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Quantification of urinary F_2-isoprostanes with 4(*RS*)-F_{4t}-neuroprostane as an internal standard using gas chromatography–mass spectrometry Application to polytraumatized patients

Emilie Mas[a], Françoise Michel[a,*], Alexandre Guy[b], Valérie Bultel[b], Yoan Falquet[c], Patrick Chardon[c], Jean-Claude Rossi[b], Jean Paul Cristol[a], Thierry Durand[b]

[a] *Department of Biochemistry, Lapeyronie Hospital, Montpellier, France*
[b] *Institut des Biomolécules Max Mousseron (IBMM) UMR 5247 CNRS/UM I/UM II, Faculty of Pharmacy, Montpellier, France*
[c] *Department of Anesthesiology and Critical Care Medicine, Lapeyronie Hospital, Montpellier, France*

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ABSTRACT

Isoprostanes are a family of prostaglandin isomers produced from oxidation of polyunsaturated fatty acids through a non-enzymatic free radical-catalyzed mechanism. Quantification of F_2-isoprostanes (F_2-IsoPs) provides a good index of oxidative stress and allows non-invasive assessment of lipid peroxidation *in vivo*. Since "interferences peaks" at *m/z* 573 co-elute with d_4-15-F_{2t}-IsoP preferentially used, we propose a new GC–NICI-MS approach to quantify urinary F_2-IsoPs by using 4(*RS*)-F_{4t}-neuroprostane as the internal standard. This method was applied to quantify urinary F_2-IsoPs excretion in healthy volunteers and polytraumatized patients. Our results showed a significant increase ($p < 0.0001$) in urinary F_2-IsoPs in polytraumatized patients compared with healthy volunteers (4.73 ± 2.75 ng/mg vs. 0.811 ± 0.359 ng/mg creatinine).

1. Introduction

Morrow et al. [1] reported that F_2-IsoPs, a complex group of prostaglandin $F_{2\alpha}$-like compounds, were produced *in vivo* by non-enzymatic free radical peroxidation of arachidonic acid. Four different series of compounds, called regioisomers, differing in the nature of their side chains, are formed. Eight isomers could be produced among each of these regioisomers, i.e. theoretically 64 F_2-IsoPs can be formed [2,3]. F_2-IsoPs are formed from esterified arachidonate present in phospholipids and are released subsequently as free acids by phospholipases [4,5]. Different studies in various pathophysiological states, including cardiovascular, pulmonary and neurological diseases, have shown a concentration increase of these compounds in body fluids supporting the concept that F_2-IsoPs measurement appears to be a sensitive and specific marker of lipid peroxidation [6,7]. One of the most abundant and best studied isomers often used as an index for F_2-IsoPs levels is 15-F_{2t}-IsoP, present in plasma and excreted in human urine [8].

Quantification of urinary F_2-IsoPs is particularly useful because sampling is non-invasive, and sample preparation is not affected by potential auto-oxidation as in the measurement of F_2-IsoPs in plasma, or lipid-containing tissues or fluids.

Various analytical methods have been developed to quantify F_2-IsoPs including enzyme immunoassay (EIA) [9], radioimmunoassay (RIA) [10], GC–MS [11,12], GC–tandem MS [13], LC–MS [14,15] and LC–tandem MS [16]. Although immunoassays are commercially available, Proudfoot et al. [17] showed that results obtained by GC–MS and EIA are not equivalent.

Quantification of F_2-IsoPs by GC–MS requires sample purification consisting of one or two solid phase extractions (SPE) [18], a combination of SPE and thin layer chromatography (TLC) [8], HPLC [19], or immunoaffinity chromatography (IAC) using immobilized antibodies raised against 15(*S*)-F_{2t}-IsoPs [20]. In general, methods using immunoaffinity-based purification followed by GC–MS or immunoassay quantification, give lower values of urinary 15-F_{2t}-IsoP than those using physicochemical purification methods.

The aim of the present work was to develop a GC–NICI-MS method using the 4(*RS*)-F_{4t}-NeuroP as an internal standard (IS) for the determination of F_2-IsoPs in human urine. d_4-15-F_{2t}-IsoP cannot be used because urinary endogenous compounds

* Corresponding author at: Department of Biochemistry, Lapeyronie Hospital, 371 Avenue du Doyen Gaston Giraud, 34295 Montpellier Cedex 5, France. Tel.: +33 67 33 83 45; fax: +33 67 33 83 93.
E-mail address: francoise-michel@chu-montpellier.fr (F. Michel).

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co-elute with this labelled compound, deteriorating quantitative results.

We have applied our method to a clinical study in polytraumatized patients. There is considerable evidence that lipid and phospholipid oxidation is a feature of inflammation. Multiple lipid oxidation products have been identified in animal models and in human syndromes associated with inflammation [21]. Multiple trauma induces an inflammatory response syndrome of the whole body. This is characterized by local and systemic release of pro-inflammatory cytokines (serum levels of Interleukin-1 (IL-1), IL-2, tumor necrosis factor alpha, IL-6, IL-12, and interferon-gamma are increased [22]), arachidonic acid metabolites, proteins of the contact phase and coagulation systems, complement factors and acute phase proteins, as well as hormonal mediators: it is defined as systemic inflammatory response syndrome (SIRS), according to clinical parameters. However, in parallel, anti-inflammatory mediators are produced compensatory anti-inflammatory response syndrome (CARS). Endothelial cell damage, accumulation of leukocytes, disseminated intravascular coagulation (DIC) and microcirculatory dysfunction finally lead to the development of multiple organ dysfunction syndrome (MODS) or multiple organ failure (MOF) [23]. This inflammatory imbalance is associated with an over production of reactive oxygen species leading to dramatic oxidative damage [24,25].

