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Tandem mass spectrometric quantification of 8-iso-prostaglandin $F_{2\alpha}$ and its metabolite 2,3-dinor-5,6-dihydro-8-iso-prostaglandin $F_{2\alpha}$ in human urine

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Abstract

Whole body synthesis of F_2-isoprostanes, a family of cyclooxygenase-independent eicosanoids formed by free-radical catalysed peroxidation, should be best assessed by quantifying their urinary metabolites. Two methods for the quantitative determination of F_2-isoprostane metabolites in human urine performing either thin-layer chromatography (TLC) (method A) or high-performance liquid chromatography (HPLC) (method B) prior to GC–tandem MS are described. Method A allows for simultaneous quantification of 8-iso-$PGF_{2\alpha}$, one prominent member of the F_2-isoprostane family, and its major urinary metabolite, 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. Mean excretion was found to be 223 and 506 pg/mg creatinine of 8-iso-$PGF_{2\alpha}$ and 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$, respectively ($n=14$). A tight correlation existed between the urinary excretion of these two isoprostanes ($r=0.86$). Method B enables quantification of dinor-dihydro metabolites of various F_2-isoprostanes including 8-iso-$PGF_{2\alpha}$. 2,3-Dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ was found to be an abundant dinor-dihydro F_2-isoprostane metabolite. Validity of method A was proven by a combination of HPLC with TLC prior to GC–tandem MS analysis. A correlation was observed between the urinary concentrations of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ measured by GC–MS and GC–tandem MS ($r=0.84$).

Keywords: 8-Isoprostaglandin $F_{2\alpha}$; 2,3-Dinor-5,6-dihydro-8-isoprostaglandin $F_{2\alpha}$

1. Introduction

Nonenzymatic, free radical-catalysed peroxidation of esterified arachidonic acid results in the formation of isoprostanes which are isomers of the cyclo-oxygenase-dependent prostanoids (reviewed in Ref. [1]). The isoprostane family includes F_2-, D_2-, E_2-isoprostanes and isothromboxanes, F_2-isoprostanes being the most abundant among these isoeicosanoids [2–4]. The mechanisms responsible for the formation of the different classes of isoprostanes are still unresolved [5]. 8-iso-Prostaglandin (PG) $F_{2\alpha}$ is one abundant F_2-isoprostane which is excreted in the urine of humans [6]. The measurement of 8-iso-$PGF_{2\alpha}$ or of total F_2-isoprostanes in human urine has

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been established as a reliable method to assess non-invasively lipid peroxidation in vivo [7–9].

Systemically administered 8-iso-$PGF_{2\alpha}$ has been shown to undergo extensive metabolism in man [10]. The most abundant urinary metabolites of 8-iso-$PGF_{2\alpha}$ have been identified as 2,3-dinor-8-iso-$PGF_{2\alpha}$ and 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ (Fig. 1) [11]. Basal excretion of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ into the urine of humans has been recently shown by gas chromatography–mass spectrometry (GC–MS) [12,13]. The measurement of urinary excretion of metabolites of prostanoids has been established as a non-invasive method to assess the whole body synthesis of cyclooxygenase-derived prostanoids in vivo [14]. For some prostanoids such as PGE_2 and thromboxane (Tx) B_2, an additional measurement of the primary molecules in human urine has been shown to be useful to assess their formation in the kidney [15]. Analogous to the cyclooxygenase-dependent prostanoids, it can be expected that a measurement of the primary isoprostanes and their corresponding metabolites would allow for the assessment of renal and systemic formation in humans. The correlation between the primary isoprostane and its metabolite could furthermore enhance knowledge about the metabolic fate of isoprostanes. Such data are not available to date. Therefore, the aim of the present work was to develop a method useful for the quantification both of 8-iso-$PGF_{2\alpha}$ and its major urinary metabolite 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ in human urine.

GC–MS and GC–tandem MS in the negative-ion chemical ionization (NICI) mode have been shown to be the most reliable analytical approaches to assess isoprostanes formation in vivo in humans [9].

