

Rapid Quantitative Analysis of 8-iso-PGF_{2α} Using Liquid Chromatography-Tandem Mass Spectrometry and Comparison to an Enzyme Immunoassay Method

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Short title

LC-MS-MS and Enzyme Immunoassay of 8-iso-PGF_{2α}

Abstract

A rapid liquid chromatography-tandem mass spectrometry (LC-MS-MS) assay was developed for the measurement of urinary 8-iso prostaglandin $F_{2\alpha}$ (8-iso-PGF $_{2\alpha}$), a biomarker of lipid peroxidation. Since urine contains numerous F_2 prostaglandin isomers, each of identical mass and similar mass spectrometric fragmentation patterns, chromatographic separation of 8-iso-PGF $_{2\alpha}$ from its isomers is necessary for its quantitative analysis using tandem mass spectrometry. We were able to achieve this separation using an isocratic LC method with a run time under nine minutes which is at least three-fold faster than previous methods—while maintaining sensitivity, accuracy, precision and reliability. The limits of detection and quantitation were 53 and 178 pg/mL urine, respectively. We compared our method with a commercially available affinity purification and enzyme immunoassay kit and found both assays in agreement. Despite the high sensitivity of the enzyme immunoassay method, it is more expensive and has a narrower dynamic range than LC-MS-MS. Our method was optimized for rapid measurement of 8-iso-PGF $_{2\alpha}$ in urine, and it is ideally suited for clinical sample analysis.

Keywords

Mass spectrometry, enzyme immunoassay, quantitative analysis, biomarker, 8-iso prostaglandin $F_{2\alpha}$,

Introduction

The lipid peroxidation product 8-iso prostaglandin $F_{2\alpha}$ (8-iso-PGF $_{2\alpha}$) forms from the non-enzymatic oxidation of arachidonic acid and is a biomarker of oxidative stress. Elevated 8-iso-PGF $_{2\alpha}$ levels are linked with a variety of conditions associated with oxidative stress [1-5]. In particular, smoking is strongly associated with higher 8-iso-PGF $_{2\alpha}$ levels [6]. Isoprostanes are present in all bodily tissues, blood and urine. In plasma, 8-iso-PGF $_{2\alpha}$ concentration ranges between 40–100 pg/mL [7] and exists in esterified and free forms, which can be measured separately [8], or the esterified form may be hydrolyzed for total isoprostane measurement [9,10]. One of the major limitations of measuring isoprostanes in plasma or tissues is the potential for formation of artifacts from spontaneous oxidation of arachidonic acid. Morrow *et al.* [11] found that plasma samples stored at $-20\text{ }^{\circ}\text{C}$ contained more than 50-fold higher isoprostane levels than fresh plasma. Special precautions must be taken if plasma or tissue measurements are desired.

Once formed in the body, 8-iso-PGF $_{2\alpha}$ is rapidly excreted in the urine [12]. Normal human urinary 8-iso-PGF $_{2\alpha}$ concentrations vary from 180 to 500 pg/mg creatinine or more [11,13]. In studies evaluating dietary antioxidants, measurements of urinary 8-iso-PGF $_{2\alpha}$ are often preferred to plasma or tissue measurements because there is no potential for artifactual formation, urine levels represent a time-averaged value reducing intra-subject variability, sampling is minimally invasive, and sample volume is abundant.

Because 8-iso-PGF $_{2\alpha}$ is an important biomarker for many clinical studies investigating oxidative stress or the effects of antioxidants, assays are needed that are robust, accurate, rapid, and cost-effective. The earliest methods for isoprostane analysis in urine were based on GC-MS and

required two stages of TLC purification and chemical derivitization [14]. Recognizing the need for a less cumbersome method, Ohashi *et al.* [8] developed an LC-MS assay with solid phase extraction followed by liquid-liquid extraction sample cleanup and high resolution selected ion monitoring measurement. Tandem mass spectrometry was found to increase the specificity of MS methods because many, though not all, isoprostane isomers with the same mass have different fragmentation patterns [15]. Since the type III F₂ isoprostanes which include 8-iso-PGF_{2α} have identical fragmentation patterns, chromatographic separation is essential for the quantitative analysis of 8-iso-PGF_{2α}. Taylor *et al.* [10] used a 32-minute LC gradient to separate the various 8-iso-PGF_{2α} isomers and their metabolites. Although their method used a one-step solid phase extraction sample cleanup which significantly reduced analysis time and cost, the long HPLC gradient still limited the number of samples that could be analyzed per day. Bastani *et al.* [16] shortened this gradient to 18 min, but the total run time was still 22 min plus an unspecified equilibration time.

As an alternative to mass spectrometry, enzyme immunoassays (EIAs) have been developed to measure 8-iso-PGF_{2α}, and commercially available affinity columns are used with these EIAs for sample purification. Advantages of affinity assays for the measurement of 8-iso-PGF_{2α} include high sensitivity and microtiter plate formats for rapid measurements of large numbers of samples, but they are expensive and exhibit cross-reactivity with other isoprostane isomers. For example, the manufacturer of the EIA kit we evaluated in this investigation reports 20.6% cross-reactivity towards 8-iso-PGF_{3α} and 4% to 2,3-dinor-8-iso-PGF_{2α}. EIAs have generated inconsistent results when compared with validated GC measurements [17], and the requirement for extensive sample cleanup negates some of the advantages of the otherwise high-throughput

format. In this study, a fast LC-MS-MS assay for 8-iso-PGF_{2α} was developed, validated and cross-validated by comparison to EIA.

Materials and Methods

Reagents

Standards, affinity columns (Cat. No. 10368), and immunoassay kits (Cat. No. 516351) were purchased from Cayman Chemical (Ann Arbor, MI). The standards included 8-iso-PGF_{2α}, 8-iso-15(R)-PGF_{2α}, PGF_{2α}, 15(R)-PGF_{2α}, 11β-PGF_{2α}, and the surrogate standard 8-iso-PGF_{2α}-d₄ (see Figure 1 for structure and location of deuterium labels). All solvents and other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ).

