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Determination of isoprostaglandin $F_{2\alpha}$ type III in human urine by gas chromatography–electronic impact mass spectrometry. Comparison with enzyme immunoassay

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Abstract

F_2-Isoprostanes are stable lipid peroxidation products of arachidonic acid, the quantification of which provides an index of oxidative stress in vivo. We describe a method for analysing isoprostaglandin $F_{2\alpha}$ type III (15-F_{2t}-IsoP) in biological fluids. The method involves solid-phase extraction on octadecyl endcapped and aminopropyl cartridges. After conversion to trimethylsilyl ester trimethylsilyl ether derivatives, isoprostaglandin $F_{2\alpha}$ type III is analysed by mass spectrometry, operated in electronic impact selected ion monitoring mode. We have compared enzyme immunoassay (EIA; Cayman, Ann Arbor, MI, USA) to this method with 30 human urine aliquots following the same extraction procedure in order to determine the agreement between both methods. Isoprostaglandin $F_{2\alpha}$ type III concentrations determined with gas chromatography–mass spectrometry (GC–MS) did not agree with those determined with EIA. Our results suggest that GC–MS and EIA do not measure the same compounds. As a consequence, comparison of clinical results using GC–MS and EIA should be avoided.

Keywords: Isoprostaglandin $F_{2\alpha}$

1. Introduction

Isoprostanes are stable lipid peroxidation products of arachidonic acid. These compounds are esterified to phospholipids and then released in free form by phospholipases [1]. These chemically stable compounds can be measured as free form in urine [2], plasma [3], cerebrospinal fluid [4], exhaled breath condensate [5], broncho–alveolar lavage fluid [6] or as esterified complexes in tissue membranes or circulating lipid particles [3]. Urinary measurements have been favoured due to the non-invasive approach and the lack of in vitro artefactual generation of isoprostanes. The most extensively studied isoprostanes are the prostaglandin $F_{2\alpha}$ isomers. Depending on which of arachidonic acid labile hydrogen atom is first abstracted by free radical attack, four prostaglandin $F_{2\alpha}$ regioisomers are formed (named type III, IV, V and VI F_2-isoprostanes in Rokach et al.'s nomenclature [7], or 15, 8, 12 and 5-F_2-isoprostanes, respectively, in Taber et al.'s nomenclature [8], Fig. 1). As each regioisomer is comprised of eight racemic diastereoisomers, 64 different F_2-isoprostanes can be generated. It remains undetermined

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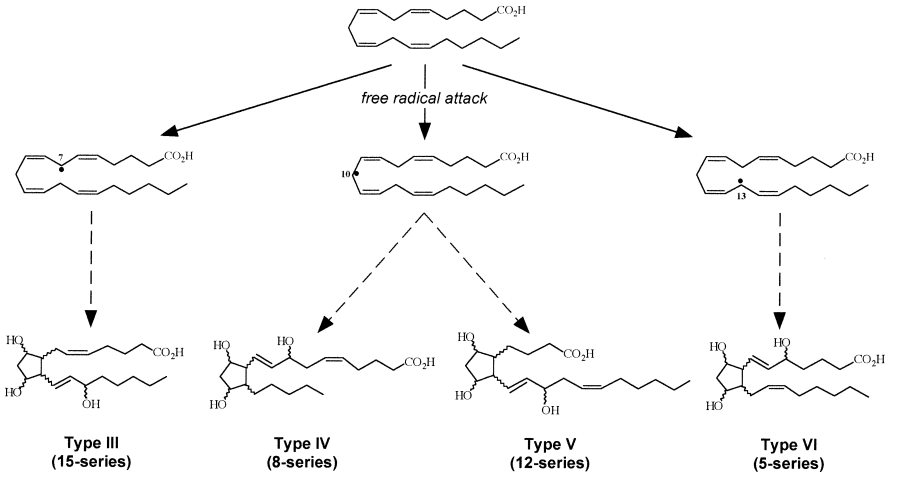

Fig. 1. F_2-Isoprostane formation from arachidonic acid, leading to four F_2-isoprostane regioisomers. Each regioisomer is comprised of eight racemic diastereoisomers. For simplicity, the intermediate compounds have not been introduced.

whether variations in F_2-isoprostane generation under conditions of oxidative stress would be reflected similarly by all detectable regioisomers. Li et al., using liquid chromatography–tandem mass spectrometry (LC–MS–MS), showed that urinary type III and VI F_2-isoprostane isomers were elevated to a comparable degree in patients with homozygous familial hypercholesterolemia, but not in patients with cardiac failure [9].

Although many isoforms are produced, most studies have focused on isoprostaglandin $F_{2\alpha}$ type III ($iPF_{2\alpha}$-III also named 15-F_{2t}-IsoP [7,8]) and type VI (5-F_{2t}-IsoP [7,8]) quantification. Their quantification in biological fluids provides an index of oxidative stress in vivo [10,11], and as such may replace previous methods that suffer from a lack of specificity, sensitivity or feasibility [12]. As recently reviewed by Tsikas [13], $iPF_{2\alpha}$-III (see Fig. 2 for stereochemistry) is currently quantified using gas chromatography–negative ion chemical ionisation mass spectrometry (GC–NICI-MS) [2,14–21] or LC–MS [22]; further methods have been developed using GC–MS–MS [23,24] or LC–MS–MS [9]. The main problems of the latter methods are their cost and their technology, which is not widely available. Immunoassays have been developed by academic and commercial investigators to enable a larger development of $iPF_{2\alpha}$-III quantification [17,25]. Immunoassays suffer from a lack of specificity and from potential interferences in biological fluids. A recent report of Proudfoot et al. has shown that comparison of levels measured by GC–NICI-MS and enzyme immunoassay (EIA) was inappropriate [21]. We have developed a gas chromatography–electronic impact mass spectrometry (GC–EI-MS) method that enables the quantification of $iPF_{2\alpha}$-III, and compared EIA to this method for 30 human urine aliquots following the same extraction procedure in order to determine the agreement between both methods.

