Major urinary metabolites of 6-keto-prostaglandin F$_{2\alpha}$ in mice

Dmitry V. Kuklev, Joseph A. Hankin, Charis L. Uhlson, Yu H. Hong, Robert C. Murphy, and William L. Smith

Abstract

Western diets are enriched in omega-6 vs. omega-3 fatty acids, and a shift in this balance toward omega-3 fatty acids may have health benefits. There is limited information about the catabolism of 3-series prostaglandins (PG) formed from eicosapentaenoic acid (EPA), a fish oil omega-3 fatty acid that becomes elevated in tissues following fish oil consumption. Quantification of appropriate urinary 3-series PG metabolites could be used for noninvasive measurement of omega-3 fatty acid tone. Here we describe the preparation of tritium- and deuterium-labeled 6-keto-PGF$_{2\alpha}$ and their use in identifying urinary metabolites in mice using LC-MS/MS. The major 6-keto-PGF$_{2\alpha}$ urinary metabolites included dinor-6-keto-PGF$_{2\alpha}$ ($\sim$10%) and dinor-13, 14-dihydro-6,15-diketo-PGF$_{1\alpha}$ ($\sim$10%). These metabolites can arise only from the enzymatic conversion of EPA to the 3-series PGH endoperoxide by cyclooxygenases, then PGI$_3$ by prostacyclin synthase and, finally, nonenzymatic hydrolysis to 6-keto-PGF$_{2\alpha}$.

The 6-keto-PGF derivatives are not formed by free radical mechanisms that generate isoprostanes, and thus, these metabolites provide an unbiased marker for utilization of EPA by cyclooxygenases.—Kuklev, D. V., J. A. Hankin, C. L. Uhlson, Y. H. Hong, R. C. Murphy, and W. L. Smith. Major urinary metabolites of 6-keto-prostaglandin F$_{2\alpha}$ in mice. J. Lipid Res. 2013. 54: 1906–1914.

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Diets enriched in polysaturated fatty acids versus saturated fatty acids are associated with a reduction in the incidence of death from coronary heart disease in healthy adults (1). Substituting long-chain omega-6 fatty acids with long-chain omega-3 fish oil fatty acids [e.g., 5,8,11,14,17-eicosapentaenoic acid (EPA) and 4,7,10,13,16,19-docosahexaenoic acid] appears to provide a benefit over that seen with omega-6 fatty acids alone (2, 3). Although additional benefits (e.g., in inflammation and depression) to dietary omega-3 fish oil fatty acids have been reported, the omega-3/omega-6 fatty acid “tone” of experimental animals or of humans ingesting nonprescription fish oil tablets or prescription supplements (Lovaza®) can be determined by measuring the ratio of omega-3 to omega-6 fatty acids in blood or in red blood cell membranes. Commercial kits are available for these relatively simple blood tests that involve quantifying fatty acid levels in plasma (4) or fatty acyl compositions of red blood cell lipids (5). Unfortunately, there is no validated method for estimating tissue levels of omega-3 versus omega-6 fatty acids noninvasively, such as by measuring appropriate metabolites in the urine.

Prostaglandins (PG) can be formed from either arachidonic acid (AA), an omega-6 fatty acid, or from EPA, an omega-3 fatty acid, through the sequential actions of a phospholipase A$_2$, cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2), and a PG synthase (6–10). Methods are available for measuring 2-series urinary PG metabolites formed from AA, including the major PGE$_2$ metabolite (PGEM) tetrnor (11), two dinor metabolites of prostacyclin (12) and PGD$_2$ (13), and thromboxane B$_2$ metabolites (14). We reasoned that quantification of a major urinary 3-series PG metabolite(s) of EPA in conjunction with measuring an appropriate 2-series PG metabolite might prove useful in estimating the AA/EPA ratio in tissue phospholipid precursors of PGs.

Previous examination of the major urinary metabolites of PGE$_2$ performed in rats indicated that one of the metabolites is the tetrnor derivative of PGE$_2$ formed via two steps of catabolism (15). The evidence is inconclusive.

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β-oxidation (15, 16). Another report indicated that the urinary metabolite of PGE₃ in the rat (7α,11α-dihydroxy-5-keto tetranorprosta-9,13-dienoic acid) is identical to that derived from PGE₂, because formation of the PGE₃ metabolite involved ω-oxidation, in which the additional double bond exists that differentiates AA from EPA (15).

