Mass Spectrometric Analysis of Four Regioisomers of F₂-Isoprostanes Formed by Free Radical Oxidation of Arachidonic Acid

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F₂-isoprostanes are complex metabolites of arachidonic acid generated via nonenzymatic free radical oxidation and are isomeric to prostaglandin F_{2 α} , enzymatically produced by prostaglandin H₂ synthase. In theory, four distinct regioisomeric families are possible. These regioisomeric families have a common 1,3-diol cyclopentane structural feature, but differ by the comparative length of two attached alkyl chains and the position of a third hydroxyl group. Eight synthetic PGF_{2 α} isomers were found separable by capillary gas chromatography (GC) and reversed-phase high-performance liquid chromatography (HPLC). Electrospray ionization tandem mass spectrometry was used to detect the elution of these isomers from the HPLC column by monitoring the characteristic loss of 44 u (C₂H₄O) from the 1,3-diol cyclopentane ring. Catalytic reduction, derivatization, and electron ionization mass spectrometric techniques were used to obtain definitive information as to the location of the side chain hydroxyl position in these isomers through abundant α -cleavage ions. Free radical oxidation of arachidonic acid was used to generate a complex mixture of F₂-isoprostanes, which were separated by HPLC and capillary GC. Members of each of the four specific regioisomeric isoprostane families could be identified in this mixture from the predicted α -cleavage ions. Although many epimers within a single family type could be separated, the four regioisomeric families were substantially superimposed in their HPLC and GC elution. The Type I and Type IV regioisomers were the major F₂-isoprostane products, but the complexity of the isomers required more than a simple GC-mass spectrometry assay to precisely identify a particular stereoisomer within a regioisomeric family (e.g., 8-*epi*-PGF_{2 α}). Type I F₂-isoprostanes are unique noncyclooxygenase products and may be more specific targets to measure lipid peroxidation in vivo. (*J Am Soc Mass Spectrom* 1996, 7, 490-499)

The formation of oxygen free radical species in biological systems is known to take place by both enzymatic as well as nonenzymatic processes [1]. The phospholipid bilayer that surrounds the cell serves as an initial barrier for most of the reactive species, yet highly reactive species such as hydroxyl radical profoundly alter cellular biochemistry as the result of lipid peroxidation [2]. Lipid peroxidation leads to a large number of products, some of which are quite simple stable species such as pentane, malonyldialdehyde, and 4-hydroxynonenal [3]. However, recently more complex structures have been identified as initial oxidation products of lipids. Certain free radical products of arachidonic acid peroxidation have been observed that are isomeric to the enzymatically produced prostaglandin F₂ formed by prostaglandin H (PGH) synthase [4-7]. These isomeric prostaglandins or F₂-

isoprostanes are formed through a cyclic endoperoxide after initial formation of a bisallylic radical site at one of three sites in arachidonic acid, namely, at carbon atoms 7, 10, or 13.

Interest in F₂-isoprostanes stems not only from the similarity of these eicosanoids to the endogenous cyclooxygenase products, but also, as a result of the observation, one F₂-isoprostane—8-*epi*-PGF_{2 α} —had significant biological activity that caused potent contraction of the renal artery of the rat and reduced the glomerular filtration rate of the rat kidney [8, 9]. Therefore, such molecules could play an important role in mediation of the biological response to damage caused by oxygen free radicals, such as that observed to be characteristic of atherosclerosis, ischemic reperfusion injury, inflammatory disease, and possibly even cancer and aging [10-12].

The free radical process involved in the formation of the F₂-isoprostanes leads to the formation of four regioisomers or family subtypes (Scheme I) dependent on the initial site of hydrogen atom abstraction from

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Scheme I.

arachidonic acid. Each of the regioisomers consists of a mixture of eight racemic diastereoisomers that potentially yields a total of 64 different F₂-isoprostanes possible after free radical oxidation of arachidonic acid [4, 13].

The specific assay for F₂-isoprostanes has employed negative ion chemical ionization gas chromatography mass spectrometry (GC/MS) that uses the carboxylate anion at *m/z* 569 to detect the elution of the isomeric F₂-isoprostanes from the gas chromatographic column [14]. Use of this assay with various biological extracts typically enables detection of a mixture of isomeric compounds as a number of closely eluting compounds close to a deuterium-labeled internal standard [5, 15]. Recently, evidence has been presented for the identification of one specific isomer, 8-*epi*-PGF₂α, which is formed by a free radical mechanism based upon four separate high-performance liquid chromatography (HPLC) purifications followed by capillary gas chromatography (GC) [16]. Although a mixed mass spectrum that contains predicted ions for all four regioisomers has been previously reported [5], to date no mass spectral evidence for the individual regioisomers has been reported.

The four F₂-isoprostane regioisomers are structurally similar in that each contains a cyclopentane ring that contains a 1,3-diol moiety with each hydroxyl on the same side of the ring. Two alkyl chains extend from the ring; one chain contains the original carboxylic acid moiety of the arachidonic acid and the second chain contains the methyl terminus. These isomers also differ by the position of one additional hydroxyl substituent on either of the alkyl chains and each family member is uniquely identified by the location of the hydroxyl-containing alkyl chain relative to the cyclopentane ring. The enzymatic product of PGH synthase action on arachidonic acid results in regioisomer Type IV with the hydroxyl group at carbon atom 15. Type I has the hydroxyl substituent at carbon atom 5; Type II has the hydroxyl substituent at carbon atom 12; Type III has the hydroxyl substituent at carbon

atom 8. Mass spectrometric evidence for the formation of each of these regioisomer subtypes has been obtained in detailed studies of the free radical oxidation of arachidonic acid described herein.

