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ENZYMATIC SYNTHESIS OF DIOXYGEN-18 LABELED 8-EPI-PROSTANGLANDIN $F_{2\alpha}$ AND ITS USE IN QUANTITATIVE GC-TANDEM MS

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Summary

8-epi-Prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$) is an endogenous potent vasoconstrictor, non-cyclooxygenase-derived prostanoid which may be suitable as an index of oxidative stress in living organisms. For quantitative determination of 8-epi-$PGF_{2\alpha}$ in biological fluids we describe here the one-step enzymatic synthesis of [1,1-$^{18}O_2$]-8-epi-$PGF_{2\alpha}$ starting from commercially available unlabeled 8-epi-$PGF_{2\alpha}$, $H_2{}^{18}O$ and an unspecific porcine liver esterase. The isolated and purified reaction product was found to contain 80.3% of [1,1-$^{18}O_2$]-8-epi-$PGF_{2\alpha}$, 17.7% of [1,1-$^{18}O^{16}O$]-8-epi-$PGF_{2\alpha}$ and only 2.0% of unlabeled 8-epi-$PGF_{2\alpha}$. [1,1-$^{18}O_2$]-8-epi-$PGF_{2\alpha}$ is demonstrated to be a suited internal standard for quantitative determination of 8-epi-$PGF_{2\alpha}$ in human urine by GC-MS/MS. In 5-ml aliquots of human urine samples from spontaneous micturation on different days, 8-epi-$PGF_{2\alpha}$ was found to be present at concentrations of 300 and 490 pg/mg creatinine, respectively.

Keywords

Enzymatic carboxyl-^{18}O-labelling; [1,1-$^{18}O_2$]-8-epi-$PGF_{2\alpha}$; gas chromatography-tandem mass spectrometry; human urine; isoprostanes

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Introduction

Prostaglandins (PGs) are arachidonic acid metabolites formed via the enzymatic action of cyclooxygenase (COX). Recently, Morrow et al. have reported the discovery of a series of bioactive PGF_2-like compounds [1,2]. These isoprostanes termed substances arise spontaneously in membrane phospholipids by free radical-catalyzed peroxidation of arachidonic acid independent of the COX enzyme [1,2]. F_2-Isoprostanes and metabolites of them have recently been identified in human urine and plasma [3,4]. 8-epi-$PGF_{2\alpha}$ is among the most abundant of the F_2-isoprostanes formed under physiological conditions in humans [4]. This compound induces potent vasoconstriction in the renal and pulmonary circulations [5,6]. Also, 8-epi-$PGF_{2\alpha}$ is a mitogen in 3T3 cells and vascular smooth muscle cells [7], and it may play a role in pulmonary oxygen toxicity [8]. In addition to its generation by free radical-catalyzed mechanisms, Pratico et al. have shown that 8-epi-$PGF_{2\alpha}$ may also be formed enzymatically in activated human platelets [9]. The free radical-catalyzed formation of F_2-isoprostanes has been supposed as an indicator of oxidative stress of an organism [1,2]. Quantification of endogenous production of 8-epi-$PGF_{2\alpha}$ as the major product of the F_2-isoprostanes in humans [4] may therefore provide a valuable analytical tool to assess oxidant stress in vivo in humans. Analysis of 8-epi-$PGF_{2\alpha}$ and other F_2-isoprostanes in human plasma and urine has been accomplished by FAB-MS/MS [2,10], GC-MS [9,11] and enzyme or radioimmunoassay [12] analogous to COX-dependent F_2-prostanes and other prostanoids [13-15]. Over the last few years many isoprostanes but not stable-isotope labeled analogs of them became commercially available. In the present study we describe the enzymatic preparation of $^{18}O_2$-8-epi-$PGF_{2\alpha}$ and its utility for quantitative determination of endogenous 8-epi-$PGF_{2\alpha}$ in human urine by GC-MS/MS.

