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Quantification of F_2-isoprostanes as a reliable index of oxidative stress *in vivo* using gas chromatography–mass spectrometry (GC-MS) method

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ABSTRACT

Free radical-induced lipid peroxidation has been implicated in a number of human diseases including atherosclerosis, cancer, and neurodegenerative diseases. F_2-Isoprostanes (IsoPs) are isomers of prostaglandin $PGF_{2\alpha}$ that are generated *in vivo* from the free radical-initiated peroxidation of arachidonic acid independent of cyclooxygenase enzymes. Since the discovery of the IsoPs in the early 1990s, a large body of evidence has been accumulated to indicate that quantification of these F_2-IsoPs represents the most reliable biomarker to assess oxidative stress *in vivo*. A variety of analytical approaches have been developed for the quantification of these novel compounds; these methods include mass spectrometry (MS) detection coupled to gas chromatography (GC) or liquid chromatography (LC) separation, and detection using immunological approaches. This article summarizes our current methodology to quantify F_2-IsoPs in biological fluids and tissues using GC-MS. This method includes solid-phase extraction (SPE), thin-layer chromatography (TLC) purification, chemical derivatization, and MS detection using negative ion chemical ionization (NICI) coupled with GC. The protocol described herein has been optimized and validated to provide the best sensitivity and selectivity for quantification of F_2-IsoPs from a variety of biological sources.

Introduction

Free radical-induced autooxidation of polyunsaturated fatty acids (PUFAs) has been linked to numerous human disorders, including atherosclerosis, cancer, neurodegenerative diseases, and aging [1–5]. Isoprostanes (IsoPs) are a series of prostaglandin (PG)-like compounds that are formed nonenzymatically *in vivo* via the peroxidation of arachidonic acid by a free radical-initiated mechanism [6–8]. An important aspect of the formation of IsoPs is that they are generated *in situ* from arachidonic acid esterified in phospholipids and are subsequently released by phospholipases, such as platelet-activating factor (PAF) acetylhydrolases [9]. On the other hand, the PGs are generated from the oxidation of free arachidonic acid by cyclooxygenases (COXs) [10]. Over the past two decades, quantification of F_2-IsoPs, isomers of $PGF_{2\alpha}$, has emerged as one of the most accurate approaches to assess oxidant injury *in vivo* [11,12]. In fact, in the Biomarkers of Oxidative Stress Study (BOSS) sponsored by the National Institute of Health (NIH), F_2-IsoPs were shown to be the most accurate biomarker to assess *in vivo* oxidant stress status when compared against other well-known biomarkers [13]. In addition to F_2-IsoPs, D_2/E_2-IsoPs which contain E/D prostane rings analogous to COX-derived PGD_2 and PGE_2, and isomers of thromboxane, termed isothromboxanes (IsoTxs), are also generated in significant amounts *in vivo* [14,15]. Some IsoPs exert potent biological activity by acting as ligands for either plasma membrane bound PG receptors or for nuclear receptors and thus are likely also mediators of oxidant injury [16–18].

F_2-IsoPs are stable and robust molecules that are detectable in all human tissues and biological fluids analyzed, including plasma, urine, bronchoalveolar lavage (BAL) fluid, cerebrospinal fluid (CSF), and bile. The quantification of F_2-IsoPs in urine and plasma is most convenient and least invasive. Several methods have been developed to analyze F_2-IsoPs; these include chromatographic separation involving solid-phase extraction (SPE) or affinity chromatography with or without thin-layer chromatography (TLC) followed by final determination by gas chromatography–mass spectrometry (GC-MS), liquid chromatography (LC)–MS, or enzyme immunoassay [19–24]. GC–negative-ion chemical ionization mass spectrometric (GC/NICI-MS) employing stable isotope dilution is the preferred method for

