Research Note

An Improved Procedure for the Enrichment of Plasma F$_2$-Isoprostanes Prior to Final Determination by GC-MS/NICI

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Abstract. *One of the most popular approaches to quantify oxidative injury is to measure lipid peroxidation products and, in particular, F$_2$-isoprostanes (F$_2$-IPs). F$_2$-IPs is a group of prostaglandin F$_2$-like compounds derived from the non-enzymatic oxidation of arachidonic acid. Of these, the 15-F$_{2t}$-isoprostane (8-iso-PGF$_{2\alpha}$) has received considerable attention, because it possesses adverse biological activities. Previous Gas Chromatographic-Mass Spectrometric (GC-MS) methods for measuring plasma F$_2$-IPs from this laboratory involved two chromatography steps on C$_{18}$ and NH$_2$-cartridges. Problems may, however, arise with chromatography on C$_{18}$ cartridges, as it can be time-consuming and losses may occur depending upon the pH and efficiency of the sample loading. Therefore, it was decided that the C$_{18}$ chromatography step be replaced with a single lipid partitioning step and the NH$_2$-chromatography be simplified. In 70 plasma samples from healthy individuals, total (sum of free and esterified) 15-F$_{2t}$-isoprostane concentrations ranged from 0.5 to 3.13 nM. This assay meets all predefined method performances in terms of specificity and sensitivity. The improved method is suitable for the analysis of samples from larger clinical trials investigating the role of oxidant injury under conditions associated with oxidative stress.*

INTRODUCTION

F$_2$-isoprostanes are a series of prostaglandin F$_2$ (PGF$_2$)-like compounds produced by non-enzymatic peroxidation of arachidonic acid via a mechanism independent of the cyclooxygenase (COX) pathway [1,2]. Four possible subfamilies of F$_2$-isoprostanes are formed as a result of a radical attack at positions C$_7$, C$_{10}$ and C$_{13}$ (Figure 1). Series 5 (Type VI) F$_2$-isoprostanes are derived from a free radical attack at positions C$_7$, whilst series 8 (Type V) and series 12 (Type IV) result from a free radical attack at position C$_{10}$. Series 15 (Type III) is derived from a free radical attack at position C$_{13}$. Each subfamily comprises

16 diastereoisomers, since the hydroxy group on the cyclopentane ring can be arranged in the 2^3 configuration. In total, sixty-four F$_2$-isoprostanes isomers can be formed during the peroxidation of arachidonic acid. The nomenclature of isoprostanes are comprehensively discussed elsewhere [3,4]. Of these, 15-F$_{2t}$-isoprostane (also know as 8-epi-PGF$_{2\alpha}$ or 8-iso-PGF$_{2\alpha}$) has received most attention, because it has been shown to possess certain adverse biological activities [5-7]. Circulating 15-F$_{2t}$-isoprostane is mainly present esterified to phospholipids in situ and are released by the action of phospholipase A$_2$ [8].

Existing procedures for the enrichment of F$_2$-isoprostanes from plasma, prior to final determination by gas chromatographic/mass spectrometric-negative ion chemical ionization (GC/MS-NICI), include:

1. Chromatography on a C$_{18}$-cartridge followed by thin layer chromatography (TLC) [2,9,10];

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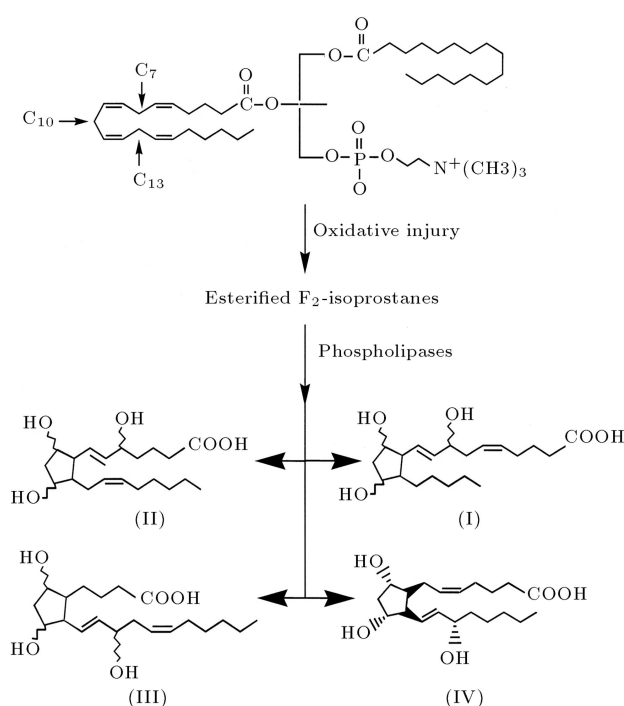
Figure 1. Proposed pathways for the formation of F_2-isoprostanes families during auto-oxidation of arachidonic acid.

