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Separation and identification of F_2-isoprostane regioisomers and diastereomers by novel liquid chromatographic/mass spectrometric methods

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Abstract

Isoprostanes are isomers of prostaglandins that are generated from free radical-initiated autoxidation of arachidonic acid. Quantification of F_2-isoprostanes is regarded as the "gold standard" to assess oxidative stress in various human diseases. There are 32 possible racemic isoprostane isomers that exist as four sets of regioisomers. Each regioisomer is composed of eight diastereomers. We report liquid chromatographic/mass spectrometric methods to separate and identify F_2-isoprostane stereoisomers. These methods have been applied to the analysis of F_2-isoprostanes derived from tissues of rats exposed to an oxidative stress and are useful to assess the relative formation of various regioisomers and stereoisomers generated in vitro and in vivo. The delineation of the more abundant isomers formed will allow for studies to examine the biological relevance of selected compounds in vivo.

Keywords: Isoprostanes; Lipid peroxidation; Liquid chromatography; Mass spectrometry; Free radical

1. Introduction

Free radical-induced autoxidation of polyunsaturated fatty acids (PUFAs) has been linked to numerous human disorders, including atherosclerosis and neurodegenerative diseases [1–4]. Isoprostanes (IsoPs) are prostaglandin (PG)-like compounds that are formed non-enzymatically in vivo via the peroxidation of arachidonic acid by a free radical-initiated mechanism. A number of IsoPs have been identified, such as including those containing either F-type, E/D-type, or A/J-type prostane rings [5,6]. A novel aspect of IsoP formation is that, unlike cyclooxygenase-derived PGs, they are generated in situ esterified to phospholipids and cholesterol. In addition, they possess different stereochemical features compared to enzymatically derived PGs [7]. Quantification of IsoPs has emerged as one of the most accurate approaches to assess oxidant injury in vivo [5,8]. Furthermore, some IsoPs exert potent biological activity by acting as ligands for either plasma membrane bound PG receptors or for nuclear receptors [7,9–11].

Abbreviations: APCI, atmospheric pressure chemical ionization; BHT, butylated hydroxytoluene; CID, collision-induced dissociation; ESI-MS, electrospray mass spectrometry; GC, gas chromatography; HPLC, high-performance liquid chromatography; IsoP, isoprostane; IPA, isopropanol; LC, liquid chromatography; MS, mass spectrometry; MeOAMVN, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile); PFB, pentafluorobenzyl; PG, prostaglandin; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; HpETE, hydroperoxyeicosatetraenoate; SIM, selective ion monitoring; SRM, selective reaction monitoring; TLC, thin layer chromatography

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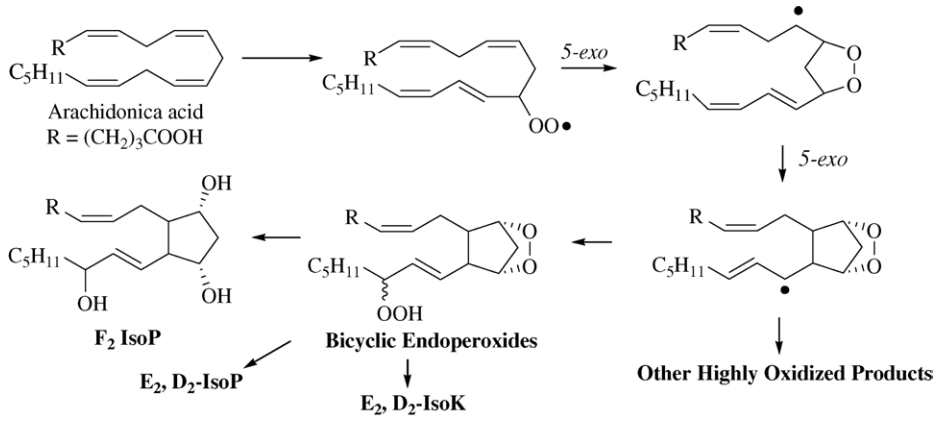
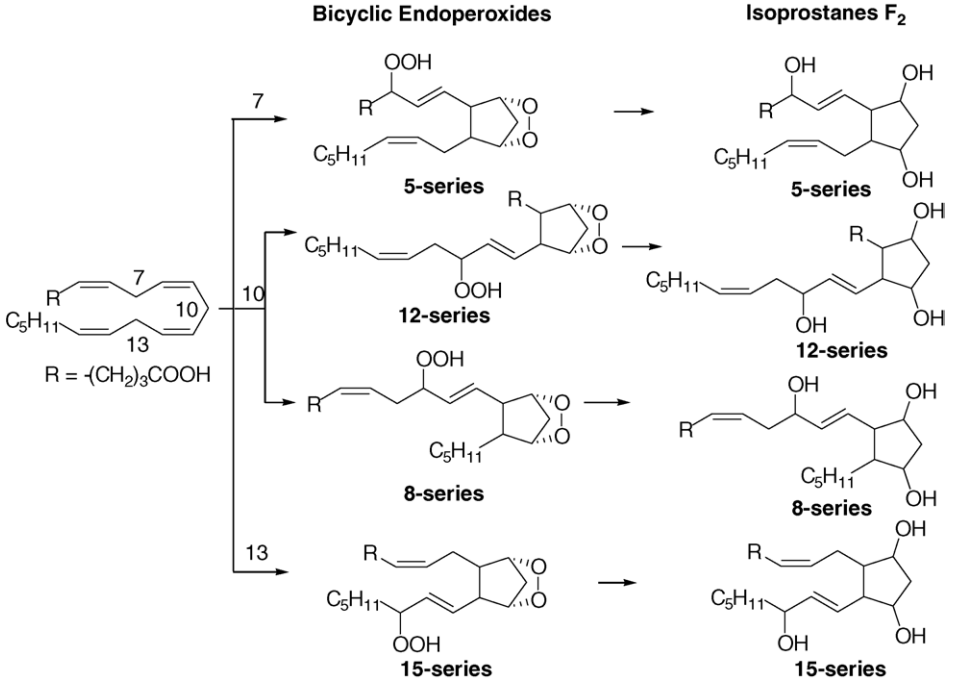


