Quantification of F_2-isoprostanes as a biomarker of oxidative stress

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Oxidant stress has been implicated in a wide variety of disease processes. One method to quantify oxidative injury is to measure lipid peroxidation. Quantification of a group of prostaglandin $F_{2\alpha}$-like compounds derived from the nonezymatic oxidation of arachidonic acid, termed the F_2-isoprostanes (F_2-IsoPs), provides an accurate assessment of oxidative stress both *in vitro* and *in vivo*. In fact, in a recent independent study sponsored by the National Institutes of Health (NIH), F_2-IsoPs were shown to be the most reliable index of *in vivo* oxidant stress when compared against other well known biomarkers. This protocol details our laboratory's method to quantify F_2-IsoPs in biological fluids and tissues using gas chromatography-mass spectrometry (GC-MS). This procedure can be completed for 12–15 samples in 6–8 h.

INTRODUCTION

Free radicals, largely derived from molecular oxygen, have been implicated in a variety of human conditions and diseases, including atherosclerosis and associated risk factors, cancer, neurodegenerative diseases and aging. Damage to tissue biomolecules by free radicals is postulated to be a major contributor to the pathophysiology of oxidative stress[1,2]. Measuring oxidative stress in humans requires accurate quantification of either free radicals or damaged biomolecules. The targets of free radical–mediated oxidant injury include lipids, proteins and DNA. A number of methods exist to quantify free radicals and their oxidation products, although many of these techniques suffer from lack of sensitivity and specificity, especially when used to assess oxidant stress status *in vivo*. In the Biomarkers of Oxidative Stress Study (BOSS), a recent multiinvestigator study sponsored by the National Institutes of Health (NIH), it was found that the most accurate method to assess *in vivo* oxidant stress status is the quantification of plasma or urinary F_2-isoprostanes (F_2-IsoPs)[3]. First discovered by our laboratory in 1990 (ref. 4), F_2-IsoPs, also referred to as 8-iso-$PGF_{2\alpha}$, are a series of prostaglandin $F_{2\alpha}$-like compounds produced by the free radical–catalyzed peroxidation of arachidonic acid independent of the cyclooxygenase. **Figure 1** shows the structures of arachidonic acid and these oxidation products.

F_2-IsoPs are stable, robust molecules detectable in all human tissues and biological fluids analyzed, including plasma, urine, bronchoalveolar lavage fluid, cerebrospinal fluid and bile[5]. The quantification of F_2-IsoPs in urine and plasma, however, is most convenient and least invasive. And, based on available data, quantification of these compounds in either plasma or urine is representative of their endogenous production and thus gives a highly precise and accurate index of *in vivo* oxidant stress. Analysis of these compounds in urine in particular is an index of systemic or 'whole body' oxidant stress integrated over time. (The measurement of free F_2-IsoPs in urine can be confounded by the potential contribution of local IsoP production in the kidney, although the extent to which this occurs is unclear. In light of this issue, we have identified the primary urinary metabolite of 15-F_{2t}-IsoP to be 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP, and we have developed a highly sensitive and accurate mass spectrometric assay to quantify this molecule[6]. Thus, the quantification of 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP might represent a truly noninvasive, time-integrated measurement of systemic oxidation status that can be applied to living subjects.)

Normal levels of F_2-IsoPs in healthy humans have been defined (**Table 1**)[5,7,8]. Defining these levels is particularly important in that it allows for an assessment of the effects of diseases on endogenous oxidant tone and permits the determination of the extent to which various therapeutic interventions affect levels of oxidant stress. Elevations of F_2-IsoPs in human body fluids and tissues have been found in a diverse array of human disorders, some of which include atherosclerosis, hypercholesterolemia, diabetes, obesity, cigarette smoking, neurodegenerative diseases, rheumatoid arthritis and many others. Furthermore, treatments for some of these conditions, including antioxidant supplementation, antidiabetic treatments, cessation of smoking and even weight loss, have been shown to decrease production of F_2-IsoPs. Thus, the clinical utility of F_2-IsoPs has been great and continues to grow in importance.