2. Immuno-affinity chromatography [9,11,12];

3. A single solid-phase extraction step [13,14];

4. Chromatography on a C_{18} cartridge followed by an NH_2-cartridge [15-17].

Of the above-mentioned procedures, the one combining C_{18}- and NH_2- chromatography is the method of choice, because it is less labour intensive than the others and is acceptable with respect to the efficiency of extraction ($\sim$ 75%) [15]. Problems may, however, arise with chromatography on a C_{18} cartridge; it can be time consuming and losses may occur depending upon the pH and efficiency of the sample loading. The objective of the present study was to improve the existing protocol for the enrichment of plasma F_2-isoprostanes (i.e. by replacing chromatography on a C_{18}-cartridge with total lipid extraction and simplifying the procedure for NH_2-chromatographic step), prior to final determination by GC-MS.

MATERIALS AND METHODS

Reagents

9α, 11α-8-iso-PGF$_2$ and $3,3',4,4'$-tetradeuterated 9α, 11α-PGF$_2$ (PGF$_2$-d$_4$) standards were obtained from SPI Bio (Massy Cedex, France). [^{3}H]-9α, 11α-PGF$_2$ was purchased from Amersham Inc. (Amersham, UK). N,O-bis(trimethylsilyl) trifluoroacetamide, pentafluorobenzyl-bromide, diisopropylethy-

lamine and butylated hydroxytoluene were purchased from Sigma (Poole, England). Solid-phase extraction cartridges (500 mg) including aminopropyl (NH$_2$) and octadecylsilane (C_{18}) were obtained from Waters Corporation (Milford, MA, USA). All other general-purpose chemicals and organic solvents were obtained from BDH, Poole, England.

Blood Collection and Plasma Preparation

After a 14-hr fast, blood was collected by a venipuncture into sampling vials (10 mL) containing ethylenediaminetetraacetic acid at a final concentration of 1% and plasma obtained by centrifugation at 2500xg at room temperature for 10 min. Aliquots (1 mL) of plasma were combined with the chain breaking antioxidant, butylated hydroxytolouene, at a final concentration of 25 μM and stored at -85°C until analysis.

Folch Extraction Procedure

Plasma samples (0.5 mL) were transferred to glass tubes. Subsequently, water (500 μL), PGF$_{2\alpha}$ (2.5 ng in 100 μL ethanol) and [^{3}H]-PGF$_{2\alpha}$ as tracer (100 μL containing $\sim$ 10000 cpm) were added. Three mL of chloroform/methanol (2/1; V/V) followed by water (0.5 mL) were next added and the samples vortex mixed for 30 seconds. After centrifugation for 5 min at 2500xg, the organic (lower) layers were transferred to new glass-tubes. Three mL of chloroform/methanol (2/1; V/V) were added to the remaining aqueous phases, the extraction procedure repeated as described above and the organic layers pooled. The solvent was dried under a stream of nitrogen (N$_2$) and the residues re-suspended in ethanol (250 μL). The samples were transferred to beta vials and three millilitres of scintillation cocktail (Pico-Fluor^{TM} 40) were added. The radioactivity was monitored using a Beckman LS-6000IC instrument.

Ethyl Acetate Extraction

Plasma samples (0.5 mL) were transferred to glass tubes and PGF$_{2\alpha}$ (2.5 ng in 100 μL ethanol) and [^{3}H]-PGF$_{2\alpha}$ (100 μL containing $\sim$ 10000 cpm) were added. Water (1.5 mL) and ethyl acetate (2, 4, 6, 8 or 10 mL) were added. The samples vortex was mixed for 30 seconds and, subsequently, centrifuged at 2500xg for 5 min. The organic (upper) layers were transferred to new glass tubes and the solvent dried under N$_2$. The residues were re-dissolved in ethanol (250 μL) and subsequently transferred to beta vials. A scintillation cocktail (3 mL) was added and the radioactivity was monitored.

