Key issues in F$_2$-isoprostane analysis

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Abstract

A large body of evidence indicates that measurement of F$_2$-isoprostanes, specific prostaglandin F$_2$-like compounds derived from the non-enzymatic peroxidation of arachidonic acid, is a reliable biomarker of oxidant stress in the human body. Since the discovery of F$_2$-isoprostanes in the early 1990s, a variety of analytical approaches has been introduced for the quantification of these novel compounds. The aim of the present review is to shed light on the available gas chromatographic–mass spectrometric assays for the measurement of plasma or urinary F$_2$-isoprostanes and to highlight a number of issues which need to be addressed in order to implement F$_2$-isoprostane measurement as a gold-standard biomarker of oxidative stress in biological samples.

Introduction

The term oxidative stress was first employed as the title of a book edited by Sies [1]. In the introduction to the second edition of this book, oxidative stress was defined as a disturbance in the pro-oxidant/antioxidant balance in favour of the former, which may lead to tissue damage [2]. Halliwell has defined oxidative stress as a serious imbalance between production of ROS (reactive oxygen species)/RNS (reactive nitrogen species)/RCS (reactive chlorine species) and antioxidant defences [3]. Baynes and Thorpe [4], on the other hand, have introduced a definition comprising both quantitative aspects of oxidative stress and chemical modification of target molecules. Oxidative stress was defined as a measure of the prevailing levels of ROS in biological systems [4]. They also emphasized that this definition takes account of the continuous detection of ROS in biological systems determined by the relative rates of their formation and their removal by cellular repair mechanisms [4].

An array of analytical tools is available for lipid peroxidation products, including TBARSs (thiobarbituric acid-reactive substances), lipid hydroperoxides, hydroxylated fatty acids, volatile hydrocarbons, aldehydes and cholesterol auto-oxidation products, protein carbonyls, aldehyde-modified proteins, oxidatively damaged DNA and total antioxidant capacity for the precise measurement of oxidative stress *in vitro* [5–7]. However, problems arise when the above-mentioned methods are applied to *in vivo* samples.

A major step forward regarding the measurement of lipid peroxidation products came with the discovery of F$_2$-isoprostanes [8,9]. The F$_2$-isoprostanes are a family of PG (prostaglandin)-like compounds produced by non-enzymatic peroxidation of arachidonic acid. The formation of F$_2$-isoprostanes from arachidonic acid involves allylic hydrogen abstraction, formation of an arachidonyl radical, insertion of molecular oxygen and endocyclization to form a bicyclic endoperoxide followed by insertion of a second oxygen molecule.

Four possible subfamilies of F$_2$-isoprostanes are formed as a result of radical attack at C7, C10 and C13 (Figure 1). Series 5 (Type VI) F$_2$-isoprostanes are derived from free radical attack at C7. Series 8 (Type V) and series 12 (Type IV) result from free radical attack at C10. Series 15 (Type III) is derived from free radical attack at C13. Each subfamily comprises 16 diastereoisomers, since the hydroxy group on the cyclopentane ring can be arranged in eight different configurations. In total, 64 F$_2$-isoprostane isomers can be formed during peroxidation of arachidonic acid [7,10]. Of these, 8-epi-PGF$_{2\alpha}$ (also known as 8-iso-PGF$_{2\alpha}$ or 15-F$_{2t}$-isoprostane) has received most attention because it has been shown to possess certain adverse biological activities [11–14]. Circulating 8-epi-PGF$_{2\alpha}$ is mainly present bound to phospholipids *in situ* and is released by the action of phospholipase A$_2$ [15]. Figure 1 shows proposed pathways for the generation of the different F$_2$-isoprostane families during non-enzymatic peroxidation of arachidonic acid.