Abbreviations: AA, arachidonic acid; BAL, bronchoalveolar lavage; BHT, butylated hydroxyl toluene; BOSS, Biomarkers of Oxidative Stress Study; BSTFA, *N,O*-bis (trimethylsilyl)-trifluoroacetamide; COX, cyclooxygenase; DMF, dimethylformamide; DIPE, diisopropylethylamine; GC, gas chromatography; IsoPs, isoprostanes; IsoTx, isothromboxane; LC, liquid chromatography; MS, mass spectrometry; NICI, negative-ion chemical ionization; NIH, National Institute of Health; PAF, platelet-activating factor; PFB, pentafluorobenzyl; PFBB, pentafluorobenzyl bromide; PGs, prostaglandins; PPh_3, triphenylphosphine; PUFA, polyunsaturated fatty acid; SIM, selective-ion monitoring; SPE, solid-phase extraction; TLC, thin-layer chromatography; TMS, trimethylsilyl; Tx, thromboxanes.

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the quantification of F_2-IsoPs and several alternative GC-MS assays have been developed by different investigators including FitzGerald and colleagues [25]. For quantification purposes, we measure 15-F_{2t}-IsoP (also known as 8-Iso $PGF_{2\alpha}$), and other F_2-IsoPs that coelute with this compound. The advantages of GC-MS include the high resolution of GC separation on fused silica capillary columns and the specificity and sensitivity of MS, which yield quantitative results in the low picogram range [26,27].

A substantial body of evidence indicates that measurement of F_2-IsoPs in body fluids provides a reliable approach to assess lipid peroxidation *in vivo*. 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 [24,28,29]. Normal levels of F_2-IsoPs in healthy humans have been defined [28–30]. Defining these levels is particularly important in that it allows for an assessment of the effects of diseases on endogenous oxidant tone and allows for the determination of the extent to which various therapeutic interventions affect levels of oxidant stress. Elevations of IsoPs in human body fluids and tissues have been found in a diverse array of human disorders, including atherosclerosis, diabetes, obesity, cigarette smoking, neurodegenerative diseases, and many others [31]. Further, 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 [32].

Principles

As has been noted, F_2-IsoPs are formed nonenzymatically as a result of the free radical-mediated peroxidation of arachidonic acid. The mechanism outlined in Fig. 1 is based on the generation of bicyclic endoperoxide intermediates [33]. Arachidonic acid initially undergoes hydrogen atom abstraction by a free radical at the three bisallylic positions of C7, C10, or C13 to yield a delocalized pentadienyl carbon-centered radical. Subsequently, there is an insertion of molecular oxygen to yield peroxyl radicals. These peroxyl radicals undergo further 5-exo cyclization, followed by the addition of another molecule of oxygen to yield the bicyclic endoperoxide (PGG_2-like) [34]. These intermediates are then reduced to form F_2-IsoPs. Based on this mechanism, four F_2-IsoP regioisomers are generated. These are named the 5-, 8-, 12-, and 15-series F_2-IsoPs, based on the location of the side chain hydroxyl group in the final product [35,36]. Each series contains 16 possible diastereomers and a total of 64 stereoisomers of F_2-IsoPs can be generated.

The F_2-IsoPs are initially formed esterified to phospholipids after the reduction of the bicyclic endoperoxides and the GC-MS can only analyze the derivatives of free F_2-IsoPs. A derivatization protocol has been developed to make the samples suitable for GC-MS analysis (Fig. 2). Both esterified and free F_2-IsoPs can be measured. To analyze the esterified F_2-IsoPs in tissue samples, the F_2-IsoPs are first released under basic conditions. After addition of deuterated internal standard, solid-phase extraction is carried out using both C18 and silica Sep-Pak to remove the contaminants. Pentafluorobenzyl (PFB) moiety is then introduced to the molecule to enhance the sensitivity of detection using electron capture chemical ionization techniques [37]. The last step is to cap the hydroxyl groups by trimethylsilyl (TMS) derivatization. Analysis of the derivatives of F_2-IsoPs and the internal standard is carried out using selective-ion monitoring (SIM) techniques; the ions monitored are m/z 569 for F_2-IsoPs and m/z 573 for the internal standard respectively. The detailed procedures for the purification and derivatization steps will be described in the Protocol section.