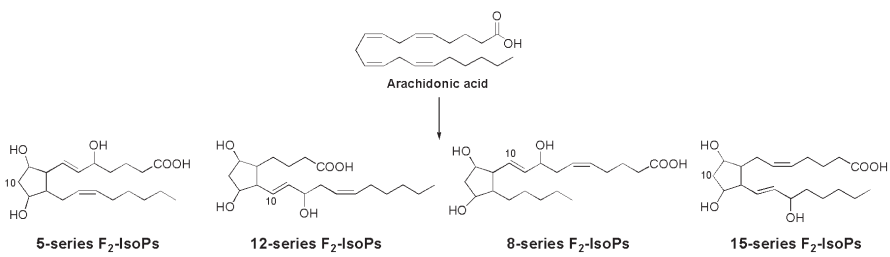

Figure 1 | Structures of arachidonic acid and of its oxidation products, 5-series F_2-IsoPs, 12-series F_2-IsoPs, 8-series F_2-IsoPs, and the analyte measured in this protocol, 15-series F_2-IsoPs.

Table 1 | Basal levels of free F_2-IsoPs in various body fluids and tissues from normal humans.

Body fluid	Level (mean ± 1 s.d.)
Plasma	35 ± 6 pg ml^{-1}
Urine	1.6 ± 0.6 ng mg^{-1} creatinine
Cerebrospinal fluid	23 ± 1 pg ml^{-1}

Several methods have been developed to quantify the F_2-IsoPs. Our laboratory uses a gas chromatography–negative ion chemical ionization–mass spectrometry (GC-NICI-MS) approach employing stable isotope dilution that will be detailed herein[5]. For quantification purposes, we measure the F_2-IsoP, 15-F_{2t}-IsoP and other F_2-IsoPs that co-elute with this compound. The advantages of this technique over other approaches include its high sensitivity and specificity, which yields quantitative results in the low picogram range. Its drawbacks are that it is labor intensive and requires considerable expenditures on equipment.

Note that different investigators, including FitzGerald and colleagues, have developed several alternative mass spectrometric assays[9–11]. Like our assay, these methods quantify F_2-IsoPs using stable isotope dilution GC-NICI-MS and require solid-phase extraction using a C18 column, thin-layer chromatography (TLC) purification and chemical derivatization. These assays, however, measure F_2-IsoP isomers other than 15-F_{2t}-IsoP but are comparable to ours in terms of utility. In addition to these gas chromatography–mass spectrometry (GC-MS) assays, a number of liquid chromatography–mass spectrometry (LC-MS) methods for F_2-IsoPs have been developed. One advantage of LC-MS methods is that the sample preparation for analysis is simpler than that for GC-MS because it requires no derivatization of the molecule. The method reported earlier this year by Taylor and colleagues is the first of these LC-MS methods to be validated for quantitation of IsoPs in biological fluids[12]. A concern with LC-MS assays, however, relates to the limits of detection in biological fluids, which are often higher than those employing GC-MS.

Alternative methods also have been developed to quantify IsoPs using immunological approaches[5,13]. Antibodies have been generated against 15-F_{2t}-IsoP, and at least three immunoassay kits are available commercially. A potential drawback of these methods is that information regarding their precision and accuracy is currently limited. In addition, little data exist comparing IsoP levels determined by immunoassay to MS. Analogous to immunological methods to quantify cyclooxygenase-derived PGs, it might be predicted that immunoassays for IsoPs will suffer from a lack of specificity. Furthermore, the sensitivity and/or specificity of these kits might vary substantially between manufacturers. Although mass spectrometric methods of IsoP quantification are considered the best methods for analysis, immunoassays have expanded research in this area due to their low cost and relative ease of use.

Experimental design

As can be noted from this protocol, quantifying F_2-IsoPs using this methodology is complicated. Further, arachidonic acid in biological samples is susceptible to *ex vivo* oxidation, which can generate F_2-IsoPs. In the processing of plasma samples and tissues samples, which both contain an abundance of arachidonic acid, take care when the samples are obtained and during processing to prevent *ex vivo* oxidation. For best results, samples should be flash frozen in liquid nitrogen immediately upon collection and not thawed until analysis. For those interested in setting up this assay in their lab, investigators should first undertake studies with unoxidized control samples to ensure that F_2-IsoPs are not generated during sample processing. Also, each investigator should determine assay precision and accuracy before beginning routine sample analysis. Finally, note that experienced and trained personnel are required to set up and operate the GC-MS.

