Identification of Two Major F2 Isoprostanes, 8,12-Isoprostanes, 8,12-Isoprostane F2α-VI, in Human Urine*

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Isoprostanes (iPs) are nonenzymatic, free radical-derived compounds isomeric with enzymatically formed eicosanoids such as prostaglandins, leukotrienes, and thromboxanes. One group formed by the auto-oxidation of arachidonic acid, the F2-ipFs, consists of four classes of isomers of prostaglandin F2α (PGF2α). They are relatively abundant in human urine. This fact, along with their chemical stability and excellent characteristics for quantitation by gas chromatography/mass spectrometry, has made them attractive indices of oxidative stress in humans. We developed a specific assay using gas chromatography/mass spectrometry for the first identification of F2-ipF, iPF2α-III (previously called 8-iso-PGF2α or 8-epi-PGF2α), which demonstrated the utility of monitoring a specific isomer. Recently, we described an assay for another isomer, IPF2α-VI, which is present in urine in greater concentration than iPF2α-III and which is particularly amenable to quantitation. We now describe the identification in human urine of two more isomers, 8,12-iso-IPF2α-VI and 5-epi-8,12-iso-IPF2α-VI, using high performance liquid chromatography/tandem mass spectrometry and gas chromatography/mass spectrometry. These compounds are each present in ∼5-fold greater concentrations than IPF2α-VI (∼20-fold greater than iPF2α-III). They share the unique chemical characteristics of class VI compounds, which make them attractive targets for quantitation by gas chromatography/mass spectrometry and immunoassay development.

Since oxidant stress has been implicated in many diseases as well as in physiological processes such as aging, there is interest in the rational development of antioxidant drugs. A paucity of noninvasive, sensitive, specific methods for monitoring the oxidative status of individual patients has constrained such efforts and limited insight into the role of reactive oxidant species in human disease. Since the discovery of F2 isoprostanes (F2-ipFs)1 (1, 2), the quantitation of these compounds in urine and serum has shown promise as an approach to the study of oxidative stress (3, 4). Immunoassay and GC/MS techniques have been used for quantitation of iPs. Immunoassays are relatively quick and technically simple (5), but assays for iPs in urine require, due to nonspecific reactivity, some degree of purification before quantitation, introducing the possibility of uncontrolled loss of analyte. Also, antibodies raised against a specific F2-ipF may cross-react with other isomers or their largely uncharacterized metabolites. Since only a few of the 64 potential F2-ipF and only one metabolite have been synthesized, this cross-reactivity is difficult to assess. GC/MS assays to date have utilized capillary gas chromatography/negative ion electron capture chemical ionization mass spectrometry and have targeted either F2-ipFs as a class or have attempted to measure a single isomer. Those approaches that measure multiple isomers (6) compare the area under a number of overlapping peaks to that under a single peak corresponding to a stable isotope-labeled internal standard and are relatively straightforward, technically. However, inadequate purification of such samples can lead to the presence of spurious peaks in the integrated region, whereas stringent purification can lead to the loss of some isomers. On the other hand, assays attempting to measure a single isomer from the myriad present require a high degree of sample purification. This is labor-intensive and may result in low yields and/or chromatographically heterogeneous peaks. We have published descriptions of two stable isotope dilution assays for specific F2-ipFs: iPF2α-III,2 previously called 8-iso-PGF2α, 8-epi-PGF2α, or IPF2α-IV (7), and IPF2α-VI, previously called IPF2α-I (8). The former is very difficult to purify to homogeneity. The latter is present in greater quantities and takes advantage of the unique characteristics of class VI F2-ipFs to form a cyclic lactone (Fig. 1), which provides a means of separating them from class III–V F2-ipFs (8). Unidentified peaks in the GC/MS chromatograms from the iPF2α-VI urinary assay made us suspect that other members of class VI might be present in even greater concentrations than iPF2α-VI.

Since little information concerning the composition of the array of F2-ipFs in urine is available (9, 10), we have developed...
methods utilizing HPLC/ESI/MS/MS for characterizing them with the hope of identifying individual isomers that are more amenable to quantitation than the two for which we have developed assays to date. We now identify 8,12-iso-\(iPF_{2\alpha,-VI}\) and 5-epi-8,12-iso-\(iPF_{2\alpha,-VI}\) (Fig. 2), using our synthetic standards, as the two most abundant \(F_2\)-iPs yet observed in human urine. Given their abundance, these compounds represent a logical target for \(F_2\)-iP quantitation by GC/MS, HPLC/ESI/MS/MS, or immunoassay.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless otherwise stated, all solvents were B&J High Purity (Burdick & Jackson, Muskegon, MI). \(N,N\)-Diisopropylethylamine and pentafluorobenzyl bromide were purchased from Sigma. 1-Butanenboronic acid was purchased from Aldrich. Bis(methylsilyl) trifluoroacetamide was purchased from Supelco Inc. (Bellefonte, PA).

