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Isolation and measurement of urinary 8-iso-prostaglandin $F_{2\alpha}$ by high-performance liquid chromatography and gas chromatography–mass spectrometry

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Abstract

8-iso-Prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) is a product of free radical-catalyzed peroxidation of arachidonic acid. Measurement of its urinary excretion has been proposed as an index of oxidative status in vivo. A stable isotope dilution method for its quantification by gas chromatography–electron capture chemical ionization mass spectrometry is described. Sample cleanup required the combined use of high-performance liquid chromatography and thin-layer chromatography. The inter-assay R.S.D. in two separate determinations was 1.6 ($n=4$) and 2.3% ($n=4$). The accuracy of the assay was evaluated through recovery experiments. The equation of the regression plot correlating the amounts added and recovered was $y=0.91x-0.31$, $r=0.9916$ ($n=12$). The pair of fragment ions ($[M-181]^-$) at m/z 569 and m/z 573 was monitored for quantification. The mean 8-iso-$PGF_{2\alpha}$ excretion rate was 528±127 (S.D.) ng per day in five male volunteers and 730±305 ng per day in six females. Intake of 80 mg of lycopene per day by eleven volunteers for four weeks resulted in a non-significant reduction of 8-iso-$PGF_{2\alpha}$ excretion.

Keywords: Prostaglandins; 8-iso-Prostaglandin $F_{2\alpha}$

1. Introduction

It is generally accepted that oxygen radicals play a significant role in several human pathologies [1]. Lipid is a major substrate of free radical injury through peroxidation processes [2]. In the late 1980s Wendelborn et al. [3] and Morrow and coworkers [4,5] demonstrated that a series of prostaglandin F_2-like compounds, named F_2-isoprostanes, are produced in vivo by a non-cyclooxygenase, free radical-catalyzed mechanism. They immediately recognized that quantification of F_2-isoprostane production could offer a novel approach for the assessment of oxidative stress [6]. This approach, which implies the use of endogenous arachidonic acid as an internal substrate, obviates the inherent difficulties and pitfalls associated with the use of external substrates, e.g., salicylate and 4-hydroxybenzoate [7,8]. In addition to greater relative simplicity, the isoprostane approach, if properly validated, promises greater accuracy compared to technologies based on the measurement of protein or DNA bases oxidation products, of short-chain alkanes, or of the thiobarbituric acid reactive substances.

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Early efforts described the measurement of F_2-isoprostanes as a group [6,9,10]. However, soon attention was focused on 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), a prominent member of the isoprostane family and a potent renal and pulmonary vasoconstrictor [4,11,12], whose formation in vivo has been conclusively demonstrated [13]. Thus, Wang et al. [14] developed an enzyme immunoassay and a radioimmunoassay for measuring urinary 8-iso-$PGF_{2\alpha}$, and Bachi et al. [15] described a method based on immunoaffinity extraction/gas chromatography–mass spectrometry. Here we describe a procedure to quantify the urinary excretion of 8-iso-$PGF_{2\alpha}$ where sample enrichment relies primarily on the use of high-performance liquid chromatography.

2. Experimental

2.1. Materials

We purchased the following materials from the indicated vendors: Sep-Pak C_{18} cartridges, Waters (Milford, MA, USA); pentafluorobenzyl bromide (PFBBr) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), Pierce (Rockford, IL, USA); Whatman LK6D silica gel 5 cm×20 cm TLC plates, 250 μm thickness, Government Scientific Sources (Vienna, VA, USA). $PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$, 15(*R*)-$PGF_{2\alpha}$, 9β,11α-PGF_2, 9α,11β-PGF_2, 8-iso-9β,11α-PGF_2, 5-*trans*-$PGF_{2\alpha}$ and 8-iso-[3,3,4,4-$^2H_4$]$PGF_{2\alpha}$ were purchased from Cayman (Ann Arbor, MI, USA). All analytical operations were carried out in silanized glassware. The 8-iso-[3,3,4,4-$^2H_4$]$PGF_{2\alpha}$ stock solution of approximately 500 μg was mass spectrometrically titrated against a reference stock solution (10 mg) of 8-iso-$PGF_{2\alpha}$ whose exact concentration was gravimetrically determined.