Sample Preparation

Human urine samples stored at −80 °C were thawed, vortexed, and centrifuged for 3 min at 3500 × *g* to remove particulates. Sample extraction was based on the method by Taylor *et al.* [13], with modifications. Briefly, 2 mL of 50 mM Tris-HCl buffer (adjusted to pH 6.00 (±0.05) using 50 mM Tris-base) was added to each 2.5 mL urine sample. Then, 10 ng of the surrogate standard, 8-iso-PGF_{2α}-d₄, was added along with 750 μL of methanol. For quality control and method validation, the appropriate amount of standard was spiked into a pooled urine sample. Strata X-AW (60 mg, 3 mL) SPE cartridges (Phenomenex, Torrance, CA) were preconditioned with 2 mL methanol containing 2% formic acid followed by 2 mL water. Samples were then loaded and the cartridges washed sequentially with 2 mL water, 4 mL of 25% methanol in water, and then 2 mL of 100% acetonitrile. After washing, the column was dried under vacuum and eluted with 3 × 1 mL methanol with 15 s vacuum drying between portions. The methanol eluates

were combined, evaporated to dryness, reconstituted in 50 μ L of mobile phase (described below), and centrifuged to remove particulates prior to LC-MS-MS analysis.

LC-MS-MS

LC-MS-MS analysis was carried out using a Shimadzu (Kyoto, Japan) LC-20AD system interfaced to an Applied Biosystems (Foster City, CA) API 4000 triple quadrupole mass spectrometer controlled by Analyst version 1.5 software. A Shimadzu Shim-Pack XR-ODS column (2 \times 50 mm, 2.2 μ m) was used with an isocratic flow of 2 mM aqueous ammonium acetate/acetonitrile/methanol (81:18:1, v/v/v) at 0.45 mL/min and a temperature of 45 $^{\circ}$ C. Alternatively, an Agilent Zorbax Eclipse XDBC18 (2.1 \times 50 mm, 3.5 μ m) was used at 0.45 mL/min and ambient temperature. The mobile phase was freshly and accurately prepared using volumetric glassware to ensure optimum chromatographic separation and repeatability. Negative ion electrospray was used to form deprotonated molecules of the analyte and its surrogate standard followed by collision-induced dissociation and selected reaction monitoring of the transitions m/z 353 \rightarrow 193 (8-iso-PGF_{2 α}) and m/z 357 \rightarrow 197 (surrogate standard, 8-iso-PGF_{2 α} -d₄). The mass spectrometer parameters were optimized as follows: source temperature 500 $^{\circ}$ C; spray voltage -4200 V; declustering potential -90 V; curtain gas 12; gas 1 45; entrance potential -10 V; capillary exit potential -19 V; collision energy -36 V; and a dwell time of 250 ms per ion transition.

Affinity purification and EIA

To compare the performance of the new LC-MS-MS method to a commercially available affinity purification and EIA kit, a set of identical urine samples was prepared and assayed by SPE followed by LC-MS-MS as above or by affinity purification followed by EIA. Sample prepa-

ration and analysis for EIA was carried out in accordance with the manufacturer's instructions. Briefly, aliquots of a pooled urine sample spiked with 0, 50, 100, 200, and 500 pg/mL were applied to a 20 mL size affinity column equilibrated with 4 mL buffer included in the kit. Next, columns were washed with 10 mL column buffer followed by 10 mL water. Columns were eluted with 2×5 mL elution solution (ethanol/water, 95:5, v/v), and the eluent was evaporated under a stream of nitrogen. Samples were reconstituted in 100 μ L methanol and split into two equal portions. The first portion was dried and reserved for LC-MS-MS measurement to determine whether the affinity column retained any cross-reacting isoprostanes. The second portion was reconstituted and diluted 400-fold with EIA buffer provided in the immunoassay kit. Finally, 50 μ L aliquots of the diluted samples were analyzed in duplicate using the EIA kit as directed. To test affinity purified samples for the presence of cross-reacting isoprostane isomers, the affinity purified pooled urine sample was analyzed by using LC-MS with negative ion electrospray with scanning of the range m/z 300 to 400, which is the expected mass range for F_2 isoprostane isomers.

Validation

Method validation was carried out in accordance with FDA guidelines for bioanalytical method validation [18]. Since all urine contains isoprostanes, seven point calibration curves were prepared from pooled urine using the method of standard addition [19] over the range of 50 pg/mL to 10 ng/mL 8-iso-PGF_{2 α} spiked into urine. Intraday precision was determined by measuring four replicates each at low, intermediate, and high concentration, and interday precision was measured by comparing the same set of samples analyzed on three different days. Recovery was measured by spiking known amounts of 8-iso-PGF_{2 α} into pooled urine samples

and comparing against unextracted standards representing 100% recovery. Absence of matrix effects was confirmed by injecting a prepared pooled urine sample onto the LC column and infusing a post-column addition of 2.5 µg/mL 8-iso-PGF_{2α} at 100 µL/min.

