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Analysis of F_2-isoprostanes as indicators of non-enzymatic lipid peroxidation in vivo by gas chromatography–mass spectrometry: development of a solid-phase extraction procedure

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Abstract

Recently, it has been reported that a series of prostaglandin F_2-like compounds (F_2-isoprostanes) are produced in vivo during peroxidation of arachidonic acid by a mechanism independent of the cyclooxygenase pathway. Of these, 8-epi-PGF_{α}^{2} is shown to be a potent vasoconstrictor. We describe an improved method for analysing F_2-isoprostanes in biological fluids. The method involves solid-phase extraction on an octadecylsilane (C_{18}) and an aminopropyl (NH_2) cartridge. After conversion to pentafluorobenzyl ester and trimethylsilyl ether derivatives, F_2-isoprostanes are analysed by negative-ion chemical ionization mass spectrometry using tetradeuterated PGF_{α}^{2} as the internal standard. The limit of detection of the assay was 10 pg/ml, with a coefficient of variation ranging from 9.4 to 15.1%. Analysis of plasma samples from healthy volunteers ($n = 7$) revealed no quantifiable levels of free (unesterified) 8-epi-PGF_{α}^{2}. However, the plasma samples contained 58 to 166 pg/ml of 8-epi-PGF_{α}^{2} when analyzed for the total (sum of free and esterified) F_2-isoprostanes. The main advantages of the method lie in the improved recovery, gas chromatographic separation and speed compared to existing techniques.

1. Introduction

Non-enzymatic peroxidation of polyunsaturated fatty acids (PUFAS) is thought to play an important role in the pathophysiology of various diseases [1]. The process involves a bisallylic hydrogen abstraction, conjugation of the double bonds and insertion of molecular oxygen. Rearrangement of the generated peroxy fatty acid radical yields a variety of intermediate and end products such as aldehydes, short-chain alkanes and hydroxylated fatty acids [2].

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The most widely used assay for the assessment of lipid peroxidation in biological material is based on measurement of thiobarbituric acid reactive substances (TBARS). The TBARS assay measures malondialdehyde formed during decomposition of PUFS/PUFS ester endoperoxides. Malondialdehyde is also formed as a secondary decomposition product of non-lipid biomolecules [3]. Thus, it is unclear to what extent plasma lipoprotein peroxidation assessed by this method accounts for biological changes associated with oxidative stress.

Recently, the formation of a series of novel F_2-isoprostanes by a mechanism independent of the cyclooxygenase pathway, has been described as an indicator of oxidative stress in vivo [4,5]. Non-enzymatic oxidation of arachidonic acid yields four regioisomers of PGF_2 having a *cis*-oriented side chain (F_2-isoprostanes) with the 8-epi-$PGF_{2\alpha}$ isomer as the major product. The levels of these abnormal F_2-isoprostanes were found to be 10 to 500 fold higher in two animal models of free radical injury and lipid peroxidation compared to controls.

The 8-epi-$PGF_{2\alpha}$ isomer has been shown to be a potent and selective vasoconstrictor of the renal and pulmonary artery [6,7]. The vasoconstricting actions in both the renal and pulmonary vascular sites have been shown to be mediated through interaction with a unique receptor on vascular smooth muscle and through inhibition of thromboxane receptors on platelets [8,9]. As atherosclerosis has been associated with increased oxidative stress, the measurement of plasma levels of the F_2-isoprostanes could be a useful tool in assessing the role of non-enzymatic lipid peroxidation in the pathogenesis of the disease.

Published techniques for analysing the F_2-isoprostanes in plasma include solid-phase extraction on a C_{18} and a silica cartridge followed by thin layer chromatography (TLC), formation of pentafluorobenzyl (PFB) ester and trimethylsilyl (TMS) ether derivatives. Quantitative analysis is accomplished by gas chromatography–mass spectrometry (GC–MS) using negative-ion chemical ionisation (NICI) [10,11].

The present study describes the development of a novel and rapid approach for the isolation of F_2-isoprostanes from plasma or LDL prior to GC–MS analysis. Our enrichment procedure involves solid-phase extraction on a C_{18} and an NH_2 cartridge. The assay is simple, reliable and could be easily adapted for analyzing different biological materials.

2. Experimental

2.1. Materials

Prostaglandin F_2 (9,11,15-trihydroxy-5,11-eicosadienoic acid) standards including 9α,11α-, 9α,11β-, 9β,11α-, 8-epi- and 3,3′,4,4′-tetradeuterated 9α,11α-PGF_2 (PGF_2-d_4) were obtained from Cascade Biochem (Reading, UK). [^{3}H]-9α,11α-PGF_2 was purchased from Amersham Incorporation (Amersham, UK). Sep-Pak C_{18} (500 mg) cartridges were obtained from Millipore Incorporation (Milford, MA, USA). Aminopropyl (NH_2) cartridges (Supelclean LC-NH_2, 500 mg) were supplied by Supelco (Bellefonte, PA, USA). *N,O-bis*(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce Chemical Company (Rockford, IL, USA) while deuterated *N,O-bis*(trimethyl-[$^2H_9$]-silyl)trifluoroacetamide was obtained from CDN Isotopes (Quebec, Canada). Indomethacin, butylated hydroxytoluene (BHT), pentafluorobenzyl bromide (PFB-Br) and di-isopropylethylamine (DIPEA) were obtained from Sigma (St Louis, MO, USA). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Polysciences Incorporation (Warrington, PA, USA). Xylenol orange [*o*-cresolsulfonphthalein-3′,3″-bis(methyliminodiacetic acid salt)] and *N*-butyl boronic acid were purchased from Aldrich (Gillingham, UK). All commercially available chemicals and reagents were of analytical grade or greater purity.