Historic background

We owe our knowledge of isoprostanes to Nugteren [16] for his pioneering investigations into the metabolism of prostaglandins. Nugteren was interested in developing a less cumbersome procedure for the determination of the excretion rate of prostaglandins and their metabolites in humans using a strategy that would limit the number of detected compounds in the final lipid extract, exhibit higher recovery and allow preparation of stable derivatives before final determination by GC–MS [16]. His approach involved hydrogenation to eliminate double bonds and removal of hydroxy and keto moieties from the molecule while leaving the prostanoid backbone intact. In healthy volunteers, excretion of unmetabolized PGF$_{2\alpha}$ was estimated to be approx. 300 μg/day. This

Figure 1 | Proposed pathways for the formation of the different F$_2$-isoprostane families during auto-oxidation of arachidonic acid
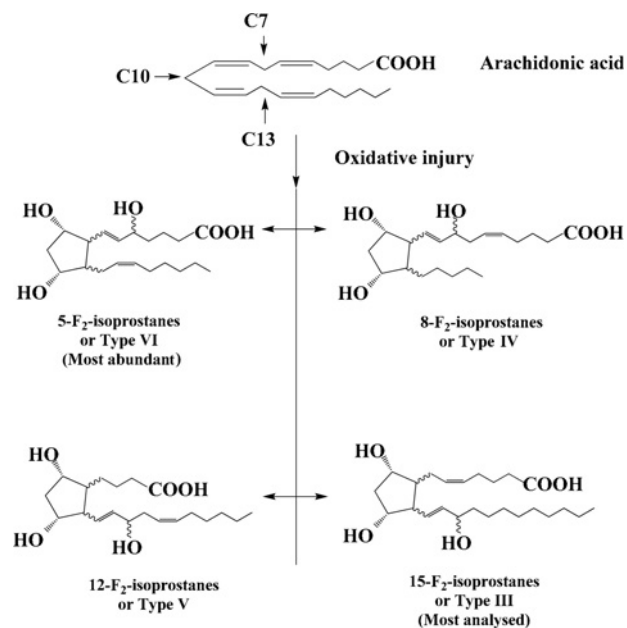
quantity was substantially higher than the sum of PGF$_{2\alpha}$ (males, $24.0 \pm 17.2 \, \mu$g/day; females, $10.5 \pm 2.1 \, \mu$g/day) and PGE$_2$ (males $15.5 \pm 13.6 \, \mu$g/day; females, $4.1 \pm 0.8 \, \mu$g/day)

reported previously by other investigators [17,18]. It was also reported that excretion of various tetranorprostanedioic acid derivatives was 0.2 ± 0.08 mg/mg of creatinine. In addition, it was found that only 15–30 % of intravenously administrated PGF$_{2\alpha}$ was excreted as tetranorprostanedioic acid derivatives. Therefore it was proposed that prostanoids (partially or fully degraded) other than tetranorprostanedioic acid derivatives should be present in human urine. Figure 2 shows the pathways proposed by Nugteren [16] for the biosynthesis and metabolism of prostaglandins in humans.

In 1988, Wendelborn et al. [19] reported that healthy subjects exhibited 48–460 pg/ml of urinary PGF$_2$-like compounds. The corresponding levels for circulating PGF$_2$-like compounds was in the range 1–12 pg/ml. Interestingly, they found that the mean concentration of PGF$_2$-like compounds increased 800-fold in patients with systemic mastocytosis. The initial explanation for the presence of markedly elevated levels of PGF$_2$-like compounds in patients with systemic mastocytosis was isomerization of PGD$_2$ to PGF$_2$ involving reduction of the keto moiety (C11) on PGD$_2$ and thus yielding more stable PGF$_2$-like compounds.