Results and Discussion

A rapid, sensitive, and accurate LC-MS-MS assay was developed for the determination of 8-iso-PGF_{2α} in urine. Calibration curves were linear over the range of 50 pg/mL to 10 ng/mL 8-iso-PGF_{2α} spiked into urine ($r^2 > 0.99$). A typical calibration curve is shown in the supplementary data. The sensitivity and reproducibility of our method are comparable with published LC-MS-MS methods [13,16]. The limit of detection (signal-to-noise of 3:1) was 53 pg/mL 8-iso-PGF_{2α} in urine and the lower limit of quantitation (signal-to-noise of 10:1) was 178 pg/mL in urine. The inter-day precision and intra-day precision as well as accuracy were tested at three concentrations, and the results are reported in Table 1. The recoveries at low, intermediate, and high concentrations (0.1, 1.0 and 10 ng/mL) were 90%, 83%, and 79%, respectively. 8-iso-PGF_{2α} was found to be stable in urine by Morrow et al.[21] who found no change in 8-iso-PGF_{2α} concentration after a one week incubation of samples at 37 °C. To test the stability of the analyte after extraction, standard solutions of 8-iso-PGF_{2α} in the HPLC mobile phase were measured after being allowed to stand at room temperature for one week. Measured concentrations were $\geq 95\%$ of the starting concentration, indicating stability at room temperature for at least one week. Nevertheless, sample preparation and analysis was usually carried out within 24 h. As shown in Figure 3, absence of a matrix effect was confirmed.

Because long gradient separations would be cost-prohibitive for large numbers of samples such

as those collected during clinical trials, our method offers significant advantages over previous mass spectrometry-based methods without sacrificing chromatographic resolution. Using a chromatography column packed with 2.2 μm particles instead of the more typical 3.5 to 5 μm particles used in previous methods, the separation efficiency was improved and the run time reduced by use of a carefully selected combination of mobile phase, column length, temperature, and flow rate. In comparison to the method of Taylor *et al.*, [10, 13] which uses a 32 min gradient followed by 5 min column equilibration, our method fully resolves the major F_2 isoprostane peaks in less than 10 min (Figure 2). There is no column equilibration in our method because HPLC separations use an isocratic mobile phase. Our method does not require ultra-high performance liquid chromatography (UHPLC) equipment and can be run on standard HPLC hardware.

In addition to 8-iso-PGF_{2 α} , LC-MS-MS methods have been developed to measure related species such as 2,3-dinor-8-iso-PGF_{2 α} with the hypothesis that such measurements might offer additional information about lipid peroxidation levels. Although detailed measurements of many isoprostane isomers and their metabolites have been made [10,15,20], only 8-iso-PGF_{2 α} has gained wide acceptance as a lipid peroxidation biomarker. There are no commercially available stable isotope labeled standards for many of these metabolites, and chromatographic separation of the full spectrum of metabolites would have to be made at the expense of speed and efficiency of 8-iso-PGF_{2 α} , which is the target of most antioxidant investigations. For expediency, we have optimized our LC-MS-MS method for 8-iso-PGF_{2 α} measurement.

Comparison to Immunoassay Method

Commercial enzyme immunoassays, alone or in combination with affinity purification, are avail-

able for 8-iso-PGF_{2α} measurement. For urine samples, the manufacturer of the EIA kit used for comparison in this study recommends either TLC followed by solid phase extraction for sample cleanup or affinity column purification. Here, affinity purification was used followed by EIA to cross-validate our LC-MS-MS based method. A set of pooled urine samples spiked with 0, 50, 100, 200, and 500 pg/mL were analyzed by either SPE followed by LC-MS-MS or affinity purification followed by EIA, and the results are compared in Table 2. The values we obtained by EIA are in agreement with the LC-MS-MS values within the measurement error.

8-iso-PGF_{2α} affinity sorbents have been reported to cross-react with other isoprostane isomers. To determine whether the affinity purification or EIA we used could be affected by cross-reactivity, we analyzed the affinity purified samples by LC-MS for the presence of various cross-reacting isoprostane isomers. Using LC-MS, no signals were observed corresponding to the deprotonated molecules of PGF_{2α}, 8-iso-PGF_{3α} (*m/z* 351), PGF_{1α} (*m/z* 355) or 2,3-dinor-8-iso-PGF_{2α} (*m/z* 325) which are reported by the immunoassay manufacturer to cross react with 8-iso-PGF_{2α}. These observations indicate that, for the pooled urine sample tested, known cross-reacting isoprostanes did not interfere with the EIC, even though 2,3-dinor-8-iso PGF_{2α} and PGF_{2α}, for example, are normally present in urine. It should be noted that these cross-reacting isoprostane isomers do not interfere with our LC-MS-MS assay because the masses, retention times, or both did not overlap with the analyte 8-iso-PGF_{2α}. The possibility remains that samples might sometimes contain high enough levels of these cross-reacting isoprostanes to cause interference with the EIA.

Despite the potential for cross-reactivity, the 8-iso-PGF_{2α} affinity columns were effective in removing contaminants from the urine samples. Our LC-MS-MS analysis of affinity column

purified samples showed low background and only a single peak in the chromatogram. If affinity columns were used in place of the usual weak anion exchange SPE, then the sensitivity and speed of the subsequent LC-MS-MS assay could be dramatically improved. However, the cost of affinity columns is several-fold higher than SPE columns, and this cost disadvantage will probably prohibit their use for the analysis of large numbers of samples.

Measurement of urinary 8-iso-PGF_{2α} using LC-MS-MS has several advantages when compared to EIA. Although both methods require sample preparation, the weak anion exchange SPE columns used in our new LC-MS-MS method are less expensive than the affinity purification columns used for EIA. Additionally we found that SPE columns could be used up to four times each without carryover or loss of column capacity while the manufacturer of the 8-iso-PGF_{2α} affinity columns recommends only single use for urine samples. Although sensitive, the linear portion of the standard curve for the EIA is narrower than its usable range of 2–500 pg/mL. Therefore, careful dilution is required to bring the sample concentration into this range. Stable isotope labeled surrogate standards may be used with SPE to correct for sample loss, matrix effect, or instrument variability whereas such standards interfere with affinity methods. Lastly, the cross-reactivity of the affinity reagents might lead to an overestimation of 8-iso-PGF_{2α} concentration in the EIA method. Our LC-MS-MS method addresses these limitations of the EIA assay.