2.2. Sample collection

Blood (10 ml) was collected in a sampling tube containing 3.8% sodium citrate (blood/anticoagulant ratio 9:1). Indomethacin in 5% sodium

bicarbonate as cyclooxygenase inhibitor and BHT as free radical scavenger were present at final concentrations of 14 and 20 μM, respectively. The sample was allowed to stand for 45 min at 4°C to achieve complete inhibition of cyclooxygenase enzymes. Platelet-poor plasma was obtained by centrifugation at 2400 g for 15 min at 4°C. Aliquots (1 ml) of the plasma were transferred to Eppendorf tubes containing BHT at a final concentration of 20 μM and were stored at −70°C until analysis.

2.3. Low density lipoprotein (LDL) preparation

LDL (floating at a relative density of 1.063) was isolated from the platelet-depleted plasma by sequential ultracentrifugation in presence of ethylenediaminetetraacetic acid (EDTA) to chelate pro-oxidant transition metals [12]. The EDTA was removed by dialysis of the LDL against phosphate-saline buffer at 4°C.

2.4. Lipid peroxidation procedure

A water-soluble azo-initiator (AAPH) or copper sulphate was used to stimulate lipid peroxidation. In the case of the azo-initiator, plasma (1 ml) or LDL (300 μg/ml) was incubated with AAPH (final concentration 54 mM) at 37°C for 24 h. For metal-catalyzed peroxidation, LDL (300 μg/ml) was incubated with copper sulphate at a final concentration 40 μM and was treated as described above.

Formation of lipid peroxidation products was routinely monitored using the FOX2 assay [13]. Briefly, plasma or LDL (100 μl) was transferred into an Eppendorf tube, FOX2-reagent (900 μl) was added and the sample was incubated at room temperature for 30 min. After centrifugation at 12 000 g for 5 min, absorbance of the supernatant was monitored at 560 nm.

2.5. Solid-phase extraction procedure

Free (unesterified) F_2-isoprostanes

Plasma (1 ml) or LDL (300 μg/ml) was spiked with PGF_2-d_4 (5 ng in 50 μl of ethanol) as an internal standard. The sample was acidified using 2 ml of water pH 3.0 and allowed to equilibrate at 4°C for 15 min. The sample was applied on a C_{18} cartridge (500 mg) preconditioned with methanol and water (pH 3.0). The cartridge was sequentially washed with 10 ml of water (pH 3.0) and acetonitrile–water (15:85, v/v) [10]. Lipids were eluted by washing the cartridge with 5 ml of hexane–ethyl acetate–propan-2-ol (30:65:5, v/v). This eluate was then applied to an NH_2 cartridge (500 mg), preconditioned with hexane (5 ml). The NH_2 cartridge was sequentially washed with 10 ml of hexane–ethyl acetate (30:70, v/v), acetonitrile–water (9:1, v/v) and acetonitrile. F_2-Isoprostanes were eluted from the NH_2 cartridge with 5 ml of ethyl acetate–methanol–acetic acid (10:85:5, v/v). The sample was immediately transferred into a screw-cap vial and the solvent was evaporated under nitrogen at room temperature.

Total (sum of free and esterified) F_2-isoprostanes

Plasma (1 ml) or LDL (300 μg/ml) was incubated with 1 ml of aqueous solution of potassium hydroxide (1.0 M) at 45°C for 30 min. Water (1 ml) was added and the pH was adjusted to 3 using concentrated HCl. PGF_2-d_4 (5 ng in 50 μl of ethanol) was added as an internal standard and the sample was centrifuged at 2400 g for 5 min. The supernatant was removed and solid-phase extraction procedure was carried out as described above.

2.6. Derivatisation

The PFB-ester was prepared by adding 40 μl of PFB-Br (10% in acetonitrile) and 20 μl of DIPEA (10% in acetonitrile) to the dried sample following solid-phase extraction. The vial was sealed with a PTFE-lined cap and kept at 40°C for 45 min. The solvent was removed under a stream of nitrogen.

BSTFA (50 μl) followed by 5 μl DIPEA (10% in acetonitrile) were added. The vial was sealed and stored at 4°C for 12 h. The solvent was removed under nitrogen and the residue was reconstituted in iso-octane (20 μl) containing

10% BSTFA. All the samples were stored at −20°C until GC–MS analysis.