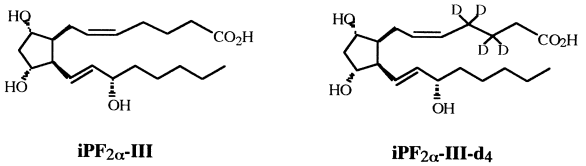

Fig. 2. Chemical structures of isoprostaglandin $F_{2\alpha}$ type III (15-F_{2t}-IsoP) and isoprostaglandin $F_{2\alpha}$ type III-d_4.

2. Experimental

2.1. Materials

Prostaglandin $F_{2\alpha}$, 15 epi-prostaglandin $F_{2\alpha}$, prostaglandin $F_{2\beta}$, 11β prostaglandin $F_{2\alpha}$, isoprostaglandin $F_{2\alpha}$ type III, 15 epi-$iPF_{2\alpha}$-III (8-iso-15 R prostaglandin $F_{2\alpha}$), 9β-$iPF_{2\alpha}$-III (8-iso-prostaglandin $F_{2\beta}$), $iPF_{2\alpha}$-III-d_4 were purchased from Cayman (Ann

Arbor, MI, USA) and were used without further purification. The $iPF_{2\alpha}$-III EIA kits were purchased from Cayman, and were read using a Bio-Rad plate reader (Hercules, CA, USA). Acetonitrile (for pesticide analysis), ethyl acetate (for trace analysis), hexane (for trace analysis), isopropanol (HPLC grade), methanol (for pesticide analysis) were purchased from SDS (Valdonne-Peypin, France). Glacial acetic acid (100%) was purchased from Merck (Darmstadt, Germany). Hydrochloric acid was purchased from Prolabo (Paris, France). Deionized water was obtained on a Milli-Q water purification system (Millipore, Bedford, MA, USA). Octadecyl, endcapped (C_{18}EC) cartridges (100 mg/10 ml) and aminopropyl (NH_2) cartridges (100 mg/10 ml) were purchased from International Sorbent Technology (Mid-Glamorgan, UK). Solid-phase extraction (SPE) columns were positioned on a Vac Elut Vacuum Manifold (Varian Sample Preparation Products, Harbor City, CA, USA). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) added was obtained from Sigma (Saint Quentin Fallavier, France). The GC column was a fused-silica capillary column (HP-5MS, 5% phenyl methyl siloxane, 30 m×0.25 mm I.D., 0.25 μm film thickness) from Hewlett-Packard (Wilmington, DE, USA).

Working solutions of $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-III-d_4 were prepared by diluting the standards in methanol to obtain concentrations of 10 and 1 mg/l. The solutions were kept in the dark at −18°C for 1 month. Solutions of concentration 0.1 mg/l were prepared daily (for each experiment).

2.2. Urine samples

Urine samples were collected at 8 a.m. from 30 volunteers. The samples (20 ml) were kept refrigerated during the collection period, after which they were transferred to the laboratory, aliquoted and stored at −20°C until they were analysed. The investigation conforms to the principles outlined in the Declaration of Helsinki.

To prepare urinary calibration, seven aliquots of 4 ml healthy human urine were spiked with $iPF_{2\alpha}$-III at concentrations of 0, 40, 100, 200, 400, 600, 800 pg/ml. The deuterated standard ($iPF_{2\alpha}$-III-d_4, 2000 pg/ml) was added before the extraction. For GC–MS and EIA comparison, the deuterated standard was added after the extraction for the GC–MS assay, in order to allow quantification of the same extracts with both methods, and to avoid EIA cross reactivity. The samples were allowed to equilibrate for 15 min.

2.3. Extraction

The extraction procedure that follows has been performed with a methodology derived from Nourooz-Zadeh et al. [15]. The samples were acidified (pH 3) using HCl and diluted with the same volume of water (pH 3). They were extracted on a Vac Elut Vacuum Manifold, using C_{18}(EC) cartridges. These cartridges were preconditioned with 2 ml methanol and 2 ml water (pH 3). The solvent program included washes with 10 ml each of water (pH 3) and CH_3CN–water (15:85, v/v). An elution was performed with 4 ml of hexane–ethyl acetate–propan-2-ol (30:65:5, v/v). These eluates were then applied to NH_2 cartridges, preconditioned with hexane (5 ml). The NH_2 cartridges were sequentially washed with 5 ml of hexane–ethyl acetate (30:70, v/v) and 5 ml acetonitrile. $iPF_{2\alpha}$-III was eluted from the NH_2 cartridges with 5 ml of ethyl acetate–methanol–acetic acid (10:85:5, v/v). For the GC–MS and EIA comparison, 1 ml was kept for EIA, 4 ml was kept for GC–MS analysis, and $iPF_{2\alpha}$-III-d_4 (2000 pg/ml) was added to these 4 ml at this stage. The solution was evaporated under N_2. Residues were reconstituted in 0.5 ml of ethyl acetate, transferred to a vial and again evaporated under N_2 at room temperature. To the dried residues, 30 μl of the silylating reagent BSTFA (1% TMCS) were added to obtain the trimethylsilyl (TMS) ester TMS ether derivatives of $iPF_{2\alpha}$-III. Three μl of the aliquot were injected into the chromatographic system. The researchers performing the analysis denied the results obtained with the alternative method.