This study aimed to demonstrate an overproduction of urinary F_2-IsoPs in polytraumatized patients compared to healthy volunteers. It has been reported that F_2-IsoPs are significantly increased in brain tissue of rats [26] and in cerebrospinal fluid (CSF) of patients [27] after traumatic brain injury (TBI). Pratico et al. [28] demonstrate that experimental TBI induces widespread brain oxidative damage, which is also reflected by a similar increase of 8,12-*iso*-iP$F_{2\alpha}$-VI in plasma and urine with a significantly reduced systemic levels of vitamin E and ascorbic acid. These findings suggest that the consumption of endogenous antioxidants reflect an attempt to neutralize excessive reactive oxygen species formation due to systemic inflammation in the whole body following TBI.

2. Experimental

2.1. Materials

15-F_{2t}-IsoP (8-*iso*-P$GF_{2\alpha}$ or iP$F_{2\alpha}$-III), [9-^{3}H] 15-F_{2t}-IsoP (15 Ci/mmol), [3,3′,4,4′-$^2H_4$] P$GF_{2\alpha}$ (d_4-P$GF_{2\alpha}$) (≥98 atom% ^{2}H) and [3,3′,4,4′-$^2H_4$] 15-F_{2t}-IsoP (d_4-15-F_{2t}-IsoP) (≥98 atom% ^{2}H) were purchased from Cayman Chemical (Cayman Chemicals, SPI-BIO, Montigny le Bretonneux, France). 5-F_{2t}-IsoP was synthesized as previously described [29]. Methanol, hexane, and hydrochloric acid (37%) (HCl) were purchased from Prolabo (Paris, France). Acetonitrile and ethyl acetate were purchased from Carlo Erba (Val de Reuils, France). Glacial acetic acid and propan-2-ol were received from Merck (Darmstadt, Germany). 2,3,4,5,6-Pentafluorobenzylbromide (99%, PFBBr) was obtained from ALDRICH (Saint-Quentin Fallavier, France). *N,O-bis*(Trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) silylating agent, *N,N*-diisopropylethylamine (DIPEA), *n*-decane and *N,N*-dimethylformamide (DMF) were purchased from Sigma Chemicals (Saint-Quentin Fallavier, France). Sep-Pak® Vac RC C_{18} (500 mg) and Vac RC NH_2 (500 mg) cartridges were purchased from Waters Corp. (Waters S.A., Guyancourt, France). HPLC-grade water (MilliQ-water, purification system Millipore) was used for the preparation of all aqueous solutions. All commercially available chemicals and reagents were of analytical grade or greater purity.

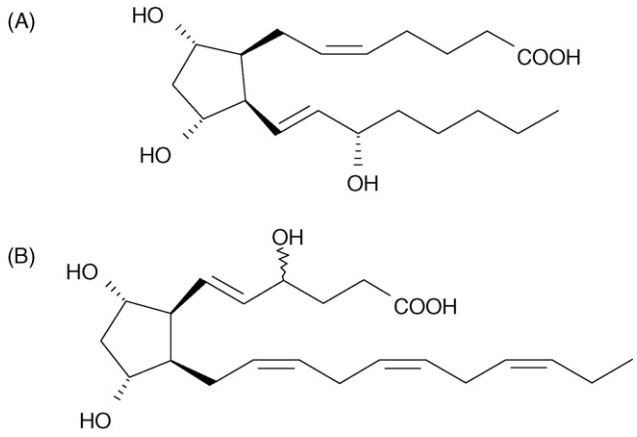

Fig. 1. Structures of (A) 15-F_{2t}-IsoP and (B) 4(*RS*)-F_{4t}-NeuroP.

2.2. Internal standard

The 4(*RS*)-F_{4t}-NeuroP (Fig. 1) was synthesized in our laboratory as previously described [30].

2.3. Clinical study

The study included 16 patients aged 38 ± 16 years suffering from polytraumatized injury (Acute Physiology and Chronic Health Evaluation (APACHE II) score was 43 (min. 38, max. 57) and Injury Severity Score (ISS) was 38 (min. 25, max. 45)) and 16 healthy volunteers aged 40 ± 10 years.

Polytraumatized patients were recruited from the Department of Anesthesiology and Critical Care Medicine, Lapeyronie Hospital, Montpellier, France. They had no previous history of significant disease (diabetes, cardiovascular pathology) and took no corticoids. Healthy volunteers were non-smokers.

The study, approved by the institutional Ethics Committee of Montpellier, was conducted in accordance with the Declaration of Helsinki as revised in 2000 and the Guidelines for Good Clinical Practice.

2.4. Urine samples

24-h urines of healthy humans and polytraumatized patients were collected in polypropylene bottles and centrifuged at 1500 × *g*, 4 °C for 10 min. The samples were stored at −80 °C until analysis.

Urinary creatinine was determined by kinetic Jaffe with an automatic analyzer (Beckman Coulter).

2.5. Sample preparation

F_2-IsoPs were determined as follows. The extraction procedure was performed according to the methodology of Nourooz-Zadeh et al. [31] and Bessard et al. [11]. Aliquots of acidified 24 h urine samples (1 ml, pH 3) were treated with 5000 pg of 4(*RS*)-F_{4t}-NeuroP as an IS, diluted with 9 ml of acidified water (pH 3) and subjected to two extractions using successively a Sep-Pak® Vac RC C_{18} and a Vac RC NH_2 cartridges (500 mg). The C_{18} cartridge was preconditioned with 5 ml of methanol and 5 ml of acidified water (pH 3). After washes with 10 ml of acidified water (pH 3) and 10 ml of acetonitrile/water (15:85; v/v), elution of the compounds was performed with 4 ml of hexane/ethyl acetate/propan-2-ol (30:65:5; v/v). The eluate was then applied to the NH_2 cartridge, which was preconditioned with 5 ml of hexane. The cartridge was washed successively with 5 ml of hexane/ethyl acetate (30:70;

v/v) and 5 ml of acetonitrile. The isoprostanes were then eluted with 5 ml of ethyl acetate/methanol/glacial acetic acid (10:85:5; v/v).