2.2. HPLC equipment and procedure

Reversed-phase HPLC was conducted at 31°C on a Partisil ODS-3 column (Whatman), 250×4.6 mm I.D., 10 μm particle size. We used an SP8700 solvent delivery system (Spectra-Physics) consisting of a control module, a pump assembly and a dynamic mixer. The injector was a Rheodyne Model 7125 equipped with a 20-μl loop. The system was coupled to an SP8440 UV–Vis variable-wavelength detector and an SP4270 integrator. Chromatographies were conducted isocratically with water–acetonitrile (7:3, v/v) containing 0.01% acetic acid as the mobile phase whose pH was 4.2. 9α,11β-PGF_2 (1 μg) was used as a reference marker whose elution time was determined daily. The analyte, 8-iso-$PGF_{2\alpha}$, was isolated by trapping the HPLC effluent during the 2.2 min immediately preceding the elution time of 9α,11β-PGF_2, which was 11.3±0.1 (S.D.) min, n=14. The elution time of 8-iso-$PGF_{2\alpha}$ was 10.3±0.2 min, n=9. The flow-rate was 1.5 ml/min. 9α,11β-PGF_2 was monitored at 193 nm and the urine samples were run at 240 nm.

2.3. Urine samples

Urine samples were collected in ice-cold 4-l polyethylene bottles. After the 24-h collections were completed, portions were stored at −23°C until they were analyzed.

2.4. Procedure

A 2-ml volume of urine was diluted with 8 ml of water, spiked with 5 ng of 8-iso-[3,3,4,4-$^2H_4$]$PGF_{2\alpha}$ internal standard, then was acidified to pH 3 with 4 *M* hydrochloric acid, and passed through a prewashed (5 ml of MeOH then 10 ml of acidified water) C_{18} Sep-Pak cartridge. The cartridge was sequentially rinsed with 10 ml of acidified (pH 3.0) water and 10 ml of methyl formate–petroleum ether (5:95, v/v). The isoprostanes and the internal standard were eluted with 10 ml of HCOOMe–petroleum ether (1:1, v/v). The residue after solvent evaporation (under N_2) was treated with 75 μl of ethyl acetate, vortex mixed and then centrifuged. The supernatant, after reduction to dryness, was dissolved in 20 μl of water–acetonitrile (1:1, v/v) and injected into the liquid chromatograph. The fraction containing 8-iso-$PGF_{2\alpha}$ (3.3 ml) was trapped as described above, then was diluted with 5 ml of water and extracted twice with 3 ml of ethyl acetate. The combined extracts were reduced to dryness and the residue was dissolved in 40 μl of acetonitrile, then treated with 10 μl of diisopropylethylamine + 20 μl of 10% PFBBr in CH_3CN and allowed to stand at room temperature for 30 min. After thorough evaporation under dry N_2, the residue was dissolved in 20 μl of AcOEt and subjected to TLC with AcOEt as

the solvent. A reference plate, spotted with the pentafluorobenzyl derivative of 8-iso-$PGF_{2\alpha}$, was developed alongside. A 1.3-cm band, centered around the reference material (R_F 0.22), was scraped off the TLC plate, loaded on a Pasteur pipet and eluted with 2 ml of AcOEt. After solvent evaporation (dry N_2), the residue was treated with 10 μl of BSTFA in pyridine (1:1, v/v) and heated at 40°C for 15 min. The dry residue obtained after evaporation was dissolved in 20 μl of 1% BSTFA–pyridine (1:1, v/v) in 2,2,4-trimethylpentane, and was thus ready for injection into the GC–MS system.

2.5. Gas chromatography–electron capture chemical ionization–mass spectrometry (GC–ECCI-MS)

GC–MS was performed with a Varian 3400 chromatograph coupled to a Finnigan-MAT TSQ-70B mass spectrometer. GC–MS, ECCI and GC column conditions have been described previously [16] except that the GC column was 20-m long, with a flow velocity of 54 cm/s. The pair of anions ($[M-PFB]^-$) at m/z 569 and m/z 573 were used for quantification. The retention time of 8-iso-$PGF_{2\alpha}$ was 9.4 min and that of 9α,11β-PGF_2 was 10.9 min. For the determination of low-energy (E_{lab}=32 eV) collision induced dissociation (CID) spectra of synthetic isomeric PGF_2 compounds (Table 1), we used the same MS conditions described previously [16].