2.4. GC–MS method

The quantitative analysis were performed in electronic impact mode at 70 eV using a Hewlett-Packard benchtop GC–MS system consisting of a HP 5973 mass-selective detector, a HP 6890 series GC instrument, and a HP 6890 series automatic liquid sampler. HP ChemStation was used for data acquisition and

processing. The initial oven temperature of 130°C was maintained for 1 min and then increased at a rate of 18°C/min to reach a maximum temperature of 300°C, which was held for 2 min. There was a final isotherm at 310°C for 5 min to purge the column. The injector system mode was splitless (45 s). The carrier gas was helium at a constant flow-rate of 1 ml/min. GC–MS temperatures were as follows: injector 250°C, interface 300°C, source 220°C, and quadrupole 100°C. The MS instrument was operated in the selected ion monitoring (SIM) mode. The mass spectrometer was autotuned daily with perfluorotributylamine (PFTBA). Ions m/z 571 and 575 (M−71, loss of C_5H_{11} from the molecular ions [26]) and ions m/z 481 and 485 (M−71−90), loss of C_5H_{11} and trimethylsilanol (TMSOH) [26] were selected for identification: ions at m/z 481 and 571 served for $iPF_{2\alpha}$-III identification, and m/z 485 and 575 served for $iPF_{2\alpha}$-III-d_4 identification. Ions at 481 and 485 were selected for quantification. The dwell time for the different ions was set at 50 ms. Identification was established by taking into account both retention times and relative abundance of the ions. Concentrations were evaluated in patient samples with calibration curves calculated using peak-height ratios (analyte/standard) plotted versus concentration ratios. The degree of unlabelled $iPF_{2\alpha}$-III-d_4 was determined for each calibration and was always near 1%, i.e., a contribution of 20 pg. This contribution, as well as the basal level, were taken into account for $iPF_{2\alpha}$-III quantification.

2.5. GC–MS cross-reactivity of structurally related F_2-isoprostane compounds

A number of structurally related F_2-isoprostane compounds were analysed using GC–MS to examine the retention times in comparison with $iPF_{2\alpha}$-III. The standards of prostaglandin $F_{2\alpha}$, 15 epi-prostaglandin $F_{2\alpha}$, prostaglandin $F_{2\beta}$, 11β prostaglandin $F_{2\alpha}$, $iPF_{2\alpha}$-III, 15 epi-$iPF_{2\alpha}$-III (8-iso-15 R prostaglandin $F_{2\alpha}$) and 9β-$iPF_{2\alpha}$-III (8-iso-prostaglandin $F_{2\beta}$), $iPF_{2\alpha}$-III-d_4 were derivatized and injected into the chromatographic system.

2.6. EIA method

EIA was performed with a methodology comparable to Proudfoot et al.'s [21]. The dried samples obtained after extraction were reconstituted in Cayman's EIA buffer (1 ml). The assays are derived from a standard competition immunoassay procedure using microtiter plates pre-coated with mouse anti rabbit immunoglobulin G (IgG) (96-well plates). The samples were assayed in duplicate with two dilutions, with standards and blanks. After an incubation period of 18 h the plate was washed and the fraction bound to the antibody was reacted with Ellman's reagent which provides the substrate for acetylcholinesterase enzyme. The resultant colour reaction was read using a plate reader at 405 n*M*, the colour development being inversely proportional to the concentration of $iPF_{2\alpha}$-III measured. To optimise the assay, ultrapure water was used for all aqueous reagents and the plates were manually washed five times. The intra- and inter-assay variations for the EIA were <10%. The detection limit was 5.3 pg/ml, the IC_{50} (concentration required to inhibit initial binding by 50%) was 54.4 pg/ml.

2.7. Statistical analysis

The data were analysed by non-parametric methods to avoid assumption about the distribution of the measured variables. Correlation analysis were performed using the Spearman rank correlation test. Bland and Altman plots were constructed to analyse the agreement between the two methods [27]. Data are expressed as mean±standard error of the mean (S.E.M.). Values of $P<0.05$ were considered significant.

3. Results

GC–EI-MS spectra of TMS ester TMS ether derivatives of isoprostaglandin $F_{2\alpha}$ type III (A) and isoprostaglandin $F_{2\alpha}$ type III-d_4 (B) are presented in Fig. 3. Fig. 4 shows typical $[M-C_5H_{11}-TMSOH]^+$ ion chromatograms of $iPF_{2\alpha}$-III (m/z 481) and $iPF_{2\alpha}$-III-d_4 (m/z 485) obtained from urine extracts. The mean retention times for $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-III-d_4 were 10.77 and 10.76 min, respectively.