2.6. Derivatization procedure

The derivatization was performed according to Morrow et al. [8]. After purification, the solvents were evaporated under nitrogen. Samples were incubated twice with a mixture of 40 μl of PFBBr (10 vol.%, acetonitrile) and 20 μl of DIPEA (10 vol.%, acetonitrile) at 37 °C for 5 min and dried under nitrogen. This procedure was repeated to ensure quantitative esterification. To the dried samples, 20 μl of BSTFA with 1% TMCS and 10 μl of DMF were added and the mixture was incubated at 40 °C for 20 min to accomplish complete silylation of the F_{2t}-IsoP. The samples were dried under a stream of nitrogen and dissolved in 35 μl of decane.

2.7. GC–NICI-MS analysis

The derivatized samples were analyzed on a ThermoFinnigan Trace DSQ mass selective detector interfaced with a Trace GC Ultra 2000 gas chromatograph, equipped with an AS 2000 automatic sampler (ThermoFinnigan, Courtaboeuf, France SA). Xcalibur© was used for data acquisition and processing. The mass spectrometer was used in the negative ion chemical ionization (NICI) mode with the ion source at 225 °C and MS transfer line at 280 °C. Electron energy was 70 eV and emission current was 100 μA. Isobutane was used as reagent gas with a flow rate of 1 ml/min. GC Separations were carried out on a "FactorFOUR™ capillary column VF-1 ms": WCOT fused silica capillary column (30 m × 0.25 mm ID), film thickness 0.25 μm (Varian, Les Ulis, France). Helium was the carrier gas with a flow rate of 1.5 ml/min. Derivatized samples (3.5 μl) were injected splitless into the GC at an injection port temperature of 280 °C. The initial column temperature was maintained at 50 °C for 1 min, increased to 250 °C at a rate of 40 °C/min, to 280 °C at a rate of 2 °C/min and to 300 °C at a rate of 30 °C/min, and finally held to 300 °C for 15 min.

The mass spectrometer was used in SCAN mode (mass range 420–650) and the carboxylate ion (M-181: loss of pentafluorobenzyl, $CH_2C_6F_5$) at *m/z* 569 for 15-F_{2t}-IsoP and *m/z* 593 for 4(*RS*)-F_{4t}-NeuroP (IS) were extracted as selected ions. Quantification was done by using the peak area ratios *m/z* 569/593 of the PFB–TMS (pentafluorobenzyl–trimethylsilyl)-derivatized compounds. Peak area ratios were determined from calibration solutions comprising 15-F_{2t}-IsoP at various concentrations and a fixed concentration of NeuroP (5000 pg for each sample). Equimolar concentrations of 15-F_{2t}-IsoP and 4(*RS*)-F_{4t}-NeuroP yielded a peak area ratio (*m/z* 569/593) of three. Urinary concentration values are expressed as F_2-IsoPs pg per mg of creatinine.

3. Results

3.1. Precision and accuracy of the method

The precision and accuracy of the method were investigated by spiking a urine sample (1 ml) in triplicate with various concentrations of 15-F_{2t}-IsoP (50, 100, 250, 500, 1000 and 2000 pg/ml) and a fixed concentration of 4(*RS*)-F_{4t}-NeuroP (5000 pg/ml).

Table 1 shows measured concentrations of each sample, mean, R.S.D. (relative standard deviation) and accuracy for each spiked 15-F_{2t}-IsoP concentration. The equation for the linear regression plot correlating the amounts spiked and the mean of amount recovered was $y = 166.7 + 0.947x$; $r^2 = 0.9998$. The assay was linear over the range of 50–2000 pg of 15-F_{2t}-IsoP in 1 ml urine aliquots.

Table 1
Precision and accuracy of the method

15-F_{2t}-IsoP spiked (pg/ml)	15-F_{2t}-IsoP measured (pg/ml)			Mean	R.S.D. (%)	Accuracy (%)
	1	2	3			
50	207	220	239	222	7.25	90
100	273	271	242	262	6.62	96
250	380	416	408	401	4.71	100
500	656	571	645	624	7.41	104
1000	1103	1143	1132	1126	1.84	102
2000	2135	2027	2015	2059	3.21	104

Analyses were performed in triplicate. The concentration of internal standard 4(*RS*)-F_{4t}-NeuroP was 5000 pg/ml.

3.2. Within- and between-day precision

Within-day precision was calculated from repeated analysis ($n = 6$) of spiked urines with three different concentrations of 15-F_{2t}-IsoP (0, 500, and 1000 pg/ml) during 1 working day, by the same operator (Table 2).

Between-day precision was calculated from analysis ($n = 6$) of spiked urines at the same concentrations of 15-F_{2t}-IsoP (0, 500 and 1000 pg/ml), one analysis being performed a day. The detection limit of the method was found to be 250 fg by injection of pure synthetic 15-F_{2t}-IsoP (S/N = 43). The quantification limit (SCAN mode) in urine was evaluated with d_4-$PGF_{2\alpha}$ as 50 pg/ml (5 pg injected on GC-column, S/N > 20).

3.3. Extraction recovery (columns)

Extraction recovery, expressed as a percentage, was determined by addition of [9-^{3}H] 15-F_{2t}-IsoP (8000 dpm) to urine samples. After extraction with C_{18} cartridges alone ($n = 10$) or a combination of C_{18} and NH_2 cartridges ($n = 10$), the recovery was measured by scintillation counting using a liquid scintillation analyzer (TRI-CARB 2300 TR, PACKARD A Canberra Company).