In order to elucidate whether PGF$_2$-like compounds were derived from the rearrangement of endogenous PGD$_2$, the configuration of the hydroxy groups on the cyclopentane ring was determined. Total lipid extracts from plasma and urine from the above-mentioned samples were therefore treated with n-butylboronic acid. n-Butylboronic acid forms

Figure 2 | Proposed pathways for biosynthesis and metabolism of prostaglandins

X, Y and Z = O, OH or H. (**1**) Arachidonic acid. (**2**) PGG$_2$. (**3**) PGH$_2$. (**4**) PGE$_2$. (**5**) PGF$_2$. (**6**) Hypothetical metabolite(s). (**7**) 15-Oxo-13,14-dihydrotetranor-PGF$_2$ derivative. (**8**) Tetranorprostadioic acid. (**9**) 15-Oxo-13,14-dihydrotetranor-PGE$_2$ derivative. (**10**) Tetranorprostenodioic acid. Solid arrow, established enzymatic conversion; broken arrow, minor pathway; crossed arrow, minor reaction; double-lined arrow, chemical conversion. Based on [16].
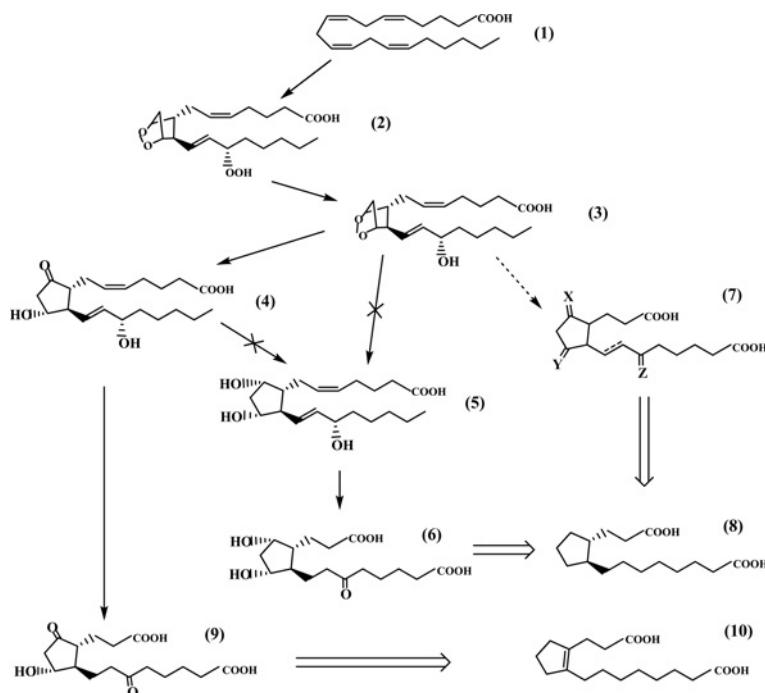
Table 1 | Events that led to the emergence of F$_2$-isoprostanes as a novel biomarker of oxidative stress

LDL, low-density lipoprotein.

Date	Event
1975	The determination of prostaglandin metabolites in human urine [16]
1988	Discovery of PGF$_2$ isomers derived from PGD$_2$ metabolism in human plasma and urine [19]
1990	Proposed mechanism for the free-radical-catalysed formation of a series of novel prostaglandins *in vivo* [8]
1991	Quantification of F$_2$-isoprostanes as a marker of oxidative stress [9]
1992	Formation of F$_2$-isoprostanes in CCl$_4$-induced hepatoxicity [45]
	Evidence for *in situ* formation of F$_2$-isoprostanes in phospholipids [12]
1993	Evidence for the existence of F$_2$-isoprostane receptors on vascular smooth muscle cells [46]
	Measurement of a marked increase of F$_2$-isoprostanes in the hepatorenal syndrome [47]
	Identification of F$_2$-isoprostane metabolites in human urine [48]
1994	Measurement of the formation of F$_2$-isoprostanes during exposure of LDL to peroxynitrite [49]
	Detection of esterified F$_2$-isoprostanes in native LDL [50,51]
1995	Detection of increased levels of F$_2$-isoprostanes in smokers [52]
	A new GC–MS assay for the analysis of F$_2$-isoprostanes [23]
	Measurement of an enhanced level of 8-epi-PGF$_{2\alpha}$ in Type 2 diabetic subjects [53]
	Immunological characterization of urinary 8-epi-PGF$_{2\alpha}$ [54]
	Evidence for the generation of 8-epi-PGF$_{2\alpha}$ by human platelets [55]
1996	Modulation of oxidant stress in chronic cigarette smokers [56]
	Measurement of F$_2$-isoprostanes generated by human monocytes [57]