3.1. Linearity

The linearity of the assay was verified by spiking aliquots of human urine samples at concentrations of

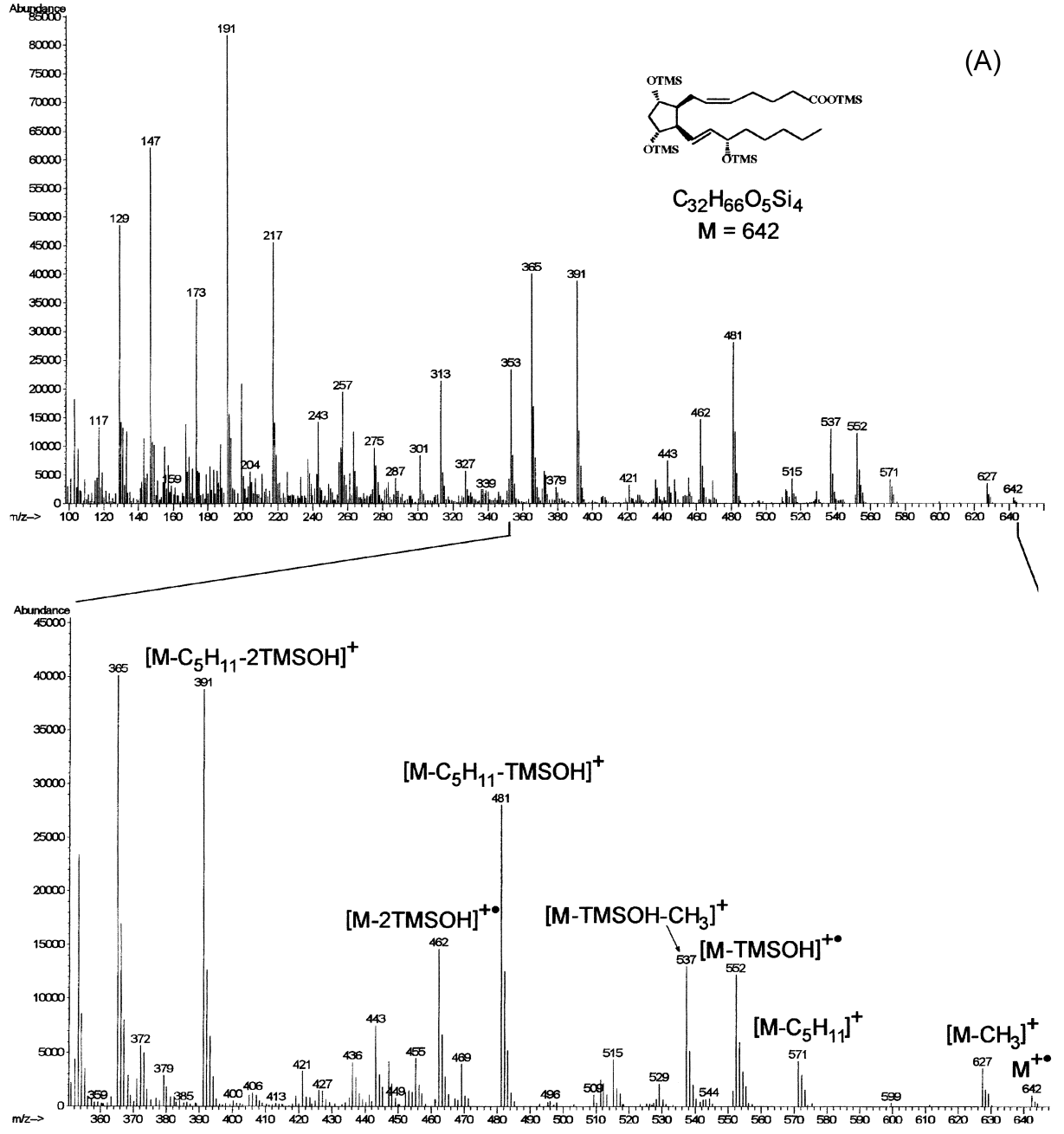

Fig. 3. GC–EI-MS spectrum of TMS ester TMS ether derivatives of isoprostaglandin $F_{2\alpha}$ type III (A) and isoprostaglandin $F_{2\alpha}$ type III-d_4 (B).

0, 40, 100, 200, 400, 600, 800 pg/ml. All samples were spiked with 2 ng of iP$F_{2\alpha}$-III-d_4. Plotting the ratio height of iP$F_{2\alpha}$-III/height of deuterated standard versus concentration ratio provides a linear relationship described by an equation of slope= 1.09±0.09 (correlation coefficient 0.996±0.001; n= 8). Intercept values (y-axis intercept) were a function of the endogenous concentration of iP$F_{2\alpha}$-III.