Extraction recovery was 94.8 ± 5.3% following extraction with the C_{18} cartridges alone and 90.6 ± 8.3% after extraction with the combined C_{18} and NH_2 cartridges.

The calibration curve slopes obtained from non-extracted analytes and urinary samples spiked with these analytes are identical, in all cases the recovery of the neuroprostane and 15-F_{2t}-IsoP are similar.

3.4. GC–NICI-MS

The fragmentation patterns of 15-F_{2t}-IsoP and 4(*RS*)-F_{4t}-NeuroP during NICI-MS were very similar. The most intense ion was M-181 (loss of $CH_2C_6F_5$) representing about 85% of the total ion current. The retention times of 15-F_{2t}-IsoP, d_4-15-F_{2t}-IsoP, $PGF_{2\alpha}$, d_4-$PGF_{2\alpha}$, and 4(*RS*)-F_{4t}-NeuroP were 15.74, 15.68, 16.49, 16.45 and 18.33 min, respectively. As observed with d_4 compounds, the retention times of labelled compounds are approximately 0.05 min shorter than that of the corresponding non-labelled compounds.

Table 2
Intra- and inter-day precision of 15-F_{2t}-IsoP in human urine

Urine spiked with 15-F_{2t}-IsoP (pg/ml)	Within-day			Between-day		
	Mean	S.D.	R.S.D. (%)	Mean	S.D.	R.S.D. (%)
0	457	26.4	5.8	452	29.3	6.48
500	898	42	4.7	848	84.5	9.97
1000	1441	73.1	5.1	1395	65.4	4.69

A representative chromatogram obtained from the analysis of human urine is depicted in Fig. 2. Fig. 2A shows *m*/*z* 569 representing F_2-IsoPs at 15.67 min and prostaglandins as overlapping peaks at 16.48 min. In Fig. 2B, the *m*/*z* 573 chromatogram shows two not fully separated peaks at 15.66 and 15.76 min, near the retention time of d_4-15-F_{2t}-IsoP. In the *m*/*z* 593 chromatogram (Fig. 2C), there were negligible peaks detected between 17 and 20 min representing the retention time of 4(*RS*)-F_{4t}-NeuroP (18.33 min).

Fig. 3 shows chromatograms obtained with the same urinary sample spiked with 15-F_{2t}-IsoP, $PGF_{2\alpha}$ (A), d_4-15-F_{2t}-IsoP, d_4-$PGF_{2\alpha}$ (B) and 4(*RS*)-F_{4t}-NeuroP (C). The d_4-15-F_{2t}-IsoP (*m*/*z* 573) has a retention time of 15.68 min and elutes in the vicinity of two impurity peaks at 15.66 and 15.76 min (Fig. 2B). We have tried to resolve the peak representing d_4-15-F_{2t}-IsoP from the impurity two peaks using different GC conditions. However, neither changing the retention times between 9 and 26 min, nor the use of a DB-5 column under multiple temperature regions enabled different retention times of the impurities compared to the d_4-15-F_{2t}-IsoP. This drawback was efficiently overcome by using 4(*RS*)-F_{4t}-NeuroP as IS (Fig. 3C).