a cyclic derivative when reacting with *cis*-1,3-dihydroxy moieties on a cyclopentane ring. GC–MS analysis revealed that approx. 67% of the 16 detected PGF$_2$-like compounds contained *cis*-oriented hydroxy groups, thus suggesting that a predominant number of PGF$_2$-like compounds present in plasma or urine was not derived from the reduction of PGD$_2$. Although several possible mechanisms were suggested to explain the presence of PGF$_2$-like compounds (i.e. having *cis*-oriented cyclopentane hydroxy groups), none seemed to be entirely satisfactory.

In 1990, Roberts and co-workers reported the discovery of a new series of PGF$_2$-like compounds (F$_2$-isoprostanes) formed independently of PGD$_2$ isomerization and the cyclo-oxygenase pathway [8,9]. They proposed that F$_2$-isoprostanes were generated following an increase in free-radical-mediated oxidation of arachidonic acid. The events that led to the emergence of F$_2$-isoprostanes as a novel biomarker of oxidative stress are summarized in Table 1.

Quantification of F$_2$-isoprostanes

There are several approaches to the enrichment and final determination of F$_2$-isoprostanes in biological samples. These include chromatographic separation involving SPE (solid-phase extraction) or affinity chromatography with or without TLC followed by final determination by GC–MS, LC (liquid chromatography)–MS, RIA or EIA (enzyme immunoassay). GC–MS/NICI (negative-ion chemical ionization) employing stable isotope dilution is the preferred method for the quantification of F$_2$-isoprostanes, combining

the high resolution of GC separation on fused silica capillary columns with the specificity and sensitivity of MS [20].

Assessment of plasma F$_2$-isoprostanes

Mainstream analytical approaches for the enrichment of plasma 8-epi-PGF$_{2\alpha}$ before final determination by GC–MS/NICI include: (i) chromatography on a C$_{18}$ cartridge followed by TLC [21]; (ii) two chromatography steps (i.e. C$_{18}$ and silica cartridges) followed by HPLC [22]; and (iii) chromatography steps combining C$_{18}$ and NH$_2$ cartridges [23]. Advantages of the procedure combining C$_{18}$ and NH$_2$ cartridges are that it is less labour-intensive than the other method involving TLC and is acceptable with respect to efficiency of extraction ($\sim$75%). It is noteworthy that the original enrichment procedure developed by Nourooz-Zadeh et al. [23] has recently been simplified in order to increase sample output (Figure 3). The modifications involved the use of a single extraction step of total lipids with ethyl acetate and the simplification of the NH$_2$-chromatographic step by omitting two cartridge washes [24]. Figure 3 compares values for plasma total (sum of free and esterified) 8-epi-PGF$_{2\alpha}$ using the modified enrichment by Nourooz-Zadeh [24] with those obtained by other investigators [22,25–28]. A slightly different approach has been undertaken by Schwedhelm et al. [29] for the quantification of F$_2$-isoprostanes by GC–MS/NICI. The enrichment procedure involves combining chromatography on a C$_{18}$ cartridge with TLC before the final determination by GC–MS/MS (tandem MS).