Conclusions

We have developed an LC-MS-MS assay for measurement of 8-iso-PGF_{2α} in urine, which is the most common lipid peroxidation biomarker used in clinical antioxidant studies. Compared with

existing 8-iso-PGF_{2α} assays, our new assay increases the speed and lowers the cost per analysis while maintaining accuracy, precision, and sensitivity. By chromatographically resolving 8-iso-PGF_{2α} from its isomers in less than ten minutes, this new assay provides more accurate measurements than LC-MS-MS assays that do not resolve 8-iso-PGF_{2α} from all of its isomers. Our assay is more than three-fold faster than previously published LC-MS-MS methods which require 32-minute and 22-minute gradients in addition to the time necessary for column equilibration. The use of isocratic chromatography during our LC-MS-MS method saves time by eliminating the need for column re-equilibration between runs, and yet complete chromatographic separation of 8-iso-PGF_{2α} from its isomers is achieved in less than 10 minutes. Although we did not detect in our samples any cross-reacting isoprostanes that could lead to an overestimation of 8-iso-PGF_{2α} when using EIA, our LC-MS-MS assay eliminates this risk by chromatographically resolving these compounds and distinguishing them by mass and by MS-MS. We are applying this new assay to measure changes in urinary 8-iso-PGF_{2α} in 150 human subjects in an ongoing clinical intervention with lycopene, the tomato carotenoid and antioxidant thought to offer protection from prostate cancer. With this new 8-iso-PGF_{2α} assay, this important lipid peroxidation biomarker can be measured accurately, quickly, and cost effectively.

Acknowledgement

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Figure Legends

Figure 1: LC-MS-MS analysis of standards showing chromatographic separation. The deuterium labeled surrogate standard is shown in the bottom chromatogram.

Figure 2: LC-MS-MS chromatogram of a typical urine sample. Top: 8-iso-15(R)-PGF_{2α}, 3.3 min; 8-iso-PGF_{2α}, 3.8 min; 15(R)-PGF_{2α}, 4.9 min; PGF_{2α}, 7.0 min; and bottom: 8-iso-PGF_{2α}-d₄, 3.8 min. The peaks were identified by using co-injection with authentic standards.

Figure 3: Injections of blank (top) and a prepared pooled urine sample (bottom) with LC-MS-MS analysis and post-column infusion of 8-iso-PGF_{2α} confirm the absence of matrix effect.

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Standards, affinity columns (Cat. No. 10368), and immunoassay kits (Cat. No. 516351) were purchased from Cayman Chemical (Ann Arbor, MI). The standards included 8-iso-PGF_{2α}, 8-iso-15(R)-PGF_{2α}, PGF_{2α}, 15(R)-PGF_{2α}, 11β-PGF_{2α}, and the surrogate standard 8-iso-PGF_{2α}-d₄ (see Figure 1 for structure and location of deuterium labels). All solvents and other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ).

Sample Preparation

Human urine samples stored at −80 °C were thawed, vortexed, and centrifuged for 3 min at 3500 × *g* to remove particulates. Sample extraction was based on the method by Taylor *et al.* [13], with modifications. Briefly, 2 mL of 50 mM Tris-HCl buffer (adjusted to pH 6.00 (±0.05) using 50 mM Tris-base) was added to each 2.5 mL urine sample. Then, 10 ng of the surrogate standard, 8-iso-PGF_{2α}-d₄, was added along with 750 μL of methanol. For quality control and method validation, the appropriate amount of standard was spiked into a pooled urine sample. Strata X-AW (60 mg, 3 mL) SPE cartridges (Phenomenex, Torrance, CA) were preconditioned with 2 mL methanol containing 2% formic acid followed by 2 mL water. Samples were then loaded and the cartridges washed sequentially with 2 mL water, 4 mL of 25% methanol in water, and then 2 mL of 100% acetonitrile. After washing, the column was dried under vacuum and eluted with 3 × 1 mL methanol with 15 s vacuum drying between portions. The methanol eluates

were combined, evaporated to dryness, reconstituted in 50 μ L of mobile phase (described below), and centrifuged to remove particulates prior to LC-MS-MS analysis.

LC-MS-MS

LC-MS-MS analysis was carried out using a Shimadzu (Kyoto, Japan) LC-20AD system interfaced to an Applied Biosystems (Foster City, CA) API 4000 triple quadrupole mass spectrometer controlled by Analyst version 1.5 software. A Shimadzu Shim-Pack XR-ODS column (2 \times 50 mm, 2.2 μ m) was used with an isocratic flow of 2 mM aqueous ammonium acetate/acetonitrile/methanol (81:18:1, v/v/v) at 0.45 mL/min and a temperature of 45 $^{\circ}$ C. Alternatively, an Agilent Zorbax Eclipse XDBC18 (2.1 \times 50 mm, 3.5 μ m) was used at 0.45 mL/min and ambient temperature. The mobile phase was freshly and accurately prepared using volumetric glassware to ensure optimum chromatographic separation and repeatability. Negative ion electrospray was used to form deprotonated molecules of the analyte and its surrogate standard followed by collision-induced dissociation and selected reaction monitoring of the transitions m/z 353 \rightarrow 193 (8-iso-PGF_{2 α}) and m/z 357 \rightarrow 197 (surrogate standard, 8-iso-PGF_{2 α} -d₄). The mass spectrometer parameters were optimized as follows: source temperature 500 $^{\circ}$ C; spray voltage -4200 V; declustering potential -90 V; curtain gas 12; gas 1 45; entrance potential -10 V; capillary exit potential -19 V; collision energy -36 V; and a dwell time of 250 ms per ion transition.