MATERIALS

REAGENTS

- [$^2H_4$]-15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) internal standard (Cayman Chemical, cat. no. 316351)
- 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) (Cayman Chemical, cat. no. 16350)
- Butylated hydroxytoluene (BHT) (Sigma-Aldrich, cat. no. B1378)
- Pentafluorobenzyl bromide (PFBB) (Sigma-Aldrich, cat. no. 10105-2)
- N,N′-Diisopropylethylamine (DIPE) (Sigma-Aldrich, cat. no. D3887)
- N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, cat. no. 33084)
- Dimethyformamide (DMF) (Sigma-Aldrich, cat. no. 6407) (store over calcium hydride to prevent water accumulation)
- Undecane (Sigma-Aldrich) (store over calcium hydride to prevent water accumulation)
- Ultrapure water (triply distilled or its equivalent)
- Methanol
- Chloroform (containing ethanol as a preservative)
- Ethyl acetate
- Heptane
- Acetonitrile
- Ethanol
- HCl (American Chemical Society certified or equivalent grade)
- Sodium chloride
- Potassium hydroxide pellets
- Anhydrous sodium sulfate
- 10% Phosphomolybdic acid in ethanol (Sigma-Aldrich, cat. no. P4869)

EQUIPMENT

- React-a-vials with Teflon-lined cap (Supelco, cat. no. 33299)
- Blade homogenizer-PTA 10S generator (Brinkman)
- Nitrogen gas
- C-18 plus solid-phase extraction (or Sep-Pak) cartridges; each cartridge contains 500 mg of C-18 (Waters, cat. no. WAT036575)
- Silica plus solid-phase extraction (or Sep-Pak) cartridges; each cartridge contains 500 mg of silica (Waters, cat. no. WAT036580)
- Disposable plastic syringes (10 ml) (Laboratory Supply, cat. no. SMJ512878)
- Glass scintillation vials (20 ml)
- TLC plates: LK6D silica, marked lanes, glass backed (Whatman, cat. no. WC486562IV)
- Dessicator
- Glass TLC tank (11.5 in w × 10 in h × 3.5 in d)
- TLC paper, cut to 11 in w × 9 in h (VWR, cat. no. 28298-020)
- Capillary GC column (DB-1701, Agilent, cat. no. 21512067)
- GC-MS with capabilities for NICI-MS

REAGENT SETUP

pH 3 water (0.002N HCl) Mix 40 ml 1N hydrochloric acid with 20 L of water.

Folch solution Combine 2 volumes of chloroform with 1 volume of methanol. Dissolve BHT crystals in solution to make a final concentration of 0.005% BHT (wt/vol). Cool to 4 °C and store solution in the dark in a brown bottle to prevent light degradation.

PFBB solution Dilute PFBB to 10% (vol/vol) in dry acetonitrile. Store over calcium hydride to keep solution free of water. **! CAUTION** PFBB is a potent lachrymator. Do not work outside of a well-ventilated fume hood.

DIPE solution Dilute DIPE to 10% (vol/vol) in acetonitrile. Store over calcium hydride to keep solution free of water.

Internal standard The internal standard [$^2H_4$]-15-F_{2t}-IsoP is quantified using a 5-point calibration curve. Each sample contains 0.50 ng of 15-F_{2t}-IsoP, which has been accurately quantified by weighing, and either 0.05 ng, 0.25 ng, 0.50 ng, 2.50 ng or 5.00 ng of [$^2H_4$]-15-F_{2t}-IsoP. Actual [$^2H_4$]-15-F_{2t}-IsoP/15-F_{2t}-IsoP ratios are compared with the expected ratios to quantify the [$^2H_4$]-15-F_{2t}-IsoP.

TLC standard, 15-F_{2t}-IsoP methyl ester Dissolve 1 mg of 15-F_{2t}-IsoP (fatty acid) in 50 ml of dry methanol (solution will be clear). Methylate by adding dry diazomethane dropwise into the solution until the solution is a permanent yellow color. Allow to stand 5 min. Dry solution under a stream of nitrogen. For use, dilute to a concentration 1 mg ml^{-1} in 3/2 chloroform/methanol (vol/vol).

EQUIPMENT SETUP

TLC plate preparation Prewash all TLC plates with a solution of ethyl acetate and ethanol (90:10, vol/vol). One hour before using plates, dry in a 90 °C oven for 10 min and then cool in a dessicator before sample application.