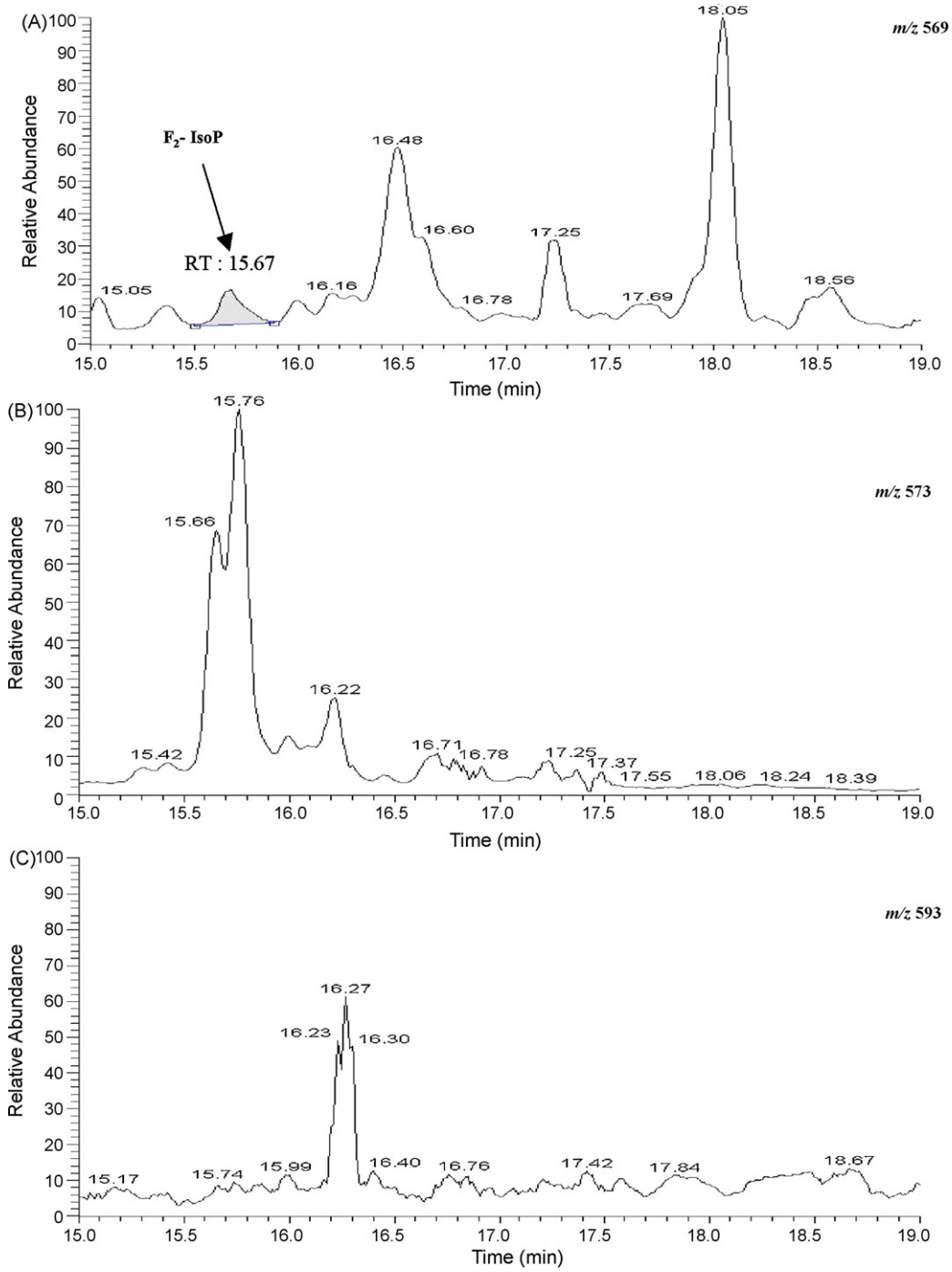

Fig. 2. Partial chromatograms from GC–NICI-MS analysis on unspiked urine sample (1 ml): (A) (*m*/*z* 569) endogenous F_2-IsoPs, (B) (*m*/*z* 573) interference peaks with a retention time close to d_4-15-F_{2t}-IsoP and (C) (*m*/*z* 593) no peak detectable at the retention time of 4(*RS*)-F_{4t}-NeuroP (18.33 min).

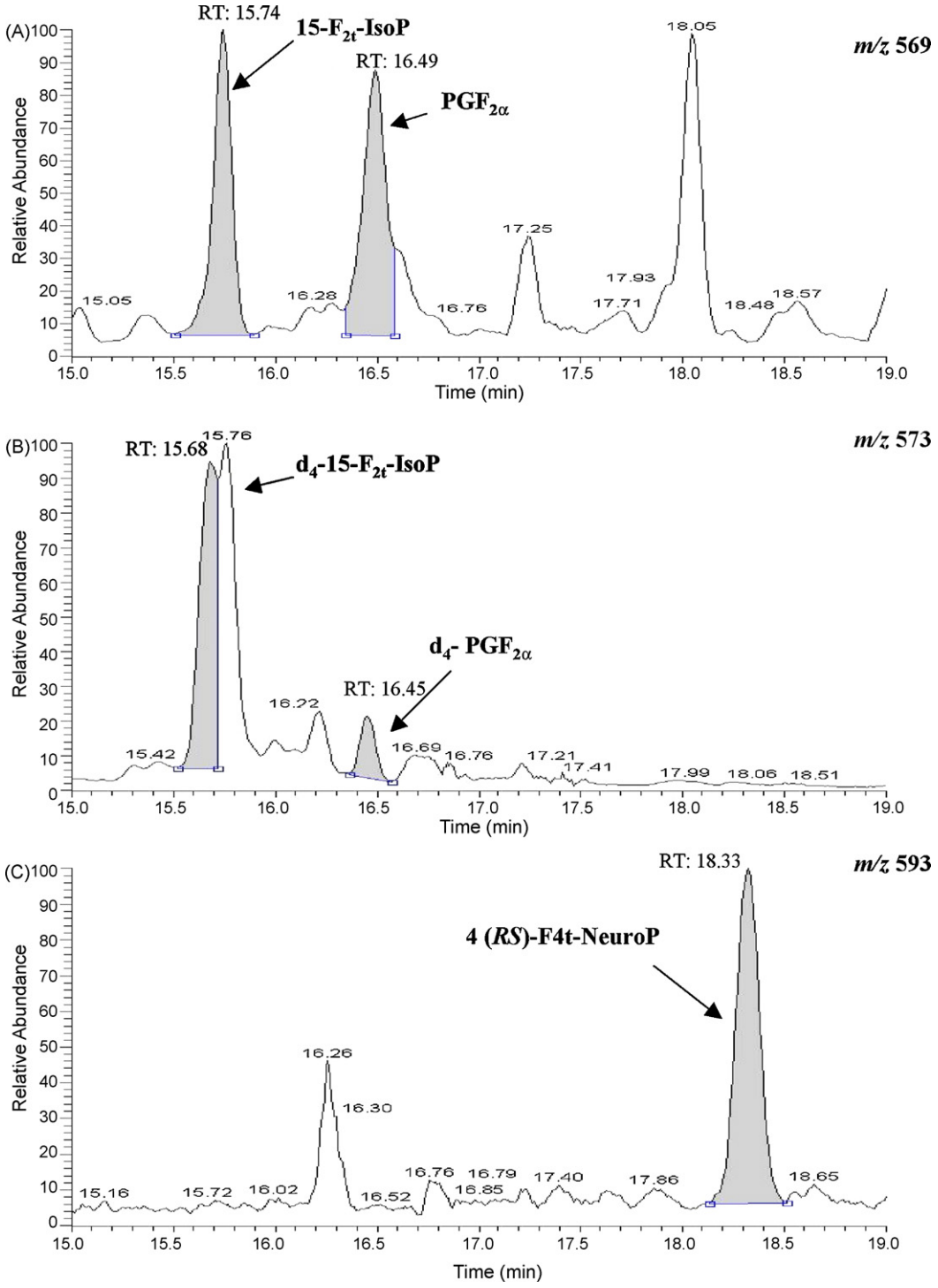

Fig. 3. Partial ion chromatograms from GC–NICI-MS analysis of urinary sample (1 ml) spiked with (A) (*m/z* 569) 15-F_{2t}-IsoP, $PGF_{2\alpha}$, (B) (*m/z* 573) d_4-15-F_{2t}-IsoP, d_4-$PGF_{2\alpha}$ (2000 pg/ml each) and with (C) (*m/z* 593) 4(*RS*)-F_{4t}-NeuroP (5000 pg/ml).