N-Butyl boronate (NBB) derivatisation was carried out as follows: 9α,11α-, 9α,11β- 9β,11α- and 8-epi-PGF_2 (50 ng of each component or as a mixture in ethanol) were transferred to screw-cap vials and the solvent was dried under nitrogen. The authentic PGF_2 compounds were subsequently converted to PFB-esters as described above. The solvent was removed under nitrogen and the residue was redissolved in dimethoxypropane (10 μl) and 2% boronic acid in dimethoxypropane (50 μl). The vials were sealed with PTFE-lined caps and the samples were incubated at 60°C for 5 min. Subsequently, the solvent was evaporated and samples were converted to TMS-ether derivatives prior to GC–MS analysis. In the case of final extracts from oxidized plasma and LDL, the samples were esterified to PFB derivatives and subsequently converted to NBB-TMS ether derivatives as described above.

2.7. Hydrogenation

Rhodium on alumina powder (10 mg) was tranferred into a screw-cap vial and was sequentially washed with methanol (1 ml × 2). 9α,11α-, 9α,11β-, 9β,11α- and 8-epi-PGF_2 (50 ng of each component or as a mixture in 1 ml of methanol) were added. The vial was sealed with a PTFE-lined cap. The sample was kept on ice and was flushed with hydrogen for 30 min. The solvent was transferred into a new vial and was removed under a stream of nitrogen. The reduced (hydrogenated) samples were converted to PFB-ester/TMS-ether derivatives as described above. In the case of oxidized plasma or LDL, the final extracts were redissolved in methanol (1 ml) and hydrogenation was carried out as described above. Subsequently, the reduced samples were then converted to PFB-ester/TMS-ether.

2.8. Gas chromatography–mass spectrometry

GC–NICI-MS analysis was carried out on a Hewlett-Packard 5890 GC linked to a VG70SEQ mass spectrometer (Fisons Instruments, Manchester, UK) using ammonia as reagent gas. F_2-Isoprostanes were separated on an SPB-1701 column (30 m × 0.25 mm I.D.; 0.25 μm film thickness, Supelco Incorporation, Bellefonte, PA, USA). Samples were injected in iso-octane into a temperature programmed Gerstel injector (Thames Chromatography, Maidenhead, UK). The GC oven was programmed from a temperature of 175 to 270°C at a rate of 30°C/min. Quantitative analysis was performed using selected-ion monitoring (SIM) of the carboxylate anion $[M-181]^-$ at m/z 569 for the F_2-isoprostanes and m/z 573 for PGF_2-d_4 as the internal standard.

For mass spectrometric characterisation of F_2-isoprostanes as cyclic *N*-butylboronate/PFB-ester/TMS-ether derivatives, the instrument was programmed for single-ion monitoring at m/z 569 (15.0–25.0 min), m/z 401 (25.0–29.46 min), m/z 491 (29.46–37.0 min) and m/z 401 (37.0–40.0 min). The signals at m/z 491 and 401 correspond to $[M-180]^-$ and $[M-180-90]^-$, respectively.

2.9. Recovery experiments

Plasma (1 ml) was spiked with about 20 000 dpm of [^{3}H]-9α,11α-PGF_2 as a tracer. The sample was processed using the solid-phase extraction procedure described above. Final recovery was determined by comparing the counts in an extract obtained after passage through C_{18} and NH_2 cartridges, respectively, to those of the original sample. Efficiency of the solid-phase extraction procedure was determined by GC–MS of the final extract from aqueous and plasma samples containing increasing amounts of the authentic PGF_2 analogues ranging from 50 to 1000 pg/ml, including a fixed amount of the PGF_2-d_4 (5 ng/ml) as an internal standard.

For calibration plots, plasma samples (1 ml) were spiked with increasing amounts of a mixture of the authentic PGF_2 compounds (50 to 1000 pg/ml) containing PGF_2-d_4 (5 ng/ml) as internal standard. The PGF_2 compounds were

isolated and quantified by GC–MS as described above.

3. Results

3.1. Assay validation

F_2-Isoprostanes isolated from oxidized LDL were initially analyzed using an Rt_x-1 column (15 m × 0.25 mm I.D., 0.25 μm film thickness, Restek Corp., PA, USA) as described earlier [10]. We found that this GC phase provided complete separation of authentic 9β,11α-, 9α,11β- and 9α,11α-PGF_2. However, 9β,11α- and 8-epi-PGF_2 were not resolved. In the present study, we used a more polar column (SPB-1701) for the analysis of F_2-isoprostanes since it was found superior to the Rt_x-1 column in providing a better separation of 9β,11α-, 9α,11β-, 8-epi- and 9α,11α-PGF_2. Fig. 1 shows a typical selected-ion monitoring chromatogram of a mixture of authentic PGF_2-compounds after solid-phase extraction on a C_{18} and an NH_2 cartridge. The signal monitored at *m/z* 569 represents the $[M - 181]^-$ ion intensity from the authentic PGF_2 compounds whereas the signal at *m/z* 573 corresponds to that of the PGF_2-d_4 as internal standard.