Experimental

Synthetic prostaglandins which include (5Z, 9α, 11α, 13E, 15S) -9, 11, 15-trihydroxyprosta-5, 13-dien-1-oic acid (prostaglandin F₂α, PGF₂α) and synthetic epimers (named as PGF₂α derivatives), 8-*epi*-PGF₂α, 5-*trans*-PGF₂α, 11β-PGF₂α, 15-*epi*-PGF₂α, 9β-PGF₂α, 8-*epi*-9β-PGF₂α, 5-*trans*-9β-PGF₂α, [²H₄]8-*epi*-PGF₂α, and [²H₄]PGF₂α, were purchased from Cayman Chemical (Ann Arbor, MI) and used without further purification. All solvents used were HPLC grade purchased from Fisher Scientific (Fairlawn, NJ). Hydrogen peroxide (30%, w/v) and phosphate-buffered saline (PBS) were purchased from Sigma Chemical (St. Louis, MO). Copper (II) chloride, pentafluorobenzyl bromide, diisopropylethylamine, and 5% rhodium absorbed on alumina powder were purchased from Aldrich Chemical (Milwaukee, WI). Bis(trimethylsilyl)trifluoroacetamide was purchased from Supelco (Bellefonte, PA). Water was purified via a Millipore water ultrafiltration system (Millipore, Bedford, MA).

Free Radical Oxidation of Arachidonic Acid

Three micromoles of arachidonic acid (5,8,11,14-eicosatetraenoic acid) in 0.01 M sodium hydroxide (100 μL) was placed in a 15 mL polypropylene conical tube. The fatty acid immediately was suspended in 9.7 mL of 50 mM PBS, pH 7.3, by vortexing. To the solution, H₂O₂ (100 μL) and 1 mM CuCl₂ (100 μL) were added to result in a final concentration of 70 mM and 100 μM, respectively. The solution was capped and mixed gently at 5°C for 8.5 h. Progress of the oxidation was monitored by negative ion chemical ionization mass spectrometry from aliquots (1%) taken at various time points.

The reaction mixture was loaded onto a C₁₈ (1 mL, 100 mg) reverse phase 40 μM Bond Elut extraction cartridge (Varian, Harbor City, CA). The cartridge was preconditioned with 10 mL of methanol followed by 10 mL of water. The inorganic salts and remaining H₂O₂ were eluted with 10 mL water. The isoprostanes and other products of arachidonate oxidation were eluted with 10 mL methanol and then taken to near dryness with rotary evaporation.

Hydroperoxides or endoperoxides formed during the oxidation reaction were reduced with stannous chloride to prevent further rearrangement and degradation of these products. Stannous chloride (3 mM, 1 mL) was added to the methanol fraction that contained oxidized arachidonic acid and allowed to stand for 30 min at ambient temperature. Water (8 mL) was added and the mixture was acidified to pH 3 (1 N HCl). The reaction mixture was then subjected to solid phase

extraction as before. The oxidized arachidonic acid was eluted with 10 mL methanol, the volume was reduced in vacuo, and solvent was added to yield a final volume of 1 mL (9:1 water:methanol).

Reverse Phase High-Performance Liquid Chromatography

An Ultracarb ODS 5 μm , 4.6 mm $\times$ 25 cm column (Phenomenex, Torrance, CA) was used to separate the oxidized arachidonic acid. The mobile phase consisted of 0.05% acetic acid adjusted to pH 5.7 with ammonium hydroxide (system A) and acetonitrile:methanol (95:5; system B). The separation was carried out with a linear gradient starting with 10% B and ramping to 100% B in 40 min. The flow rate was 1 mL/min and fractions were collected at 0.5 mL/min.

Derivatization for Gas Chromatography

For negative ion chemical ionization GC/MS, a portion of the HPLC fractions was derivatized as the pentafluorobenzyl ester trimethylsilyl ethers as described previously [17]. For positive ion electron ionization GC/MS, samples were methylated with ethereal diazomethane and then hydrogenated by bubbling hydrogen gas through a methanol solution of the sample for 20–30 min after the addition of Rh/ Al_2O_3 (1 mg) as catalyst. Samples were then taken to dryness and derivatized as the trimethylsilyl ethers by using bis(trimethylsilyl)trifluoroacetamide (20 μL) and injected directly onto the gas chromatographic column in the derivatization mixture, which contained an equal volume of acetonitrile.