Ethyl Acetate Extraction/NH$_2$-Chromatography

To evaluate the efficiency of the ethyl acetate/ NH$_2$-chromatography procedure, plasma (0.5 mL) was spiked with PGF$_{2\alpha}$ (2.5 ng in 100 μL ethanol) as a carrier and [^{3}H]-PGF$_{2\alpha}$ (100 μL containing $\sim$ 10000 cpm) as a tracer. An extraction of total lipid with ethyl acetate/NH$_2$-chromtrography was carried out, as described above. All fractions following the NH$_2$-chromatography step were collected and the solvents removed under N$_2$. The residues were re-suspended in ethanol (250 μL) and transferred to beta vials, and the radioactivity was monitored.

F$_2$-ISOPROSTANE ANALYSIS

Alkaline Hydrolysis

Plasma samples (0.5 mL) were transferred into glass-tubes and 4 M aqueous potassium hydroxide (250 μL) was added. The samples were incubated for 30 min at 45°C to hydrolyze the esterified lipids. Subsequently, the pH was adjusted to 2 by adding 4 M HCl (250 μL). Internal standard (PGF$_{2\alpha}$-d$_4$; 2.5 ng in 100 μL ethanol) was added and the samples were vortex mixed.

Total Lipid Extraction

Ethyl acetate (10 mL) and water (1 mL) were added to samples following the alkaline hydrolysis step. The samples were vortex mixed for 30 seconds and then centrifuged at 2500xg for 5 min at room temperature.

NH$_2$-Chromatography

Total lipid extracts from the previous step were applied to NH$_2$-cartridges pre-conditioned with hexane (5 mL), and the cartridges were washed with ethyl acetate (10 mL). Isoprostanes were eluted by washing the cartridges with 5 mL of ethyl acetate/methanol/acetic acid (10/85/5, V/V/V).

Pentaflurorobenzyl Derivatization

The final extracts from the NH$_2$-chromatography step were dried under N$_2$ at 45°C. Pentafluorobenzyl bromide (40 μL, 10% in acetonitrile) and diisopropylethylamine (20 μL; 10% in acetonitrile) were added and the samples were kept at 45°C for 30 min.

Trimethylsilyl Ether Derivatisation

Samples following the pentaflurorobenzyl derivatization step were dried under N$_2$ at 45°C. N,O-bis(trimethylsilyl)trifluoroacetamide (50 μL) and diisopropylethylamine (5 μL; 10% in acetonitrile) were added and the samples heated for 30 min at 45°C. The samples were then dried under N$_2$ at 45°C and the residue reconstituted in iso-octane (40 μL).

GAS CHROMATOGRAPHY-MASS SPECTROMETRY/NEGATIVE ION CHEMICAL IONISATION (GC-MS/NICI)

The analysis was carried out on a Hewlett Packard 5890 GC linked to a VG70SEQ, using the NICI with ammonia as the reagent gas. Samples (2 μL) were injected into a temperature programmed Gerstel injector. Separation was carried out on an SPB-1701 column (30 m × 0.25 mm ID; 0.25 μm film thickness, SUPLECO Dorset, England) using a temperature programme; initial temperature 175°C; initial time: 2 min; rate: 30°C/min; final temperature: 270°C; final time 30 min. A quantitative analysis was performed using a Selected Ion Monitoring (SIM) of the carboxylate anion [M-181]$^-$ at m/z 569, 573 for 8-iso-PGF$_{2\alpha}$ and PGF$_{2\alpha}$-d$_4$, respectively.

RESULTS AND DISCUSSION

Folch extraction (chloroform/methanol) is the most commonly used procedure for the partition of total lipids including F$_2$-isoprostanes from tissue and biological fluids. To the best of my knowledge, however, no information is available on the recovery of F$_2$-isoprostanes using the Folch extraction described by Morrow et al. [2,9]. Therefore, it was decided to evaluate the suitability of this extraction procedure for the partitioning of plasma F$_2$-isoprostanes. Radioactivity measurements revealed that only 64 ± 5% ($n = 3$) of the [^{3}H]-PGF$_{2\alpha}$ tracer was recovered from the plasma, following three sequential extractions with chloroform/methanol. On the contrary, a quantitative extraction (98 ± 3%; $n = 3$) of the tracer added to the plasma was achieved at a ratio of an ethyl acetate/aqueous phase of 5/1 (V/V). As indicated in Figure 2, the recovery of the tracer was dependent on the ratio of the ethyl acetate/aqueous phase. These data suggest that the ethyl acetate extraction procedure is simpler and more efficient than the Folch extraction for the partitioning of F$_2$-isoprostanes from biological fluids. The ratio of an ethyl acetate/aqueous phase of 5/1 was used in all further experiments.