Experimental

Chemicals and reagents

8-epi-$PGF_{2\alpha}$ was obtained from Biomol (Hamburg, Germany). Pentafluorobenzyl (PFB) bromide was purchased from Aldrich (Steinheim, Germany). N,N-Diisopropylethylamine, $H_2{}^{18}O$ (95 atom % ^{18}O) and porcine liver esterase (PLE, EC 3.1.1.1) provided as a suspension in 3.2 M

ammonium sulfate, pH 8, were bought from Sigma Chemie (Deisenhofen, Germany). N,O-bis(Trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL, USA). $Li^{18}OH$ (0.36 M) was prepared by dissolving the appropriate amount of lithium in $H_2{}^{18}O$ (97.8 atom % ^{18}O) which was bought from MSD Isotopes, Merck Frosst (Montreal, Canada). All other chemicals were purchased from Merck (Darmstadt, Germany).

Enzymatic synthesis of $^{18}O_2$-8-epi-$PGF_{2\alpha}$

$^{18}O_2$-8-epi-$PGF_{2\alpha}$ was synthesized by a similar procedure recently described for PGE_1 [16]. Briefly, 8-epi-$PGF_{2\alpha}$ (500 µg) was converted to its methyl ester using a freshly prepared etheral solution of diazomethane. After esterification diethylether was evaporated under nitrogen and the residue was diluted in 10 µl of ethanol. A 200-µl aliquot of the PLE suspension (1000 units) was dried under a stream of nitrogen, the residue was resuspended in 300 µl of $H_2{}^{18}O$ and the pH of the resulted suspension was adjusted to about 7 by treating with approx. 10 µl of 0.36 M $Li^{18}OH$. The ethanolic solution of 8-epi-$PGF_{2\alpha}$ methyl ester was diluted with this suspension and the mixture was incubated at 37°C for 30 min. After cooling to room temperature the suspension was acidified by 5 M HCOOH to pH 3 and reaction products were extracted three times each with 1 ml of ethylacetate. The combined organic phases were dried over sodium sulfate, then evaporated to dryness under a stream of nitrogen and the residue was reconstituted in 1 ml of ethanol. Aliquots of this solution which was stored at - 80°C were taken and derivatized for structure elucidation by GC-MS as described below.

Derivatization procedures

Methyl esters were prepared by incubation of solvent-free prostaglandins by incubation with 1 ml of a freshly prepared etheral solution of diazomethane at room temperature for 15 min. PFB esters of the compounds were prepared by addition to their solvent-free residues of 100 µl of acetonitrile, 10 µl of N,N-diisopropylethylamine and 10 µl of PFB bromide, and by incubation of the reaction mixtures for 60 min at 30°C. Solvents and reagent excess were removed under a stream of nitrogen. The residues were treated with 20 or 100 µl of BSTFA and heated at 60°C for 60 min or they were incubated at room temperature overnight in order to obtain the trimethylsilyl (TMS) ether derivatives.

Gas chromatography-mass spectrometry (GC-MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS)

Both electron impact (EI) and negative-ion chemical ionization (NICI) GC-MS and GC-MS/MS were performed on a triple-stage quadrupole mass spectrometer Finnigan MAT TSQ 45 interfaced with a Finnigan MAT gas chromatograph 9611 (San Jose, CA, USA). The gas chromatograph was equipped with a fused-silica capillary column DB-1 (15 m x 0.25 mm I.D., 0.25 μm film thickness) from J &W Scientific (Rancho Cordova, CA, USA). The following oven temperature program was used with helium (55 kPa) as the carrier gas: 2 min at 80°C, then increased to 250°C at a rate of 25°C/min, followed by increases to 280°C and 320°C at rates of 2°C/min and 4°C/min, respectively. Interface, injector, and ion source were kept at 280, 280, and 140°C, respectively. Electron energy was set to 70 eV in EI and to 90 eV in NICI. In both ionization modes electron current was set to 220 μA. In NICI, methane (65 Pa) and argon (0.2 Pa collision pressure) were used as reagent and collision gases, respectively. Collision energy was set to 18 eV. Electron multiplier voltage was set to 1400 - 2000 V. Aliquots of up to 2 μl were injected in the splitless mode.