Mass Spectrometry

On line HPLC analysis of the F_2 -isoprostanes was performed in the negative ion mode by utilizing the multiple reaction monitoring mode of the Sciex API-III⁺ triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Thornhill, Canada) to monitor the loss of 44 u from the carboxylate anion. The HPLC was operated at a flow rate of 1 mL/min with a linear gradient of 25–50% B over 25 min and a postcolumn split that yielded 15 $\mu\text{L}/\text{min}$ flow into the mass spectrometer. The remaining 985 $\mu\text{L}/\text{min}$ was collected by using a fraction collector operated at 0.25 mL/min. The elution of each component relative to the retention time (t_R) for [$^2\text{H}_4$]8-*epi*-PGF $_{2\alpha}$ and [$^2\text{H}_4$]PGF $_{2\alpha}$ mixed with the samples was calculated by the following equation for relative retention index (RRI):

$$\text{RRI} = \frac{t_R \text{ unknown} - t_R[\text{}^2\text{H}_4]\text{8-epi-PGF}_{2\alpha}}{t_R[\text{}^2\text{H}_4]\text{PGF}_{2\alpha} - t_R[\text{}^2\text{H}_4]\text{8-epi-PGF}_{2\alpha}} + 1 \quad (1)$$

For all electrospray ionization analyses, the curtain gas flow rate was 1.2 L/min, nebulizer pressure was 42 lb/in², turbospray flow was 6 L/min, and turbospray

temperature was held at 400 °C. The collision cell was operated with a gas thickness of 172×10^{13} molecules/cm² and the orifice potential was typically held at –70 V.

Gas chromatography/mass spectrometry of the HPLC fractions was carried out in both the electron capture negative ion mode and by electron ionization via either a Finnigan SSQ or a TSQ-700 mass spectrometer (Finnigan-MAT, San Jose, CA). Both instruments were interfaced with a capillary DB-1 column (15 m $\times$ 0.25 mm; J & W Scientific, Folsom, CA) with a 0.25 μm film thickness. The injector temperature was maintained at 275 °C and the transfer line at 300 °C. For electron capture negative ionization mass spectrometry the instrument was maintained with an ion source pressure of approximately 1 mtorr with methane. The F_2 -isoprostanes were detected by using selected ion monitoring of the ion at m/z 569, which corresponded to the loss of pentafluorobenzyl radical from the intact F_2 -isoprostane anion. The corresponding ion for either deuterium-labeled PGF $_{2\alpha}$ or 8-*epi*-PGF $_{2\alpha}$ was m/z 573, and these two molecules were well separated by gas chromatography. Electron ionization mass spectra were recorded at 70 eV; the instrument was scanned over a mass range of 50–600 u/s. The ion source temperature was maintained at 180 °C.

Gas chromatographic retention is expressed as equivalent fatty acid ester carbon number, determined as previously described for either the pentafluorobenzyl or methyl esters [18].

Results

Synthetic prostaglandins epimeric to the PGF $_{2\alpha}$ Type IV regioisomer were partially separated by reversed phase HPLC (Figure 1). These epimeric prostaglandins were detected as they eluted from the HPLC by colli-

Figure 1. Direct liquid chromatography tandem mass spectrometry analysis via electrospray ionization of a mixture of synthetic Type IV regioisomers of PGF $_{2\alpha}$ separated by reversed-phase HPLC. (a) The loss of 44 u ($\text{C}_2\text{H}_4\text{O}$) from the carboxylate anion (m/z 353) was used to detect the elution of each synthetic isomer. (b) The loss of 44 u from m/z 357 was used to detect the elution of the deuterium-labeled internal standards.

sion-induced dissociation of the carboxylate anions formed by electrospray ionization with loss of C₂H₄O (44 u) as described previously for the characteristic decomposition ions observed for PGF_{2α} [19]. Elution of these synthetic compounds occurred between 12 and 17 min: 8-*epi*-PGF_{2α} eluted in the earliest peaks and PGF_{2α} eluted last. The elution of these two species also was marked by the deuterium-labeled internal standards [²H₄]8-*epi*-PGF_{2α} and [²H₄]PGF_{2α} at 13.55 and 16.37 min, respectively (Figure 1b). The relative retention characteristics for each of the epimeric prostaglandins relative to [²H₄]PGF_{2α} in this reversed phase system are presented in Table 1.

Gas chromatographic separation of the epimeric prostaglandins was carried out after conversion either to the pentafluorobenzyl (PFB) ester trimethylsilyl (TMS) ether or methyl ester trimethylsilyl ether derivatives. The former derivative was used for a sensitive negative ion GC/MS assay whereas the latter derivative was used for positive ion electron ionization mass spectrometric analysis. Partial separation of each of the Type IV regioisomers epimeric to PGF_{2α} was achieved as the PFB/TMS derivative (Figure 2a) and, as expected, the elution order of each individual epimer was different from that observed for the HPLC separation (Table 1). Derivatized 8-*epi*-PGF_{2α} did elute prior to PGF_{2α}; however, there was no obvious structural determinant to the elution order of each epimer.

Because the last component to elute had a double bond at C-5 of the *trans* configuration, but was otherwise identical to the next to last component of the mixture to elute, namely PGF_{2α}, this suggested that the geometry of each double bond played an important role in determination of the gas chromatographic retention behavior in this set of isomeric molecules. Catalytic reduction of the epimeric PGF_{2α} standards

Figure 2. (a) GC/MS analysis via electron capture ionization of the pentafluorobenzyl trimethylsilyl ether derivatives of the synthetic epimeric PGF_{2α} Type IV regioisomers. The carboxylate anion at *m/z* 569 was used in selected ion monitoring mode to detect the elution of the components in the mixture. The elution is expressed as equivalent fatty acid carbon number (PFB ester). (b) GC/MS analysis via electron ionization of catalytically reduced methyl ester trimethylsilyl ether derivative of the Type IV regioisomers epimeric to PGF_{2α}. The ion at *m/z* 498 (M-90) was used to monitor the elution of the epimers. The retention time is expressed as equivalent fatty acid carbon number (methyl ester).