Materials

1. Deuterated international standard [$^2H_4$]-15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$-d_4) (Cayman Chemical, Cat. No. 316351)

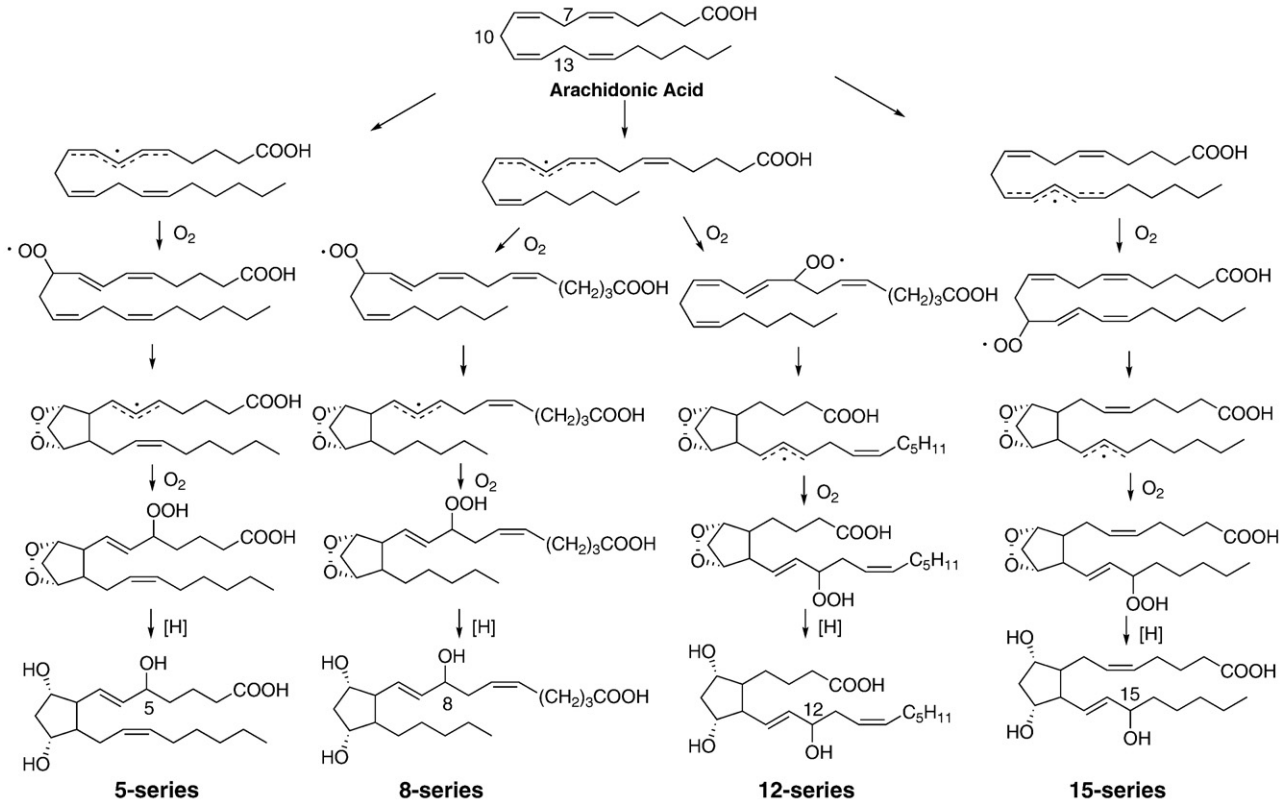

Fig. 1. Mechanism of formation of F_2-isoprostanes. Four regioisomers are formed each consisting of 16 stereoisomers.