Figure 3 | Data obtained by different research groups on plasma 8-epi-PGF$_{2\alpha}$ concentrations in control subjects

Studies are (A) J. Nourooz-Zadeh, unpublished work ($n = 71$); (B) Morrow et al. [25] ($n = 8$); (C) Gopaul et al. [26] ($n = 9$); (D) Gopaul et al. [27] 2001 ($n = 39$); (E) Mori et al. [22] ($n = 10$) and (F) Handelman et al. [28] ($n = 23$). Results are means ± S.E.M.
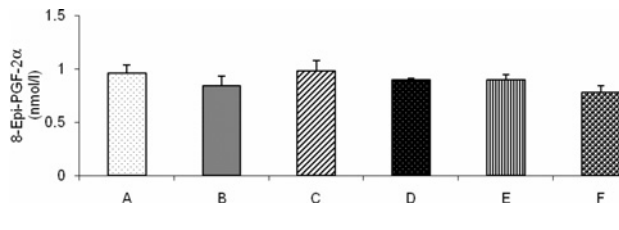
Assessment of urinary F$_2$-isoprostanes

A number of disadvantages, however, are associated with the measurement of plasma 8-epi-PGF$_{2\alpha}$ because it is cleared rapidly from the circulation (within approx. 16 min). This means that its quantification in the circulation will only provide information regarding a discrete point in time [8]. As a result, if large changes occur in the production of lipid peroxides over a particular period, the levels of 8-epi-PGF$_{2\alpha}$ measured in a single sample of blood will not provide an accurate integrated assessment of oxidative stress. Secondly, artefactual generation of 8-epi-PGF$_{2\alpha}$ can occur with improper sample handling and/or prolonged storage, leading to spurious results. The quantification of urinary 8-epi-PGF$_{2\alpha}$ has therefore been proposed as being superior to the measurement of circulating 8-epi-PGF$_{2\alpha}$ levels, as it is believed to represent a more accurate systemic index of oxidative stress.

Various analytical approaches are available for the enrichment of urinary F$_2$-isoprostanes before final determination by GC–MS/NICI. These include: (i) conventional procedures involving chromatography on C$_{18}$ and silica cartridges followed by TLC [21]; (ii) a combination of C$_{18}$ and silica cartridges followed by HPLC [22]; (iii) the inclusion of an HPLC step between C$_{18}$-chromatography and TLC [30]; and (iv) immunoaffinity chromatography [31]. It is worth noting that the FitzGerald group has employed chromatography on C$_{18}$ cartridges followed by two steps of TLC for the enrichment of urinary F$_2$-isoprostanes (Type I) before final quantification by GC–MS/NICI [32].

Nourooz-Zadeh et al. [33,34] have adopted another approach for the rapid enrichment of urinary F$_2$-isoprostanes before final determination by GC–MS/NICI. The assay involved total lipid extraction and chromatographic sample enrichment on NH$_2$ and silica cartridges before final determination by GC–MS/NICI. Inter- and intra-assay coefficients of variation for urinary 8-epi-PGF$_{2\alpha}$ were acceptable at 5 and 7 % respectively. Schematic outlines of the improved analytical procedures for the enrichment of plasma or urinary F$_2$-isoprostanes are shown in Figure 4. Using the combined ethyl acetate extraction/NH$_2$/silica cartridges procedure, it was found that absolute 8-epi-PGF$_{2\alpha}$ excretion in healthy subjects ($n = 12$) was 2.02±0.39 nmol/l. The respective value for 8-epi-

Figure 4 | Analytical procedures for the enrichment and quantification of plasma or urinary F$_2$-isoprostanes by GC–MS/NICI