GC-MS setup For quantification of F_2-IsoPs by GC-MS, we routinely use an Agilent 5973 mass spectrometer with a computer interface, but other mass spectrometers can be utilized. The F_2-IsoPs are chromatographed on a 15-meter DB1701 fused silica capillary column because we have found this column gives excellent separation of individual regioisomers compared to other columns. The column temperature is programmed from 190 °C to 300 °C at 20 °C per mm. Methane is used as the reagent gas, and helium is used as the carrier gas for NICI. The ion source temperature is 200 °C. The ion monitored for endogenous F_2-IsoPs is the carboxylate anion *m/z* 569 (M-181, loss of $CH_2C_6F_5$). The corresponding carboxylate anion for the deuterated intemal standard is *m/z* 573. Each day the sensitivity of the mass spectrometer is checked by injecting a standard consisting of 40 pg each $PGF_{2\alpha}$ and [$^2H_4$]-15-F_{2t}-IsoP. Note that $PGF_{2\alpha}$ elutes at a sufficiently different retention time from the F_2-IsoPs quantified using this procedure. Therefore, this cyclooxygenase-derived prostaglandin does not interfere with the signal of the non-cyclooxygenase-derived IsoPs.

PROCEDURE

Sample preparation

1| F_2-IsoPs can be quantified in biological samples from a variety of sources to afford an accurate index of *in vivo* oxidant stress status. In plasma, the present methodology measures only free fatty acid. Although other methodologies can quantify esterified F_2-IsoPs, with values obtained for free F_2-IsoPs added to that for esterified F_2-IsoPs to measure total F_2-IsoPs in plasma, we have found no advantage in measuring total rather than free F_2-IsoPs. Measuring F_2-IsoPs in a 24-h urine collection also represents a reliable probe of the oxidant stress status. With regard to tissue samples, given that F_2-IsoPs from biological sources can only be quantified as free compounds using GC-MS (Morrow *et al.*[7]), to measure levels of F_2-IsoPs esterified in glycerophospholipids in tissues, the glycerophospholipids must first be extracted from the sample and then be subjected to alkaline hydrolysis to release free F_2-IsoPs as described below in option C. We recommend preparing blood, urine and tissue samples for F_2-IsoPs quantitation by following the steps in options A, B and C, respectively.

(A) Plasma

(i) Collect blood (5–10 ml) in a tube containing EDTA. (An example of an appropriate collection tube is cat. no. BD367844 from Laboratory Supply Company. This is a 4 ml tube containing 7.2 mg of EDTA.)

(ii) Centrifuge 10 min at 4,000g to yield plasma. 2–3 ml of plasma is necessary for the quantification of F_2-IsoPs.
■ **PAUSE POINT** Store samples at –80 °C until analysis.

(iii) For analysis, after thawing, dilute plasma in 7 ml of pH 3 water and acidify to pH 3 with 1N HCl.
▲ **CRITICAL STEP** As plasma contains a large amount of arachidonic acid, which can oxidize *ex vivo* to generate F_2-IsoPs, the samples should be frozen at –80 °C immediately. It is imperative that samples not be thawed before analysis.

(B) Urine

(i) Volunteers should collect all urine voided during a 24-h period in a sterile container, storing it at 4 °C between collections.
■ **PAUSE POINT** Store sample at –80 °C until analysis.

(ii) For analysis, after thawing, dilute 0.250 ml urine in 10 ml pH 3 water and acidify to pH 3 with 1N HCl.

(C) Tissue samples

(i) Weigh out 50–100 mg of either fresh tissue or frozen tissue.
▲ **CRITICAL STEP** Arachidonic acid in tissue samples can oxidize *ex vivo* to generate F_2-IsoPs. If tissue is not to be used immediately after collection, flash freeze the sample in liquid nitrogen and store at –80 °C.

(ii) Add tissue to 20 ml of ice-cold Folch solution in a 50 ml centrifuge tube with cap. (If sample is <15 mg, reduce the volume of Folch solution to 10 ml.)
▲ **CRITICAL STEP** Polypropylene tubes are recommended because polystyrene is not resistant to chloroform. Keep on ice.

(iii) Homogenize tissue with blade homogenizer at full speed for 30 s or until fully homogenized.

(iv) Flush centrifuge tube with a stream of nitrogen or argon for 30–60 s to remove air from tube, then cap. Let solution stand at room temperature (22–25 °C) for 1 h to allow maximal extraction of lipids from ground tissue. Shake tube occasionally for several seconds during this period of time.