Initial hydrolysis of the bicyclic prostaglandin PGI₃ results in the formation of 6-keto-PGF₁α, whereas the hydrolysis of PGI₄ yields 6-keto-PGF₂α. The major urinary metabolites of AA-derived 6-keto-PGF₁α have been described in both man (12, 17, 18) and rats (19) but not in mice. In contrast, no careful, quantitative analysis of the metabolites of PGI₃ have been performed in any species, although homologs of the major PGI₃ metabolites have been measured in urine of primates following dietary fish oil (20–22) and rats (19) but not in mice. In the present study, we prepared 6-keto-PGF₂α from EPA bearing tritium and deuterium labels to form two different isotopomers in order to facilitate metabolite isolation from the urine matrix and product identification using mass spectrometry, and we studied the metabolism of 6-keto-PGF₂α in mice.

**EXPERIMENTAL PROCEDURES**

**Materials**

All fatty acid methyl esters with purities of greater than 98% were purchased from NuCheck Prep (Elysian, MN). Anhydrous ethyl ether and tetrahydrofuran (THF) with water contents of less than 50 ppm were purchased from EMD Chemicals (Gibbstown, NJ) and used without further purification. Chemicals used for metabolite extraction and solvents used for HPLC/MS analysis were purchased from Fisher Scientific (Pittsburgh, PA) as “HPLC grade” and used without further purification. Stable isotopes were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Radioactive isotopes were purchased from and custom radioactive labeling was performed by Vitrax Radiochemicals (Placentia, CA). Silica gel “Selecto” 32–63 μm for preparative column chromatography was purchased from Selecto Scientific (Suwanee, GA). Lipid standards used as mass spectrometric and chromatographic references (6-keto- PGF₂α; 2,3-dinor-6-keto-PGF₂α; and 6,13-diketo- PGF₂α) were purchased from Cayman Chemicals (Ann Arbor, MI).

**Enzymes and tissues**

Human recombinant COX-2 was prepared as described previously (23) and used as a 400 μg/ml solution in 50 mM phosphate buffered saline at pH 7.0. Bovine aorta was purchased from Animal Technologies Inc. (Tyler, TX). Soybean lipoxygenase type 1 was from Sigma-Aldrich Chemical Co. (St. Louis, MO).

**Synthesis of d5-eicosapentaenoic acid**

Analytical and separation procedures used in the synthesis of d5-EPA are presented in the supplementary data. The first step in the synthesis of d5-EPA was the synthesis of (5Z,8Z,11Z,14Z)-methyl 16-(3-ethyloxiran-2-yl)hexadeca-5,8,11,14-tetraenoate (Scheme 1, structure [2]), which was performed as detailed in the supplementary data. The mixture of monoepoxides (Scheme 2, structure [6]) has an isomer composition that is comparable to that described previously: 5,6-isomer (18%); 8,9-isomer (14.5%); 11,12-isomer (15.5%); 14,15-isomer (17%); and 17,18-isomer (35%) (24). The target compound (5Z,8Z,11Z,14Z)-methyl 16-(3-ethyloxiran-2-yl)hexadeca-5,8,11,14-tetraenoate [6] was separated from the other isomers by preparative C18-RP-HPLC (Kromasil, acetonitrile/H₂O/acidic acid, 65:35:0.1, v/v/v), peak with K’ = 4 min.

**Scheme 1.** Synthesis of 19,19,20,20,20-d5 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid [5]. (a) 1 eq m-Chloroperbenzoic acid/CH₂Cl₂, rt. (b) NaBH₄/ethanol, rt (75% yield), then I₂, triphenylphosphine (Ph₃P), imidazole/toluene, 65 °C, and then Ph₃P/acetonitrile, reflux. (c) C₂D₆/D₂O, NaN(SiMe₃)₂/THF-hexamethylphosphoramide (HMPA), −78 °C. Experimental details are provided in the text and supplementary data. Synthetic intermediates [1] through [5] are noted in the text.

**Scheme 2.** Synthesis of [³H]-eicosapentaenoic acid. v) Acetyl bromide/methanol, −20 °C. v) Dess-Martin periodinane/CH₂Cl₂, rt. vii) hydroquinone, tosyl-hydrazide (Tos-NHNH₂)/CH₂Cl₂, acetic acid, rt. Experimental details are provided in the text and supplementary data. Synthetic intermediates [6] through [9] are noted in the text.
the crude oil [5] was purified by column chromatography on 20 g of silica gel with 4% ethyl acetate in hexane used as a mobile phase to yield a colorless oil of prepurified [5] (20.3 mg, 28%).