Table 1
Low-energy CID spectra of synthetic isomeric prostaglandin F_2 compounds

m/z	Compound						
	A	B	C	D	E	F	G
569[a]	13[b]	42	34	12	13	7	12
479	5	7	9	12	14	15	4
389	24	14	24	16	10	[c]	11
363	8	7	[c]	[c]	1	[c]	4
317	27	24	33	18	13	6	12
299	100	100	100	100	75	49	100
281	18	13	24	6	13	15	7
273	12	42	52	26	23	13	38
255	55	68	30	48	40	23	36
247	13	8	10	7	3	3	7
243	10	7	10	9	7	8	6
229	4	3	1	3	3	[c]	4
219	24	70	26	16	9	[c]	18
215	41	31	44	12	100	100	41
201	17	15	10	23	20	8	15
193	11	4	7	8	3	[c]	7
191	9	8	22	12	18	[c]	6
177	4	[c]	4	[c]	6	16	[c]
173	3	[c]	[c]	13	6	4	[c]
161	43	57	47	14	7	6	5

[a] Parent ion $[M-PFB]^-$.
[b] Relative intensity.
[c] Less than 1%.
Compounds: A=$PGF_{2\alpha}$; B=8-epi-$PGF_{2\alpha}$; C=15(*R*)-$PGF_{2\alpha}$; D=9β,11α-PGF_2; E=9α,11β-PGF_2; F=8-iso-9β,11α-PGF_2; G=5-*trans*-$PGF_{2\alpha}$.

3. Results

Fig. 1 shows typical $[M-PFB]^-$ ion chromatograms of derivatized endogenous 8-iso-$PGF_{2\alpha}$ and 8-iso-[3,3,4,4-$^2H_4$]$PGF_{2\alpha}$ obtained from a urine extract purified as described.

3.1. Daughter ion spectra

Table 1 shows CID spectra of $PGF_{2\alpha}$, of two isoprostanes as defined by Morrow and Roberts [17] – 8-iso-$PGF_{2\alpha}$ and 15(*R*)-$PGF_{2\alpha}$ – and of four isomeric PGF_2s.

3.2. Standard curves

Standard curves were developed by injecting increasing amounts (0–10 ng) of synthetic 8-iso-$PGF_{2\alpha}$ in the presence of a constant amount (5 ng) of 8-iso-[3,3,4,4-$^2H_4$]$PGF_{2\alpha}$ internal standard, and by plotting the response ratio, ($^2H_0/^2H_4$)×ng $^2H_4$, against the amount of analyte injected. A typical linear regression curve followed the equation $y=0.88x+0.01$ ($r=0.9998$).

3.3. Precision

The precision of the method was assessed by analyzing four identical 2-ml samples of urine. The mean concentration of 8-iso-$PGF_{2\alpha}$ was 1.26±0.02 (S.D.) ng/ml and the R.S.D. was 1.6%. When an identical test was carried out with urine from another donor, the mean concentration was 0.87±0.02 (S.D.) ng/ml and the R.S.D. was 2.3%. The minimum amount of analyte that we could measure accurately was 25 pg/ml of urine.