Scheme 1.

IsoP formation involves the initial autoxidation of arachidonic acid with hydrogen atom abstraction at either carbon 7, 10, or 13 of arachidonate, followed by molecular O_2 addition and radical cyclization to form unstable bicyclic endoperoxides (Scheme 1) [12–14]. A number of different parent IsoPs can be generated from these unstable endoperoxide precursors by either reduction or rearrangement. F_2-IsoPs are formed upon reduction of the bicyclic peroxides and hydroperoxide moieties in these molecules. Four regioisomeric F_2-IsoPs are formed according to this mechanism (Scheme 2). Although five stereogenic centers are generated in the process of F_2-IsoP formation, only eight possible racemic diastereomers can be formed from one precursor peroxyl radical because the two hydroxyl groups on the prostane ring have to adapt a *cis* configuration. Therefore, a total of 32 F_2-IsoP stereoisomers can be generated theoretically since four sets of regioisomeric IsoPs are formed [6]. The routine analysis of F_2-IsoPs by previously published gas chromatography (GC)/mass spectrometry (MS) methods quantifies all possible regioisomers and stereoisomers. It may be of importance, however, to separate and identify selected different F_2-IsoPs because they may have different biological activities and be formed and metabolized differently under human disease conditions which are linked to oxidative stress [15–17].

We report herein methods based on liquid chromatography (LC)/MS to separate and identify the different regioisomers and diastereomers of F_2-IsoPs that are generated in vitro and in vivo. In this protocol, reverse-phase LC is coupled to electrospray (ESI) MS to analyze free acid F_2-IsoPs, whereas normal phase LC is coupled to atmospheric pressure chem-



Scheme 2.

ical ionization (APCI)-MS to analyze the pentafluorobenzyl (PFB) derivatives. These two methods are complimentary and need only minimal sample workup compared to laborious GC/MS methods. The four IsoP regioisomers can be distinguished based on their characteristic fragmentation in collision-induced dissociation (CID). In addition, all eight diastereoisomers of 15-series F_2-IsoPs can be separated and identified by comparison with known synthetic standards. These methods can thus be applied to study the regioselectivity and diastereoselectivity of F_2-IsoPs formed via the autoxidation of arachidonic acid and will allow for studies of the biological relevance of the more abundant F_2-IsoP isomers.

2. Experimental procedures

2.1. Materials

All lipid autoxidation reactions were carried out under an atmosphere of oxygen unless otherwise noted. Air and argon were passed through a bed of calcium sulfate desiccant. Benzene was distilled from sodium and stored over 4A molecular sieves. Tetrahydrofuran and dichloromethane were dried by Solv-Tek (Berryville, VA) solvent purification columns using activated alumina for drying and Q-5 packing for deoxygenating the solvents.