Purification of 19,19,20,20-d5 methyl 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid [5]

Twenty milligrams of [5] was dissolved in 5 ml of CH2Cl2, cooled to 0 °C, and 4-methyl-3H-1,2,4-triazole-3,5(4H)-dione solution (1 mg/ml CH2Cl2) was added dropwise until a solution having a stable pink color was formed. Then, the reaction mixture was evaporated under vacuum and purified by flash chromatography on 1 g of silica gel eluting with 4% ethyl acetate in hexane. After purification and saponification with 5 eq of LiOH in ethanol-water (1:1, v/v) the target acid 19,19,20,20-d5 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid was purified by C-18 RP-HPLC eluting with acetonitrile/H2O/acetic acid, 70:30:0.1 (v/v/v).

Synthesis of uniformly tritium-labeled EPA

The first step in the synthesis of [3H]EPA (Scheme 2) was the preparation of a mixture of isomeric epoxides (Scheme 2, [6]) from which were prepared a mixture of isomeric bromohydrins [7] as described in the supplementary data. The products [7] had properties very similar to that we described previously for bromohydrins (27). A mixture of isomeric bromoketones [8] was prepared, and converted to a mixture of isomeric monoacetylenes [9] as described in the supplementary data. The regioisomeric distribution of [9] established by GC-FID-MS analysis of the resulting mixture, the sample was mixed well, and was incubated at 37°C for 15 min, and the supernatant was separated from the precipitate. This supernatant was used immediately after preparation, but it could be kept on ice (0–5°C) for 1–3 h. To prepare the substrate, 1 g of [12] and d5-6-keto-PGF2α [13] as a mixture with uniformly tritium-labeled 6-keto-PGF2α (at a ratio 2 μg μCi) was injected onto an HPLC system equipped with an C18-RP-HPLC (Shodex RSpack DE-413L, 250 mm × 4.6 mm, 5 mm) column operated at 1 ml/min with a binary gradient of solvent A with solvent B, in which solvent A was acetonitrile/water/ acetic acid (30:70:0.1, v/v/v) and solvent B was acetonitrile/water/acetic acid (90:10:0.1, v/v/v). The gradient was solvent A (100%, 5 min), followed by solvent B (100%, 15 min), then solvent B (100%, 8 min) and finally, solvent A (100%, 6 min). The peak of d0/d5 6-keto-PGF2α was collected between 9 and 11 min. This sample was evaporated under vacuum and redissolved in ethanol. HPLC-MS (ES, negative ion mode), m/z (1%) structural data for d0 and d5-6-keto-PGF2α, respectively, were: 392.2 (100%) [d0, M+H], 372.2 (92%) [d5, M+H]. High-resolution mass measurements of starting d5-6-keto-PGF2α were carried out by ESI/MS using a Synapt G2-S quadrupole/time-of-flight mass spectrometer (Waters, Milford, MA). A solution of the d5-6-keto-PGF2α was mixed with PGE2 (used as an internal calibrant) at final concentrations of 1 μM each and infused into the instrument at a flow rate of 2 μl/min using ESI. The data was collected in full scan, negative ion mode at high resolution, centroided, and calibrated to the calculated mass of PGE2 ([M+H]+ m/z 351.2172). The mass of the d5-6-keto-PGF2α starting material was measured at m/z 372.2442 (calculated for C20H26D5O6, m/z 372.2435), which is within a 1.9 ppm error.

Injection of mice with d0/d5 [3H]6-keto-PGF2α

A solution of d0/d5 [3H]6-keto-PGF2α containing 0.2 μg of d0-6-keto-PGF2α, 0.2 μg of d5-6-keto-PGF2α, and 2 μCi of [3H]6-keto-PGF2α per ml of 0.9% NaCl was prepared. Briefly, an ethanol solution of the d0/d5 [3H]6-keto-PGF2α prepared as described in the supplementary data was added to a sterile 10 ml glass vial and the sample dried under a stream of N2 gas, then sterile saline was added, and the samples were shaken vigorously. Eight 8-week-old C57BL/6j male mice purchased from Jackson Laboratories were divided into two groups. Each mouse was injected with 0.2 μg (1 μCi) of d0/d5 [3H]6-keto-PGF2α in 0.5 ml of sterile 0.9% NaCl. Following the injections, the mice were placed, four per cage, in metabolic cages for 24 h urine collections. Mice were allowed free access to water and mouse chow (LabDiet 5K67) during urine collections. The mice were euthanized by isoflurane inhalation. The urine was centrifuged to remove contaminating feces and stored at −80 °C until analyzed. All animal protocols were conducted in conformity with USPHS policy, and the experimental protocols were approved by the University Committee on Use and Care of Animals at the University of Michigan.