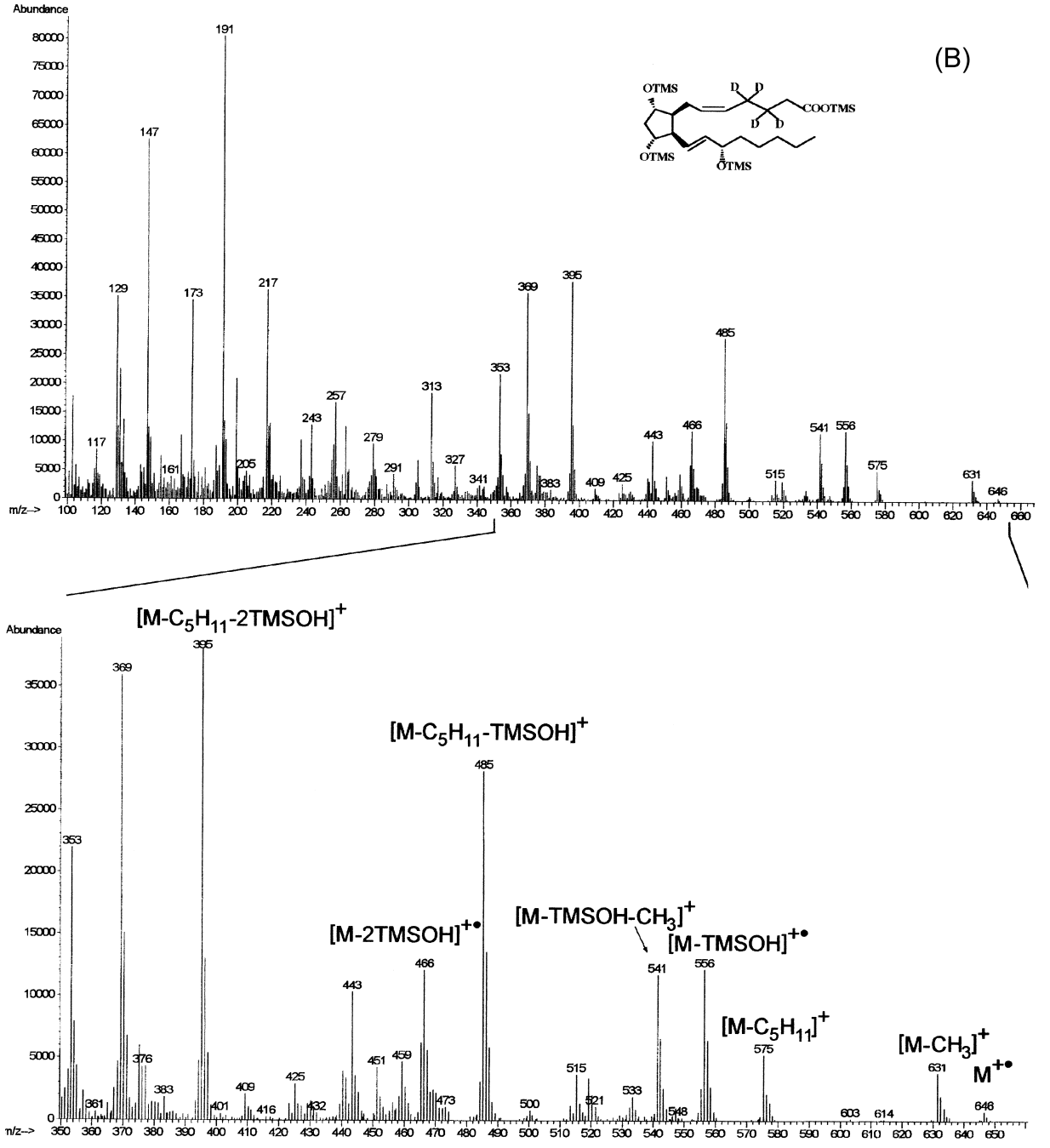

Fig. 3. (*continued*)