Oxygen (medical grade) was obtained from A.L. Compressed Gas (Nashville, TN). HPLC grade solvents were purchased from Burdick & Jackson (Muskegon, MI) or EM Science (Gibbstown, NJ). All lipids were purchased from Nu Chek Prep (Elysian, MN) and were of the highest purity (>99+%). The free radical initiator 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeOAMVN) was generously donated by Wako Chemicals USA Inc. (Richmond, VA). $PGF_{2\alpha}$, 15*R*-$PGF_{2\alpha}$, 15*R*-8-*iso*-$PGF_{2\alpha}$ and 8-*iso*-$PGF_{2\alpha}$-d_4 were purchased from Cayman Chemicals (Ann Arbor, MI). Other F_2-IsoP diastereomers in 15-series were synthesized by Taber et al. [18]. All other reagents were purchased from Aldrich Chemical Company (Milwaukee, WI) and used without further purification.

2.2. Hydroperoxide reaction analysis

Reactions involving hydroperoxides were visualized by TLC using a stain of 1.5 g *N*,*N*′-dimethyl-*p*-phenylenediamine dihydrochloride/25 mL H_2O/125 mL MeOH/1 mL acetic acid. Hydroperoxides yield an immediate pink color, while cyclic peroxides exhibit a pink color after mild charring. General TLC staining was accomplished by iodine or use of a phosphomolybdic acid stain prepared as a 20% (w/v) solution in EtOH. In general, hydroperoxides were stored as dilute solutions with 1 mol% BHT in either hexanes or benzene at −78 °C and were never exposed to temperatures >40 °C. Flash column chromatography was performed using 35–70 μm silica gel. Thin layer chromatography was performed using 0.2 mm layer thickness silica gel coated aluminum (60 F_{254}, EM Industries), and TLC plates were analyzed using UV light (254 nm) with a Mineralight UVSL-25 hand lamp. Preparative TLC was performed on silica gel 60ALK6D plates (Whatman International Ltd., Maidstone, UK).

2.3. HPLC separation of IsoP isomers

Analytical HPLC was carried out using a Waters Model 600E pump with a Waters 996 Photodiode array detector. Millenium32 chromatography software (Waters Corp., Milford, MA) was used to control the array detector and to collect and process data. PFB esters of F_2-IsoPs were analyzed by normal phase HPLC using 12% IPA in hexanes and a single Beckman Ultrasphere 5 μm (4.6 mm × 25 cm) silica column or two narrowbore Beckman Ultrasphere 5 μm (2.0 mm × 25 cm) Si columns. A flow rate of 1 mL/min was used for analytical normal phase HPLC, whereas 0.2 mL/min was used for narrowbore columns. Preparative HPLC was performed using a Dynamax-60 Å 8 μm (83-121-C) silica column (21.4 mm × 25 cm) with a flow rate of 10 mL/min. For reverse-phase HPLC, a Phenomenex Luna® ODS 5 μm (2.1 mm × 25 cm) column (Torrence, CA) was utilized at 0.2 mL/min flow rate with a gradient starting with 80% solvent A (2 mM NH_4Ac) and 20% solvent B (MeOH:CH_3CN = 5:95), holding for 2 min and increasing to 65% B in 30 min and holding for ten min.

2.4. LC/MS analysis of IsoPs

LC/MS was carried out using a ThermoFinnigan TSQ Quantum 1.0 SR 1 mass spectrometer in negative ion mode. The ESI and APCI sources were fitted with a deactivated fused silica capillary (100 μm I.D.). Nitrogen was used as both the sheath gas and the auxiliary gas, at 45 and 17 psi, respectively. For ESI-MS, the capillary temperature was 280 °C. The spray voltage was 4.3 kV, and the tube lens voltage was 80 V. For APCI-MS, the mass spectrometer was operated in negative ion mode with a capillary temperature of 300 °C, vaporizer temperature of 460 °C, discharge current 20 and −94 V tube lens voltage. Collision-induced dissociation (CID) was performed from 20 to 30 eV under 1.5 mTorr of argon. Spectra that are shown were obtained at 25 eV. Spectra were displayed by averaging scans across chromatographic peaks. Selective reaction monitoring (SRM) was performed according to characterizing fragmentation patterns of isoprostanes (20). Data acquisition and analysis were performed using Xcaliber software, version 1.3.

2.5. Oxidation of arachidonic acid in vitro

Arachidonic acid (25 mg, 80.24 μmol) was dissolved in 1.0 mL benzene. To the mixture was added 0.1 equivalent of MeOAMVN as radical initiator. The mixture was kept

under atmospheric oxygen at 37 °C for 24 h. The reaction was quenched by adding 2 mg of BHT. The reaction mixture was stored at −80 °C until analysis. F_2-isoprostanes are generated by PPh_3 reduction in situ.