Table 1. Relative retention indices of synthetic prostaglandins and F₂-isoprostanes Type IV

Epimers	F ₂ -isoprostane relative retention index ^a	HPLC retention time ^b (min)
[² H ₄]8- <i>epi</i> -PGF _{2α}	1.00	13.55
[² H ₄]PGF _{2α}	2.00	16.37
5- <i>trans</i> -9β-PGF _{2α}	0.89	13.25
8- <i>epi</i> -9β-PGF _{2α}	1.00	13.55
8- <i>epi</i> -PGF _{2α}	1.00	13.55
9β-PGF _{2α}	1.07	13.75
11β-PGF _{2α}	1.39	14.65
5- <i>trans</i> -PGF _{2α}	1.71	15.55
15- <i>epi</i> -PGF _{2α}	1.71	15.55
PGF _{2α}	2.00	16.37

^aElution of F₂-isoprostane relative to [²H₄]8-*epi*-PGF_{2α} and [²H₄]PGF_{2α} according to the equation given in Experimental Procedures.

^bOn-line reversed-phase HPLC analysis monitored with a Sciex API-III⁺ triple quadrupole mass spectrometer. HPLC was operated at a flow rate of 1 mL/min with a linear gradient of 25–50% B (A = pH 5.7 H₂O, 0.05% CH₃CO₂H; B = 95:5, CH₃CN:MeOH) and a postcolumn split that yielded 15 mL/min flow into the mass spectrometer.

followed by gas chromatographic separation of the methyl ester trimethylsilyl ether derivative simplified to some extent the separations of individual isomers by reducing the total number of isomers present and affecting separation of the 9β-PGF_{2α} from the 8-*epi*-PGF_{2α} and 8-*epi*-9β-PGF_{2α} epimers (Figure 2b). For the fully saturated compounds, the earliest eluting epimer had adjacent substituents of the cyclopentane ring *trans* to each other, whereas the last eluting epimers had three substituents on the same α side of the cyclopentane ring.

The combination of HPLC and gas chromatographic separation could effect resolution of most of the available epimeric PGF_{2α} Type IV regioisomers. For example, separation of 5-*trans*-PGF_{2α} and 15-*epi*-PGF_{2α} was not possible by reversed phase HPLC (Table 1); however, they were well separated by gas chromatography (Table 2). PGF_{2α} was well separated from 15-*epi*-PGF_{2α} by HPLC, but both co-eluted in capillary GC (Table 2) either as the reduced or nonreduced molecules. Some epimers could not be separated by either technique

Table 2. Gas chromatographic equivalent carbon numbers^a of synthetic Type IV F₂-isoprostane^b

Epimers	PFB/TMS equivalent carbon number ^c		CH ₃ /TMS equivalent carbon number ^d
	Unreduced	Reduced	
9β-PGF _{2α}	23.05	23.45	23.80
5- <i>trans</i> -9β-PGF _{2α}	23.12	23.45	23.80
8- <i>epi</i> -PGF _{2α}	23.04	23.60	23.90
8- <i>epi</i> -9β-PGF _{2α}	23.04	23.60	23.90
11β-PGF _{2α}	23.32	23.86	24.20
15- <i>epi</i> -PGF _{2α}	23.44	23.97	24.30
PGF _{2α}	23.44	23.97	24.30
5- <i>trans</i> -PGF _{2α}	23.69	23.97	24.30

^aEquivalent carbon numbers were calculated by using the corresponding fatty acid derivatives according to the method previously described (ref 17).

^bThese standards were derivatized as pentafluorobenzyl ester trimethylsilyl ethers or catalytically reduced with Rh/Al₂O₃ and H₂ gas prior to derivatization as PFB ester TMS ethers or methyl ester TMS ethers.

^cElectron capture negative ion chemical ionization mass spectrometry. Operating parameters are as described in Experimental Procedures and a linear temperature program of 230–235 °C in 10 min.

^dElectron ionization mass spectrometry. Operating parameters are as described in Experimental Procedures and a linear temperature program of 210–215 °C in 10 min.

(such as 8-*epi*-PGF_{2α} from 8-*epi*-9β-PGF_{2α}), yet a 30 m capillary GC column was reported to achieve this separation [20]. However, this latter compound would not be an expected F₂-isoprostane, which requires the 1,3-diol substituents to be on the same side of the cyclopentane ring.