Initially, the procedure described by Wendelborn et al. [10] was employed to isolate the F_2-isoprostanes from plasma. Recovery experiments with [^{3}H]-PGF_2 as a tracer indicated that both the C_{18} (350 mg) and the silica (350 mg) cartridges were highly efficient for enrichment of the [^{3}H]-PGF_2. Final extracts, following chromatography on C_{18} and silica cartridges, contained 80% of the originally added [^{3}H]-PGF_2 as a tracer. However, significant amounts of the labelled PGF_2 (70–80%) were lost during the TLC steps. The overall recovery of the [^{3}H]-PGF_2 following solid-phase extraction and TLC was only about 20%.

Therefore, the possibility of replacing the silica cartridge and the TLC step(s) with an NH_2 cartridge was investigated. The NH_2 cartridge was found to be highly efficient in retaining the

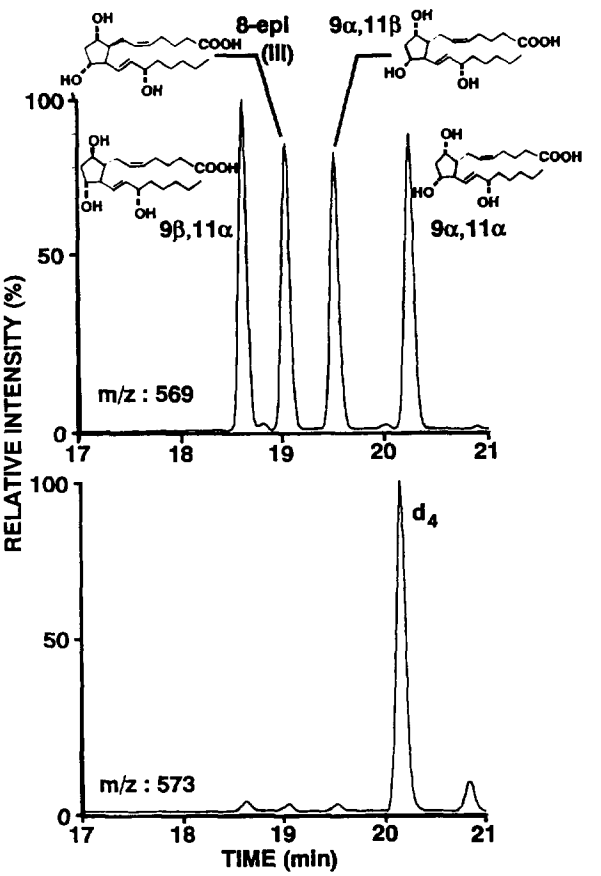

Fig. 1. GC–NICI-MS chromatograms of authentic PGF_2 compounds as PFB-ester/TMS-ether derivatives. The signal monitored at *m/z* 569 represents the carboxylate anion [M − 181]$^-$. The signal at *m/z* 573 corresponds to PGF_2-d_4 as the internal standard. Separation was carried out on an SPB-1701 column as described in the Experimental section.

labelled PGF_2 when applied in hexane–ethyl acetate–propan-2-ol (30:65:5, v/v). The [^{3}H]-PGF_2 was quantitatively eluted by washing the cartridge with 5 ml of ethyl acetate–methanol–acetic acid (10:85:5, v/v). Recovery of the [^{3}H]-PGF_2 from the NH_2 cartridge was approximately 70% ± 2.2% ($n = 4$). In separate recovery experiments, plasma (1 ml) was spiked with [^{3}H]-PGF_2 as a tracer and the solid-phase extraction procedure was carried out as illustrated in Fig. 2. Scintillation counting indicated that the final eluate obtained after the combined C_{18} and NH_2

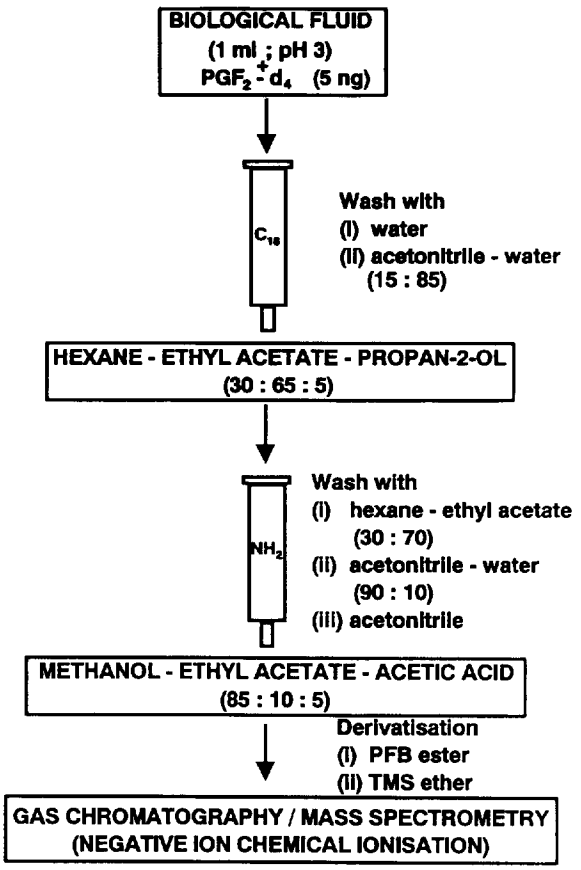

Fig. 2. Solid-phase extraction procedure for the isolation of F_2-isoprostanes from biological fluids.

solid-phase extraction steps contained 65% ± 4% ($n = 4$) of the added [^{3}H]-PGF_2.