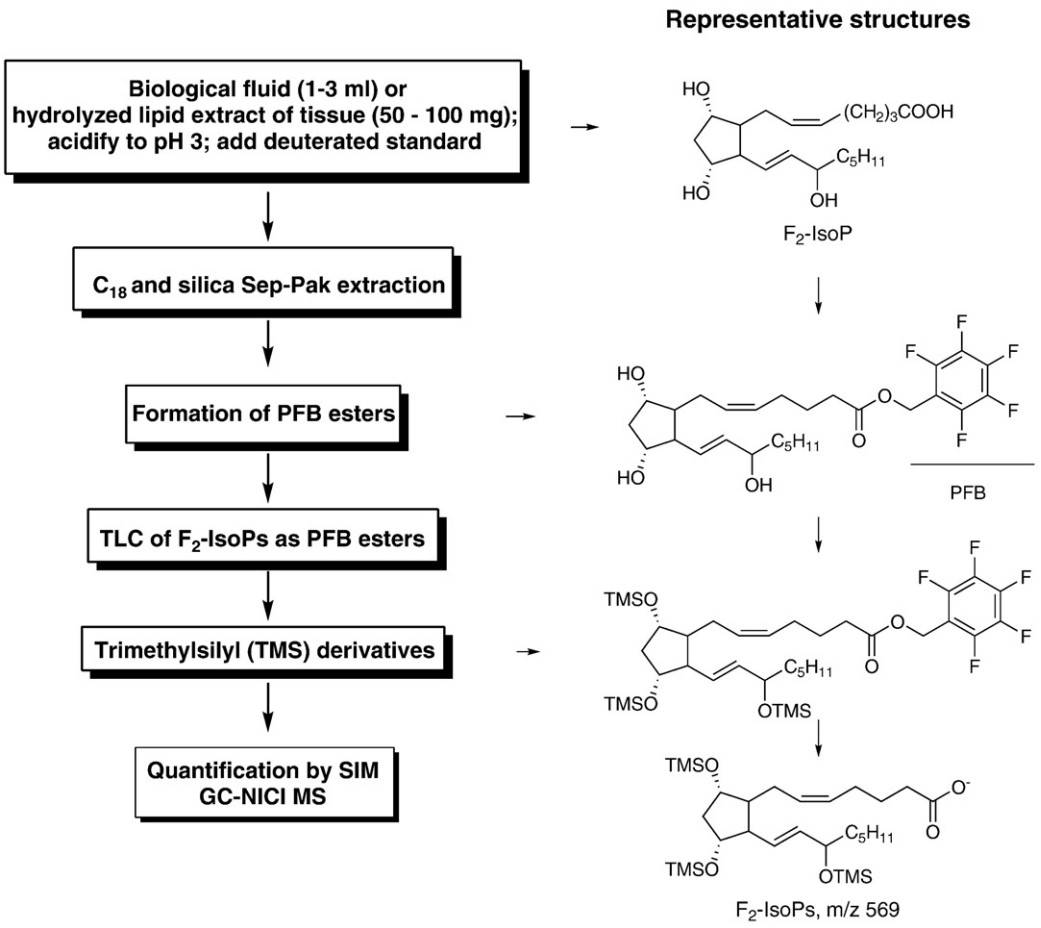

Fig. 2. Outline of the procedures used for the extraction, purification, derivatization, and mass spectrometric analysis of F_2-IsoPs from biological sources. The representative chemical structures for each derivatization step are also included.

2. TLC standard, $PGF_{2\alpha}$ methyl ester (Cayman Chemical, Cat. No. 16011)
3. Butylated hydroxytoluene (BHT) (Sigma-Aldrich, Cat. No. B1378)
4. Triphenylphosphine (PPh_3) (Sigma-Aldrich, Cat. No. 93090 Fluka)
5. Pentafluorobenzyl bromide (PFBB) (Sigma-Aldrich, Cat. No. 10105-2)
6. *N*,*N*′-Diisopropylethylamine (DIPE) (Sigma-Aldrich, Cat. No. D3887)
7. N,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco., Cat. No. 33084)
8. Dimethylformamide (DMF) (Sigma-Aldrich, Cat. No. 6407) (store over calcium hydride to remove water)
9. Undecane (Sigma-Aldrich) (store over calcium hydride to prevent water accumulation)
10. Ultrapure water (triply distilled or its equivalent)
11. Methanol
12. Chloroform (containing ethanol as a preservative)
13. Ethyl acetate
14. Heptane
15. Acetonitrile
16. Ethanol
17. HCl (American Chemical Society certified or equivalent grade)
18. Sodium chloride
19. Potassium hydroxide pellets
20. Anhydrous sodium sulfate
21. 10% Phosphomolybdic acid in ethanol (Sigma-Aldrich, Cat. No. P4869)
22. Nitrogen gas