Isolation of 6-keto PGF2α metabolites from urine

Metabolites of 6-keto PGF2α with a 2,3-dinor structure were chemically isolated from urine using a protocol developed by Falardeau et al. (12). These dinor metabolites have a unique configuration of a hydroxyl on carbon 9, a ketone at carbon 6, and a carboxylate at carbon 3 that can be manipulated with pH to form a ketal with subsequent lactone structure that can be more selectively purified from the complex urine matrix.

Chemical derivatization of metabolites

Ketone functionalities of metabolites and standards were derivatized with methoxylamine using the following protocol: From 10 ng to 1 μg of sample was dried at the bottom of a 10 ml screwtop glass tube. In a matching tube, 1 ml of 1N NaOH and then approximately 5 mg of methoxylamine hydrochloride were added, and the tubes were quickly joined with a female-female union, liquid side down, and incubated at 67 °C for 2 h. The sample end of the apparatus was removed and suspended in HPLC solvents for RP LC/MS analysis.

Analysis and identification of metabolites

Reverse-phase HPLC analysis was carried out for lipid analysis using a 2 × 150 mm 5 μm particle size C-18 Luna column.
Mobile phase A consisted of water and 10 mM ammonium acetate buffered to pH 4.5 with acetic acid. Mobile phase B was composed of acetonitrile and methanol at a ratio of 65:35. Flow rate was 200 μl/min, and the gradient used for analysis started at 10% B, isocratic for 5 min, then to 40% B in 20 min, to 95% B in 5 min, then isocratic at 95% B for 5 min. Aliquots of the isolated metabolites from urine were dried and resuspended in HPLC solvents for analysis. The flow was split 3:1 between an in-line radioactivity counter (Flo-One Beta; Radiomatic, Tampa, FL) and mass spectrometer (QTRAP® 5500; AB SCIEX, Foster City, CA) to establish correlation between chromatographic radioactivity and mass spectra of the component.

Mass spectrometer conditions included the following conditions for all negative ion analyses: ion spray voltage, -4,000; curtain gas, 30; nebulizer gas, 30; source temperature, 300°C; declustering potential, -30; and exit potential, -25. Q3 scan range was 290–700 Da at a rate of 200 Da/s. The linear trap functionality was used intermittently in complimentary enhanced mass spectra mode, which enhanced mass resolution and sensitivity. In this modality, a scan rate of 1,000 Da/s was used with collision gas set at “low.” Product ion spectra were acquired in enhanced product ion mode at a rate of 1,000 Da/sec, fill time 1 msec, and collision energy of -30.

**RESULTS**

**Overview**

The metabolism of 6-keto-PGF₂α was carried out by peritoneal injections of this eicosanoid into male mice (four per group) with 1 μCi/mouse of tritium-labeled eicosanoid that additionally had a deuterium-labeled isotopomer so that the d0/d5 ratio was approximately 1:1. Urine from each group of mice was collected for 24 h. Therefore, these studies had three different isotopic variants of 6-keto-PGF₂α that facilitated development of optimal extraction and purification protocols using radioactivity to detect elution from the HPLC and structural characterization of metabolites by mass spectrometry detected as isotope doublets. For these studies, the appearance of an isotopic doublet in the mass spectrum enabled assignment of an eluting component as an eicosanoid metabolite. Because the site of tritium label was not on the identical carbon atoms as the deuterium label, it was possible that a change in the isotop doublet pattern could be observed for a radiolabeled metabolite but unlikely that any reasonably large molecular weight metabolite would have lost all tritium atoms. Approximately 50% of the injected 6-keto-PGF₂α-associated radioactivity was eliminated in the urine; no obvious behavioral effects were noted following administration of this amount of 6-keto-PGF₂α.