Affinity purification and EIA

To compare the performance of the new LC-MS-MS method to a commercially available affinity purification and EIA kit, a set of identical urine samples was prepared and assayed by SPE followed by LC-MS-MS as above or by affinity purification followed by EIA. Sample prepa-

ration and analysis for EIA was carried out in accordance with the manufacturer's instructions. Briefly, aliquots of a pooled urine sample spiked with 0, 50, 100, 200, and 500 pg/mL were applied to a 20 mL size affinity column equilibrated with 4 mL buffer included in the kit. Next, columns were washed with 10 mL column buffer followed by 10 mL water. Columns were eluted with 2×5 mL elution solution (ethanol/water, 95:5, v/v), and the eluent was evaporated under a stream of nitrogen. Samples were reconstituted in 100 μ L methanol and split into two equal portions. The first portion was dried and reserved for LC-MS-MS measurement to determine whether the affinity column retained any cross-reacting isoprostanes. The second portion was reconstituted and diluted 400-fold with EIA buffer provided in the immunoassay kit. Finally, 50 μ L aliquots of the diluted samples were analyzed in duplicate using the EIA kit as directed. To test affinity purified samples for the presence of cross-reacting isoprostane isomers, the affinity purified pooled urine sample was analyzed by using LC-MS with negative ion electrospray with scanning of the range m/z 300 to 400, which is the expected mass range for F_2 isoprostane isomers.

Validation

Method validation was carried out in accordance with FDA guidelines for bioanalytical method validation [18]. Since all urine contains isoprostanes, seven point calibration curves were prepared from pooled urine using the method of standard addition [19] over the range of 50 pg/mL to 10 ng/mL 8-iso-PGF_{2 α} spiked into urine. Intraday precision was determined by measuring four replicates each at low, intermediate, and high concentration, and interday precision was measured by comparing the same set of samples analyzed on three different days. Recovery was measured by spiking known amounts of 8-iso-PGF_{2 α} into pooled urine samples

and comparing against unextracted standards representing 100% recovery. Absence of matrix effects was confirmed by injecting a prepared pooled urine sample onto the LC column and infusing a post-column addition of 2.5 µg/mL 8-iso-PGF_{2α} at 100 µL/min.

Results and Discussion

A rapid, sensitive, and accurate LC-MS-MS assay was developed for the determination of 8-iso-PGF_{2α} in urine. Calibration curves were linear over the range of 50 pg/mL to 10 ng/mL 8-iso-PGF_{2α} spiked into urine ($r^2 > 0.99$). A typical calibration curve is shown in the supplementary data. The sensitivity and reproducibility of our method are comparable with published LC-MS-MS methods [13,16]. The limit of detection (signal-to-noise of 3:1) was 53 pg/mL 8-iso-PGF_{2α} in urine and the lower limit of quantitation (signal-to-noise of 10:1) was 178 pg/mL in urine. The inter-day precision and intra-day precision as well as accuracy were tested at three concentrations, and the results are reported in Table 1. The recoveries at low, intermediate, and high concentrations (0.1, 1.0 and 10 ng/mL) were 90%, 83%, and 79%, respectively. 8-iso-PGF_{2α} was found to be stable in urine by Morrow et al.[21] who found no change in 8-iso-PGF_{2α} concentration after a one week incubation of samples at 37 °C. To test the stability of the analyte after extraction, standard solutions of 8-iso-PGF_{2α} in the HPLC mobile phase were measured after being allowed to stand at room temperature for one week. Measured concentrations were $\geq 95\%$ of the starting concentration, indicating stability at room temperature for at least one week. Nevertheless, sample preparation and analysis was usually carried out within 24 h. As shown in Figure 3, absence of a matrix effect was confirmed.

Because long gradient separations would be cost-prohibitive for large numbers of samples such

as those collected during clinical trials, our method offers significant advantages over previous mass spectrometry-based methods without sacrificing chromatographic resolution. Using a chromatography column packed with 2.2 μm particles instead of the more typical 3.5 to 5 μm particles used in previous methods, the separation efficiency was improved and the run time reduced by use of a carefully selected combination of mobile phase, column length, temperature, and flow rate. In comparison to the method of Taylor *et al.*, [10, 13] which uses a 32 min gradient followed by 5 min column equilibration, our method fully resolves the major F_2 isoprostane peaks in less than 10 min (Figure 2). There is no column equilibration in our method because HPLC separations use an isocratic mobile phase. Our method does not require ultra-high performance liquid chromatography (UHPLC) equipment and can be run on standard HPLC hardware.

In addition to 8-iso-PGF_{2 α} , LC-MS-MS methods have been developed to measure related species such as 2,3-dinor-8-iso-PGF_{2 α} with the hypothesis that such measurements might offer additional information about lipid peroxidation levels. Although detailed measurements of many isoprostane isomers and their metabolites have been made [10,15,20], only 8-iso-PGF_{2 α} has gained wide acceptance as a lipid peroxidation biomarker. There are no commercially available stable isotope labeled standards for many of these metabolites, and chromatographic separation of the full spectrum of metabolites would have to be made at the expense of speed and efficiency of 8-iso-PGF_{2 α} , which is the target of most antioxidant investigations. For expediency, we have optimized our LC-MS-MS method for 8-iso-PGF_{2 α} measurement.

Comparison to Immunoassay Method

Commercial enzyme immunoassays, alone or in combination with affinity purification, are avail-

able for 8-iso-PGF_{2α} measurement. For urine samples, the manufacturer of the EIA kit used for comparison in this study recommends either TLC followed by solid phase extraction for sample cleanup or affinity column purification. Here, affinity purification was used followed by EIA to cross-validate our LC-MS-MS based method. A set of pooled urine samples spiked with 0, 50, 100, 200, and 500 pg/mL were analyzed by either SPE followed by LC-MS-MS or affinity purification followed by EIA, and the results are compared in Table 2. The values we obtained by EIA are in agreement with the LC-MS-MS values within the measurement error.