2.6. Oxidation of 15(S)-HpETE

15(*S*)-HpETE was generated using the 15-lipoxygenase enzyme according to previous procedures [19]. The product oxidation mixture was obtained by incubation of 15-HpETE under free radical conditions using 0.1 equivalent of MeAMVN as an initiator. A typical oxidation experiment was carried out as follows: ∼1.0 mg of 15-HpETE was mixed with 10% (molar) of MeAMVN in 1 mL anhydrous benzene. The mixture was stirred at 37 °C under an air atmosphere for 24 h. The oxidized mixture was diluted in benzene and stored at −78 °C with BHT.

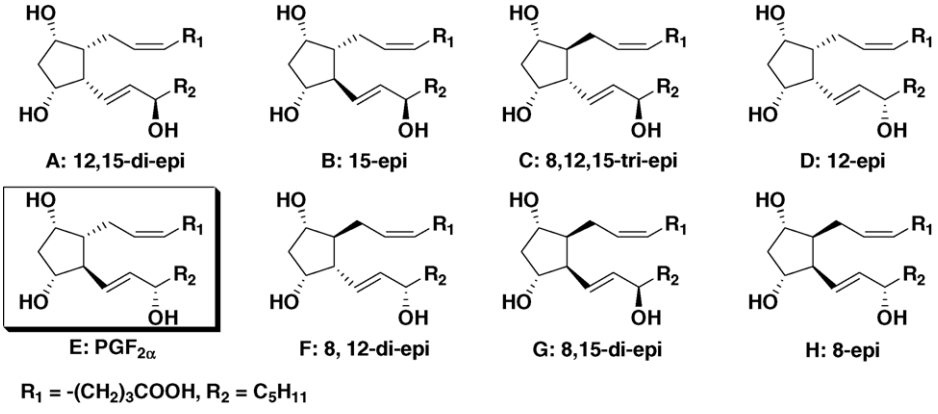
2.7. Extraction of oxidized lipids from the livers of rats

The extraction of lipids from a liver of a rat has been reported previously [20]. In brief, after intragastric administration of CCl_4 (2 mg/kg) in corn oil to Sprague–Dawley rats for 2 h, the animals were anesthetized with pentobarbital (60 mg/kg) intraperitoneally and sacrificed; subsequently, the livers were removed. Approximately 1.0 g of tissue was immediately homogenized and extracted in Folch solution (MeOH:$CHCl_3$ = 1:2) to obtain a crude phospholipid extract. Basic hydrolysis was carried out in 0.5 mL MeOH and 0.5 mL of 15% KOH at 37 °C for 30 min. After adjusting the pH to 3, the mixture was loaded on a C18 Sep-Pak (Waters Associates, Milford, MA) cartridge that was preconditioned with 5 mL methanol and then 5 mL water. The column was washed with 5 mL of water and 5 mL of heptane. The eluent of 10 mL ethyl acetate was collected. After evaporation of the solvent, the residue was reconstituted in 80% water and 20% (MeOH:CH_3CN = 5:95).

3. Results

F_2-IsoPs are one of the major oxidation of products of arachidonic acid generated in vitro and in vivo. Extensive research has been carried out to determine the isomeric distribution of IsoPs formed from the oxidation of arachidonic acid. Among the four possible regioisomers of IsoPs, it has been noted that the 5- and 15-series are generated in greater amounts than IsoPs of the 8- and 12-series. This selectivity can be explained based on the formation of dioxolane-IsoPs from peroxyl radical precursors which lead to 8- and 12-series IsoPs, whereas the peroxyl radical precursors of 5- and 15-series cannot form the more highly oxygenated dioxolane-IsoPs [21]. In theory, eight possible diastereoisomers of each series can be formed and they each may have different biological activities. However, systematic separation and identification of the eight diastereomers has not been reported for any of the IsoP regioisomers. The structures of the eight diastereomers of the 15-series F_2-IsoPs are illustrated in Scheme 3. The nomenclature of these compounds is based on the structure of $PGF_{2\alpha}$. For example, compound A is named 12,15-di-epi, because the configuration of carbons of 12 and 15 is opposite to those found in $PGF_{2\alpha}$.

In order to simplify the IsoP mixtures formed from arachidonate oxidation, we have adapted the strategy of conversion of a single peroxyl radical to its IsoP products. Thus, 15-HpETE was prepared from enzymatic reaction or autoxidation and converted to its oxidation products under typical free radical conditions. According to the proposed mechanism for IsoP formation, only 15-series F_2-IsoPs can be formed from the oxidation of 15-HpETE. Employing this strategy significantly simplifies the oxidation mixture formed compared to that observed from the oxidation of arachidonic acid. Reverse-phase HPLC was then used to separate the diastereomers and ESI-MS was used as a detection method. CID was carried out by selecting the parent ion of interest in the first quadrupole of a triple quadrupole mass spectrometer and fragmenting it in the second quadrupole to give structural information of the parent ion. The CID result of $PGF_{2\alpha}$



Scheme 3.