**Metabolites of 6-keto-PGF₂α**

Direct analysis of an aliquot of the pooled urine sample collected 24 h after injection of 6-keto-PGF₂α into the mice was surveyed by reversed-phase LC using a radioactivity monitor to detect elution of metabolites (Fig. 1A). Several metabolites were apparent with some components eluting early (before 4 min), indicative of very weakly lipophilic metabolites not well retained on the column and two abundant lipophilic metabolites eluting at 14 and 16 min, respectively (labeled 6K1 and 6K2). Other minor radioactive metabolites were also observed in the urine eluting at 16.5, 18, and 25 min. The two major metabolites became the target to drive purification strategies because they represented over 20% of the initial 6-keto-PGF₂α administered to the mice based on the summed radioactivity in these two peaks compared with the total radioactivity eluting in Fig. 1A. Based on the observed retention times of the 6K1 and 6K2 being shorter than the starting 6-keto-PGF₂α, we reasoned that these were chain-shortened metabolites, and we essentially followed a procedure previously published by Falardeau et al. (12) that purified the dinor metabolites of 6-keto-PGF₂α. This strategy made use of the unique chemistry innate to a 2,3-dinor 6-keto eicosanoid that could form a hemiacetal lactone stable to mild basic conditions (supplementary Fig. I). Recovery of radioactivity at each step of the purification procedure is presented in Table 1. An aliquot of urine (1 ml) was adjusted to pH 10 and let react for 15 min prior to adjusting to pH 3 in order to cyclize a metabolite as a hemiacetal lactone. This lactone-containing solution was subjected to reversed-phase solid-phase extraction (SPE), and the radioactivity was eluted with methanol (recovery 53%). This SPE extract was dried, redissolved in dichloromethane, and extracted with pH 8 borate buffer that ionized any free carboxylic

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**Fig. 1.** Isolation of ω-3 prostacyclin metabolites of obtained from collecting the 24 h urine from four mice injected with d0/d5 [³H]6-keto-PGF₂α. (A) Reversed-phase HPLC separation of radioactive urinary metabolites (cpm) without prior treatment of the crude urine and elution of two major metabolites 6K1 and 6K2. (B) Reversed-phase HPLC separation of urinary (radioactive) metabolites after differential solvent extraction of hemiacetal lactone structures following the method of Falardeau et al. (12). The two major metabolites were significantly purified from the crude urine. (C) Extracted ion chromatogram (m/z 339) obtained following LC-MS analysis of the purified metabolites by differential solvent extraction and the resultant molecular ions [M-H]⁻ (insets: molecular ion region for 6K1 and 6K2) revealing isotope doublet characteristic of the d0/d5 ratio of the injected d0/d5 [³H]6-keto-PGF₂α.
acid groups present in any unrelated molecules excreted into the urine but was not sufficiently strong to saponify the hemi-acetal lactone (Table 1, yield 38%). The organic layer was dried, then treated with pyridine/borate buffer to saponify the hemi-acetal lactone, and then this aqueous solution was extracted with ethyl acetate to remove neutral, unrelated molecules still present from the urine (yield 20%). The radioactive metabolites remaining in the aqueous layer were extracted into dichloromethane after adjusting to pH 2 using HCl to yield 13% purified recovery based on the starting radioactivity in the urine (Table 1). This extract was analyzed by reversed-phase LC (Fig. 1B) to reveal the same abundant metabolites eluting at 14 and 16 min as seen in the crude urine analysis. The total yield for this extraction protocol for these hemi-acetal lactones was approximately 13% of the radioactivity in the pooled urine sample, which corresponded to 6.5% of the injected 6-keto-PGF₂α. Even though there was significant loss of radioactivity at each step (Table 1), the solvent extraction yielded fairly clean metabolite isolation.

This extracted fraction was then subjected to LC-MS to detect the major negative ions for the components eluting from the HPLC (Fig. 1C). At the 14 min elution period, an isotopic doublet was observed at m/z 339/344, and at the 16 min retention time, a more complex multiplet was observed, including a doublet at m/z 339/344 (Fig. 1C, inset). Clearly there were radioactive metabolites as well as other components eluting at the 16 min time period, as evident by peaks at m/z 338 and 340. Nonetheless, these two urine components did reveal isotopic doublets, thus strongly supporting the radioactivity data that there were metabolites of 6-keto-PGF₂α eluting at this time.