The established protocol for the enrichment of F$_2$-isoprostanes, employing NH$_2$-chromatograpy, involves three sequential washes with ethyl acetate/hexane (30/70; V/V), acetonitrile/water (90/10; V/V) and acetonitrile (10 mL each). F$_2$-isoprostanes were eluted by washing the cartridge in 5 mL of ethyl acetate/methanol/acetic acid (10/85/5; V/V/V) [11,12]. It was found that omitting two washing steps (i.e. acetonitrile/water (90/10; V/V) and acetonitrile pro-
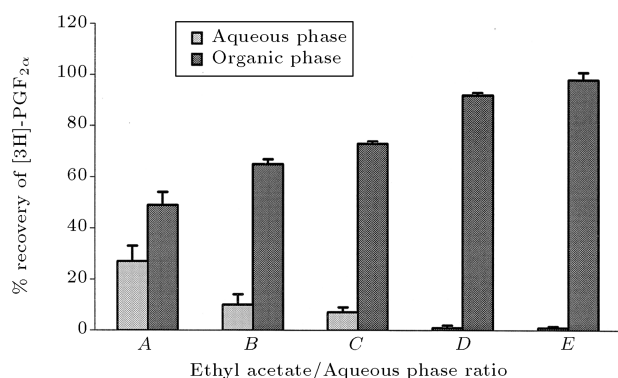
Figure 2. Percentage recovery of [^{3}H]-PGF$_{2\alpha}$ added to plasma following ethyl acetate extraction. Plasma samples (0.5 mL) were transferred to glass tubes. [^{3}H]-PGF$_{2\alpha}$ as a tracer was added. Total lipid extraction was carried out as described under the material & method section. The radioactivity was monitored and yield was calculated relative to controls. Data are presented as the mean of triplicate analysis ± SD. Ethyl acetate/water ratios: $A = 1/1$; $B = 2/1$; $C = 3/1$; $D = 4/1$ and $E = 5/1$.

duced) had an impact on the recovery of radiolabel PGF$_{2\alpha}$ when compared to existing methods [15]. The overall recovery of radiolabel PGF$_{2\alpha}$, following total lipid extraction and chromatography on NH$_2$-cartridges, was $74 \pm 4\%$ ($n = 3$). This is in good agreement with the recoveries reported previously for the combined C$_{18}$- and NH$_2$-cartridge chromatographic procedure [15].

Figure 3 shows the SIM of the carboxylate anion

[M-PFB]$^-$ chromatograms of plasma F$_2$-isoprostanes employing the combined ethyl acetate extraction/NH$_2$-cartridge chromatographic procedure. The signals at m/z 569 and 573 represent the SIM of the carboxylate anion [M-181]$^-$ for the F$_2$-isoprostanes and PGF$_{2\alpha}$-d$_4$ as the internal standards, respectively. The [M-PFB]$^-$ ion chromatograms obtained, using two types of extraction procedure (i.e. ethyl acetate extraction/NH$_2$-cartridge vs C$_{18}$- and NH$_2$-cartridge), were virtually identical. These data indicated that the combined ethyl acetate extraction followed by the simplified NH$_2$-chromatography step is as efficient as the conventional but more time consuming C$_{18}$ cartridge/NH$_2$-cartridge method for the isolation of plasma F$_2$-isoprostanes.