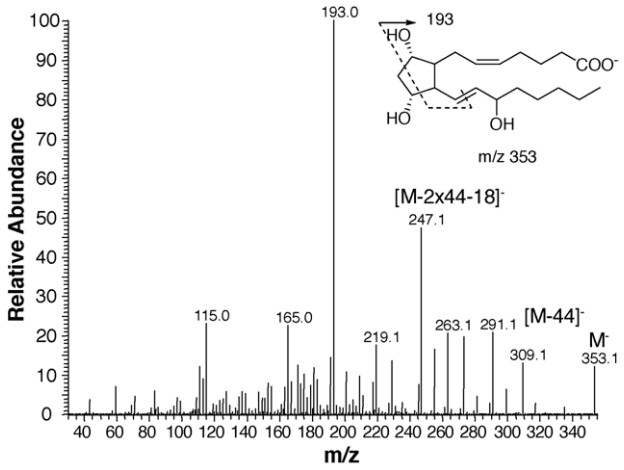


Fig. 1. CID results of $PGF_{2\alpha}$ using ESI-MS in the negative ion model (parent ion *m/z* 353 @ 30 eV).

is illustrated in Fig. 1. Besides non-selective fragmentations, such as dehydration and loss of *m/z* 44, a fragment of *m/z* 193 is observed and was confirmed to result from multiple bond cleavages in the gas phase [22,23]. This fragmentation is characteristic of 15-series F_2-IsoPs. A SRM experiment was then carried out by selecting the parent ion *m/z* 353 to daughter ion *m/z* 193 transformation. The results of the SRM experiment of an oxidation mixture of 15-HpETE are shown in Fig. 2. There are seven distinguishable peaks in the SRM chromatogram and the structure giving rise to each peak was assigned by comparison with synthetic standards. The diasteromers B and F cannot be separated under these conditions.

It is well documented that $PGF_{2\alpha}$ is not a major diastereomer in the autoxidation process although it is thermodynamically favored. The chromatogram shown in Fig. 2 shows that diastereomers with two *cis* alkyl chains, such as A, D, G and H, are formed in greater amounts than compounds having

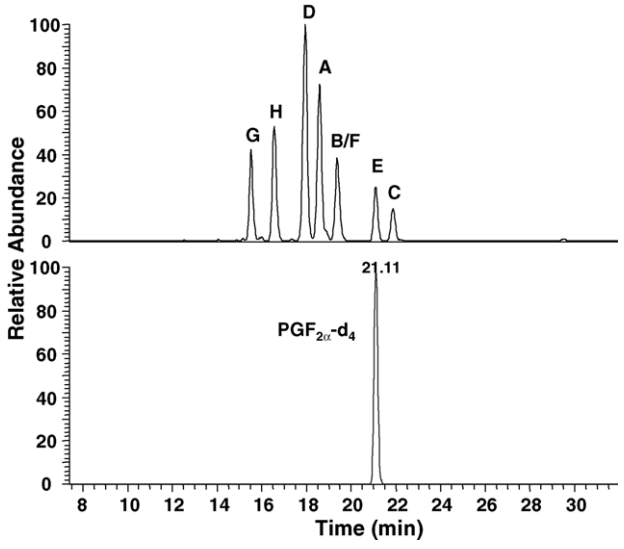


Fig. 2. SRM of F_2-IsoPs from 15-HpETE oxidation (reverse-phase LC/ESI-MS. SRM: *m/z* 353–193 @ 30 eV, *m/z* 357–197 @ 30 eV).