3.5. Urinary F_2-IsoPs of polytraumatized patients compare to healthy volunteers

Statistical analyses were performed with the StatView statistical package for Windows (version 5.0). Results are given as mean ± S.D. The Mann–Whitney–Wilcoxon non-parametric test was used to compare differences in urinary F_2-IsoP levels.

Urinary excretion of the F_2-IsoPs was significantly ($p < 0.0001$) higher in polytraumatized patients ($n = 16$) than healthy volunteers ($n = 16$) (4.73 ± 2.75 ng/mg creatinine vs. 0.811 ± 0.359 ng/mg creatinine) (Fig. 4).

4. Discussion

The aim of this study was to demonstrate that the use of 4(*RS*)-F_{4t}-NeuroP as IS is suitable to quantify IsoP by GC–NICI-MS after an extraction–purification step.

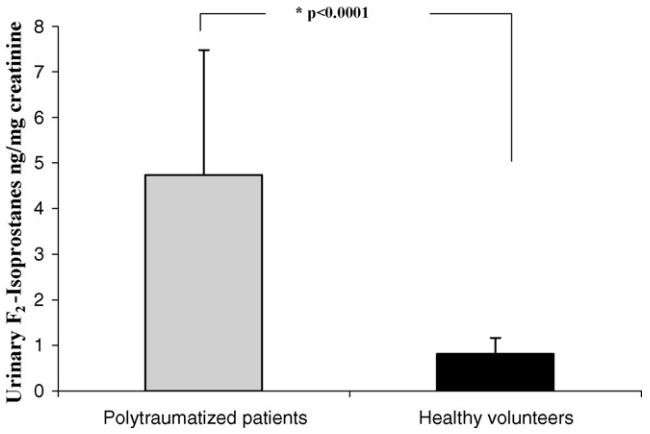

Fig. 4. Urinary levels of F_2-isoprostanes in polytraumatized patients (4.73 ± 2.75 ng/mg creatinine) compare to healthy volunteers (0.811 ± 0.359 ng/mg creatinine). Data are presented as mean ± S.D.

In our study, we used two SPE extractions: C_{18} and NH_2 cartridges as described by Nourooz-Zadeh et al. [31] and Bessard et al. [11]. The recovery for F_2-IsoPs ($89.3 \pm 6.7\%$) was better than those previously reported by Nourooz-Zadeh et al. [31] ($65 \pm 4\%$) and Bessard et al. [11] (72%). Lee et al. [18] using a quick sample purification method with an anion exchange SPE showed a recovery of $53.4 \pm 2.2\%$. Mori et al. [12] obtained a recovery of 70% with combination of C18 and silica cartridges.

The most commonly used internal standard in quantitative determination of F_2-IsoPs is a stable-isotope-labelled analogue of one of the isomers. The commercially available corresponding tetradeuterium-labelled [3,3′,4,4′-$^2H_4$] 15-F_{2t}-IsoP has a few seconds shorter retention time on GC compared to the unlabelled compound, thus facilitating the identification of the analyte. However, in our hands, d_4-15-F_{2t}-IsoP could not be used as internal standard since the analysis of PFB and TMS derivatives showed analytical interferences at m/z 573 with a retention time near d_4-15-F_{2t}-IsoP (Figs. 2 and 3). The total area (m/z 573) for these two peaks is greater than 2 ng/ml of d_4-15-F_{2t}-IsoP spiked in urinary sample (Figs. 2B and 3B). Despite, others GC conditions, we are unsuccessful to separate unknown peaks. These peaks were present in all human urine samples from controls and polytraumatized patients. In our laboratory, these interferences were not observed in other biological samples such as (i) plasmas obtained from elderly and diabetics patients, (ii) cerebrospinal fluid, or (iii) in tissues like muscles.

It is quite possible that other protocols for clean up of urinary isoprostanes prior to GC–MS also suffer from this interference at m/z 573, which may artifactually increase the peak area of the heavy isotope and consequently underestimate the true value of urinary isoprostanes. The peak areas of these impurities were large and not reproducible in samples, thus preventing the use of d_4-15-F_{2t}-IsoP as IS. We, therefore, used 4(*RS*)-F_{4t}-NeuroP as IS (Fig. 1, m/z 593). Its structure is similar to 15-F_{2t}-IsoP and both compounds have similar physicochemical properties. In urine samples chromatograms (m/z 593), no peak was detectable in the region of the IS. To our knowledge, there are no literature reports of NeuroPs in human urine. Lawson et al. [32] were unable to detect nPF4a-VI in urine sample from healthy subjects ($n = 20$) by LC/MS/MS. Furthermore, measurement by Montine et al. [33] in Alzheimer disease (AD) patients of urine NeuroP by GC–NICI-MS were below the limit of detection, NeuroP are elevated in diseased region of brain and CSF from patients with advanced AD.