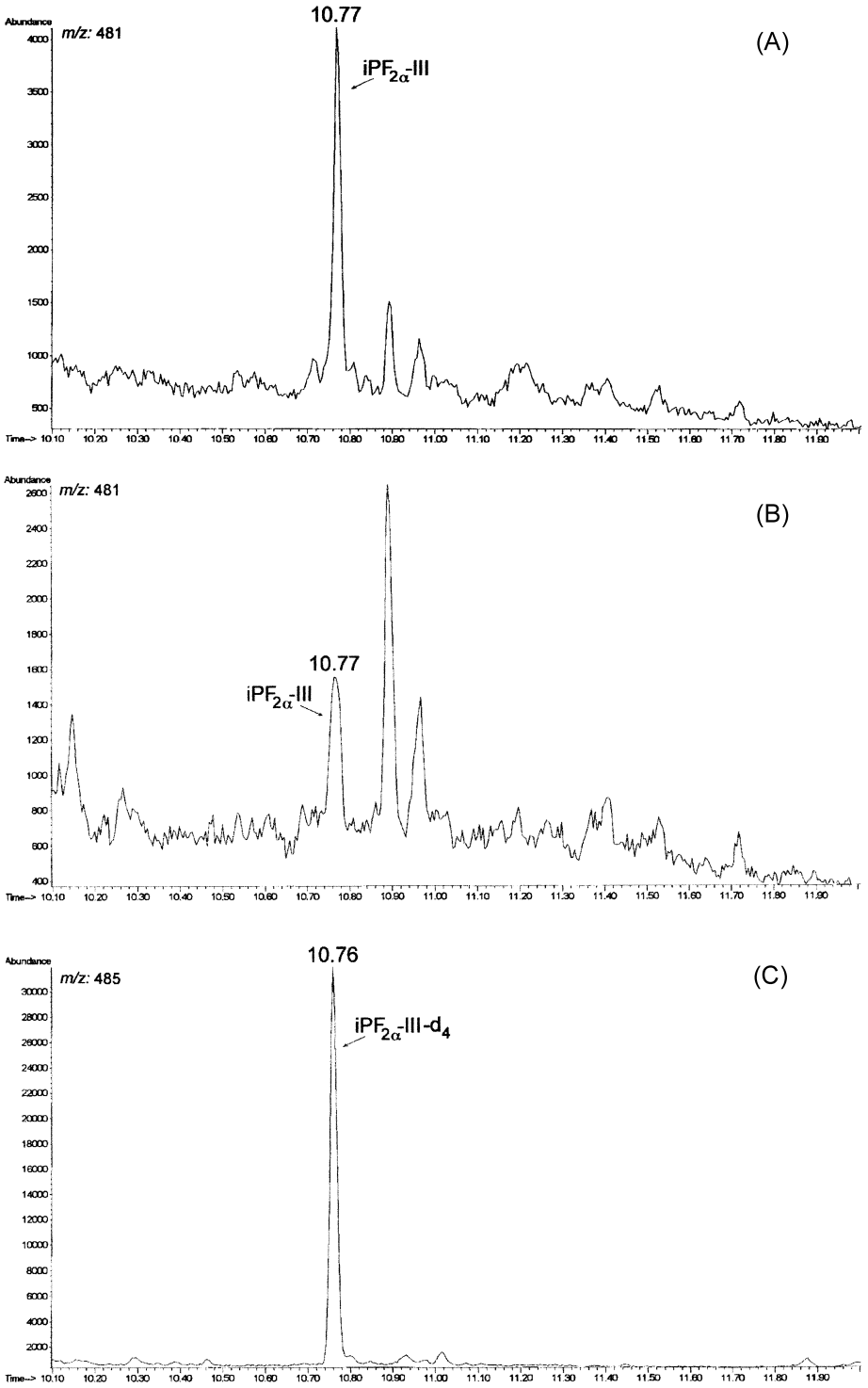

Fig. 4. 481 ion chromatograms registered the same experiment from: (A) a derivatized extract of healthy human urine spiked with 200 pg/ml of isoprostaglandin $F_{2\alpha}$ type III; (B) a urine extract of a patient. (C) 485 ion chromatogram registered from the latter extract (isoprostaglandin $F_{2\alpha}$ type III-d_4 at the concentration of 2000 pg/ml).

Linear correlation was verified for $iPF_{2\alpha}$-III in the range 0 to 6000 pg/ml in urine.

3.2. Within- and between-day precision

Within-day precision was calculated from repeated analysis of spiked urines during 1 working day, by the same operator. Between-day precision was calculated from analysis of spiked urines at the same concentration of $iPF_{2\alpha}$-III, one analysis being performed a day. The RSDs are given in Table 1. The minimum amount of analyte that we could measure accurately was 25 pg/ml of urine. Fig. 5 shows 481 ion chromatograms registered from: (A) double blank healthy human urine; (B) blank urine: the same human urine sample spiked with deuterated internal standard; (C) and blank urine spiked with isoprostaglandin $F_{2\alpha}$ type III at the concentration of 25 pg/ml. Fig. 5D shows the 485 ion chromatogram of isoprostaglandin $F_{2\alpha}$ type III-d_4.

3.3. Extraction recovery

Extraction recovery, expressed as a percentage, was defined as the ratio of calibration curve slope of extracted analyte to calibration curve slope of non-extracted analytes. In all cases, $iPF_{2\alpha}$-III-d_4 was added just before derivatization. The recovery was 72%.

3.4. Cross reactivity of structurally related F_2-isoprostane compounds

Chromatograms and retention times of $iPF_{2\alpha}$-III and structurally related F_2-isoprostanes and F_2-prostaglandins are presented in Fig. 6. Their analysis showed that the isoprostane diastereoisomers 15 epi-$iPF_{2\alpha}$ and 9β-$iPF_{2\alpha}$-III coeluted with $iPF_{2\alpha}$-III (retention time 10.77 min). The retention time of the

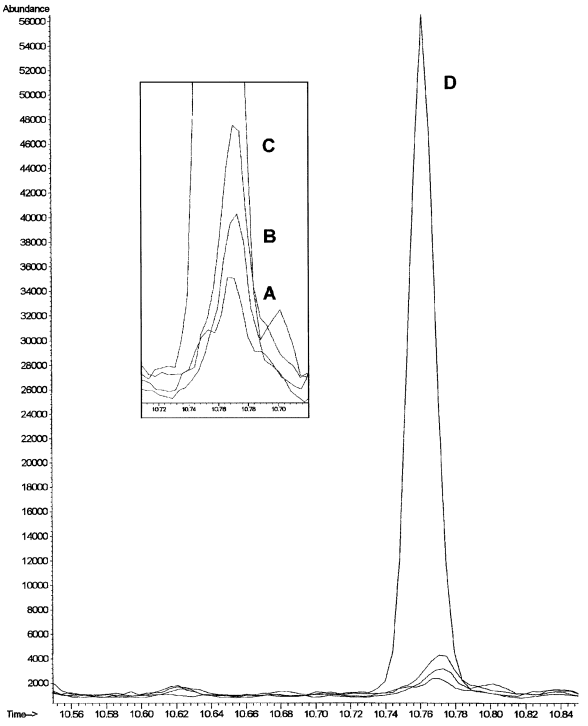

Fig. 5. 481 ion chromatogram registered from: (A) double blank healthy human urine sample of low endogenous concentration of isoprostaglandin $F_{2\alpha}$ type III (55 pg/ml) further used for calibration. No deuterated standard added; (B) blank urine: the same human urine sample spiked with deuterated internal standard at the concentration of 2000 pg/ml; (C) blank urine spiked with isoprostaglandin $F_{2\alpha}$ type III at the concentration of 25 pg/ml; (D) 485 ion chromatogram of isoprostaglandin $F_{2\alpha}$ type III-d_4 added at the concentration of 2000 pg/ml in B and C.