8-iso-PGF_{2α} affinity sorbents have been reported to cross-react with other isoprostane isomers. To determine whether the affinity purification or EIA we used could be affected by cross-reactivity, we analyzed the affinity purified samples by LC-MS for the presence of various cross-reacting isoprostane isomers. Using LC-MS, no signals were observed corresponding to the deprotonated molecules of PGF_{2α}, 8-iso-PGF_{3α} (*m/z* 351), PGF_{1α} (*m/z* 355) or 2,3-dinor-8-iso-PGF_{2α} (*m/z* 325) which are reported by the immunoassay manufacturer to cross react with 8-iso-PGF_{2α}. These observations indicate that, for the pooled urine sample tested, known cross-reacting isoprostanes did not interfere with the EIC, even though 2,3-dinor-8-iso PGF_{2α} and PGF_{2α}, for example, are normally present in urine. It should be noted that these cross-reacting isoprostane isomers do not interfere with our LC-MS-MS assay because the masses, retention times, or both did not overlap with the analyte 8-iso-PGF_{2α}. The possibility remains that samples might sometimes contain high enough levels of these cross-reacting isoprostanes to cause interference with the EIA.

Despite the potential for cross-reactivity, the 8-iso-PGF_{2α} affinity columns were effective in removing contaminants from the urine samples. Our LC-MS-MS analysis of affinity column

purified samples showed low background and only a single peak in the chromatogram. If affinity columns were used in place of the usual weak anion exchange SPE, then the sensitivity and speed of the subsequent LC-MS-MS assay could be dramatically improved. However, the cost of affinity columns is several-fold higher than SPE columns, and this cost disadvantage will probably prohibit their use for the analysis of large numbers of samples.

Measurement of urinary 8-iso-PGF_{2α} using LC-MS-MS has several advantages when compared to EIA. Although both methods require sample preparation, the weak anion exchange SPE columns used in our new LC-MS-MS method are less expensive than the affinity purification columns used for EIA. Additionally we found that SPE columns could be used up to four times each without carryover or loss of column capacity while the manufacturer of the 8-iso-PGF_{2α} affinity columns recommends only single use for urine samples. Although sensitive, the linear portion of the standard curve for the EIA is narrower than its usable range of 2–500 pg/mL. Therefore, careful dilution is required to bring the sample concentration into this range. Stable isotope labeled surrogate standards may be used with SPE to correct for sample loss, matrix effect, or instrument variability whereas such standards interfere with affinity methods. Lastly, the cross-reactivity of the affinity reagents might lead to an overestimation of 8-iso-PGF_{2α} concentration in the EIA method. Our LC-MS-MS method addresses these limitations of the EIA assay.

Conclusions

We have developed an LC-MS-MS assay for measurement of 8-iso-PGF_{2α} in urine, which is the most common lipid peroxidation biomarker used in clinical antioxidant studies. Compared with

existing 8-iso-PGF_{2α} assays, our new assay increases the speed and lowers the cost per analysis while maintaining accuracy, precision, and sensitivity. By chromatographically resolving 8-iso-PGF_{2α} from its isomers in less than ten minutes, this new assay provides more accurate measurements than LC-MS-MS assays that do not resolve 8-iso-PGF_{2α} from all of its isomers. Our assay is more than three-fold faster than previously published LC-MS-MS methods which require 32-minute and 22-minute gradients in addition to the time necessary for column equilibration. The use of isocratic chromatography during our LC-MS-MS method saves time by eliminating the need for column re-equilibration between runs, and yet complete chromatographic separation of 8-iso-PGF_{2α} from its isomers is achieved in less than 10 minutes. Although we did not detect in our samples any cross-reacting isoprostanes that could lead to an overestimation of 8-iso-PGF_{2α} when using EIA, our LC-MS-MS assay eliminates this risk by chromatographically resolving these compounds and distinguishing them by mass and by MS-MS. We are applying this new assay to measure changes in urinary 8-iso-PGF_{2α} in 150 human subjects in an ongoing clinical intervention with lycopene, the tomato carotenoid and antioxidant thought to offer protection from prostate cancer. With this new 8-iso-PGF_{2α} assay, this important lipid peroxidation biomarker can be measured accurately, quickly, and cost effectively.

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Figure Legends

Figure 1: LC-MS-MS analysis of standards showing chromatographic separation. The deuterium labeled surrogate standard is shown in the bottom chromatogram.

Figure 2: LC-MS-MS chromatogram of a typical urine sample. Top: 8-iso-15(R)-PGF_{2α}, 3.3 min; 8-iso-PGF_{2α}, 3.8 min; 15(R)-PGF_{2α}, 4.9 min; PGF_{2α}, 7.0 min; and bottom: 8-iso-PGF_{2α}-d₄, 3.8 min. The peaks were identified by using co-injection with authentic standards.

Figure 3: Injections of blank (top) and a prepared pooled urine sample (bottom) with LC-MS-MS analysis and post-column infusion of 8-iso-PGF_{2α} confirm the absence of matrix effect.

