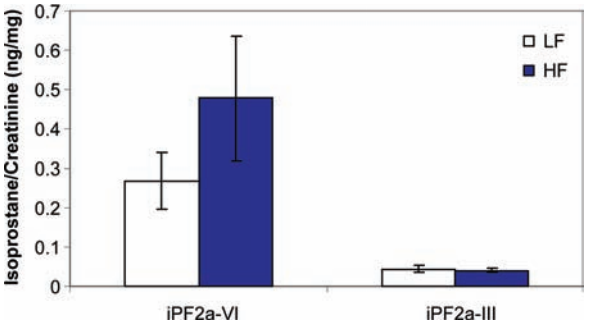

Figure 5. F_2-isoprostane concentrations in hamster urine samples following 12 weeks feeding with either a 10% kcal low-fat (LF) or a 45% kcal high-fat (HF) diet.

were calculated to be 0.104, 0.088, and 0.087 ng/mL and the LOQs were determined to be 0.348, 0.295, and 0.292 ng/mL for $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-IV, and $iPF_{2\alpha}$-VI, respectively.

The linear range was found to be 0.05−50 ng/mL. The average recoveries ranged from 73 to 115% depending upon the individual F_2-isoprostane isomers $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-IV, and $iPF_{2\alpha}$-VI in rat urine (**Table 2**). The pooled precisions based on 36 determinations over 2 days were 10.1, 10.0, and 15.8% RSD for $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-IV, and $iPF_{2\alpha}$-VI, respectively, with no significant difference between days. Overall, the assay presented in this paper has been found to perform well, showing good linearity, recovery, and sensitivity.

The developed methodology was further applied to different urine samples from different animals, including hamsters. A series of calibration standards (0.05−50 ng/mL) were analyzed, and peak areas for analytes and SILs were determined. For each $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-IV, and $iPF_{2\alpha}$-VI an internal standard quantitation was used. Similarly, a linear fit was applied to the calibration data, and analyte concentrations (ng/mL) were calculated using the linear equation. It should be noted that all of the spiked matrices have been processed in the same way, thus unifying the methodology when urine samples from different animals are screened.

Low-Fat and High-Fat Fed Hamster Study. To determine changes of the F_2-isoprostane levels induced by different diets compared to background levels, the urine isoprostane levels were measured from hamsters fed either a 10% kcal low-fat diet or a 45% kcal high-fat diet. Hamsters were fed their respective diet for 12 consecutive weeks. The concentrations of F_2-isoprostanes were measured from hamster urine (see **Figure 4**). The treatment group fed with a 45% kcal high-fat diet demonstrated an increase ($P = 0.0959$) in $iPF_{2\alpha}$-VI relative to the 10% kcal low-fat group (see **Figure 5**). However, the $iPF_{2\alpha}$-III levels did not show a significant difference when the low-fat and high-fat diets were compared. In addition, a significant correlation between $iPF_{2\alpha}$-VI and $iPF_{2\alpha}$-III was observed ($r = 0.698$, $P = 0.0006$). The concentrations of $iPF_{2\alpha}$-IV were below the LOQ in hamster urine samples. Furthermore, these results suggest that the application of the described method in a hamster study demonstrates that the detection range for at least $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI is sufficient for the determination of the basal levels within hamster urine. These preliminary results indicate that F_2-isoprostane levels can be measured using the assay method to evaluate the effects of different types of supplemented diets on oxidative stress.

In conclusion, an online SPE-LC-MS/MS method was developed for $iPGF_{2\alpha}$-III, $iPF_{2\alpha}$-IV, and $iPF_{2\alpha}$-VI in rat urine and was applied successfully to a metabolic stressed animal model. The method allowed minimal sample handling, automated analysis, and short analysis cycles for rapid data collection. In addition, the combination of pretreatment with β-glucuronidase and online SPE-LC-MS/MS analysis made it possible to determine $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI at physiological urine concentrations. Although this method seems suitable for the F_2-isoprostanes in hamster urine in this study, further method validation would be required for expanding the detection and quantification of different isomers of F_2-isoprostanes. Furthermore, the current method for F_2-isoprostanes may be adaptable to other animal models and potentially for human urine samples.

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