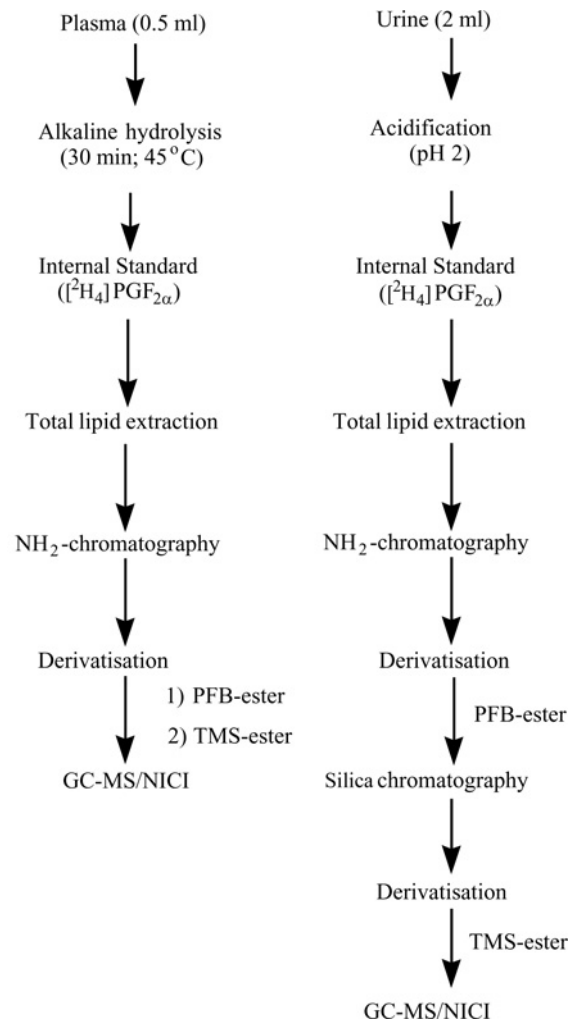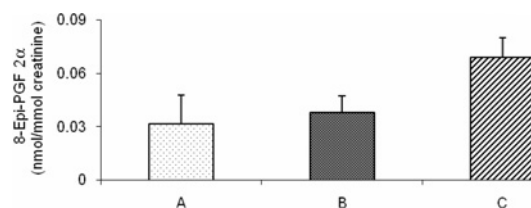PFB, pentaflurobenzyl; TMS, trimethylsilyl.

Figure 5 | Data obtained by various laboratories on urinary 8-epi-PGF$_{2\alpha}$/creatinine ratios in control subjects

Studies are (A) J. Nourooz-Zadeh, unpublished work ($n = 14$); (B) Richelle et al. [35] ($n = 4$) and (C) Schwedhelm et al. [29] ($n = 14$). Results are means ± S.E.M.

PGF$_{2\alpha}$ concentration relative to that of creatinine excretion was 31.8±15.9 pmol/mmol. As seen in Figure 5, the values obtained by us were in the same range as those reported previously by other investigators employing fairly similar GC–MS/NICI-based assays [29,35]. Also, the values for absolute

urinary 8-epi-PGF$_{2\alpha}$ concentrations, employing the combined total lipid/C$_{18}$/NH$_2$ procedure, were also comparable with those reported previously for male and female control subjects (1.49±0.36 and 2.05±0.87 nmol/l respectively) [30].

In addition to mainstream techniques in the field, a variety of other mass spectrometric (i.e. GC or LC) assays have been devised for the measurement of F$_2$-isoprostanes or simultaneous determination of F$_2$-isoprostanes and other lipid peroxidation products [36–44]. Further studies are, however, needed to evaluate the reliability and specificity of these methods and their usefulness for clinical trials.

In conclusion, a large body of evidence suggests that measurement of F$_2$-isoprostanes is a reliable biomarker of oxidative stress in the human body. However, there are several key issues which need to addressed: (i) different conventions with regard to the nomenclature of isoprostanes are used by leading laboratories in the field; (ii) many types of GC–MS or LC–MS assays with variable quality have also been developed for the assessment of F$_2$-isoprostanes, but firm data are still missing with regard to the specificity, accuracy and validity of the assays; (iii) no standard protocol is available for handling and/or storage of biological samples; and (iv) confusion exists concerning the metabolic fate of 8-epi-PGF$_{2\alpha}$. Thus resolving the above-mentioned issues would open new avenues for the implementation of F$_2$-isoprostanes as a gold-standard biomarker of oxidative stress.

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