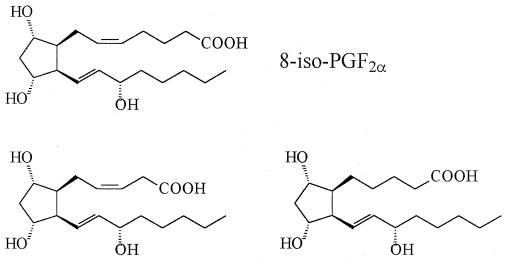

Fig. 1. Chemical structures of 8-iso-$PGF_{2\alpha}$, 2,3-dinor-8-iso-$PGF_{2\alpha}$ and 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$.

We have recently shown that GC–tandem MS enables specific quantification of 8-iso-$PGF_{2\alpha}$ in human urine with a minimum of labour involved requiring a single thin-layer chromatographic (TLC) step for sample purification [16]. We extended this method for the specific quantification of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ in human urine. For this purpose, we synthesised a dioxygen-18 labelled analogue starting from newly synthesised *ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. We show that this method allows for rapid and specific quantification of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ in human urine samples. In addition, the use of high-performance liquid chromatography (HPLC) instead of TLC enables quantification by GC–tandem MS of several dinor-dihydro F_2-isoprostane metabolites including 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ in human urine.

2. Experimental

2.1. Chemicals and materials

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 2,3-dinor-5,6-dihydro-$PGF_{2\alpha}$ (2,3-dinor-$PGF_{1\alpha}$), and [3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$ (98 atom% ^2H) were obtained from Cayman (Ann Arbor, MI, USA). Pentafluorobenzyl (PFB) bromide, 4-fluorobenzylbromide, and *N*,*N*-diisopropylethylamine were obtained from Aldrich (Steinheim, Germany). *N*,*O*-bis(Trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce (Rockford, IL, USA). $H_2^{18}O$ (99 atom% ^{18}O) was supplied by Campro Scientific (Berlin, Germany). Porcine liver esterase (PLE, EC 3.1.1.1) provided as a suspension in 3.2 *M* ammonium sulfate, pH 8, was obtained from Sigma (Deisenhofen, Germany). Acetonitrile of HPLC grade, all other chemicals, and 20×20 cm silica gel 60 plates were from Merck (Darmstadt, Germany). Octadecyl silica (ODS) cartridges (500 mg) were purchased from Macherey–Nagel (Düren, Germany).

2.2. Chemical synthesis of ent-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$

The total synthesis of the enantiomer of (15*S*)-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ was carried out as previously described [17]. After methylation of the

carboxyl group with diazomethane, the 15*R*-epimer was separated from the 15*S*-epimer by flash chromatography on silica gel using cyclohexane–ethyl acetate (30:70, v/v) as solvent. Relative stereochemical assignments were made by Nuclear Overhauser Effect (NOE) ^{1}H-nuclear magnetic resonance (NMR) spectroscopy.

2.3. Enzymatic synthesis of [1,1′-$^{18}O_2$]-ent-2,3-dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$

[1,1′-$^{18}O_2$]-*ent*-2,3-Dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$ was synthesised by a similar procedure previously described for the enzymatic labelling of 8-iso-PGF$_{2\alpha}$ [18]. Briefly, *ent*-2,3-dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$ (200 μg) was converted into its methyl ester using freshly prepared diazomethane. A PLE suspension (1000 units) was dried in vacuum and the residue was redissolved in 200 μl of $H_2^{18}O$. This solution was used to reconstitute the methyl ester of *ent*-2,3-dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$ and reaction was started by adjustment of the pH to 7.5. After 30 min of incubation at 37°C reaction products were solid-phase extracted. A stock solution of ^{18}O-labelled *ent*-2,3-dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$ was prepared in acetonitrile and stored at −78°C. Standard curves were generated by GC–MS and GC–tandem MS analysis of the PFB-trimethylsilyl (TMS) derivatives of mixtures, each containing 10 ng of [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$ and various amounts of *ent*-2,3-dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$ (0, 1, 2, 5, 10 and 20 ng, $n=2$).

2.4. Biological samples

Urine from spontaneous micturition by healthy adults was collected in polypropylene bottles, containing 1 m*M* each of 5-hydroxy-tempo (HTMP) and ethylenediaminetetraacetic acid (EDTA). Urine samples were divided into 5-ml aliquots and stored at −20°C until use. Creatinine was determined spectrophotometrically by an assay based on the alkaline picric acid reaction with an automatic analyser (Beckman, Galway, Ireland) [19].