**Synthesis of 8,12-Iso-\(iPF_{2\alpha,-VI}\) and Its 5R- Epimer**—Briefly, the first total synthesis of 8,12-iso-\(iPF_{2\alpha,-VI}\) and its 5R-epimer, (5R)-8,12-iso-\(iPF_{2\alpha,-VI}\), was performed using a key intermediate, the all-syn six-membered ring lactone (4aR,5S,7R,7aS)-hexahydro-3-oxo-5,7-bis(tert-butyldimethylsilyl)oxyyclopentapyran, which was prepared from diacetone-\(\delta\)-glucose. The details of the syntheses of these two class VI isoprostanes will be published elsewhere.

**Solid-phase Extraction**—A RapidTrace SPE workstation (Zymark Corp., Hopkinton, MA) was used for all extractions. Reverse-phase cartridges (C18 EC, 100 mg; International Sorbent Technology, Mid Glamorgan, United Kingdom) were primed with 3 ml of methanol followed by 0.5 ml of 0.8 M phosphate buffer, pH 7.0. Urine samples (1–3 ml) were applied at 1 ml/min, and the cartridge was washed with 1 ml of 25% ethanol/buffer at 1 ml/min. The cartridge was dried with 3 ml of air (30 ml/min) and eluted with 1 ml of ethyl acetate at 1 ml/min.

**HPLC**—HPLC was performed on an ABI 140B solvent delivery system (Perkin-Elmer) utilizing a Hypersil BDS C18 column (2 × 150 mm) packed with 3-μm particles with 130-Å pore size (Phenomenex Inc., Torrance, CA). The flow rate was 200 μl/min. The column effluent was split, with 25% going to the electrospray source and the remainder to waste. For GC/MS analysis, fractions were hand-collected from the HPLC/ESI/MS/MS data. The mobile phase was programmed from 25 to 35% mobile phase B in 20 min, held for 20 min, and then programmed to 90% mobile phase B. Mobile phase A consisted of deionized distilled water, with the pH adjusted to 5.7 with acetic acid. Mobile phase B was 95% acetonitrile and 5% methanol. Samples were injected by a Hewlett-Packard Series 1100 autosampler.

**Electrospray Ionization/Tandem Mass Spectrometry**—All MS/MS analyses were performed on a MicroMass Quadrupole II instrument interfaced with a MicroMass ESI source. Instrument control, data acquisition, and data analysis were achieved with MassLynx software. Conditions were as follows: nebulizer gas flow, 30 liter/h; drying gas flow, 300 liter/h; spray potential, ~3000 V; cone potential, ~40 V; and collisional offset potential, 23 V. Argon was used for the collision gas. Q2 scans were acquired at a rate of 225 atomic mass units/s. For multiple reaction monitoring, each ion was monitored for 0.15 s.

**Derivatization**—The PFB ester was formed by adding 10 μl of \(N,N\)-disopropylethylamine and 20 μl 10% PFB bromide in acetonitrile to the dry sample. This was dried under a gentle stream of nitrogen after standing for 10 min at room temperature. The TMS ether was formed by adding 10 μl each of bis(trimethylsilyl) trifluoroacetamide and pyridine and allowing the sample to stand at room temperature for 10 min. The n-buty1 boronate TMS derivative was formed by adding 10 μl of n-butylboronic acid (20 mg/ml) to the sample and allowing it to stand for 60 min at room temperature and then adding 10 μl of bis(trimethylsilyl) trifluoroacetamide and allowing the sample to stand at room temperature for 10 min. Samples were dried and dissolved in dodecane prior to injection into the GC/MS.

**Gas Chromatography/Mass Spectrometry**—All of the GC/MS analyses were performed on an MD-800 mass spectrometer interfaced with a GC-8060 gas chromatograph and an AS-800 autosampler (Finnigan MAT, San Jose, CA). For GC, a 30 m × 0.25-mm, 0.25-μm coating, DB5-MS column (J & W Scientific, Folsom, CA) was used. The carrier gas was helium. The temperature program consisted of an initial temperature of 190 °C (1 min) and then a programmed increase to 320 °C at 20 °C/min. The mass spectrometer was operated in the negative ion (electron capture) chemical ionization mode using ammonia as the moderating gas. Ions monitored are \(m/z\) 569 for the PFB ester TMS derivative and \(m/z\) 451 for the PFB cyclic n-buty1 boronate TMS derivative.