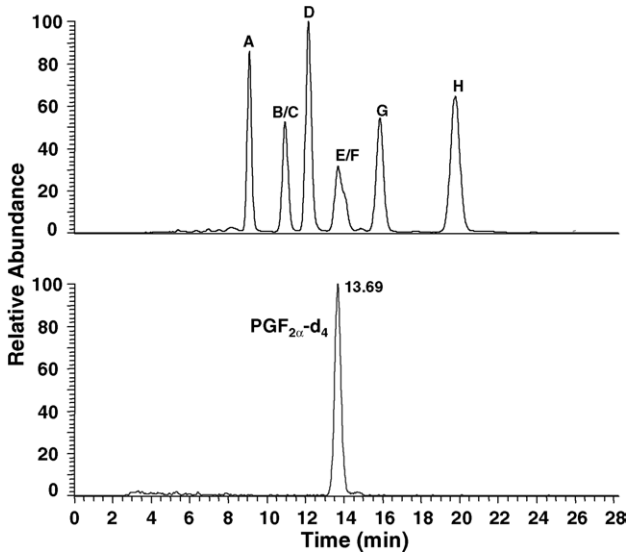
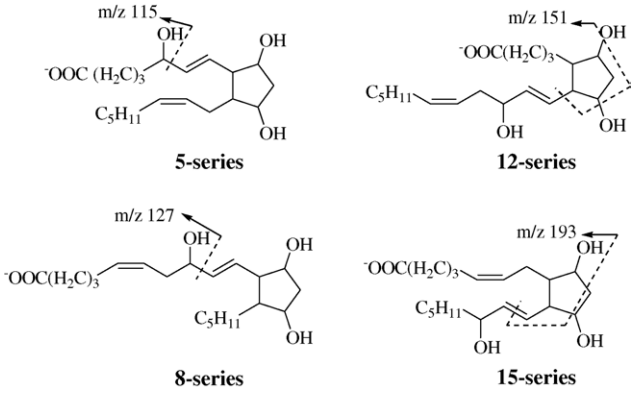


Fig. 3. SRM of F_2-IsoPs as PFB esters from 15-HpETE oxidation (normal phase LC/APCI-MS, 12% IPA/hexane, Beckman Si, 4.6 mm × 250 mm; SRM: *m/z* 353–193 @ 30 eV, *m/z* 357–197 @ 30 eV).

trans substituted alkyl chains such as $PGF_{2\alpha}$ (peak E), consistent with literature reports [24,25].

Recently, electron capture (EC) APCI-MS has been applied to study synthetic and biological molecules after electrophilic derivatization [26,27]. This technique combines the ultra-sensitivity and selectivity of electron capture with separation methods such as HPLC. The same method was employed to analyze the oxidation mixture of 15-HpETE after triphenylphosphine reduction and PFB esterification. The PFB esters of F_2-IsoPs are separated by normal phase HPLC using 12% isopropanol in hexane as mobile phase. The same type of fragmentation was observed using electron capture APCI-MS because the PFB moiety is readily lost and forms the carboxylate anion. The SRM results are summarized in Fig. 3. Six peaks were observed and have been assigned by comparison with synthetic standards. By using electrospray and electron capture APCI ionization techniques, the eight possible diastereomers of 15-series F_2-IsoP have been unequivocally identified for the first time.

Subsequently, we analyzed the complex oxidation mixture derived from the peroxidation of arachidonic acid in vitro as well as the liver lipid extract from rats that have been treated under oxidative stress using reverse-phase LC coupled with ESI-MS. Although four series of F_2-IsoPs can be formed from the oxidation of arachidonic acid, the 5- and 15-series of IsoPs are formed in more abundance than 8- and 12-series because, as noted, the precursors of the 8- and 12-series compounds can be further oxidized to form dioxolane-IsoPs [21]. This regioselectivity of F_2-IsoP formation has been observed both in vivo and in vitro [22,23]. Furthermore, these four series of F_2-IsoPs have their characteristic fragmentation in the gas phase of MS when the parent ion is collisionally activated under CID conditions (Scheme 4) [17,22,23,28]. Therefore, SRM can be employed to differentiate the four regioisomers.



Scheme 4.

The SRM results are summarized in Fig. 4. The overall chromatography is much more complex than that observed from the oxidation mixture of 15-HpETE alone because F_2-IsoPs can be generated from four possible peroxyl radical precursors. Four series of F_2-IsoPs are detectable but the 5- and 15-series are formed in much greater amounts than the 8- and 12-series. The diastereomer pattern of the 15-series IsoPs is similar to that observed in the product mixture derived from 15-HpETE. 5-Series F_2-IsoPs can be separated by this method and some of the peaks have been identified in the literature [17]. As with the 15-series, the 5-series IsoP diastereomers with *cis* alkyl chains on the prostane ring are formed in greater amount than those with *trans* configuration. The analytical results derived from the rat liver oxidation are strikingly similar to those obtained from the in vitro autoxidation of arachidonic acid.