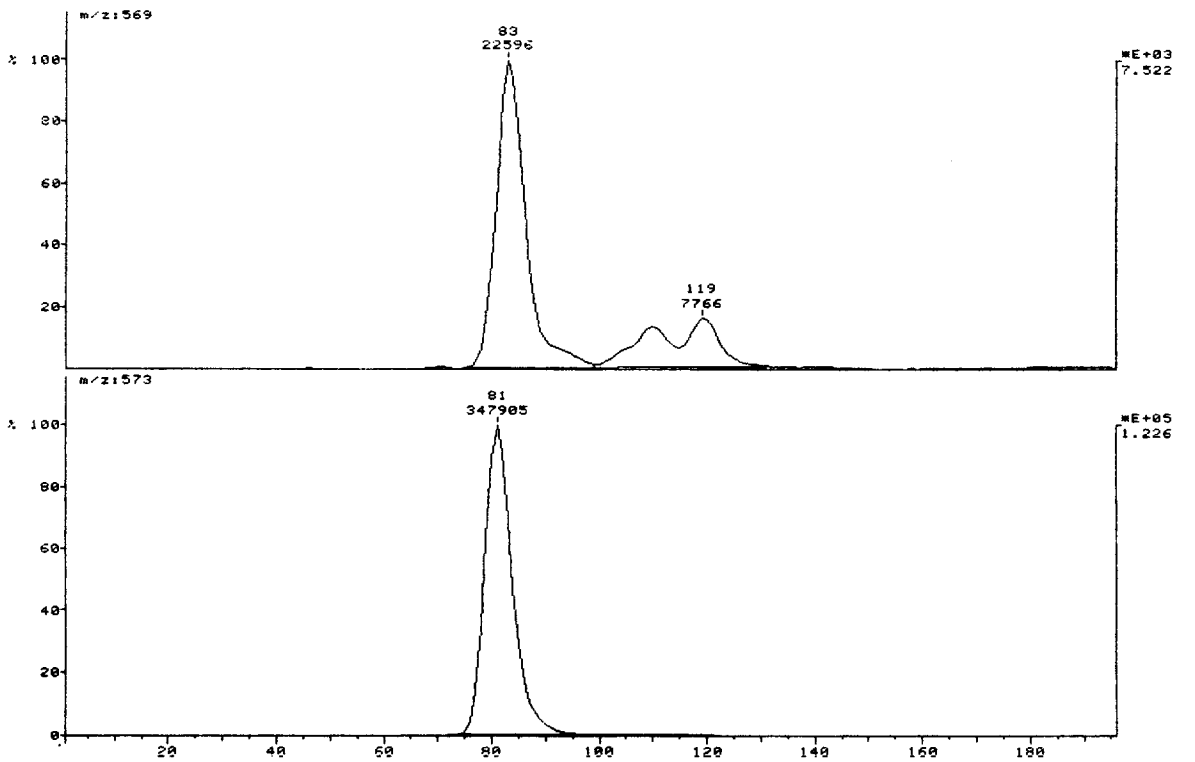

Fig. 1. Typical $[M-PFB]^-$ ion chromatograms of derivatized endogenous 8-iso-$PGF_{2\alpha}$ (upper trace, *m/z* 569) and 8-iso-[3,3,4,4-$^2H_4$]$PGF_{2\alpha}$ (lower trace, *m/z* 573) from a purified urine extract.

3.4. Evaluation of accuracy

Accuracy of the assay system was evaluated by plotting nanograms of 8-iso-$PGF_{2\alpha}$ recovered vs. nanograms of 8-iso-$PGF_{2\alpha}$ added before extraction. In a typical experiment, 8 ml of urine was spiked with 20 ng of internal standard, then was divided into four 2-ml aliquots. Exact and increasing amounts of underivatized analyte were added to three of the four aliquots, and all four were analyzed as described. This procedure was repeated with three more urine pools from different donors. The results are shown in Fig. 2 where the twelve points indicate the recovery of the spiked 8-iso-$PGF_{2\alpha}$. Endogenous 8-iso-$PGF_{2\alpha}$, measured in the nonspiked aliquots, was subtracted.

3.5. 8-iso-$PGF_{2\alpha}$ excretion in adult volunteers

The procedure described above was used to evaluate basal urinary excretion of 8-iso-$PGF_{2\alpha}$ in eleven healthy, non-smoking, free living volunteers, five males and six females (age 34 to 61 years). The mean excretion rate in males was 528±127 (S.D.) ng per day (range 431–724), $n=5$. In females it was 730±305 (S.D.) ng per day (range 371–1257), $n=6$. When the same subjects ingested approximately 80 mg per day of the carotenoid lycopene for four weeks, we observed a non-significant reduction in 8-iso-$PGF_{2\alpha}$ excretion in both males and females at the end of the supplementation period: 508±213 (S.D.) ng per day (range 250–819) and 702±288 (S.D.) ng per day (range 405–1110), respectively.