As the m/z 569 chromatogram of urine showed a tailing peak at 15.68 min (Fig. 5A) we attempted to identify the compounds which co-eluted. Lawson et al. [34] previously reported co-elution of pure d_4-15-F_{2t}-IsoP and d_4-5-F_{2t}-IsoP (d_4-15-F_{2t}-IsoP was 0.09 min later than d_4-5-F_{2t}-IsoP) and two overlapping peaks at this retention time in urine samples. The authors subsequently demonstrated by using HPLC–tandem MS, that 5-F_{2t}-IsoP and two isomers 5-F_{2c}-IsoP and 5-*epi*-5-F_{2c}-IsoP were present in urine.

We showed that spiking a urine sample with 15-F_{2t}-IsoP (Fig. 5B) increased a peak at 15.74 min, corresponding with the retention time of pure synthetic 15-F_{2t}-IsoP. Similarly, spiking the urine sample with 5-F_{2t}-IsoP (Fig. 5C) increased a peak at 15.68 min, the retention time of pure synthetic 5-F_{2t}-IsoP. Therefore, 5-F_{2t}-IsoP co-eluted with 15-F_{2t}-IsoP with a retention time difference of 0.06 min, in full agreement with the results of Lawson et al. [34] and confirming the presence of other F_2-IsoPs such as 5-F_{2t}-IsoP in urine samples.

In addition, Lee et al. [18] observed that several F_2-isoprostanes isomers co-elute on GC and form the same mass ion, which may contribute to the total endogenous F_2-IsoPs peak area measured. Others teams [12,35,36] using GC–MS also measure 15-F_{2t}-IsoP and others F_2-IsoPs that co-elute with this compound.

The urinary level of F_2-IsoPs measured in controls by our method was 0.811 ± 0.359 ng/mg creatinine, what is consistent with values reported by others authors using different extraction and purification methods before GC–NICI-MS (i.e. Mori et al. [12] using SPE/HPLC procedure, Morrow et al. [8] using SPE/TLC and Walter et al. [19] using HPLC). They found 1.143 ± 0.016 ng/mg creatinine, 1.6 ± 0.6 ng/mg creatinine and 0.75 ± 0.34 ng/ml urine, respectively. Lee et al. [18] reported a quick sample purification method using mixed anion exchange solid phase extraction (MAX-SPE cartridges) followed by GC–NICI-MS with urinary F_{2t}-IsoP concentrations of 0.502 ± 0.404 ng/mg creatinine.

Urinary concentrations of 15-F_{2t}-IsoP determined by GC–tandem MS were lower than those reported earlier. Schwedhelm et al. [37] used solid phase extraction on ODS and TLC on silica gel and reported concentrations of 0.223 ± 0.135 ng/mg creatinine. Tsikas et al. [38] quantified 15-F_{2t}-IsoP in human urine by GC–tandem MS after use of two sample treatment procedures, IAC and TLC. IAC was more selective than TLC (0.141 ± 0.041 ng/mg creatinine and 0.291 ± 0.102 ng/mg creatinine, respectively). Analysis of non-retained compounds from the IAC step showed the presence of non-immunoreactive 15-F_{2t}-IsoP at 0.128 ± 0.055 ng/mg creatinine. These authors assumed this compound as *ent*-15-F_{2t}-IsoP.

Quantification of 15-F_{2t}-IsoP in urine was also performed by LC–MS and LC–tandem MS. Ohashi and Yoshikawa [39] reported basal levels of 0.426 ng/mg creatinine, by LC–MS, while Murai et al. [40] using LC–tandem MS reported comparable values, with those measured by Tsikas et al. [38] by GC–tandem MS after TLC purification. This suggests that both GC–tandem MS (TLC purification) and LC–MS or LC–tandem MS measure the sum of 15(*S*)-F_{2t}-IsoP and *ent*-15-F_{2t}-IsoP, in human urine. Montine et al. [33] developed an assay to quantify F_4-NeuroPs utilizing a modification of the method employed to measure F_2-IsoPs. Musiek et al. [41] optimized this method by using a stable-isotope-labelled neuroprostane for use as internal standard of neuroprostanes. Recently members of the same group, Arneson et al. [42] used the labelled 15-F_{2t}-IsoP to quantify Neuroprostanes. They observed some variability in the quantification compare to original method to quantitate the F_2-IsoPs. The reasons of this can be at least partially attributed to F_2-IsoPs, structurally similar to the F_4-NeuroPs, but is slightly more polar. Therefore will have slightly different extraction and chromatographic characteristics than the F_4-NeuroPs. Also, the quantification of the F_4-NeuroPs requires the integration of multiple peaks increasing the possibility for compounds that are not F_4-NeuroPs to co-elute and interfere quantification, decreasing the