Stock solutions

1. pH 3 water (0.001 N HCl): Mix 20 ml 1 N hydrochloric acid with 20 L of water
2. Folch solution: Combine 2 vol of chloroform with 1 vol of methanol. Dissolve BHT and PPh_3 in solution to make a final concentration of 0.005% BHT (wt/vol) and 0.25 mg/ml PPh_3. The solution is stored at 4°C in the dark in a brown bottle to prevent light degradation.
3. PFBB solution: Dilute PFBB to 10% (vol/vol) in dry acetonitrile. Store over calcium hydride to keep solution free of water.
4. DIPE solution: Dilute DIPE to 10% (vol/vol) in acetonitrile. Store over calcium hydride to keep solution free of water.
5. TLC standard, $PGF_{2\alpha}$ methyl ester: Dissolve 1 mg of $PGF_{2\alpha}$ methyl ester in 1 ml of chloroform/methanol (3:2, vol/vol) to make a 1 mg/ml solution and stored at -20°C.

Instrumentation

1. Blade homogenizer-PTA 10 S generator (Brinkmann Instruments, Westbury, NY)
2. C-18 solid-phase extraction (or Sep-Pak) cartridges; each cartridge contains 500 mg of C-18 (Waters, Cat. No. WAT036575)
3. Silica solid-phase extraction (or Sep-Pak) cartridges; each cartridge contains 500 mg of silica (Waters, Cat. No. WAT036580)
4. TLC plates: LK6D silica, marked lanes, glass backed (Whatman, Cat. No. WC486562IV)
5. Capillary GC column (DB-1701, Agilent, Cat. No. 21512067)
6. GC-MS with capabilities for NICI-MS

7. TLC plate preparation: Prewash all TLC plates with a solution of ethyl acetate and ethanol (90:10, vol/vol). One hour before using plates, drying at 90°C oven for 10 min and then cool in a desiccator before sample application.
8. 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 separated on a 15-m DB1701 fused silica capillary column that gives good separation of F_2-IsoPs compared to other columns. The column temperature is programmed from 190 to 300°C at 20°C per minute. Methane is used as the reagent gas, and helium is used as the carrier gas for NICI. The ion source temperature is set at 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 internal standard is m/z 573. The sensitivity of the mass spectrometer is checked every day 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 times from the F_2-IsoPs quantified using this procedure. Therefore, this COX-derived $PGF_{2\alpha}$ does not interfere with the signal of the F_2-IsoPs.

Protocol

General precautions. Arachidonic acid in biological samples is susceptible to free radical oxidation and thus prevention of the *ex vivo* oxidation is crucial in the processing and storage of the samples especially plasma and tissue samples that contain abundant arachidonic acid. For the best results, samples should be flash-frozen in liquid nitrogen immediately on collection and not thawed until analysis. BHT and PPh_3 are added to the Folch solution to prevent *ex vivo* oxidation during the extraction of lipids.

Extraction and hydrolysis of F_2-IsoP-containing phospholipids in tissues and biological fluids

Levels of F_2-IsoPs in a variety of biological sources can be measured as an accurate index of *in vivo* oxidant stress status. Although free or esterified F_2-IsoPs (bound) can be quantified in plasma, we found no advantage in measuring total (free + bound) rather than free F_2-IsoPs. Thus the present method measures only free F_2-IsoPs. For urine sample, 24-h urine collection represents a reliable probe for the oxidant stress and only free F_2-IsoPs is measured. For the tissue samples, the majority of the F_2-IsoPs are esterified on phospholipids and basic hydrolysis is necessary to release these compounds after extraction of the total lipids from the tissue homogenates.