Quantitation of 8-epi-$PGF_{2\alpha}$ in human urine

Two 5-ml aliquots of urine samples from spontaneous micturation of a healthy volunteer on different days were spiked with 5 ng of $^{18}O_2$-8-epi-$PGF_{2\alpha}$ and acidified to pH 3 by 5 M HCOOH. Prostanoids were extracted by solid-phase extraction on octadecylsilica cartridges preconditioned with methanol and 5 M HCOOH. Elution was performed with 2 ml of ethylacetate. After solvent evaporation prostanoids were converted to their PFB esters which were subjected to thin layer chromatography (TLC) using ethylacetate/hexane, 90/10, v/v, for elution. A 0.5-cm band, centered around the reference compound (R_f = 0.27), was scraped off the TLC plate, compounds were extracted with ethanol and the suspension was centrifuged (4000 xg, 10 min). The supernatant was decanted, ethanol was removed under nitrogen and PFB esters were converted to their TMS ether derivatives as described above. 1-μl aliquots were injected into the GC-MS/MS apparatus. Selected reaction monitoring at m/z 299 (from m/z 569) and m/z 303 (from m/z 573) were performed for endogenous 8-epi-$PGF_{2\alpha}$ and $^{18}O_2$-8-epi-$PGF_{2\alpha}$, respectively. For measurements of urine samples a SPB-1701 fused silica capillary column (30 m x 0.25 mm I.D., 0.25 μm film thickness) from Supelco (Bellefonte, PA, USA) was used. The following oven temperature

program was used with helium (65 kPa) as the carrier gas: 2 min at 100°C, then increased to 250°C at a rate of 25°C/min, followed by an increase to 280°C at a rate of 2°C/min where the temperature was kept constant for 5 min.

Results and discussion

GC-MS analysis of the methyl (Me) ester TMS ether derivative of the isolated reaction product in the EI mode showed a single peak. Figure 1 shows GC-MS EI mass spectra derived from the Me ester-TMS ether derivatives of authentic 8-epi-$PGF_{2\alpha}$ and of its enzymatically prepared ^{18}O-analog which emerged from the capillary column at the same time. The GC-MS EI mass spectra shown in Figure 1 are very similar to the corresponding mass spectra of $PGF_{2\alpha}$ and [$^{18}O_2$]-$PGF_{2\alpha}$ reported by Picket & Murphy [15]. The intense mass fragments below m/z 217, which have been characterized as containing the C9, C11, and C15 oxygen atoms [17,18], do not contain any ^{18}O-label. The ion at m/z 191 most probably corresponds to $[TMSO{=}CH\text{-}OTMS]^+$ [18].

The GC-MS NICI mass spectra of the PFB-TMS derivatives of unlabeled and labeled 8-epi-$PGF_{2\alpha}$ are characterized by the most intense ions $[M\text{-}PFB]^-$ which are formed by loss of the PFB radical from the corresponding molecules. The most abundant ions were m/z 569 for 8-epi-$PGF_{2\alpha}$ and m/z 573 for its ^{18}O-labeled analog. Other less intense ions are formed from $[M\text{-}PFB]^-$ by consecutive loss of one (m/z 479), two (m/z 389) and three (m/z 299) TMSOH groups in the PFB-TMS derivatives. These observations strongly suggest that the isolated reaction product from enzymatic labeling of 8-epi-$PGF_{2\alpha}$ is identical with $^{18}O_2$-8-epi-$PGF_{2\alpha}$ from incorporation of two ^{18}O-atoms in the carboxylic group. Selected ion monitoring of the ions at m/z 569 ([1,1-$^{16}O_2$]-8-epi-$PGF_{2\alpha}$), m/z 571 ([1,1-$^{18}O^{16}O$]-8-epi-$PGF_{2\alpha}$) and m/z 573 ([1,1-$^{18}O_2$]-8-epi-$PGF_{2\alpha}$) resulted in the distribution of 2.0, 17.7 and 80.3 %, respectively (Figure 2).