Electron Ionization Mass Spectrometry

Reduction of the prostaglandins (each of which contains two double bonds) simplified the chromatographic separation of the individual Type IV epimers and furthermore altered the mass spectrometric behavior when analyzed by electron ionization techniques (EI). The EI mass spectrum of reduced PGF_{2α} is presented in Figure 3 and was characterized by a series of α-cleavage ions adjacent to the 15-trimethylsiloxy substituent. The ion at *m/z* 173 corresponded to α-cleavage of the 14,15-bond and the ion at *m/z* 427 corresponded to α-cleavage on the other side of the trimethylsiloxy moiety with the concomitant loss of trimethylsilanol. Either ion could be directly formed from the observed ion at *m/z* 498, which corresponded to the loss of trimethylsilanol from the molecular ion (*m/z* 588). The ions *m/z* 73, 191, and 217 were characteristic of trimethylsilylated polyhydric molecules, in particular *m/z* 217, which is quite abundant for those compounds that contain a 1,3 diol structure [21]. The ion at *m/z* 355 corresponded to cleavage of the alkyl chain adjacent the cyclopentane ring (carbon-8) and loss of trimethylsilanol. This was supported by experiments with *d*₉-TMS-labeled reduced PGF_{2α}. The EI mass spectrum of derivatized PGF_{2α}

Figure 3. Electron ionization mass spectrum (70 eV) of the reduced methyl ester trimethylsilyl ether derivative of authentic PGF_{2α}. Abundant α-cleavage ions are indicated.

(Figure 3) was virtually identical for all of the reduced Type IV regioisomers (data not shown).

Abundant formation of α-cleavage ions adjacent to the trimethylsiloxy substituent in the side chain would be expected to occur for each of the different regioisomers of F₂-isoprostanes. Those α-cleavage ions that retained the cyclopentane ring would appear at the mass that corresponds to a further loss of trimethylsilanol. Because the hydroxyl substituent on the alkyl portion of the F₂-isoprostane is unique for each of the regioisomers (Scheme 1), such α-cleavage ions predicted in Figure 4 would uniquely identify each of the four regioisomers present in a complex mixture such as would be expected from a free radical oxidation of arachidonic acid.

Arachidonic Acid Oxidation

Oxidation of arachidonic acid was carried out by using a modified Fenton reaction with CuCl₂ and H₂O₂ to generate hydroxyl radical and induce peroxidation [22].

Figure 4. Predicted α-cleavage ions for each of the four F₂-isoprostane regioisomeric families. The dashed lines indicate unknown stereochemistry.

Following oxidation, products were isolated by using reversed phase HPLC and individual HPLC fractions were screened for the appearance of F₂-isoprostanes by using negative ion chemical ionization GC/MS of the pentafluorobenzyl ester trimethylsilyl ether derivative (m/z 569). Those HPLC fractions that contained F₂-isoprostanes then were reduced catalytically and derivatized to the methyl ester trimethylsilyl ether for electron ionization GC/MS. Mass chromatograms for the expected α -cleavage ions for each subtype revealed those HPLC fractions that contained each of the four F₂-isoprostane regioisomers.

Type I F₂-isoprostanes would be expected to yield α -cleavage ions at m/z 203 and m/z 397 (an α -cleavage ion from the M-90). The inset to Figure 5a shows the mass chromatogram for these target ions that reveals the elution of a component from the gas chromatograph with a carbon number of 23.80. The mass spectrum of this component was consistent with an F₂-isoprostane as evidenced by the observed ion at m/z 498 (M-90) and the trimethylsiloxyl ions at m/z

73, 191, and 217. Furthermore, abundant ions were observed at m/z 203 and 397, the expected α -cleavage ions. Additional abundant ions were also present at m/z 385 and 408. The ion at m/z 385 could correspond to an α -cleavage of the M-90 ion species with charge retention at carbon-6, but is most likely the cleavage of the alkyl chain adjacent to the cyclopentane ring (carbon-12) and loss of trimethylsilanol as observed for reduced PGF_{2 α} . The ion at m/z 408 would be loss of one additional trimethylsilanol from m/z 498. This oxidation product of arachidonic acid was consistent with a Type I F₂-isoprostane.

A Type II regioisomer would have the side chain hydroxyl group at carbon-12 that would yield two abundant α -cleavage ions at m/z 215 and 385. Individual F₂-isoprostanes of this family member were substantially less abundant than either Type I or Type IV family members, but, nevertheless, it was possible to obtain a representative mass spectrum of this family subtype as seen in Figure 5b for a component that eluted from the HPLC in 22 min and a carbon number

Figure 5. Electron ionization mass spectrum of reduced methyl ester trimethylsilyl ether derivative of F₂-isoprostane regioisomeric families obtained by free radical oxidation (modified Fenton reaction) of arachidonic acid. (a) Type I F₂-isoprostane component that displays abundant α -cleavage ions at m/z 203 and 397. The inset to a shows the selected ion monitoring trace for these α -cleavage ions that indicates the elution of this component at scan number 70 presented as a. (b) Type II F₂-isoprostane that displays abundant α -cleavage ions at m/z 215 and 385. (c) Type III F₂-isoprostane that displays abundant α -cleavage ions at m/z 245 and 355. (d) Type IV F₂-isoprostane that displays abundant α -cleavage ions at m/z 173 and 427. Dashed lines in the chemical structures are used to indicate unknown stereochemistry.

of 24.17 by gas chromatography. The expected ions for derivatized F_2 -isoprostane were present at m/z 73, 129, 147, 191, 217, 408 (M-2TMSOH), and 498 (M-TMSOH) [23]. The additional ion at m/z 295 was most likely a further loss of trimethylsilanol from the α -cleavage ion at m/z 385 because this ion would retain the cyclopentane ring and associated trimethylsiloxy groups.