The major HPLC peaks (Fig. 1C) were then directly analyzed by LC-MS/MS following injection of an aliquot of this urine extract (Fig. 2). Collisional activation of each ion at m/z 339 and 344 revealed product ions that were shifted in most cases by 5 amu (d⁵ isotope data, supplementary Fig. IIA) and with relative abundances very similar, suggesting identical ion chemistry for m/z 339 and 344. Collision activation of m/z 339 from peak 6K1 (Fig. 2A) yielded losses corresponding to one water molecule (m/z 321), two water molecules (m/z 303), and two water molecules and CO₂ (m/z 259). In addition, there was an abundant ion corresponding to loss of 98 amu, which would correspond to the loss of three molecules of water and CO₂, leading to a highly stabilized structure shown in Fig. 2A. This ion structure was supported by appropriate mass shifts in the observed ions from the deuterated metabolite (m/z 246, supplementary Fig. 1A) and the homologous ion formed from authentic 2,3-dinor-6-keto-PGF₁α (m/z 243, supplementary Fig. 1B). However, the ions formed following collision activation of m/z 339 from 6K2 (Fig. 2B) showed only the losses of one and two molecules of water, but not formation of the highly conjugated product ion discussed above (m/z 241). The isolation strategy and reduction in molecular weight from the starting 6-keto-PGF₂α for each metabolite was consistent with a chain-shortened 2,3-dinor metabolite, but differences in HPLC retention time (Table 2) suggested that one metabolite could have the prostanoid side chain intact while the other metabolite could have the carbon 13-14 double bond saturated and the hydroxyl group at C-15 oxidized to a keto moiety, which are known metabolic transformations of 6-keto-PGF₁α (12).

![Fig. 2. Tandem mass spectrometry of urinary metabolites obtained by negative electrospray ionization. (A) CID of [M-H]⁻ of 6K1 metabolite at m/z 339.3. The proposed structure of the product ion m/z 241.2 is consistent with the deuterium labels and retains the α-3 double bond. (B) CID of [M-H]⁻ of 6K2 metabolite at m/z 339.3. The proposed structure of the product ion m/z 221.2 is consistent with the deuterium labels and retains the α-3 double bond.](https://example.com/fig2.png)
Because the difference between the suspected metabolites was the presence of one versus two keto groups, an aliquot of the urine extract was treated with methoxylamine hydrochloride to form the methoxime (MOX) derivative(s). Additional separation of these components was revealed in the radiochromatogram (Fig. 3A). Major radioactive peaks eluted at 16.9, 21.6, 23.0, 23.8, and 24.5 min. Because formation of a methoxime from a keto group can lead to two separable syn and anti isomers for this derivative, some of the components clearly could have been due to these geometric isomers (Table 2). Peak A at 16.9 min (Fig. 3A) was found to contain an isotope doublet at m/z 368/373 after LC-MS analysis, and extracting the ion current for m/z 368 revealed a single HPLC peak at 16.9 min (Fig. 3B). Furthermore, the ions at this retention time revealed the isotope doublet at an approximately 1:1 ratio (Fig. 3B, inset, m/z 368/373). The components at 21.6 (peak B), 23.0 (peak C), 23.8 (peak D), and 24.5 min (peak E) all revealed isotope doublets at m/z 397 and 402, and an elution profile of m/z 397 (Fig. 3C) identical to the radiochromatogram (Fig. 3A) with appropriate doublets at m/z 402 for each retention time (Fig. 3C, inset).

HPLC peak A had a [M-H]⁻ ion (m/z 368.2) 29 amu higher than metabolite 6K1 (Fig. 2A) and a collision-induced decomposition (CID) mass spectrum consistent with a singly methoxylated 2,3-dinor-6-keto-PGF₂α (Fig. 4A). Characteristic ions corresponding to the loss of the methoxime derivative (m/z 338, [M-H-30]⁻) as well as a very diagnostic ion at m/z 230.1 (structure in the inset of Fig. 4A). Furthermore, all of these ions were advanced by 5 amu in the CID mass spectrum of the ion at m/z 373 (Fig. 4B). Authentic 2,3-dinor-6-keto-PGF₁α was derivatized to the methoxime and found to have a retention time 2 min later than peak A, consistent with one less double bond (Table 2). Comparison of the CID mass spectrum from compound A to that of authentic derivatized 2,3-dinor-6-keto-PGF₁α, which lacks the αβ double bond ([M-H]⁻, m/z 370) (Fig. 4C), revealed similar CID behavior. Collectively, these data indicate that 6K1 (Figs. 1 and 2) was derivatized to peak A and that this derivatized metabolite was formed from 2,3-dinor-6-keto-PGF₂α.