Figure 1

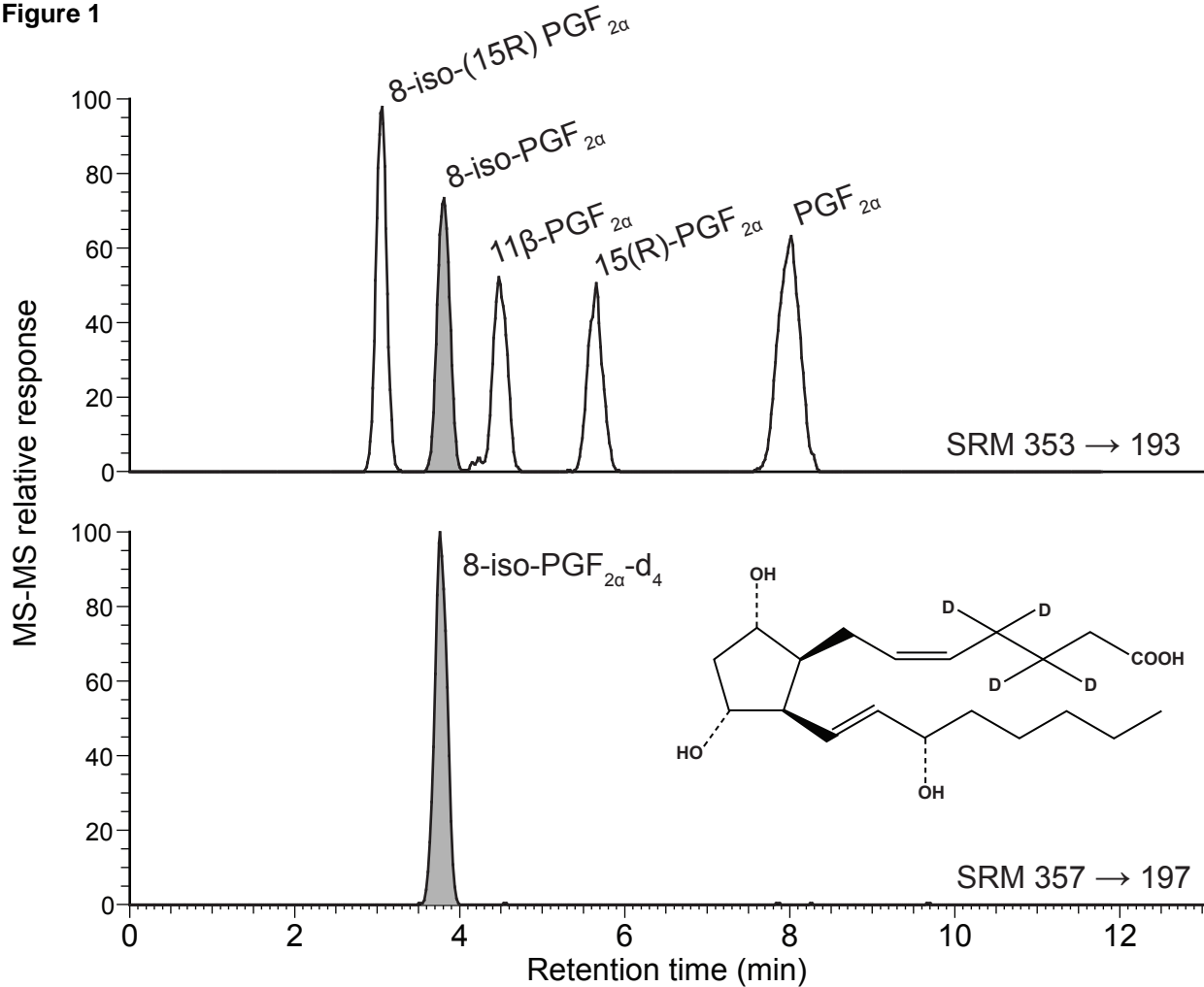
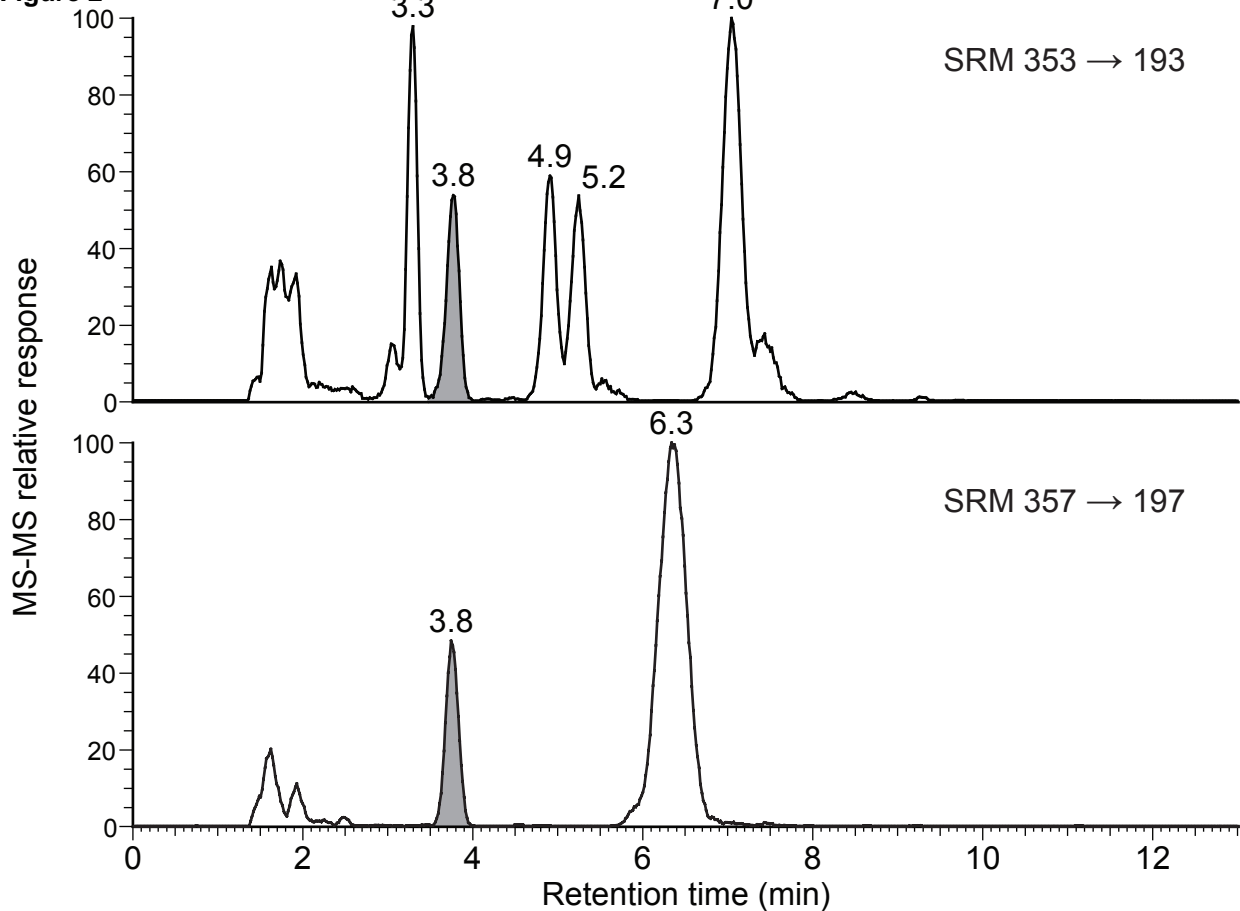