Suitability of the solid-phase extraction for isolation and final determination of F_2-isoprostanes by GC–NICI-MS was assessed by analyzing plasma samples containing mixtures of 9β,11α-, 9α,11β-, 8-epi- and 9α,11α-PGF_2 at final concentrations ranging from 50 to 1000 pg/ml, respectively. Accuracy of the assay was determined by analysing four samples (1-ml aliquots of plasma from the same donor) containing 100 pg/ml of each the authentic PGF_2 compounds. The least squares linear regressions for 9β,11α-, 9α,11β-, 8-epi- and 9α,11α-PGF_2 in plasma were $y = 0.002 + 0.178x$, $r^2 = 0.996$; $y = 0.003 + 0.161x$, $r^2 = 0.996$; $y = 0.006 + 0.103x$, $r^2 = 0.995$ and $y = 0.009 + 0.178x$, $r^2 = 0.997$, respectively. Inter-assay coefficients of variation for 9β,11α-, 9α,11β-, 8-epi- and 9α,11α-PGF_2 were 15.1%, 9.4%, 11.4% and 14.2%, respectively. Intra-assay coefficients of variation for the above mentioned PGF_2 compounds were 4.3%, 11.1%, 5.0% and 8.0%, respectively. The limit of detection for the authentic PGF_2 compounds extracted from plasma samples was 10 pg/ml. Thus, the combined C_{18} and NH_2 solid-phase extraction procedure was shown to be an efficient technique for the isolation of F_2-isoprostanes from plasma.

3.2. Model lipid peroxidation systems

Plasma was incubated with AAPH at 37°C to stimulate lipid peroxidation. After 24-h incubation with AAPH, the plasma samples ($n = 2$) accumulated on average 115 μ*M* of hydroperoxides as revealed by the FOX2-assay. The corresponding hydroperoxide levels for the control samples ($n = 2$), incubated at 37°C for 24 h, were 15 μ*M*.

GC–NICI-MS analysis of the signal at *m/z* 569 revealed no measurable levels of free (unesterified) F_2-isoprostanes in the control plasma samples (Fig. 3A). However, substantial amounts of free F_2-isoprostanes were present in the AAPH-oxidized plasma samples (Fig. 3B). Levels of the major components assigned as II, III and V (retention times around 18.45, 18.60 and 19.59 min, respectively) in the AAPH-oxidized plasma samples ranged from 6.7 to 9.0 ng/ml. When the oxidised plasma samples were analysed for total (sum of free and esterified) F_2-isoprostanes, the levels were on average 3.7-fold higher compared to that of the free levels. It is worth noting that the chromatographic patterns for the total (sum of free and esterified) F_2-isoprostanes in the oxidized plasma samples were very similar to those of the free F_2-isoprostanes (data not shown).

To demonstrate that the generation of F_2-isoprostanes in the oxidized plasma was associated with peroxidation of lipoproteins, LDL was incubated with AAPH at 37°C for 24 h. The FOX2-assay revealed hydroperoxide levels of 1145 nmol/mg protein ($n = 2$) in the oxidized

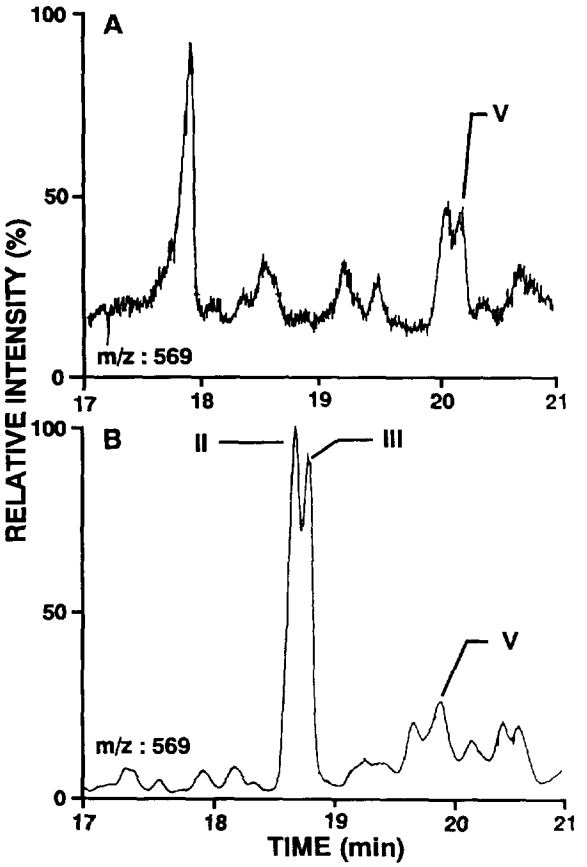

Fig. 3. GC–NICI-MS analysis of F_2-isoprostanes isolated from oxidized plasma. Chromatograms represent: (A) plasma incubated at 37°C for 24 h; and (B) plasma incubated with AAPH (54 m*M*) at 37°C for 24 h.