The same lipid mixtures were also analyzed by APCI-MS techniques coupled with normal phase HPLC. The SRM results are illustrated in Fig. 5. F_2-IsoPs of the 5- and 15-series are major regioisomers among the four possible regioisomeric series. Within each group, a number of diastereoisomers can be separated and identified. The lipid extract of oxidized rat liver showed an almost identical chromatographic pattern as those observed from the in vitro oxidation mixture of arachidonic acid. As noted and predicted, $PGF_{2\alpha}$ is not a major IsoP diastereoisomer resulting from the autoxidation of arachidonic acid both in vitro and in vivo. Rather, diastereomers having *cis* dialkyl prostane ring substitution are major products.

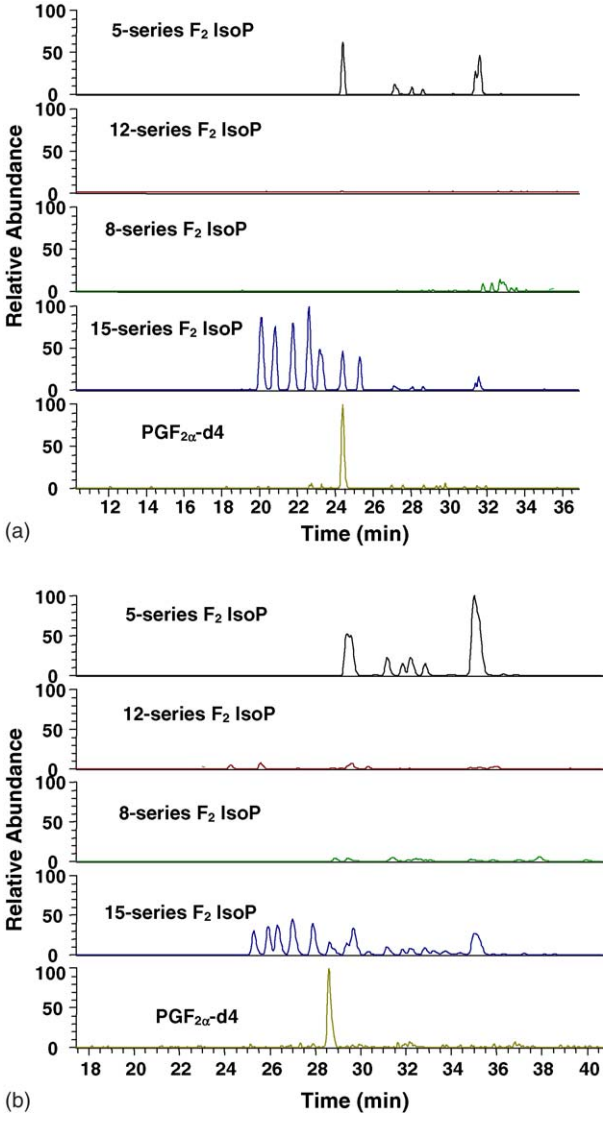


Fig. 4. SRM of F_2-IsoPs: (a) from arachidonic acid oxidation; (b) lipid extract from rat liver using reverse-phase LC/ESI-MS in negative ion mode. SRM: 5-series *m/z* 353–115 @ 30 eV; 12-series *m/z* 353–151; 8-series *m/z* 353–127 @ 30 eV; 15-series *m/z* 353–193 @ 30 eV, *m/z* 357–197 @ 30 eV. The relative intensity is normalized to 5-series.

4. Discussion

Free radical-induced autoxidation of polyunsaturated fatty acids such as arachidonic acid have been implicated in a number of human diseases. IsoPs are formed from autoxidation of arachidonic acid in vivo [6,29]. Measurement of these compounds has become a "gold standard" to assess oxidative stress in many human diseases. GC/MS approaches using chemical ionization in the negative ion mode have been widely used to measure IsoPs after PFB and TMS derivatization [30]. The assay is highly accurate and precise compared to other methods such as immunogical assays for IsoPs as well as the measurement of other lipid oxidation products. [31,32]. However, there are some drawbacks associated with GC/MS assays. Importantly, stereochemical information regarding IsoPs cannot be gained from GC/MS analysis since this method does not significantly separate different compounds.

In recent years, reports of the use of LC/MS methods to analyze IsoPs have appeared [17,33]. These protocols require minimal sample workup and avoid ex vivo lipid oxidation during sample workup and derivatization, a potential problem with GC/MS protocols. Both ESI-MS and APCI-MS have been developed to analyze IsoPs [27]. However, most reports