The parent ions $[M\text{-}PFB]^-$ of the investigated compounds were subjected to collisionally activated dissociation (CAD) with argon as the collision gas. In Table 1, the major daughter ions derived from the parent ions of the PFB-TMS derivatives of unlabeled and labeled 8-epi-$PGF_{2\alpha}$ are summarized. The most intense daughter ions of the PFB-TMS derivatives result from loss of all three TMSOH groups. 8-epi-$PGF_{2\alpha}$ and $^{18}O_2$-8-epi-$PGF_{2\alpha}$ have two identical daughter ions at

m/z 273 and m/z 255 because these ions are formed by loss of CO_2 from 8-epi-$PGF_{2\alpha}$ and $C^{18}O_2$ from $^{18}O_2$-8-epi-$PGF_{2\alpha}$. This is further strong evidence for the enzymatic incorporation of two ^{18}O-atoms in the carboxylic group of 8-epi-$PGF_{2\alpha}$.

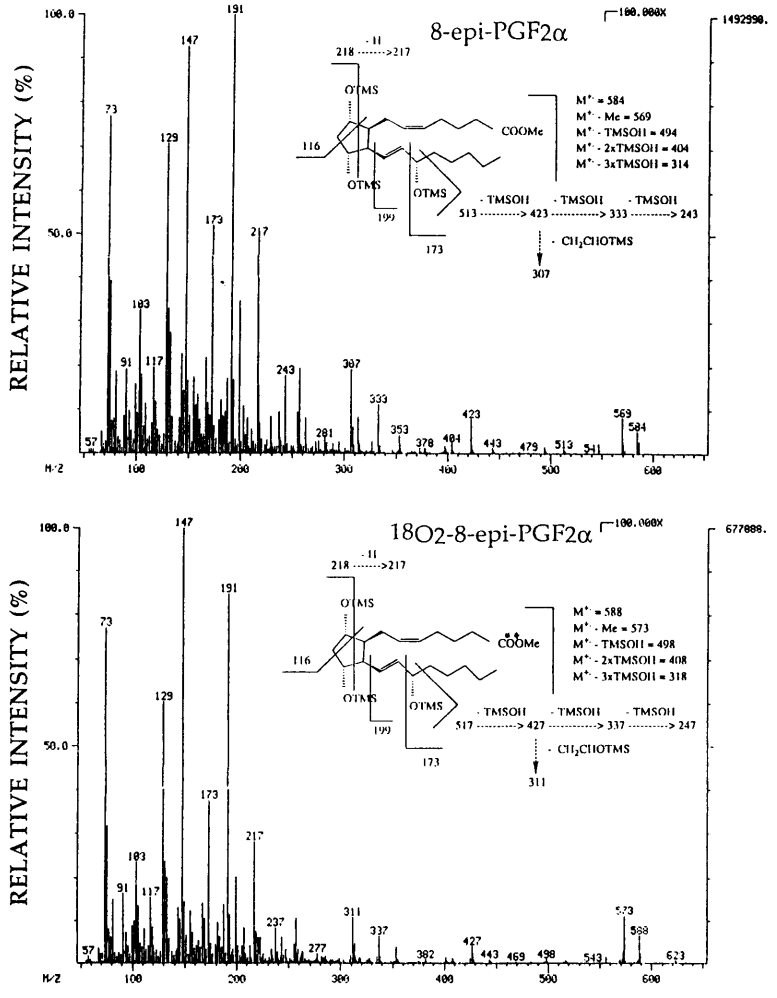

Figure 1. GC-MS EI mass spectra of the methyl (Me) ester-trimethylsilyl (TMS) ether derivatives of 8-epi-$PGF_{2\alpha}$ (upper panel) and of the enzymatically synthesized ^{18}O-labeled 8-epi-$PGF_{2\alpha}$ (lower panel). Both panels have been magnified by a factor of 100 above m/z 550. Insertions show the structures and the proposed fragmentation patterns of these compounds.