(A) Tissue samples:

(i) Weigh 50 to 100 mg of tissue and add to 20 ml of ice-cold Folch solution. (*Note*: Polypropylene tubes are recommended because polystyrene tubes are not resistant to chloroform.)

(ii) Homogenized tissue with a blade homogenizer for 30 s or until fully homogenized.

(iii) Flush tube with nitrogen or argon and allow the sealed solution to stand at room temperature for 1 h, shaking occasionally.

(iv) Add 4 ml of aqueous NaCl (0.9%) and vortex vigorously for 1 min.

(v) Centrifuge at 800 *g* for 10 min at room temperature to separate the aqueous and organic phases. After centrifugation, the upper aqueous layer is discarded and the lower organic layer is carefully separated from the intermediate semisolid protein layer.

(vi) Transfer the organic phase containing the extracted lipids to a 50-ml centrifuge tube and evaporated to dryness under a stream of N_2.

(vii) Add 2 ml of methanol containing BHT (0.005%) and an equal volume of aqueous KOH (15%). The mixture is then vortexed and flushed with nitrogen before the tube is capped.

(viii) Incubate the mixture at 37°C for 30 min to effectively hydrolyze and release free F_2-IsoPs.

(ix) Acidify the mixture to pH 3 with 1 N HCl and diluted to a final volume of at least 40 ml with pH 3 water. (*Note*: Dilution of the methanol in the solution with water to 5% or less is necessary to ensure proper column extraction of F_2-IsoPs in the subsequent purification procedure.)

(B) Biological fluids: 2–3 ml of plasma is added in 7 ml of pH 3 water or 0.25 ml of urine is added in 10 ml of pH 3 water and acidified to pH 3 with 1 N HCl.

Purification, derivatization, and quantification of free F_2-IsoPs

(i) Add accurately 1 ng of the deuterated internal standard [$^2H_4$]-15-F_{2t}-IsoP to the sample mixture and vortex. (*Note*: An accurate syringe is recommended rather than disposal pipette.)

(ii) Apply the mixture to C18 Sep-Pak column preconditioned with 5 ml methanol and 5 ml pH 3 water. (*Note*: Sample solution should be pushed through at a flow of 1–2 ml/min and a steady stream is not recommended.)

(iii) Wash the column sequentially with 10 ml pH 3 water and 10 ml of heptane.

(iv) Elute the samples from the cartridge with 10 ml of ethyl acetate/heptane (50/50, v/v).

(v) Dry the solution over using about 5 g of anhydrous Na_2SO_4 for 1 min to remove water.

(vi) Apply the solution to a silica Sep-Pak that was prewashed with 5 ml of ethyl acetate.

(vii) Wash the cartridge with 5 ml of ethyl acetate followed by elution of the F_2-IsoPs with 5 ml of ethyl acetate/methanol (50/50 v/v).

(viii) Evaporate the ethyl acetate/methanol eluant under a stream of nitrogen and add 40 μl of 10% PFBB in acetonitrile and 20 μl of 10% DIPE in acetonitrile to the residue. (*Note*: PFBB is a potent lachrymator and carry out the experiments in a well-ventilated fume hood).

(ix) Vortex and incubate the mixture at 37°C for 20 min.

(x) Add 97 ml of chloroform and 3 ml of ethanol to a TLC tank and place TLC paper in the tank. Allow the tank to equilibrate for 30 min.

(xi) Evaporate the incubation mixture thoroughly under nitrogen and reconstitute it in 50 μl chloroform/methanol (3/2, v/v).

(xii) Apply each sample mixture to a separate lane of a prewashed TLC plate. Approximately 2 to 5 μg of the methyl ester of $PGF_{2\alpha}$ is applied on a separate TLC plate.

(xiii) Place the TLC plates in the tank after the solvent is dry. Remove the plates from the tank after the solvent reaches about 13 cm on the TLC plate.