As indicated in Figure 3, the major F$_2$-isoprostanes detected in plasma were observed as a doublet of partially resolved peaks, with retention times between 17:10 and 17:25 min. Attempts to improve the GC separation of these two peaks were unsuccessful. However, peaks labelled as I and II were identified as the 15(S)-isomer of 8-epi-PGF$_{2\alpha}$ and the 15(R)-isomer of 8-epi-PGF$_{2\alpha}$ by the comparison of relative retention times and structural characteristics with the authentic 15(S)-isomer of 8-epi-PGF$_{2\alpha}$ and the 15(R)-isomer of 8-epi-PGF$_{2\alpha}$, respectively. This finding supports the notion that, free radical attacks on arachidonic acid would be expected to produce equal amounts of isomers 8-epi-PGF$_{2\alpha}$. In this study, the quantitative measurement of a 15(S)-isomer of 8-
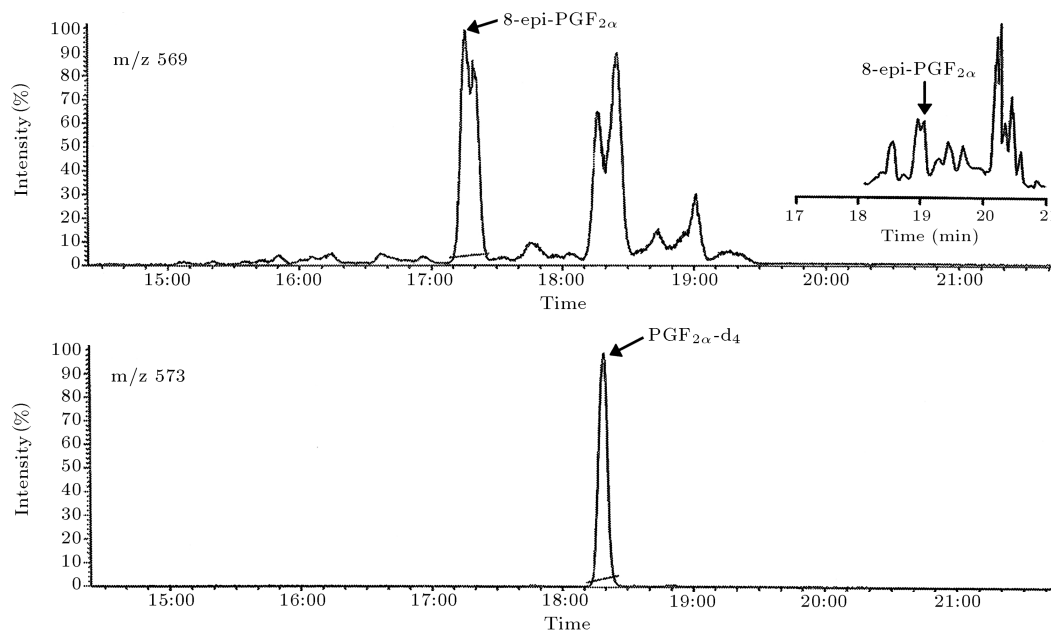

Figure 3. Gas chromatographic/mass spectrometric (GC-MS) profile of plasma F$_2$-isoprostanes using ethyl; the combined ethyl acetate/NH$_2$ procedure. Upper trace monitored at m/z 569 represents F$_2$-isoprostanes. Bottom chromatogram monitored at m/z 573 corresponds to PGF$_{2\alpha}$-d$_4$ as the internal standard. Peaks labelled I and II represent the 15(S)-isomer of 8-epi-PGF$_{2\alpha}$ and 15(R)-isomer of 8-epi-PGF$_{2\alpha}$, respectively. Insert represents GC-MS chromatograph of plasma F$_2$-isoprostanes using the combined C$_{18}$/NH$_2$ enrichment procedure [15].

epi-PGF$_{2\alpha}$ was based on integration by peak height relative to the internal standard (PGF$_{2\alpha}$-d$_4$). Thus, quantitative determinations of these two isomers may represent a useful addition to the measurement of F$_2$-isoprostanes as markers of oxidative stress in vivo.

Analysis of plasma samples from healthy control subjects ($n = 69$; age 53 ± 11 years) revealed that the mean level of the total 15(S)-isomer of 8-epi PGF$_{2\alpha}$ (peak labelled as I in Figure 3) was 0.76 nM (range 0.5-3.13 nM). These values are in good agreement with those data previously published by us and by other competing laboratories [12,16,18-20].

In conclusion, an improved method for the enrichment of plasma F$_2$-isoprostanes prior to final determination by GC-MS/NICI is described. The modifications include replacing chromatography on a C$_{18}$-cartridge by total lipid extraction with ethyl acetate and by omitting two washing steps during subsequent NH$_2$-chromatography. The improved method is sensitive, simple and less labour intensive compared to existing methods.

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