Table 1
Within- and between-day precisions

Spiked concentration (pg/ml)	*n*	Relative standard deviation (%)	
		Within-day precision	Between-day precision
0	8	5	2
200	8	10	5
800	8	4	4

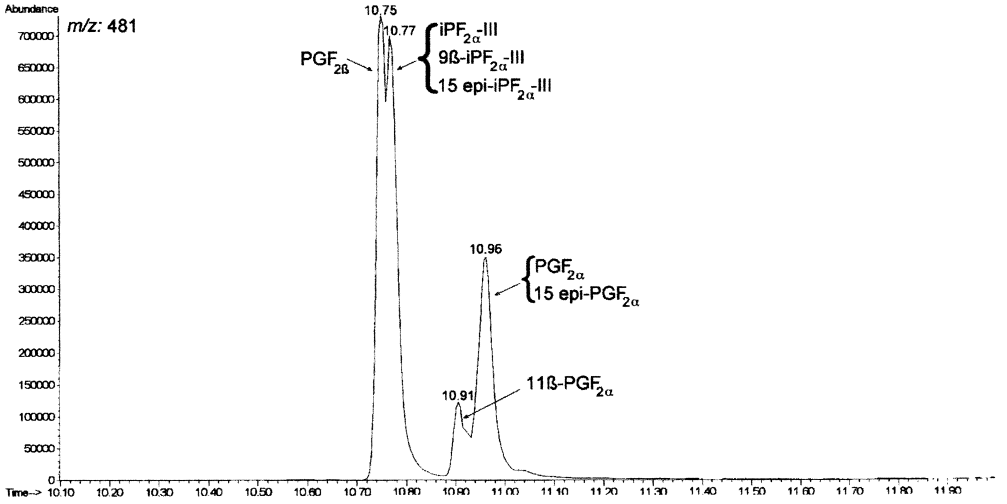

Fig. 6. Gas chromatography–mass spectrometry selected ion monitoring mode chromatograms of a mixture of standard F_2-isoprostanes and F_2 prostaglandins.

prostaglandin $F_{2\beta}$ was slightly shorter (10.75 min) than $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-III-d_4, whereas that of other F_2-prostaglandins were longer (11β prostaglandin $F_{2\alpha}$: 10.91 min; 15 epi-prostaglandin $F_{2\alpha}$ and prostaglandin $F_{2\alpha}$: 10.97 min).

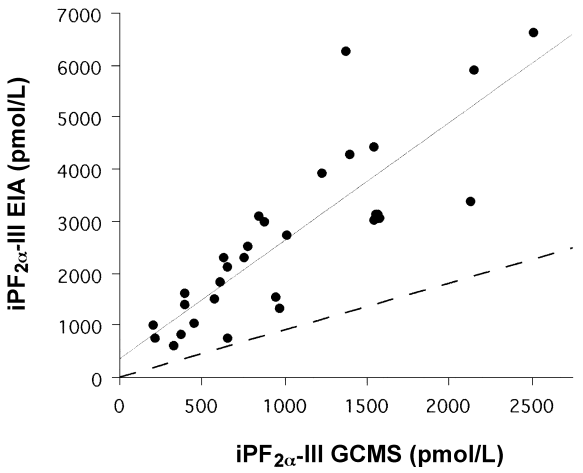

Fig. 7. Isoprostaglandin $F_{2\alpha}$ type III measurement with GC–MS and EIA method, with line of equality (dotted line) and regression line (plain line) ($r=0.863$, $P<0.001$).

3.5. Comparison of GC–MS and EIA

3.5.1. Correlation

The Spearman correlation coefficient between GC–MS and EIA measurements was 0.863 ($P<0.001$) (Fig. 7).

3.5.2. Agreement

A Bland–Altman plot was constructed to measure the agreement between both methods (Fig. 8). The EIA method gave higher values for all urines tested, with a mean difference of 1649 (1162) p*M*/l. There was a significant linear association between the differences and the mean values ($r=0.881$, $P<0.001$), suggesting the presence of proportional bias with EIA reading.