The isoprostane family member Type III would be characterized by a hydroxyl group at carbon-8 in the alkyl chain and α -cleavage ions at m/z 245 and 355 would be expected for the reduced, derivatized product. The HPLC fraction at 20.5 min contained such a Type III F_2 -isoprostane as evidenced by a component that eluted from the gas chromatograph as the methyl ester trimethylsilyl ether with a carbon number of 24.00 that yielded abundant ions at m/z 245 and 355. The observed ion at m/z 427 likely corresponded to cleavage adjacent to the cyclopentane ring at carbon-15 with loss of trimethylsilanol. Type III family members were also of lesser abundance than either Type I or Type IV F_2 -isoprostanes.

Figure 5d shows a mass spectrum of one Type IV F_2 -isoprostane that eluted from the HPLC in the fraction collected at 20 min and exhibited a gas chromatographic retention carbon number of 23.90 as the reduced trimethylsilyl methyl ester derivative. The observed mass spectrum was virtually identical to that of authentic $PGF_{2\alpha}$ (Figure 3) and the carbon number was identical to 8-*epi*- $PGF_{2\alpha}$. Several other Type IV family members were readily identified in this mixture of oxidized arachidonic acid, and Type I and Type IV F_2 -isoprostanes were the most abundant species formed in this model system of lipid peroxidation.

Liquid Chromatography–Mass Spectrometry Analysis

Limited oxidation of arachidonic acid by using the modified Fenton reaction (4 h) yielded a complex mixture of products that were analyzed by direct liquid chromatography–electrospray tandem mass spectrometry (ESI-MS/MS) after extraction of the oxidized sample and reduction of peroxy and hydroperoxy groups with $SnCl_2$. Two deuterated standards, $[^2H_4]8$ -*epi*- $PGF_{2\alpha}$ and $[^2H_4]PGF_{2\alpha}$, were added to serve as HPLC retention standards. Fractions were collected from a split of the HPLC effluent prior to electrospray and were analyzed off-line by electron ionization GC/MS as the reduced trimethylsilylated methyl ester products. The F_2 -isoprostanes that eluted from the HPLC were detected by loss of 44 u (C_2H_4O) from m/z 353 (m/z 353 $\rightarrow$ 309) and m/z 357 (m/z 357 $\rightarrow$ 313) for the deuterium-labeled internal standards as seen in Figure 6. Multiple components were obvious for the F_2 -isoprostanes (m/z 353 $\rightarrow$ 309) whereas the epimeric deuterated internal standards were clearly separated as single peaks; therefore, each collected fraction was likely to contain unresolved epimeric F_2 -isoprostanes.

Figure 6. Direct liquid chromatography–tandem mass spectrometry analysis obtained by electrospray ionization for the loss of 44 u from the carboxylate anion of F_2 -isoprostanes (m/z 353). The F_2 -isoprostanes were obtained by free radical oxidation (modified Fenton reaction) of arachidonic acid. (a) Fractions collected during the elution of the isoprostanes indicated by the numbers 1–4 were collected and analyzed by electron ionization after catalytic reduction and derivatization. The regioisomeric families identified in these fractions are indicated in Table 3. (b) A loss of 44 u from m/z 357 that corresponds to the deuterium-labeled internal standards.

Catalytic reduction and EI GC/MS analysis enabled assignment of the major F_2 -isoprostane family subtype. The HPLC fractions collected at peaks 1, 2, 3, and 4 from 15 to 20 min contained several distinct, derivatized F_2 -isoprostanes separated by the gas chromatograph, but only major epimers could be assigned unambiguously to one of the four regioisomer types (Table 3). The HPLC fractions collected between 15 and 16 min contained a F_2 -isoprostane based on the ions at m/z 498, 217, 191, and 73 in the most abundant GC component. The α -cleavage ions at m/z 173 and 427 as well as an abundant ion at m/z 355 were consistent with a Type IV regioisomer. It is important to note, however, that the gas chromatographic carbon number of 23.90 (Table 3) was identical to that of the catalytically reduced authentic 8-*epi*- $PGF_{2\alpha}$ (Table 2); however, the HPLC RRI (1.74; Table 3) of this component was significantly different from that of 8-*epi*- $PGF_{2\alpha}$ (RRI 1.00; Table 1). Thus, this component was an entirely different stereoisomer of the Type IV family than either 8-*epi*- $PGF_{2\alpha}$ or $PGF_{2\alpha}$. The HPLC fractions that eluted between 16.7 and 17.9 min contained one Type I and two Type IV regioisomers with carbon numbers of 23.80, 23.90, and 24.13, respectively. All of the common F_2 -isoprostane ions at m/z 498, 217, 191, and 73 were present with the Type I regioisomer identified by the characteristic α -cleavage ions at m/z 203 and 397 (e.g., Figure 5a). The Type-IV isomers displayed the characteristic α -cleavage ions seen in Figures 3 and 5d. The fractions from the HPLC between 18 and 18.7 min displayed mass spectra consistent with regioisomers of the Type IV (carbon number 24.13) and Type I families (carbon number 24.23) as

Table 3. Relative retention indices, gas chromatographic carbon numbers and regioisomeric family types formed by free radical oxidation of arachidonic acid.^a

HPLC fractions ^b	RRI ^c	HPLC retention time (min) ^c	CH ₃ /TMS equivalent carbon number ^{d,e}	Major regioisomeric family type
1	1.74	15.65	23.90	IV
2	2.22	17.00	23.80	I
			23.90	IV
			24.13	IV
3	2.71	18.36	24.13	IV
			24.23	I
			24.30	IV
4	3.06	19.35	24.20	I
			24.23	IV
			24.30	IV

^aThese F₂-isoprostanes were formed by Cu²⁺/H₂O₂ oxidation of 5,8,11,14-eicosatetraenoic acid and separated by reversed-phase HPLC prior to catalytic reduction and derivatization as methyl ester TMS ethers.