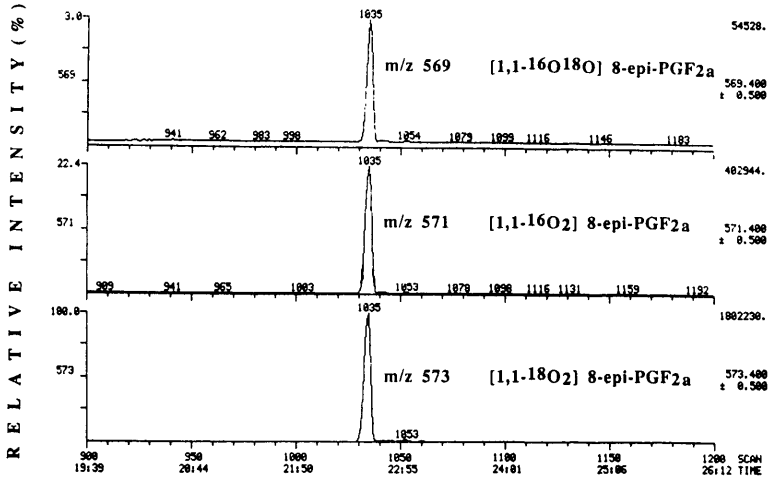

Figure 2. Partial GC-MS chromatograms from the analysis of the PFB-TMS derivative of enzymatically prepared $^{18}O_2$-8-epi-PGF$_{2\alpha}$. Selected ion monitoring of the anions [M-PFB]$^-$ at m/z 569 for [1,1-$^{16}O_2$] 8-epi-PGF$_{2\alpha}$, m/z 571 for [1,1-$^{16}O^{18}O$] 8-epi-PGF$_{2\alpha}$, and m/z 573 for [1,1-$^{18}O_2$] 8-epi-PGF$_{2\alpha}$.

Table 1. Major mass fragments (intensity ≥ 5 % is given in parenthesis) in the GC-MS/MS NICI mass spectra of the PFB-TMS derivatives of 8-epi-PGF$_{2\alpha}$ and $^{18}O_2$-8-epi-PGF$_{2\alpha}$. The ions [M-PFB]$^-$ ([P]$^-$) were subjected to collisionally activated dissociation with argon (0.2 Pa, 18 eV).

Ion assignment	8-epi-PGF$_{2\alpha}$	$^{18}O_2$-8-epi-PGF$_{2\alpha}$
[P]$^-$	569 (26)	573 (15)
[P-TMSOH]$^-$	479 (17)	483 (15)
[P-2xTMSOH]$^-$	389 (32)	393 (37)
[P-2xTMSOH-$(CH_3)_2Si{=}CH_2$]$^-$	317 (23)	321 (33)
[P-3xTMSOH]$^-$	299 (100)	303 (100)
[P-2xTMSOH-$(CH_3)_2Si{=}CH_2$-C$^{18}O_2$]$^-$	273 (33)	273 (60)
[P-3xTMSOH-C$^{18}O_2$]$^-$	255 (76)	255 (62)

Since $^{18}O_2$-8-epi-$PGF_{2\alpha}$ was prepared and isolated at a microgram quantity, the following procedure was used to precisely determine the concentration of $^{18}O_2$-8-epi-$PGF_{2\alpha}$ in its ethanolic stock solution. Each 10 ng of accurately weighed native 8-epi-$PGF_{2\alpha}$ were diluted with different amounts of $^{18}O_2$-8-epi-$PGF_{2\alpha}$ then converted to their PFB-TMS derivatives and analyzed by selected ion monitoring of m/z 569 for 8-epi-$PGF_{2\alpha}$ and m/z 573 for $^{18}O_2$-8-epi-$PGF_{2\alpha}$. Linear regression analysis between the ratio of the peak areas at m/z 573 to 569 (y) and the ratio of the supposed amounts (yield 100%) of $^{18}O_2$-8-epi-$PGF_{2\alpha}$ to the amount of 8-epi-$PGF_{2\alpha}$ (x) resulted in a linear standard curve (Figure 3, $r^2 = 0.998$). The slope of the straight line results in a mean final yield of 85.7 % of $^{18}O_2$-8-epi-$PGF_{2\alpha}$, so that the stock solution was calculated to contain 428.5 ng of $^{18}O_2$-8-epi-$PGF_{2\alpha}$ per microliter.