(v) Add 4 ml aqueous NaCl (0.9%) prepared in ultrapure water. Vortex or shake vigorously for 1 min at room temperature (22–25 °C). (If tissue sample is < 15 mg, reduce volume of NaCl solution to 2 ml.)

(vi) Centrifuge for 10 min in a tabletop centrifuge at room temperature (22–25 °C) to separate aqueous and organic layers. Following centrifugation, a semisolid protein layer should have formed between the upper (aqueous) and lower (organic) layers.

(vii) After centrifugation, carefully pipette off the top aqueous layer and discard. Carefully remove the lower organic layer from under the intermediate semisolid protein layer and transfer it to a 50 ml conical tube. Evaporate under nitrogen stream in an analytical evaporation unit until dry.

(viii) Resuspend lipids in 4 ml methanol containing 0.005% BHT and vortex. Next, add 4 ml aqueous KOH (15%, wt/vol). (If sample originally weighed <15 mg, lipid extract can be resuspended in 1 ml methanol to which 1 ml KOH is added.) Vortex, purge flask with nitrogen and cap. Incubate mixture at 37 °C for 20 min.

(ix) After incubation, acidify the mixture to pH 3 with 1N HCl and dilute the mixture to 80 ml with pH 3 water.
▲ **CRITICAL STEP** It is important to dilute the methanol in this solution to 5% or less to ensure proper extraction of F_2-IsoPs in the subsequent purification procedure. It is also important to continue with the steps under 'Sample purification for mass spectrometric analysis'. The sample should not be stored in this form because of the potential for oxidation.

Sample purification for mass spectrometric analysis

2| To sample accurately add 1 ng of the internal standard, [$^2H_4$]-15-F_{2t}-IsoP, and vortex.
▲ **CRITICAL STEP** To ensure accuracy, always add internal standard with an accurate syringe rather than a pipetteman.

3| Connect one C-18 Sep-Pak cartridge to a 10 ml disposable syringe per sample and precondition each cartridge with 5 ml methanol and 7 ml pH 3 water.

4| Apply acidified lipid mixture to the preconditioned Sep-Pak cartridge.
▲ **CRITICAL STEP** It is important that samples be corrected to pH 3 or slightly below in order for the carboxylate moiety to be protonated. If not protonated, the compounds will not chromatograph correctly on the Sep-Pak cartridges.
▲ **CRITICAL STEP** Care must be taken to avoid loss of IsoPs when applying the sample to the cartridge. The sample should be pushed through the column at ~1–2 ml per min, such that individual drops emerge from the Sep-Pak. Slightly more vigorous fluid flow is acceptable during the subsequent wash and elution steps, but a steady stream is not recommended.

5| Wash cartridge, first with 10 ml pH 3 water and then with 10 ml heptane.

6| Elute F_2-IsoPs from the cartridge with 10 ml ethyl acetate/heptane (50:50, vol/vol) into a glass scintillation vial.

7| Add 5 g anhydrous sodium sulfate to the vial and swirl gently for 10 s. This step removes residual water from the eluant. Decant eluant into another scintillation vial away from the sodium sulfate.

8| Remove used C-18 Sep-Pak cartridges from syringes and discard. Connect one silica Sep-Pak cartridge for each syringe per sample and precondition cartridge with 5 ml ethyl acetate.

9| Apply eluant from C-18 Sep-Pak cartridge to silica Sep-Pak cartridge in the same manner as in Step 4.

10| Wash the cartridge with 5 ml ethyl acetate, then elute F_2-IsoPs from the silica Sep-Pak cartridge with 5 ml ethyl acetate/methanol (1:1, vol/vol) into a react-a-vial.

11| Evaporate eluant under nitrogen in the analytical evaporation unit.

12| Convert F_2-IsoPs to the corresponding pentaflourobenzyl esters (**Fig. 2**). (This derivatization facilitates compound analysis by GC-MS). Add 40 µL of 10% (vol/vol) PFBB in acetonitrile and 20 µL 10% (vol/vol) DIPE in acetonitrile to the reactivial, vortex briefly and incubate for 20 min at 37 °C.
! **CAUTION** PFBB is a potent lachrymator. Do not work outside of a well-ventilated fume hood.