^bSee Figure 6.

^cElution of F₂-isoprostane relative to [²H₄]8-*epi*-PGF_{2α} and [²H₄]PGF_{2α} according to the equation given in Experimental Procedures. On-line reversed-phase HPLC analysis monitored with a Sciex API-III⁺ triple quadrupole mass spectrometer. HPLC was operated at a flow rate of 1 mL/min with a linear gradient of 25–50% B (A = pH 5.7 H₂O, 0.05% CH₃CO₂H; B = 95:5, CH₃CN:MeOH) and a postcolumn split that yielded 15 mL/min flow into the mass spectrometer.

^dElectron ionization mass spectrometry. Operating parameters as described in Experimental Procedures and a linear temperature program of 210–215 °C in 10 min.

^eEquivalent carbon numbers were calculated by using the corresponding fatty acid derivatives according to the method previously described (ref. 17).

well as a Type IV regioisomer (carbon number 24.30). This latter Type IV regioisomer had the same gas chromatographic retention time as PGF_{2α}; however, its HPLC elution RRI (2.71; Table 3) was not the same as any of the specific epimers available for study (Table 1). Type I (carbon number 24.20) and Type IV (carbon numbers 24.23 and 24.30) regioisomers were identified as the major components present in the HPLC fractions that eluted between 18.8 and 19.8 min (Table 3). Multiple F₂-isoprostanes were present in other HPLC fractions at an abundance that precluded definitive assignment of the elution of Type II or Type III regioisomers. The deuterium-labeled prostaglandins did co-elute in the fractions where Type-IV F₂-isoprostanes that eluted between 13 and 14 min ([²H₄]8-*epi*-PGF_{2α}) and 16–17 min ([²H₄]PGF_{2α}, as seen in Figure 6) were identified.

Discussion

Isoprostanes are a relatively recent addition to the family of lipid mediators that are derived from arachidonic acid by a free radical mechanism of oxidation and lead to a family of regioisomers each composed of multiple epimers rather than enzymatic products of arachidonate oxidation. Thus a complex mixture of isoprostanes results in tissues as a consequence of the reaction of active oxygen species with arachidonate esterified to membrane glycerophospholipids. Negative ion electron capture mass spectrometry has been instrumental in identification of the existence of epimeric isoprostanes since the pioneering work of Roberts and colleagues [4–9, 13–16]. Continued interest in these molecules stems in part from the biological activity that has been discovered for one of the prod-

ucts, namely, 8-*epi*-PGF_{2α} [8, 9]. The activity of this molecule has led to the suggestion that these free radical products may play an important pathophysiological role to mediate the events of tissue damage caused by active oxygen species [13, 14]. Measurement of isoprostanes also has been suggested to serve as a reliable marker of oxidant injury in vivo [8–10]. 8-*Epi*-PGF_{2α} also has been uniquely identified as one of the free radical products through an exhaustive isolation procedure that involved multiple HPLC as well as capillary gas chromatographic separations [16]. Recently, evidence also has emerged to suggest that 8-*epi*-PGF_{2α} can be a cyclooxygenase product also, which thus suggests that measurement of this isoprostane may not truly reflect noncyclooxygenase formation of isoprostanes [24]. However, in experiments that used a specific immunoassay, activation of platelet cyclooxygenase did not lead to a correlation between production of 8-*epi*-PGF_{2α} and PGF_{2α} [25] which suggests that 8-*epi*-PGF_{2α} formation was independent of platelet cyclooxygenase activity. However, 8-*epi*-PGF_{2α} is a member of the same regioisomeric family as native PGF_{2α} (Type IV)—the enzymatic product of PGH synthase—and the search for other regioisomeric family members of isoprostanes has continued.

Separation of most synthetic epimers of PGF_{2α} was possible both by reversed-phase HPLC as well as by capillary gas chromatography. Unfortunately, the availability of only a small number of PGF_{2α} epimers precluded definite conclusions as to unique retention properties of the Type IV regioisomer family. Nevertheless, there was a trend to suggest that an all trans arrangement of the substituents or a cis (e.g., “8-*epi*”) arrangement of the unsaturated alkyl substituents of

the cyclopentane ring did impart early elution from both HPLC and GC. Furthermore, the trans configuration of the double bond at the 5-position and the trans arrangement of the alkyl substituents on the cyclopropane ring (adjacent to the 1,3-diol) had the effect of increasing gas chromatographic retention time whilst decreasing HPLC retention time.