Peak B (Fig. 3), which eluted at 21.6 min, had a [M-H]⁻ ion (m/z 397) consistent with the addition of two methoxime groups to the metabolite 6K2 that had a [M-H]⁻ at m/z 339 (Fig. 2B). The ion at m/z 397 (peak B) was collisionally activated (Fig. 5A), and as was the case for peak A, the deuterium-labeled analog eluting at 21.6 min had an identical CID mass spectrum that revealed 5 amu shifts in most product ions (data not shown). To have 2-keto groups and a molecular mass of 340 Da, one of the hydroxyl substituents in the starting 6-keto-PGF₂α had to be oxidized to a ketone and one carbon-carbon double bond had to be reduced. This type of metabolism is quite well known for the double bond Δ³,⁴ and 15-hydroxyl moieties of many prostanoids (11, 13, 28, 29). The molecular ion for this dimethoxime derivative yielded product ions corresponding to the losses expected for each of the methoxime derivatives (sequential losses of 30 amu) as well as the loss of acetic acid (m/z 337.2), which is an interesting ion perhaps unique to these 2,3-dinor derivatives (Fig. 5A). Very few other product ions were present, making it difficult to unambiguously assign the exact structure for each of these metabolites eluting from the HPLC in this region as geometric isomers of the methoxime derivative. To test whether the dimethoxime derivative of this metabolite would form HPLC-separable geometric isomers, authentic 6,15-diketo-13,14-dehydro-PGF₁α was derivatized (MW 428) and separated by the same reversed-phase HPLC system, then analyzed by mass spectrometry (Fig. 5B). Four geometric isomers (syn/anti at both keto positions) were clearly observed in the HPLC separation (Fig. 5B, inset) at an expected 4 min longer retention time, because this synthetic eicosanoid had two additional carbon atoms. The CID spectra of four peaks in Fig. 5B-E (supplementary Fig. II) were all similar and consistent with the suggested structural features of metabolite 6K2 being 2,3-dinor-6,15-diketo-13,14-dihydro-PGF₂α.

**DISCUSSION**

Intact PGs are present only at low concentrations in urine and are not representative of whole-body PG synthesis. Measurements of circulating PGs in blood are also unreliable because of the rapid synthesis of PGs during sampling. Instead, measurements of urinary metabolites

| Synthetic Standards and Urinary Metabolites | [M-H]⁻ (m/z) | RP-HPLC Retention Time (min) | [M-H]⁻ (m/z) of Methoxime Derivative | RP-HPLC Retention Time (min) of Methoxime Derivative(s) |
|--------------------------------------------|-------------|-------------------------------|-------------------------------------|-----------------------------------------------------|
| 6-keto-PGF₁α                               | 369.2       | 17.6                          | 398.2                               | 20.5, 21.8                                           |
| 6-keto-PGF₂α (starting material)            | 367.2       | 18.5                          |                                     |                                                     |
| 15-keto-PGF₁α                              | 353.2       | 23.4                          | 382.2                               | 30.3, 33.7                                           |
| 2,3-dinor-6-keto-PGF₂α (6K1/metabolite A)   | 359.2       | 14.1                          | 368.2                               | 16.9                                                |
| 6-keto-PGF₂α                                | 341.2       | 16.8                          | 370.2                               | 18.3                                                |
| 2,3-dinor 6,15-diketo 13,14-dihydro-PGF₂α   | 339.2       | 15.7                          | 397.2                               | 21.6, 23.0, 25.8, 24.5                              |
| (6K2/metabolites B, C, D, E)                | 369.2       | 19.4                          | 427.2                               | 27.2, 28.3, 30.2, 31.2                               |
used to synthesize d5-EPA. We then synthesized d0/d5 [3H]PGI2 from a mixture of d0/d5 [3H]EPA using purified human COX-2 and a microsomal preparation of prostacyclin synthase from bovine aorta. Extraction of d0/d5 [3H]PGI2 under acidic conditions caused its hydrolysis to d0/d5 [3H]6-keto-PGF1α.