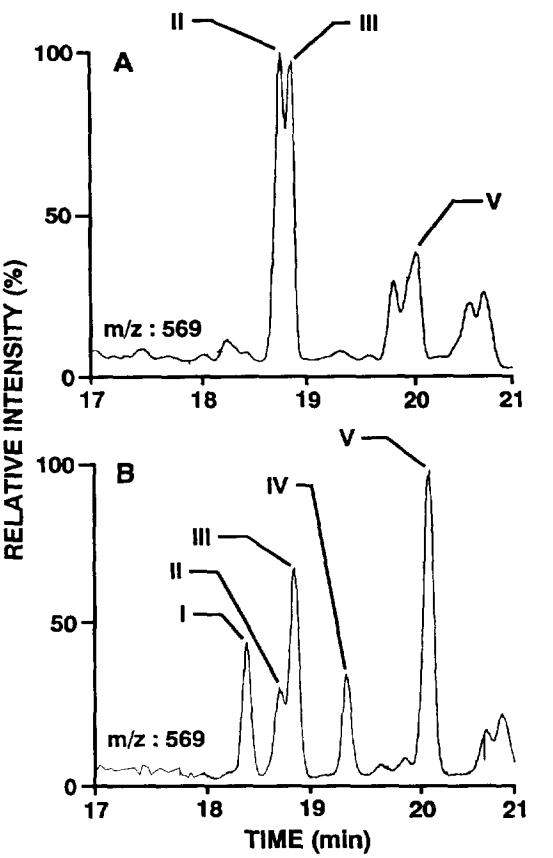

Fig. 4. GC–NICI-MS analysis of F_2-isoprostanes isolated from oxidized LDL. Chromatograms represent: (A) LDL (300 μg/ml) incubated with AAPH (54 m*M*) at 37°C for 24 h; and (B) LDL (300 μg/ml) incubated with copper sulphate (40 μM) at 37°C for 24 h.

LDL samples. The corresponding hydroperoxide levels in the control samples ($n = 2$) were 152 nmol/mg protein.

GC–MS analysis showed that substantial amounts of free F_2-isoprostanes were produced during AAPH peroxidation of the LDL samples (Fig. 4A). Levels of free (unesterified) F_2-isoprostanes (represented by peaks II, III, V) in the AAPH-oxidized LDL samples were about 8.9 ng/mg protein. In the control LDL samples, free F_2-isoprostanes were between 0.06 and 0.10 ng/mg protein. The chromatographic profiles of the free as well as the total (sum of free and esterified) F_2-isoprostanes in the AAPH-oxidized LDL samples were very similar to those obtained from the AAPH-treated plasma samples (data not shown). Free F_2-isoprostanes (10.6 ng/mg protein; $n = 2$; represented by peaks I–V) were also generated during copper-mediated oxidation of LDL (Fig. 4B).

3.3. *Structural determination*

The following criteria were used to demonstrate that the components in the final extracts from oxidised plasma and LDL samples, following solid-phase extraction and GC–NICI-MS at *m/z* 569 as PBF-ester/TMS-ether derivatives, were F_2-isoprostanes. First, no peaks were observed at *m/z* 568, indicating that the peaks were

not natural isotope peaks of compounds with m/z lower than 569 (data not shown). Second, analysis of the final extracts as PBF-ester/[$^2H_9$]TMS-ether derivatives revealed that the signal monitored at m/z 569 shifted to m/z 596 indicative of the presence of three hydroxyl moieties in the molecules (Fig. 5A,B). The differences in the gas chromatographic profile monitored at m/z 596 compared to that at m/z 569 are probably due to changes in the gas chromatographic characteristics of the F_2-isoprostanes analyzed as deuterated-TMS ether derivatives. Third, analysis of the hydrogenated final extract gave no peaks at m/z 571 and 575, which would have been indicative of either one or three double bonds in the molecule, respectively. However, this coincided with the appearance of a series of new peaks at m/z 573 confirming the presence of two double bonds in the molecules (data not shown). Fourth, formation of cyclic boronate derivatives resulted in the disappearance of the signal monitored at m/z 569 (Fig. 6A). This coincided with the appearance of new peaks at m/z 491 and 401 corresponding to $[M - PFB]^-$ and $[M - PFB -$