Figure 4 shows that $^{18}O_2$-8-epi-$PGF_{2\alpha}$ is a reliable internal standard in the quantitative determination of 8-epi-$PGF_{2\alpha}$ in urine of humans by GC-MS/MS. The level of endogenous 8-epi-$PGF_{2\alpha}$ in the urine sample analysed was determined as 300 pg/mg creatinine. In a urine sample of the same person obtained 10 days latter, the concentration of 8-epi-$PGF_{2\alpha}$ was determined to be 490 pg/mg creatinine. These values are about four times smaller than that reported by Morrow et al. using [$^2H_7$] 9α,11β-PGF_2 as internal standard [1].

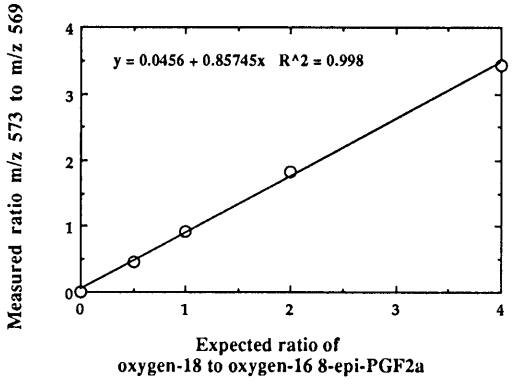

Figure 3. Standardization of $^{18}O_2$-8-epi-$PGF_{2\alpha}$ as an internal standard for quantitative GC-MS. Mixtures containing a fixed amount of 8-epi-$PGF_{2\alpha}$ (10 ng) and various amounts of $^{18}O_2$-8-epi-$PGF_{2\alpha}$ were derivatized and analysed by GC-MS. From the slope of the regression equation the concentration of $^{18}O_2$-8-epi-$PGF_{2\alpha}$ in its stock solution was calculated.

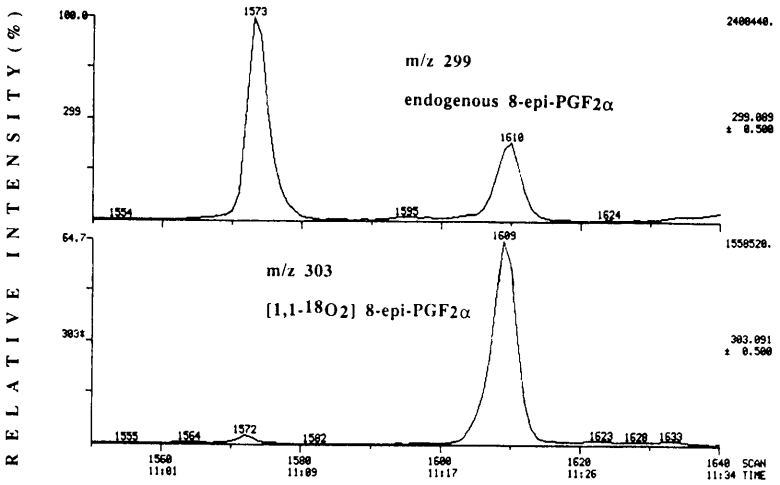

Figure 4. Partial chromatogram from the GC-MS/MS analysis of a human urine sample from spontaneous micturation by a healthy person. The urine sample was spiked with 1 ng/ml of $^{18}O_2$-8-epi-PGF$_{2\alpha}$. Selected reaction monitoring was performed at m/z 299 for endogenous 8-epi-PGF$_{2\alpha}$ and m/z 303 for $^{18}O_2$-8-epi-PGF$_{2\alpha}$. 8-epi-PGF$_{2\alpha}$ and $^{18}O_2$-8-epi-PGF$_{2\alpha}$ emerged from the column practically at the same time (scan numbers 1610 and 1609, respectively). The concentration of endogenous 8-epi-PGF$_{2\alpha}$ in the urine sample was measured to be 530 pg/ml.

Conclusions

The esterase-catalyzed incorporation of two ^{18}O-atoms in the carboxylic group of the commercially available 8-epi-PGF$_{2\alpha}$ is an elegant method to synthesize [1,1-$^{18}O_2$]-8-epi-PGF$_{2\alpha}$ at the microgram quantity in high isotopic purity and yield. [1,1-$^{18}O_2$]-8-epi-PGF$_{2\alpha}$ is a reliable internal standard for quantitative measurements in human urine by GC-MS/MS.

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