Urine from mice injected with d0/d5 [3H]6-keto-PGF1α was extracted using a method developed by Falardeau et al. (12) that is known to form a semistable hemi-acetal lactone from 2,3-dinor 6-keto eicosanoids. The radioactive metabolites were separated by HPLC, and the materials coeluting with the radioactivity were analyzed by mass spectrometry. As detailed in the Results, comparable amounts of two compounds, comprising about 20% of the urinary [3H]6-keto-PGF1α-derived radioactivity, were identified as 2,3-dinor-6-keto-PGF1α and 2,3-dinor-13,14-dihydro-6,15-diketo-PGF1α.

It is assumed from studies of other mammals that PGI2 is formed from EPA by mice (20–22). Pathways that could lead to the formation of 2,3-dinor-6-keto-PGF1α and 2,3-dinor-13,14-dihydro-6,15-diketo-PGF1α from 6-keto-PGF1α involving catabolic enzymes previously associated with PG degradation are shown in Fig. 6 (11–13, 28, 29, 33, 34); however, we have not examined the specific steps involved, and
the sequence of the various steps is not known. Data in Fig. 2A suggest that a fragment with \( m/z \) 241.2 would be an appropriate product ion for quantifying 2,3-dinor-6-keto-PGF\(_{2\alpha} \) as the dimethoxime derivative. (B) CID of the [M-H\(^-\)]\(^+\) of authentic 6,15-dimethoxyoxime-13,14-dihydro-PGF\(_{2\alpha} \) at \( m/z \) 397 showing identical mass losses to the derivatized metabolite peak B. Inset: Reversed-phase separation of the erythro and threo enantiomers of the dimethoxime derivatives of each syn and anti form of each methoxime, revealing four separable diastereoisomers.

The results of our studies of 6-keto-PGF\(_{2\alpha} \) catabolism reveal that major urinary metabolites of this eicosanoid can be found and measured and that they retain the \( \omega_3 \) double bond that distinguishes the 2-series from the 3-series prostacyclin metabolites. In the rat, where some detailed analytical work has been performed, one PGE\(_3 \) metabolite is involved that distinguishes the 2-series from the 3-series prostaglandins while isomeric metabolites arise from an initial oxidation of the 15-hydroxyl moiety by prostaglandin 15-hydroxy dehydrogenase, followed by reduction of the 13,14 double bond by a reductase (reviewed in Refs. 33 and 34). Either prior or subsequent \( \beta \)-oxidation leads to the formation of the other major metabolite 2,3-dinor-13,14-dihydro-6,15-diketo-\( \Delta^{17} \)-PGF\(_{2\alpha} \).

Fig. 6. Proposed pathway of metabolism of 6-keto-PGF\(_{2\alpha} \) in the mouse as reflected by major urinary metabolites. The 2,3-dinor metabolites arise from \( \beta \)-oxidation of the CoAester of the prostaglandin while isomeric metabolites arise from an initial oxidation of the 15-hydroxyl moiety by prostaglandin 15-hydroxy dehydrogenase, followed by reduction of the 13,14 double bond by a reductase (reviewed in Refs. 33 and 34). Either prior or subsequent \( \beta \)-oxidation leads to the formation of the other major metabolite 2,3-dinor-13,14-dihydro-6,15-diketo-\( \Delta^{17} \)-PGF\(_{2\alpha} \).

PGF\(_2\alpha\), PGD\(_2\), and thromboxane B\(_2\), can be formed via non-enzymatic, free-radical oxidation reactions (29, 38, 39). Metabolites of isoprostanes are found in urine and in some cases have properties (e.g., HPLC retention times and mass spectroscopic behaviors) of enzymatically derived PGs. This can make it complicated to discriminate between enzymatically and nonenzymatically derived urinary metabolites of PGs other than PGI\(_2\) and PGI\(_3\).

PGI\(_2\) can be formed from AA via either COX-1 or COX-2, but PGI\(_2\) is produced, at least in humans, in three times greater abundance via COX-2 (40). EPA-derived PGI\(_3\) is even more likely to be formed via COX-2 (8, 9). This is because EPA is both an effective inhibitor and a poor substrate for COX-1 (8, 23, 41, 42), but it is a modestly good substrate for COX-2 (8, 23, 41). Accordingly, measuring the formation of 6-keto-PGF\(_{2\alpha}\) metabolites may prove to be a quantitative biomarker not only for omega-3 fatty acid tone but also, independently under appropriate dietary conditions, for relative COX-2 activity. Measurements of 6-keto-PGF\(_{2\alpha}\) metabolites could also be used to assess the effectiveness of COX inhibitors on COX-2 in vivo.\[14\]

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