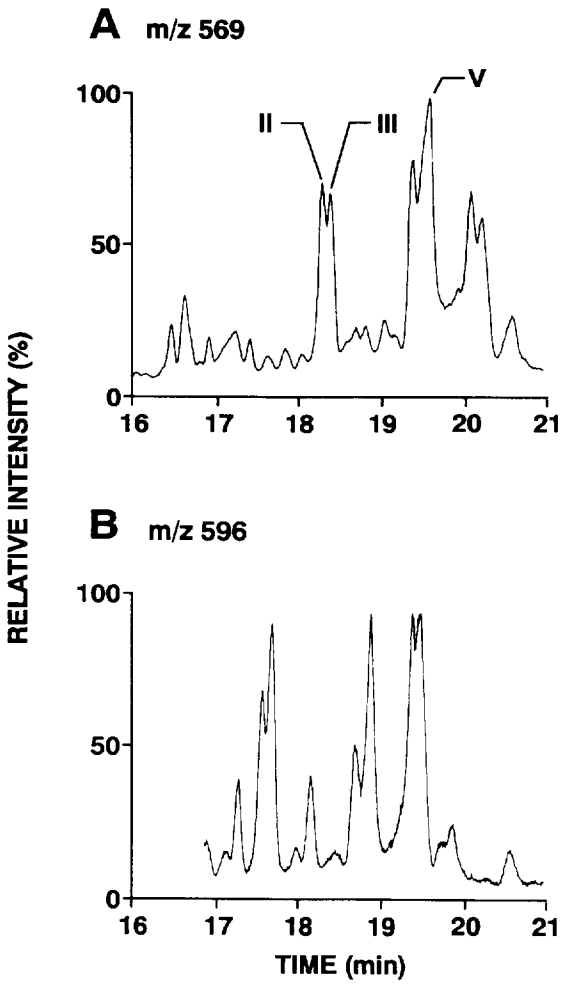

Fig. 5. Mass spectrometric characterisation of F_2-isoprostanes in oxidized LDL. Chromatograms represent: (A) F_2-isoprostanes as PFB-ester/TMS-ether derivatives; (B) F_2-isoprostanes as PFB-ester/[$^2H_4$]TMS-ether derivatives.

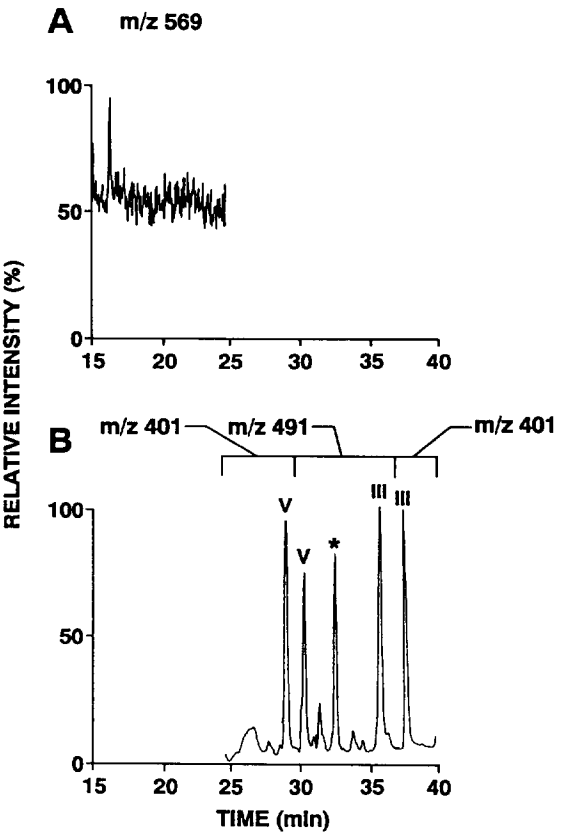

Fig. 6. Mass spectrometric characterisation of F_2-isoprostanes as cyclic *N*-butylboronate/PFB-ester/TMS-ether derivatives from oxidized LDL. Chromatograms represent signals monitored at: (A) m/z 569; (B) m/z 491 and 401, respectively. The signals at m/z 491 and 401 correspond to $[M - 180]^-$ and $[M - 180 - 90]^-$, respectively. Peaks III and V correspond to authentic 8-epi-PGF_2 and 9α,11α-PGF_2. The peak labelled as * corresponds to an unidentified F_2-isoprostane.

TMS]$^-$, respectively (Fig. 6B). PGF_2-like compounds will only form a cyclic boronate derivative when hydroxy groups on the cyclopentane ring are *cis*-oriented. Of the F_2-isoprostanes in Figs. 3 and 4, the peak assigned as III was identified as the 8-epi-$PGF_{2\alpha}$ by comparing retention time and mass spectrometric characteristics to that of authentic 8-epi-$PGF_{2\alpha}$. Peak V (Figs. 3 and 4) which co-eluted with the peak corresponding to that of authentic 9α,11α-PGF_2, also formed a boronate derivative (Fig. 6B).