**RESULTS**

**HPLC/ESI/MS/MS**—Synthetic standards for \(iPF_{2\alpha,-III}\), -IV, -V, and -VI were analyzed by HPLC/ESI/MS/MS. Product ion mass spectra of the collision-induced decomposition of the \(F_2\)-iP carboxylate anion (\(m/z\) 353) were similar for each of the four \(F_2\)-iP classes (Fig. 3) (9). However, significant exceptions occurred. The ion at \(m/z\) 115 in class VI \(F_2\)-iPs, for instance, was absent from the spectra of classes III–V. Class VI compounds could be observed, even in the presence of iPs from the other classes, by monitoring this ion (Fig. 4). Reverse-phase solid-phase extracts of human urine, when analyzed by this method, yielded a chromatogram dominated by two peaks (Fig. 5). Their retention time on reverse-phase HPLC was significantly longer than that of \(iPF_{2\alpha,-VI}\). The peaks corresponding to the two compounds always appeared as a doublet, with very similar areas. Each was approximately five times the concentration of the most abundant \(F_2\)-iP described to date, \(iPF_{2\alpha,-VI}\).
Using the specific method described above, we co-injected authentic 8,12-iso-iPF$_{2\alpha}$-VI or 5-epi-8,12-iso-iPF$_{2\alpha}$-VI with urinary extracts. 5-epi-8,12-iso-iPF$_{2\alpha}$-VI co-chromatographed precisely with compound I, and the resulting peak had no difference in peak width, shape, or symmetry from the unadulterated compound I from urine (Fig. 6). In like fashion, 8,12-iso-iPF$_{2\alpha}$-VI co-chromatographed with compound II (Fig. 7). Their collision-induced dissociation mass spectra match pre-
cisely with these compounds (Figs. 8, 9).

**GC/MS**—Preparations of unknowns I and II were obtained by subjecting reverse-phase solid-phase extracts of urine from a healthy volunteer to reverse-phase HPLC/MS/MS and collecting the peaks as they eluted. These samples were used to investigate the GC/MS characteristics of compounds I and II. When the PFB ester TMS ether derivative was used, compound I once again showed chromatographic characteristics identical to those of 5-epi-8,12-iso-iPF2α-VI (Fig. 10). An arbitrary amount of iPF2α-III PFB ester TMS was added against which to evaluate the increase in peak height when authentic standard was co-injected. In addition, the identity of compound II to 8,12-iso-iPF2α-VI was confirmed using the same techniques (Fig. 11). No significant contamination of HPLC peak I or II with other F2-iPs was observed by GC/MS. Further evidence of the equivalence of compound I to 5-epi-8,12-iso-iPF2α-VI and of compound II to 8,12-iso-iPF2α-VI was obtained by repeating this experiment with the PFB cyclic n-butyl boronate TMS derivative (data not shown).

**DISCUSSION**

F2-iPs have the potential to fill the critical need for a noninvasive marker of oxidative stress in vivo. They are believed to arise solely from the free radical-mediated peroxidation of phospholipid-bound arachidonic acid. They are cleaved, presumably by an unidentified phospholipase A2, and can be found in circulation (6). We have previously demonstrated elevation of urinary iPF2α-III and/or iPF2α-VI in cigarette smokers (8, 11), in syndromes of ischemia/reperfusion (12, 13), in the antiphospholipid syndrome (14), and in hyperlipidemia (15), all syndromes associated with excessive generation of reactive oxidant species. Immunoassays have also been used to study F2-iPs in syndromes of oxidative stress (5, 16).

Although GC/MS quantitation of F2-iPs as a group is possible with the inclusion of a single deuterated internal standard, we have taken the approach of monitoring a single isomer, using the stable isotope-labeled homolog as an internal standard. The first such method, measuring iPF2α-III, was used effectively, but purifying a single isomer from the 64 potential F2-iPs to homogeneity proved to be highly labor-intensive. We then synthesized iPF2α-VI, the class VI isomer with stereochemistry analogous to iPF2α-III, and its tetradeuterated analog to take advantage of the ability of this class to form a cyclic lactone. Formation of the lactone facilitates their separation from class III–V F2-iPs. The presence of relatively large peaks, other than iPF2α-VI, in the GC/MS chromatogram suggested that other...
isomers of class VI $F_2$-iPs were present in urine at concentrations approaching or exceeding that of $iPF_2^{8,12}$-VI. Using HPLC/ESI/MS/MS techniques that are completely, or to a large degree, specific for each of the four classes of $F_2$-iPs, we observed two large peaks in the ion chromatogram representing the class VI $F_2$-iPs. They are among the most abundant compounds of any class (data not shown).