(xiv) Visualize the TLC standard plate by spraying with a 10% solution of phosphomolybdic acid in ethanol followed by heating. (*Note*: DO NOT SPRAY THE SAMPLE PLATES.)

(xv) Scrape the silica from the sample plates in the region of the TLC standard 1 cm above and 1 cm below the standard. (*Note*: The R_f for $PGF_{2\alpha}$ methyl ester should be about 0.15; i.e., the band for the standard is about 2 cm above the original spot.)

(xvi) Place the scraped silica from each sample into separate microcentrifuge tubes and extract the sample with 1 ml ethyl acetate.

(xvii) Vortex the mixture vigorously and centrifuge the sample in a benchtop centrifuge at 13,000 rpm for 5 min.
(xviii) Carefully remove the solution and place it in a second microcentrifuge tube and evaporate the solvent.
(xix) Add 20 μl of BSTFA and 7 μl of dry DMF to the residue and incubate the mixture at 37°C for 20 min.
(xx) Dry the sample and dissolve it in 20 μl of dry undecane. Transfer the sample to an autosampler vial for GC-MS analysis.

Calculations and expected results

A representative chromatogram obtained from the analysis of F_2-IsoPs in mouse liver is shown in Fig. 3. 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. 3) 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. It should be noted that the peak denoted with "+" in Fig. 3 may also contain the COX product $PGF_{2\alpha}$ when free F_2-IsoPs are analyzed in biological fluids, such as plasma, CSF, or urine. However, caution should be exercised regarding the enzymatic origin of this peak; our studies show that the majority of this peak in human urine is actually derived from the free radical pathway [38]. Furthermore, this peak is also present when the esterified F_2-IsoPs are measured and it can only be derived from the free radical pathway in this case.

Caveats

GC-MS methods offer excellent sensitivity and selectivity for F_2-IsoPs. However, the labor-intensive purification and derivatization steps that are necessary for the GC-MS analysis make it difficult to improve the throughput of the assay. Secondly, experienced and