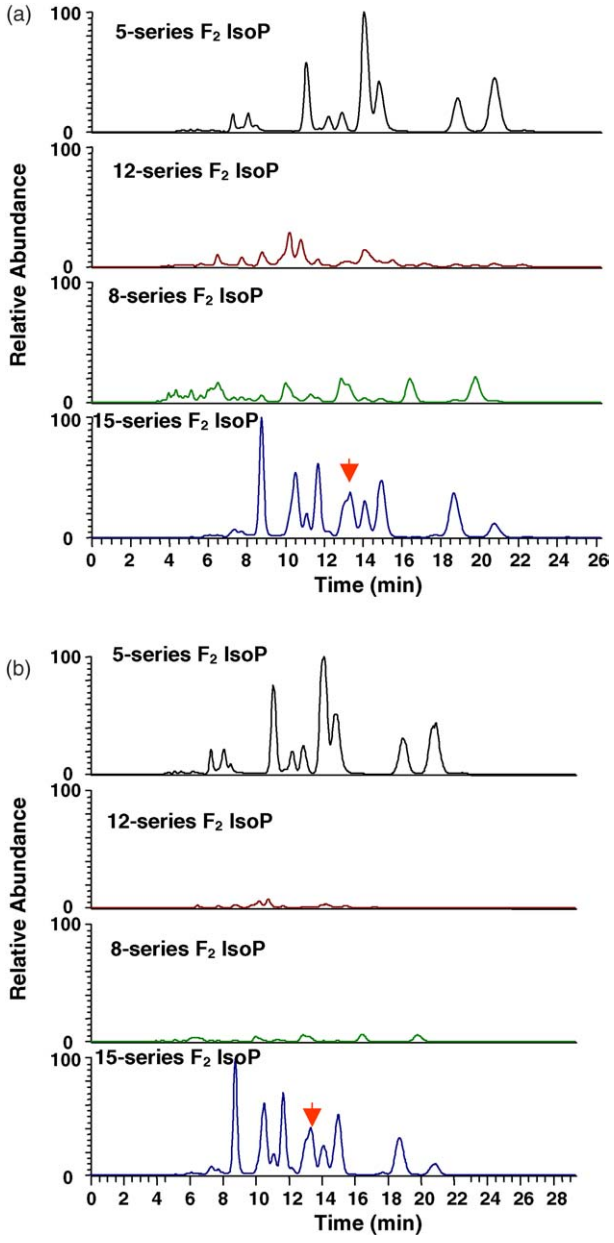


Fig. 5. SRM of F_2-IsoPs PFB esters: (a) from arachidonic acid oxidation; (b) lipid extract from rat liver using normal phase LC/APCI-MS in negative ion mode (12% IPA/hexane). SRM: 5-series *m/z* 353–115 @ 30 eV; 12-series *m/z* 353–151; 8-series *m/z* 353–127 @ 30 eV; 15-series *m/z* 353–193 @ 30 eV, *m/z* 357–197 @ 30 eV. The relative intensity is normalized to 5-series. Arrow indicates the peak of $PGF_{2\alpha}$ IsoP.

have been focused on measuring total F_2-IsoPs generated. On the other hand, determining the stereochemistry of various F_2-IsoPs has attracted significant interest because of the fact that different isomers likely possess different biological activities. The regioselectivity of IsoP formation has been studied and among the four possible series of F_2-IsoPs, the 5- and 15-series are generated in greater amount than the 8- and 12-isomers [22]. This regioselectivity was initially explained based on the stoichiometry of the abstractable hydrogen atoms. We reported, however, the formation of a novel class of IsoPs termed dioxolane-IsoPs from the peroxyl radical precursors of the 8- and 12-series of F_2-IsoPs. The peroxyl radical precursors of the 5- and 15-series cannot form dioxolane-IsoPs and for this reason, these diasteromers are formed to a greater extent than the 8- and 12-compounds [21].

The diastereoselectivity of F_2-IsoPs has been studied by using α-linolenic acid as a model compound and the diastereomers with *cis* alkyl chains on the prostane ring are major products formed, consistent with our observations reported here [24,25]. It seems likely that different isomers of F_2-IsoPs may have quite different the biological activities. Their formation, metabolism or clearance may be a biomarker reflecting different oxidative conditions or pathologies. Therefore, an enabling protocol to separate and identify the major isomers in a systematic way may have important applications. For example, FitzGerald and co-workers reported that only a limited number of F_2-IsoP isomers were elevated in a congestive heart failure patient [17]. Chiabrando et al. studied the metabolism of 15-series F_2-IsoPs by LC/MS and the diastereoisomers of $PGF_{2\alpha}$ generate quick different metabolites [34].

In summary, the LC/MS methods we report herein permit the systematic analysis of the eight diastereomers formed in the four regioisomeric sets of F_2-IsoPs. These protocols may have application in the study of the differential biological activity of these isomeric products, such as the interaction with receptors, as well as their metabolism and clearance.

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