13| Prepare TLC tank by adding 97 ml chloroform, 3 ml ethanol and TLC paper to saturate the tank. Allow the tank to equilibrate for 30 min.

14| Dry sample thoroughly under nitrogen in an analytical evaporation unit in a fume hood and resuspend material in 50 µL methanol. Vortex briefly.

15| Apply mixture from each sample to a prewashed silica TLC plate. Be sure to apply only one sample per lane on each plate. Using a separate TLC plate, apply ~2–5 µg of the methyl ester of $PGF_{2\alpha}$ to one lane for use as a standard.

▲ **CRITICAL STEP** Avoid applying sample to the first 1 cm of the plate.

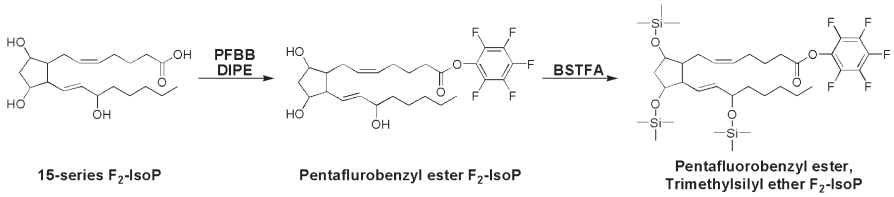

Figure 2 | Derivatization of 15-series F_2-isoprostanes for GC-MS analysis.

16| After ensuring that the application solvent has dried, place TLC plates into the TLC tank and chromatograph. When the solvent front reaches 13 cm on the TLC plate, remove from tank and allow solvent to evaporate. Visualize the TLC standard by spraying the standard plate with the phosphomolybdic acid solution and then heating on a hot plate. (Do not spray sample plates.)

17| Scrape silica from the sample TLC plates in the region of the TLC standard (R_f should be ~0.18), scraping from 1 cm above the middle of the visualized standard to 1 cm below the standard (**Fig. 3**). Retain plates until after GC-MS analysis.

18| Place scraped silica from each sample into separate microcentrifuge tubes and add 1 ml ethyl acetate to each. Vortex vigorously for 30 s to extract analytes from the silica, then centrifuge in a benchtop microcentrifuge at 13,000 r.p.m. for 3 min.

19| Carefully remove the ethyl acetate, taking care not to disrupt the silica pellet in the bottom of the tube, and place in a second microcentrifuge tube.

20| Dry under nitrogen, then add 20 µL BSTFA and 7 µL dry DMF to residue to convert to the trimethylsilyl ether derivatives (**Fig. 2**).

21| Vortex well and incubate sample at 37 °C for 20 min. Dry reagents under nitrogen.

■ **PAUSE POINT** Samples can also incubate in BSTFA and DMF at room temperature (22–25 °C) overnight.

22| Resuspend sample in 20 µL dry undecane and vortex briefly. Transfer sample to an autosampler vial for GC-MS analysis.

23| A representative chromatogram obtained from the analysis of F_2-IsoPs in plasma is shown in **Figure 4**. For quantification purposes, we compare the height of the peak containing the derivatized 15-F_{2t}-IsoP (*m/z* 569, peak labeled with * in **Fig. 4**) with the height of the deuterated internal standard peak (*m/z* 573). Levels of F_2-IsoPs in plasma are reported in picograms or nanograms per milliliter, whereas levels in tissues are reported in nanograms per gram of tissue. Levels of F_2-IsoPs in urine are normalized to creatinine clearance and reported as nanograms per milligrams of creatinine.

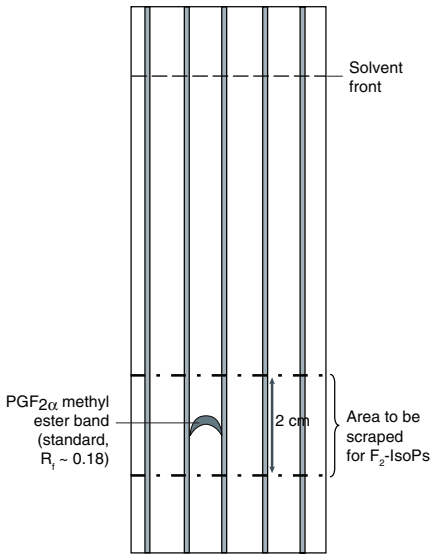

Figure 3 | Thin layer chromatography analysis of the methyl ester of $PGF_{2\alpha}$, the TLC standard, visualized with phosphomolybdic acid. Indicated on the plate are the areas to be scraped to extract F_2-IsoPs.