LC/MS/MS spectra of synthetic $iPF_2^{8,12}$-III and presumptive isomers of classes IV and VI, isolated from rat livers treated with carbon tetrachloride, have been published (9). Spectra of unambiguous synthetic representatives of classes IV–VI are presented here for the first time. These published spectra,
along with our unpublished observations, indicate that members of each class of F_2-IPs share similar MS characteristics. Thus, the four synthetic class VI compounds mentioned above show no significant differences in their collision-induced dissociation spectra.

Realizing that the only positive identification possible of any single isomer from the array of F_2-IPs is by comparison with synthetic standards, we began the process of eliminating possible candidates for the two unknowns. We first eliminated members of classes III–V by examination of the collision-induced dissociation spectra at m/z 353, which were identical to the spectra of synthetic iPF_2a-VI and 5-epi-iPF_2a-VI and the published spectrum of a putative class VI iP (9). We then applied the findings of the seminal investigations of O’Connor et al. (17) into the auto-oxidative cyclization of trienes to form PGF-ring compounds. Elimination of the isomers with trans-mono substituents, which they found to be minor components, decreased the number of possible class VI isomers from 16 to 8. They also showed that the endo,endo-bicyclo-endo peroxides, with endoperoxide and side chains on the same side of the ring (as in 8,12-iso-iPF_2a-VI), are predominant over the exo,exo-bicyclic forms, in which the peroxide and side chains are on opposite sides of the ring (as in iPF_2a-VI). This reduces the list of candidates to four, namely 8,12-iso-iPF_2a-VI and 5-epi,8,12-iso-iPF_2a-VI (which we synthesized) and their enantiomers. Identical MS/MS spectra and co-chromatography on reverse-phase HPLC/ESI/MS/MS and on GC/MS as two different derivatives confirmed their identity. When HPLC peaks were collected and analyzed by GC/MS, no significant contamination of either peak I or II by other F_2-IPs was observed. Since it would be unlikely that two or more isomers that coeluted on LC would also coelute on GC, which uses a different method of separation, this indicates that the unknown peaks are substantially pure and are not made artifically large by the coelution of two or more compounds. The present data do not allow conclusions regarding the enantiomeric composition of the unknown peaks.

The metabolism of one F_2-IP, iPF_2a-III, has been investigated to some degree, with a small, but undetermined percentage of radioactivity infused into a human volunteer being excreted unchanged (18). Nothing is known of the metabolism of class VI F_2-IPs. We and others (19–22) have observed, in the study of leukotriene C_4 and E_4 metabolism, that β-oxidation proceeded from the ω-end. This was interpreted as a slowdown of the metabolism of the side chain, possibly due to an interference by the hydroxyl group at C-5, and may explain the large amount of intact leukotriene E_4 excreted in urine (23). It is possible that the C-5 hydroxyl group of class VI isoprostanes could be a factor in the observation that this class seems to be more abundant in urine than the other series, e.g. the concentration of iPF_2b-VI is −4-fold that of the class III stereochemonal analog, iPF_2a-III. Evaluation of this hypothesis will require a more thorough quantitation of these iPs from sources that are unaffected by metabolism and/or excretion.

Knowledge of the structure of these compounds, along with the synthesis of their tetradeuterated analogs for use in stable isotope dilution assays, will allow for the development of more sensitive GC/MS and HPLC/ESI/MS/MS methods for F_2-IP analysis. Specifically, these will take advantage of the unique ability of class VI F_2-IPs to form a cyclic lactone and hence be easily separated from other F_2-IP classes. Each of these peaks is present in −5-fold greater yield, as judged by their respective LC/MS/MS areas, than the most abundant F_2-IP yet identified in human urine, iPF_2a-VI (8). Given the interest in microdetecion of iPs in mouse models of oxidant stress, these compounds have particular appeal as F_2-IP analytes. They are also potentially attractive targets for immunoassy development, immunolocalization studies (24), and extension of this methodology to large-scale clinical trials.

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