Figure 2



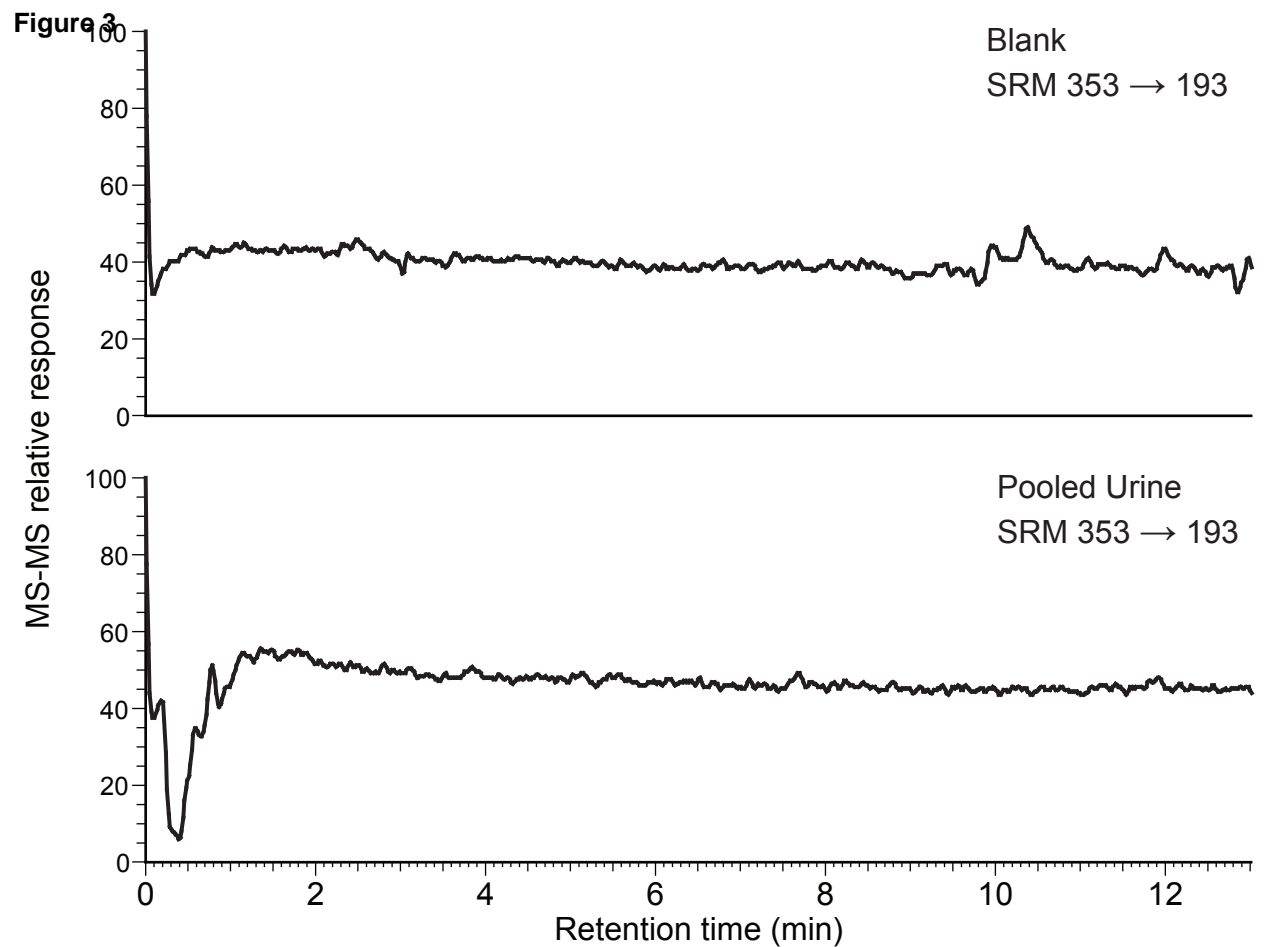


Table 1: Interday and intraday reproducibility of the LC-MS-MS quantitation of 8-iso-PGF_{2α} in urine

Concentration (ng/mL)		Intraday(n=4)		Interday (n=12)	
Expected	Measured (means, n=12)	%CV	Accuracy%	%CV	Accuracy %
0.1	0.11	15.8	108	16.1	112
1.0	0.98	4.7	96.8	7.8	98.9
10.0	10.12	5.3	103	4.4	101

Table 2: Comparison of SPE-purified urine analyzed by LC-MS-MS or affinity-purified urine analyzed by EIA for measurement of 8-iso-PGF_{2α}. Values are the mean of two replicates.

8-iso-PGF _{2α} spiked (pg/mL)	LC-MS-MS	EIA
0	382	369
50	460	477
100	517	580
200	652	793
500	960	1025