3.4. Plasma levels of 8-epi-$PGF_{2\alpha}$

Plasma from volunteers ($n = 7$), was analyzed for the content of free as well as total (sum of free and esterified) 8-epi-$PGF_{2\alpha}$. GC–MS analysis revealed no quantifiable levels of free 8-epi-$PGF_{2\alpha}$ in the plasma. The plasma samples, however, contained 58 to 166 pg/ml of the 8-epi-$PGF_{2\alpha}$ when analyzed for the total (sum of free and esterified) F_2-isoprostanes. Our findings indicate that 8-epi-$PGF_{2\alpha}$ in human plasma is mainly present esterified to functionally important phospholipids in vivo. Moreover, it also implies that the measurement of total (sum of free and esterified) levels of 8-epi-$PGF_{2\alpha}$ provides a sensitive index of non-enzymatic lipid peroxidation compared to measurement of free (unesterified) levels.

4. Discussion

We have developed and implemented a rapid and specific assay for monitoring non-enzymatic derived F_2-isoprostanes in biological materials. Our sample clean-up procedure is based on the use of a C_{18} followed by an NH_2 cartridge. Quantitative analysis of the F_2-isoprostanes as PFB-ester/TMS-ether derivatives was accomplished by GC–NICI-MS.

Morrow and co-workers [4,5] were the first to describe the formation of F_2-isoprostanes in vivo by a non-cyclooxygenase derived mechanism. They showed that F_2-isoprostanes were mainly associated with autoxidation of plasma phospholipids in vitro and in vivo. The proposed mechanism for the autoxidation of arachidonic acid leads to the formation of four regio-isomers of PGF_2, each of which is theoretically composed of a mixture of eight racemic diastereomers. Non-enzymatic oxidation of arachidonic acid yields F_2-isoprostanes with side chains predominantly *cis*-oriented whereas the hydroxyl groups on the cyclopentane ring are exclusively *cis*-oriented [14].

Established procedures for the isolation of F_2-isoprostanes in biological fluids include solid-phase extraction on a C_{18} and a silica cartridge followed by one or two steps of TLC [10,11]. Using [^{3}H]-PGF_2, we found that the recovery throughout the isolation procedure was very low, approximately 20%, with about 70% of the total radioactivity being lost during the TLC steps. Recovery data was not reported by Wendelborn et al. [10].

We have replaced the solid-phase extraction on the silica cartridge and the TLC by an extraction step on a single NH_2 cartridge. The NH_2 sorbent functions by an ion-exchange mechanism, selectively binding organic compounds containing a carboxylate anion. The binding of the F_2-isoprostanes was found to be sufficiently specific to give interference-free single-ion monitoring chromatograms without further purification (Figs. 3 and 4). Recovery of the authentic PGF_2 compounds (using [^{3}H]-PGF_2 as a tracer) following the combined C_{18} and NH_2 chromatography steps averaged 65 ± 4%, with about 30% of the total radioactivity lost during the NH_2 chromatography step. The within-day coefficient of variation of the GC–MS assay was 4.3–11.1% whereas that for day to day was 9.4–15.1%.

Suitability of the improved methodology for assessment of the F_2-isoprostanes was demonstrated by analyzing oxidized plasma and lipoproteins, respectively. Oxidation of plasma or LDL, using the azo-initiator (AAPH) or copper ions as pro-oxidants, produced a substantial amount of F_2-isoprostanes (Figs. 3 and 4). Two partially resolved peaks (II and III) with retention times between 18.40 and 18.45 min were present in abundance in the AAPH-treated samples. Attempts to optimise the GC chromatographic conditions to improve the separa-

tion of these peaks were unsuccessful. Peak III eluting at around 18.45 min was identified as the 8-epi-$PGF_{2\alpha}$ by comparison of relative retention time and mass spectrometric characteristics, following catalytic hydrogenation, formation of a cyclic boronate derivative and deuterated TMS-ether derivatization, to that of the authentic 8-epi-$PGF_{2\alpha}$.

Morrow and Roberts analysed plasma from different individuals and reported 8-epi-$PGF_{2\alpha}$ levels of 19 ± 7 pg/ml [15]. Quantitative determination by GC–NICI-MS was quoted as being extremely sensitive, with a lower limit of detection ranging from 1 to 5 pg/ml. However, the lower detection limit for the 8-epi-$PGF_{2\alpha}$ in biological fluids was not reported. We did not find quantifiable levels of free (unesterified) of 8-epi-$PGF_{2\alpha}$ at the lower detection limit of our assay (10 pg/ml). However, total (sum of free and esterified) 8-epi-$PGF_{2\alpha}$ levels in the plasma samples were found to be 112 ± 54 pg/ml ($n = 7$). Thus, measurements of total 8-epi-$PGF_{2\alpha}$ could be more valuable than those of free 8-epi-$PGF_{2\alpha}$ when assessing the extent of lipid peroxidation. Measurement of total levels of 8-epi-$PGF_{2\alpha}$ reflect changes occurring in situ on functional phospholipids which are one of the primary targets of free radical attack [16].

In conclusion, an improved method has been developed for measuring F_2-isoprostanes in plasma using GC–NICI-MS. High recoveries have been achieved using a specific solid-phase extraction procedure (97% and 67% for C_{18} and NH_2, respectively). The method is simple and could be easily adapted to a wide range of biological material.

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