● TIMING

In general, 12–15 samples per day can be processed by an experienced investigator. Lipid extraction and hydrolysis of this number of tissue samples requires ~3 h. Sep-Pak purification takes ~1.5 h; drying, derivatization and TLC purification require ~2 h; and extraction, drying and silylation require 1 h. Mass spectrometric analysis is automated, and each sample requires ~15 min of instrument time.

? TROUBLESHOOTING

If the peak signal is extremely low or if no peaks are detected by the mass spectrometer, the sample should be removed from the autosampler vial, dried thoroughly under nitrogen and Steps 20–22 should be repeated. If peaks are still not detected, it is possible that there was a problem with the thin-layer

chromatography. Scrape all silica from the bottom half of the lane, place in microcentrifuge tube and extract with ethyl acetate as described in Step 17. Continue by repeating Steps 18–22. If no result is obtained, it will be necessary to repeat the entire analysis with new sample.

If the internal standard is detected at *m/z* 573 but there are low or nonexistent peaks at *m/z* 569, then levels of F_2-IsoPs are below the limit of detection. The limit of detection for this assay is ~5 pg.

ANTICIPATED RESULTS

Figure 4 shows the selected ion current chromatogram obtained from the analysis of F_2-IsoPs in human plasma. The peaks in the upper *m/z* 569 selected ion current chromatogram represents different endogenous F_2-IsoPs. This pattern of peaks is virtually identical to that obtained from all other biological fluids and tissues that we have examined to date. The single peak in the lower *m/z* 573 chromatogram represents the $[^2H_4]$-15-F_{2t}-IsoP internal standard. For quantification purposes, the peak denoted by an asterisk (*), which co-elutes with the internal standard, is routinely measured. The concentration of F_2-IsoPs is calculated using the ratio of the intensity of this peak to that of the internal standard. In this sample, the concentration of F_2-IsoPs was calculated to 203 pg ml^{-1}.

This method of the quantification of F_2-IsoPs is highly precise and accurate. The precision is ±6 % and the accuracy is 96%.

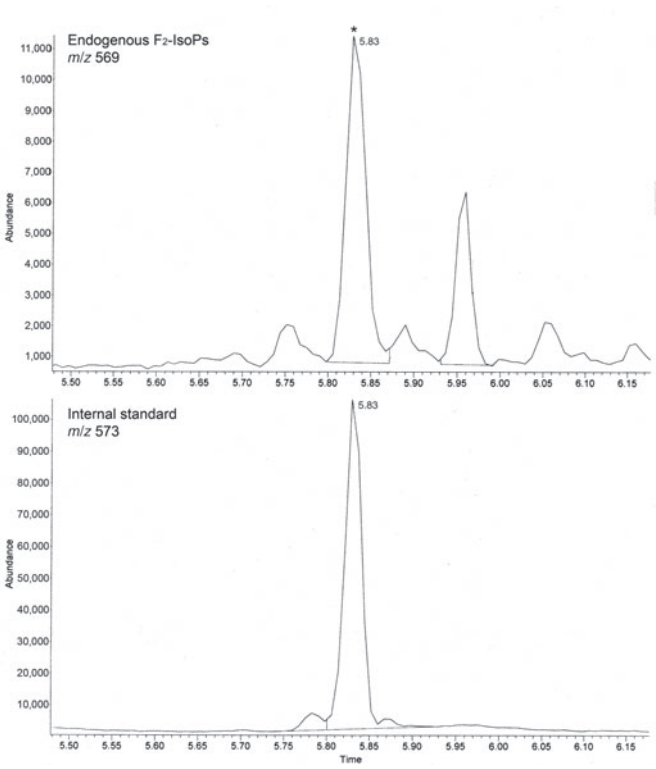

Figure 4 | Analysis of F_2-IsoPs in human plasma. The *m/z* 573 ion current chromatogram represents the $[^2H_4]$-15-F_{2t}-IsoP internal standard. The *m/z* 569 ion current chromatogram represents endogenous F_2-IsoPs. The peak in the upper chromatogram represented by the asterisk (*) is the one routinely used for quantification of the F_2-IsoPs.

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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