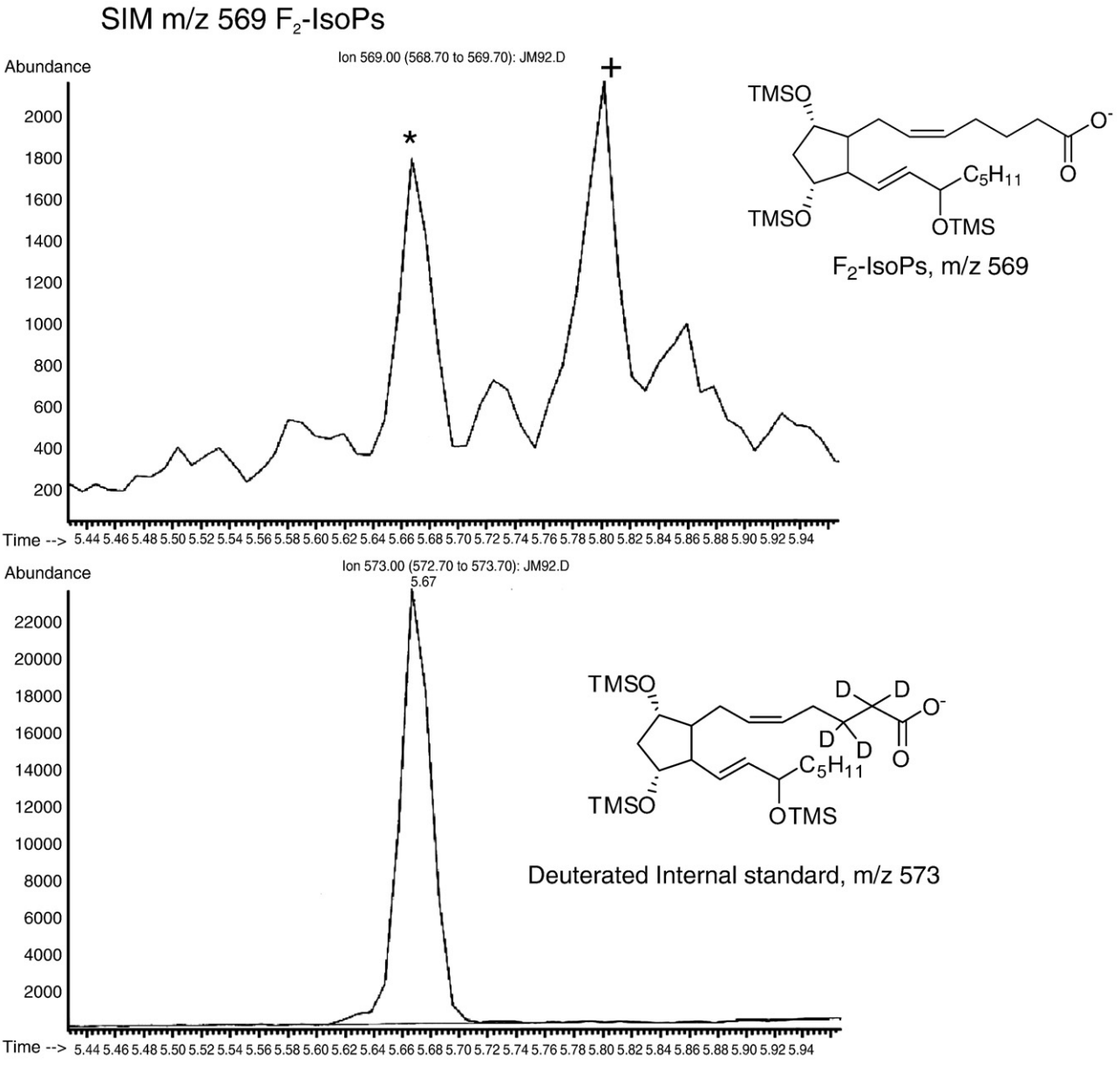

Fig. 3. Analysis of F_2-IsoPs in liver obtained from a mouse. The m/z 573 ion current chromatogram represents the [$^2H_4$] 8-iso-PGF_{2a} internal standard. The m/z 569 ion current chromatogram represents endogenous F_2-IsoPs.The peak in the upper chromatogram represented by the star (*) is the one routinely used for quantification of the F_2-IsoPs.The peak represented by the plus (+) can comprise both F_2-IsoPs and COX-derived $PGF_{2\alpha}$.

trained personnel are required to set up and operate the rather expensive instrument. In addition to GC-MS methods, a number of LC-MS methods for F_2-IsoPs analysis have been developed [39–41]. As noted, F_2-IsoPs consist of a mixture of four series of regioisomers and each has 16 diastereomers. These different stereoisomers may be formed and metabolized differently; thus the levels of these individual stereoisomers can be altered under various pathophysiological conditions [38,40]. GC-MS methods quantify all possible F_2-IsoPs stereoisomers while LC-MS methods permit separation and identification of selected regioisomers and diastereomers of F_2-IsoPs. The other advantage of LC-MS methods is that the sample preparation for analysis is simpler than that for GC-MS because no derivatization of the molecule is required. In addition to analyzing the free F_2-IsoPs, we recently also developed a LC-MS method to analyze the intact esterified F_2-IsoPs on different phospholipid head groups [42]. However, a concern with these LC-MS assays relates to the limits of detection in biological fluids that are often higher than those employing GC-MS [29].

Enzyme immunoassays have also been developed to quantify IsoPs using antibodies which generated against some selected isomers of F_2-IsoPs, such as 8-iso-15(R)-$PGF_{2\alpha}$ [43]. Although mass spectrometric methods of F_2-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 [28,29]. However, multistep sample purification is still necessary, and questions remain as to the specificity of the antibody. Studies comparing GC-MS and enzyme immunoassay assays of the same samples have demonstrated that the values from the assays are correlated but not identical [44,45].

The measurement of free F_2-IsoPs in urine can be confounded by the potential contribution of local F_2-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 [46]. 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.

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