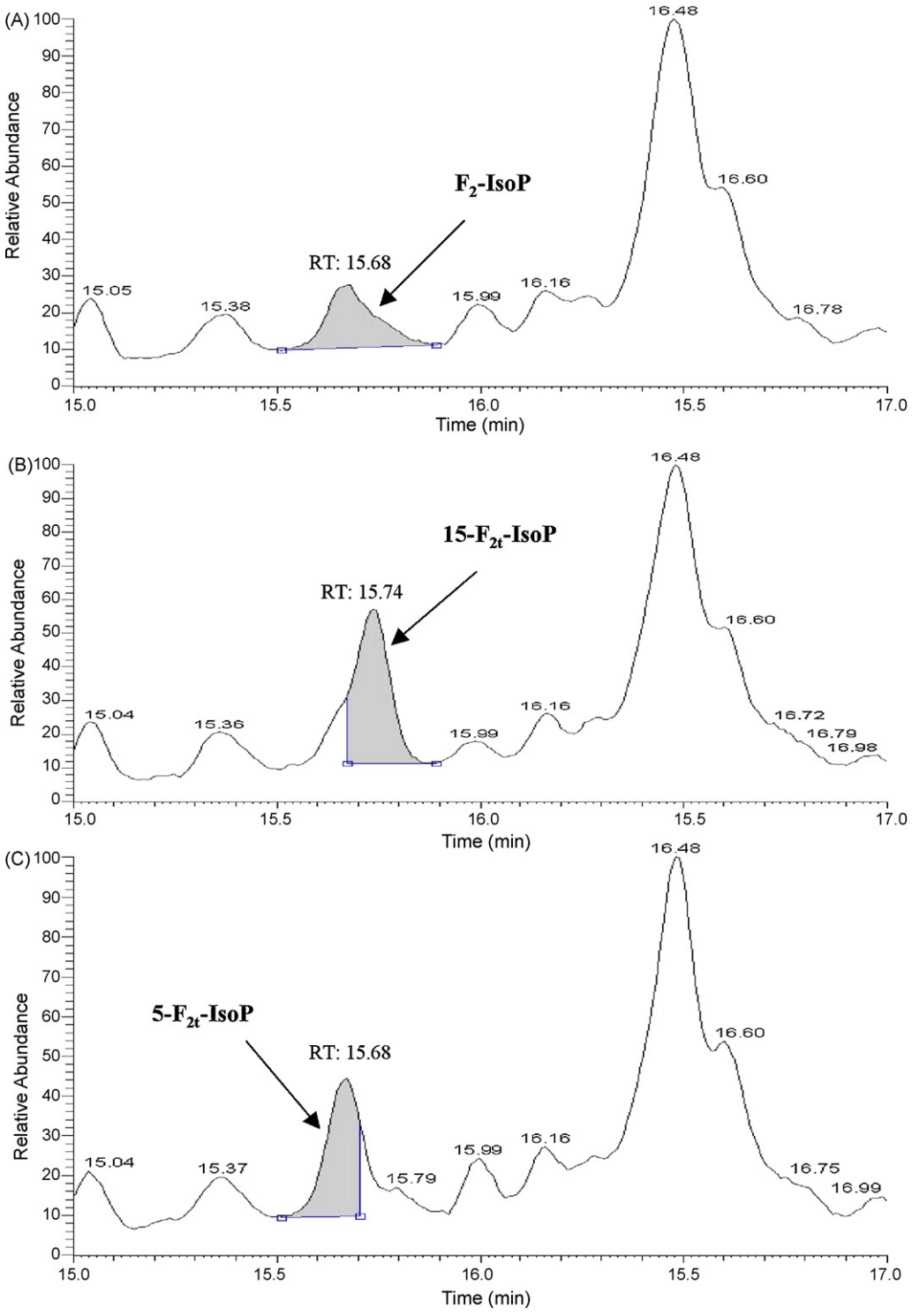

Fig. 5. Partial ion chromatograms from GC–NICI-MS analysis (m/z 569) of an unspiked urine sample (basal level of F_2-IsoPs 420 pg/ml) (A), the urine sample spiked with 500 pg of 15-F_{2t}-IsoP (B) and the urine sample spiked with 500 pg of 5-F_{2t}-IsoP (C).

accuracy and precision of this assay. The likelihood of co-eluting compounds is increased in the F_4-NeuroPs assay because of its wider TLC (thin layer chromatography) cut. TLC plates by some differences in preparations and characteristics may also introduce variability in the assay.

Therefore, we do not have the same integration of the peak areas of multiple compounds eluting from the GC over a 2-to 3-min interval. We have a tailing peak with compounds co-eluting. Our methods of extraction and purification without TLC reduce the variability observed by Anderson et al. [42] and Musiek et al. [41].

F_2-IsoP quantification by GC–MS, reflects peroxidation of arachidonic acid and is a good index of oxidative stress *in vivo*.

Our results show good within-day and between-day precision (<10%). We have quantified endogenous F_2-IsoPs using the ratio m/z 569/593, from ions extracted from scans between m/z 420 and 650. Despite a better sensitivity in selected ion monitoring

(SIM) mode , analyses were carried out using SCAN mode because it enables quantification of F_2-IsoPs as well as metabolites with different molecular mass such as 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP and 2,3-dinor-15-F_{2t}-IsoP. In recent years, additional related compounds, derived from various polyunsaturated fatty acid as eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) have been discovered. Fessel et al. [43] discovered Isofuranes, a family of free radical-induced peroxidation products of arachidonic acid (AA), formed preferentially under conditions of elevated oxygen tension. More recently Song et al. [44] described neurofuranes an analogous family of compounds formed from DHA.

The SCAN mode could provide some important information about metabolites or isoprostanes compounds in diseases. These informations could afford to develop clinical studies with some specifics IsoP compounds more present in the disease.

5. Conclusion

We have used GC–NICI-MS analysis to quantify urine F_2-IsoPs using 4(*RS*)-F_{4t}-NeuroP as an IS. This modification of previously published methods could also be applied for isoprostanes metabolites. We suggest that the use of deuterium-labelled compounds as internal standards is not essential and could be replace by compounds structurally close to F_2-IsoPs.

The advantage of our method is to avoid to underestimate the true value of urinary isoprostanes by the presence of these urinary "interferences", impurities or unknown compounds at *m*/*z* 573.

This mass spectrometric assay will prove to be a valuable analytical tool to further explore the role of lipid peroxidation in the pathophysiology of human diseases.

We quantified F_2-IsoPs in the urine of 16 polytraumatized patients and 16 healthy volunteers. Urinary F_2-IsoPs concentrations were significantly increased in polytraumatized patients compared to healthy volunteers. Our results are in accordance with Bayir et al. [45], and Varma et al. [46], which show markedly increased F_2-isoprostanes in CSF supporting a role of oxidative stress in the evolution of polytraumatized patients.

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