Electrospray ionization tandem mass spectrometry was found to be a sensitive method with which to detect the elution of F_2 -isoprostanes from an HPLC chromatographic column. Because these molecules do not have a favorable UV chromophore (two isolated double bonds), sensitive detection of these molecules that elute from an HPLC is not possible, in particular at the sensitivities required for measurement of these molecules present in biological tissues. However, electrospray mass spectrometry generated abundant carboxylate anions at m/z 353 (for all regioisomeric F_2 -isoprostanes) that further decomposed by the loss of C_2H_4O (44 u) following collisional activation in the rf-only quadrupole of the collision cell. Although this loss of 44 u is potentially identical to a loss of CO_2 from a carboxylate anion, it is particularly abundant for the isoprostanes that have a 1,3 cyclic diol structure. This collision-induced process has been described previously for prostaglandins and thromboxanes, which share this common structural modality [19, 26].

Definitive information as to the specific regioisomeric family was obtained by using electron ionization mass spectrometry of the reduced isoprostanes. Saturation of the two double bonds in these molecules decreased the total number of epimers in the complex mixture. This step did result in a loss of information with regard to the geometry of the two remaining double bonds; however, an increase in specific mass spectrometric fragmentation was observed, in particular cleavage adjacent to the hydroxyl group of the alkyl side chain. These α -cleavage ions as well as α -cleavage ions with the concomitant loss of trimethylsilanol proved to be the most abundant ions for each of the Type IV regioisomers epimeric to $PGF_{2\alpha}$ studied. Because all reduced epimers of $PGF_{2\alpha}$ gave identical EI mass spectra (with characteristic α -cleavage ions), all unknown epimers within a given regioisomer type would be expected to yield identical EI spectra (with the predicted characteristic α -cleavage ions). Thus, monitoring of characteristic α -cleavage ions of the reduced isoprostanes (after partial separation by HPLC) would be a strategy to uniquely identify compounds in complex mixtures that belong to each regioisomer type (by GC/MS after HPLC purification), but their EI mass spectra alone would not uniquely identify specific members within a family.

Free radical oxidation of arachidonic acid yields a complex mixture of isoprostanes as revealed by electrospray tandem mass spectrometry and the elution of multiple components that correspond to arachidonic acid with three oxygen atoms and four rings or double bonds (m/z 353). The detection of these isoprostanes

that elute from the HPLC chromatograph was made possible by collisional activation of m/z 353 and observation of m/z 309 that corresponds to the loss of C_2H_4O . Those fractions identified by tandem electrospray mass spectrometry were then catalytically reduced and derivatized for electron ionization mass spectrometry-capillary gas chromatography. Use of this approach enabled the four specific regioisomeric families of isoprostanes to be identified by using the specific α -cleavage ions for the alkyl-chain hydroxy groups present in each of the F_2 -isoprostanes. Each of the mass spectra were interpreted in terms of the unique isoprostane family subtype.

Type I and Type IV regioisomeric compounds could be readily identified by using this process because of their abundance in the oxidation mixture. However, it was more difficult to unambiguously detect the specific family subtypes II and III, which suggests that they were produced in substantially lower quantities. This is not surprising based upon the mechanism of formation of each of the regioisomeric families. Initial radical localization at carbon-7 can lead to only one class of isoprostane, namely, the Type I family. If oxygen is attached to carbon atom 5 from this carbon-7 radical species, a different family of oxidized arachidonic acid would be formed that includes 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and the isoleukotriene family [27]. The same is true for the initial radical attack at carbon-13, which could only lead to regioisomeric family subtype IV and 15-HPETE. Free radical attack at carbon-10 would lead to two different isoprostane families, Type II and Type III, as well as 8-HPETE and 12-HPETE. Thus it is not surprising that the Type II and Type III regioisomeric isoprostanes were of lower abundance than those of Type I and Type IV. It is important to note that more than a simple GC/MS assay is necessary to precisely identify a particular stereoisomer within a regioisomeric family, for example 8-*epi*- $PGF_{2\alpha}$ as shown by the foregoing liquid chromatography-mass spectrometry analysis when epimers with the same GC carbon number are separable by HPLC. Nevertheless, results of these studies revealed the elution of all four regioisomeric families at very similar conditions of reversed-phase HPLC and capillary gas chromatography.

It has been suggested that measurement of isoprostanes may serve as a noninvasive marker of the initial events of lipid peroxidation, rather than the extensive breakdown products of 4-hydroxy nonenal or malonyldialdehyde [13, 14]. The results of these studies suggest that development of a specific assay for isoprostanes based upon either mass spectrometric techniques or, even more critically, immunoassays that recognize unique structural characteristics, might be best for the Type I regioisomeric isoprostane family subtype. One reason for this is the other abundant isoprostane regioisomer Type IV is identical in cyclopentane ring and hydroxyl group position to that obtained following the action of PGH synthase on

arachidonic acid. This immunological cross-reactivity with endogenous prostaglandin and prostacyclin metabolites may be expected for antibodies directed against the Type IV structural isotopes. Although the biologically active 8-*epi*-PGF_{2α} isoprostane has been targeted for both direct mass spectrometric as well as immunoassay techniques, the 8-*epi*-PGF_{2α} target is largely based upon current commercial availability of this isomer, but measurement of this isoprostane is confounded by the recent finding of 8-*epi*-PGF_{2α} as a PGH synthase-derived product [23]. The regioisomeric family Type I is not derived from PGH synthase and thus would represent a totally novel structure for which specific assays could be developed.

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