4. Discussion

The measurement of F_2-isoprostanes to quantify lipid peroxidation and oxidative stress in vivo is an approach whose validity has not yet been fully established. The availability of several alternative analytical procedures should speed-up the assessment

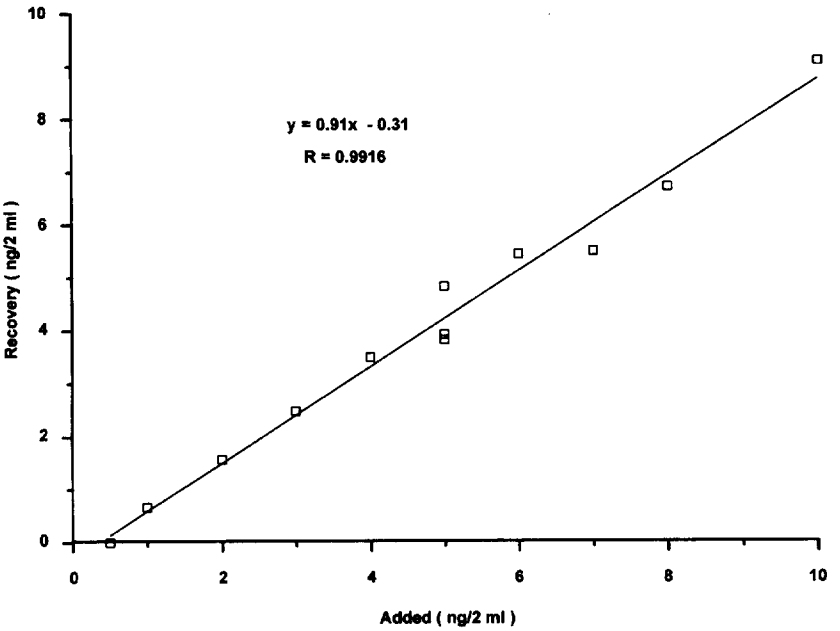

Fig. 2. Evaluation of accuracy: 8-iso-$PGF_{2\alpha}$ recovered vs. authentic 8-iso-$PGF_{2\alpha}$ added to twelve urine samples from four different donors (see Section 3.4).

process. The endogenous production of isoprostanes, like the production of their cyclooxygenase-derived counterparts, would best be assessed through the measurement of their metabolites in plasma or urine [18]. However, this approach will have to be deferred until the structure of such metabolites will be firmly established, and internal standards suitable for stable isotope dilution assays will become available. The method described here is based on conventional technologies where sample enrichment relies primarily on the use of HPLC. Therefore, it provides an alternative approach to those of Wang et al. [14] and Bachi et al. [15] to investigators who do not have access to immunotechnology. In our method, the introduction of HPLC was necessary to ensure adequate purity of the sample to be injected into the GC–MS system. We were unable to obtain acceptable recovery plots before the introduction of HPLC. TLC was necessary primarily to remove impurities introduced with pentafluorobenzyl bromide. Adequate purity is essential while assessing small variations in urinary output of the analyte.

Confidence that our procedure indeed affords the measurement of the intended target analyte, 8-iso-$PGF_{2\alpha}$, is based primarily on HPLC and GC retention times/volumes and, to a lesser extent, on the CID spectrum of the HPLC effluent obtained as described in Section 2.4. The data in Table 1 reveal extensive similarities among the low-energy CID spectra of a variety of isomeric prostaglandin F_2 compounds, including 8-iso-$PGF_{2\alpha}$. Because of such uniformity, tandem mass spectrometry could not be relied upon to further improve the specificity of the method.

Lycopene, a carotenoid found largely in tomatoes, is believed to have antioxidant capability in vivo. Therefore, it might be useful in the prevention of pathologies associated with oxidative damage of biological materials such as DNA, protein and lipid. The reduction in 8-iso-$PGF_{2\alpha}$ excretion in eleven subjects ingesting 80 mg per day of lycopene probably did not reach significance either because of the short duration or of the relatively modest level of the dietary intervention.

At the present time it is unclear whether urinary isoprostanes, including 8-iso-$PGF_{2\alpha}$, are derived from local (renal) production or, at least in part, from plasma filtration [17]. It is tempting to assume that an increase (or decrease) of 8-iso-$PGF_{2\alpha}$ excretion is indication of an increase (or decrease) of both

systemic and renal production, but it remains to be demonstrated. Further experimentation will also be necessary to ascertain whether the measurement of (unmetabolized or metabolized) urinary F_2-isoprostanes can be utilized as reliable and sensitive enough indicators of systemic isoprostane formation and oxidative stress.

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