4. Discussion

In this study, MS was operated in electronic impact ionisation mode instead of negative ion chemical ionisation mode, although most studies used the latter method. GC–EI-MS benchtop systems are widely used in hospital pharmacology and biochemical laboratories to enable drugs and biological

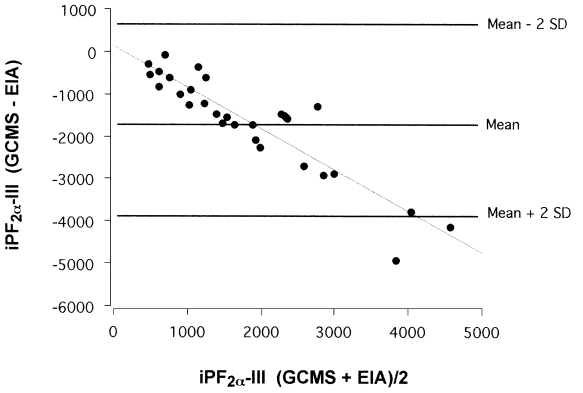

Fig. 8. Difference (GC–MS−EIA) against mean [(GC–MS−EIA)/2] for isoprostaglandin $F_{2\alpha}$ type III measurements (Bland and Altman plots), with regression line ($r=0.881$, $P<0.001$).

compounds identification and quantification. As F_2-isoprostane quantification may be of clinical interest, we aimed to develop a method of good applicability, consistent with this routine use.

In our method, isoprostanes are converted to TMS ester TMS ether derivatives. TMS derivatization is easily performed in one step with BSTFA. Derivatives are stable and can be injected without further purification. In comparison, NICI derivatization is not so easy. Generally, esterification of carboxy groups with pentafluorobenzyl (PFB) bromide precedes etherification of hydroxy groups with BSTFA to yield PFB ester TMS ether. Derivatization needs purification to eliminate side products and excess of derivatizing agent. Furthermore, NICI leads to mass spectra poor in mass fragments: an intense carboxylate anion $[M-PFB]^-$ is observed with fragments of lower intensity consecutive to the loss of trimethylsilanol (TMSOH) groups. The four isoprostanes regioisomers share the same formula and can product the same intense fragment $[M-PFB]^-$ used for quantification. This very sensitive technique can lack in selectivity and extensive purification has to be performed [13]. In contrast, EI leads to numerous fragment ions (see Fig. 3). Ions of m/z 552 and 462 corresponding to the successive elimination of molecules of TMSOH are not specific to one regioisomer. However, ions of m/z 571 are formed by cleavage of the C–C bond (Fig. 9) [26]. Loss of $^{\cdot}CH_2$-$(CH_2)_3$-CH_3 (C-16/20) from the molecular ion is specific of type III regioisomers [28]. Elimination of TMSOH

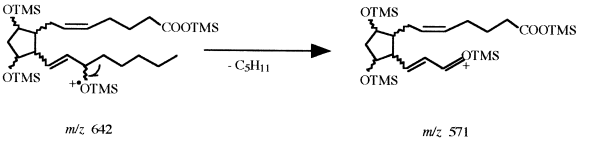

Fig. 9. Formation scheme of ion of m/z 571 from TMS derivative of isoprostaglandin $F_{2\alpha}$ type III.

from these specific ions gives abundant fragments ions of m/z 481 chosen for quantification. When authentic standards of F_2-isoprostanes of all classes are commercially available, we can hope this method will permit simultaneous analysis of the other classes by specific ion screening, especially type VI regioisomers that are the most abundant [9]. Due to the high fragmentation, EI is less sensitive than NICI, but more specific. The present method is relatively fast since it includes one step of derivatization without further thin layer purification, and allows a good SPE recovery.

We chose to quantify $iPF_{2\alpha}$-III concentrations using peak-height ratios in order to reduce interferences with prostaglandin $F_{2\beta}$, whose peak is separated but partly unresolved. All the values calculated by GC–MS were lower than those calculated by EIA, determined from an aliquot of the same extract. In contrast, Proudfoot et al. observed higher levels with GC–MS at low concentrations despite further high-performance liquid chromatography purification for the GC–MS assay [21]. This could be explained by the coelution observed between the prostaglandin $F_{2\beta}$ and the F_2-isoprostane peaks [21], minimised in our method. However, our method does not allow a separation of at least two type III F_2-isoprostane diastereoisomers (15 epi-$iPF_{2\alpha}$ and 9β-$iPF_{2\alpha}$-III).

The measurement of urinary $iPF_{2\alpha}$-III by MS is currently the reference method on account of its specificity and sensitivity. However, these methods remain expensive and their technology remains not widely available. Immunoassays have been developed to enable a larger development of F_2-isoprostane quantification [17,25]. Our study confirms the data previously shown by Proudfoot et al., that $iPF_{2\alpha}$-III concentrations determined with GC–MS do not agree with those determined with EIA. Although the correlation coefficient found was better than Proudfoot et al.'s (0.863 versus 0.628, respectively), the agreement between both methods was weak.

Immunoassays have not been checked for cross reactivity with type IV, V and VI regioisomers, and for many type III diastereoisomers. The proportional bias observed in the present study suggests that both methods do not measure the same compounds. It is possible that some of the 64 F_2-isoprostanes isomers have high cross reactivity with the EIA antibody, but are not present in the GC–MS peak measured. Conversely, some type III diastereoisomers measured in GC–MS may not cross react with the EIA antibody. Furthermore, 2,3 dinor $iPF_{2\alpha}$-III metabolites cross reactivity has not been checked, whereas these compounds will not interfere with the GC–MS method. As a consequence, we believe that comparison of clinical data using GC–MS and EIA should be avoided.

In conclusion, we have developed electronic impact GC–MS that enables the quantification of type III F_2-isoprostanes. Our results show that GC–MS and EIA do not measure the same compounds. As a consequence, comparison of clinical results using GC–MS and EIA should be made with caution.

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