2.5. Extractions and derivatization procedures

[3,3′,4,4′-$^{2}H_4$]-8-iso-PGF$_{2\alpha}$ and [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$ were added for use as internal standards to 5-ml aliquots of human urine samples resulting in final concentrations of 1 ng/ml and 2 ng/ml, respectively. Solid-phase extraction (SPE) and all derivatization steps were performed as described elsewhere for 8-iso-PGF$_{2\alpha}$ [16]. Briefly, urine samples were acidified to pH 3 and applied to ODS cartridges preconditioned with 10 ml of methanol and 3 ml of 0.05 *M* HCOOH. Cartridges were washed with 20 ml of water and 2.5 ml of heptane, and compounds were eluted with 2 ml of ethyl acetate. Analytes were converted to their PFB ester TMS ether derivatives using PFB bromide and BSTFA (100 μl) by standard derivatization procedures [16].

2.6. Thin-layer chromatography

The residues containing the PFB esters were reconstituted in ethanol (15 μl) and chromatographed on 20×20 cm silica gel plates. A TLC-applicator AS 30 and a DC-MAT from Desaga (Wiesloch, Germany) were used. Ethyl acetate–methanol (98:2, v/v) was the used eluent. Reference compounds in TLC were PGF$_{2\alpha}$-4-fluorobenzyl ester (PGF$_{2\alpha}$-4FB) ($R_F=0.31\pm0.01$, mean±SD, $n=5$) for the 8-iso-PGF$_{2\alpha}$-PFB derivative and PGF$_{2\alpha}$-methylester ($R_F=0.26\pm0.01$) for the (*ent*-)2,3-dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$-PFB derivatives. Reference compounds and derivatives of the analytes exhibited identical TLC properties, i.e., R_F values, under the conditions used. PGF$_{2\alpha}$-4FB was obtained by reaction of PGF$_{2\alpha}$ with 4-fluorobenzyl bromide similar to the PFB esterification with PFB bromide [16]. PGF$_{2\alpha}$ was converted to its methylester using a freshly prepared etheral diazomethane solution. Derivatives of the reference compounds (each at 1 μg) were spotted on a separate lane of the TLC plate and developed alongside the PFB ester derivatives of the analytes. PGF$_{2\alpha}$-4FB and PGF$_{2\alpha}$ methyl ester were used instead of 8-iso-PGF$_{2\alpha}$-PFB and 2,3-dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$-PFB, respectively, in order to avoid any contamination of urinary samples by these compounds during TLC. Each 0.6-cm band, centred around the reference compounds, were scraped off the TLC plate, compounds were extracted with ethanol (500 μl) and suspensions were centrifuged (4000 *g*, 10 min). Supernatants were decanted, ethanol was removed under nitrogen, and the PFB esters were converted to their TMS ether derivatives.

2.7. High-performance liquid chromatography

Reversed-phase high-performance liquid chromatography (HPLC) of isoprostanes was performed using a Hewlett-Packard series 1050 system (Waldbronn, Germany) equipped with a column (250×4.6 mm I.D.) packed with 100-5C_{18} Nucleosil, 5-μm particle size, from Macherey–Nagel. PFB esters of isoprostanes were analysed isocratically using a mobile phase consisting of water–acetonitrile (50:50, v/v). The flow-rate was 2 ml/min and the effluent was detected at 235 nm. The following retention times (in min) were obtained from separate analyses of 1-μg amounts of PFB esters of each isoprostane: 6.02±0.05 for 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$-PFB and 12.08±0.1 for 8-iso-$PGF_{2\alpha}$-PFB (mean±SD, n=5). For quantitative measurements using HPLC for sample purification, the following procedure was used: urine samples were subjected to SPE and PFB esterification as described. The residue containing the PFB esters was diluted in a 100-μl aliquot of the mobile phase and subjected to HPLC analysis. A 2-ml fraction at the retention time of synthetic 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$-PFB was collected and the analyte was extracted twice with 2 ml ethyl acetate.

2.8. Experiments on precision, accuracy and validation of the methods

Inter-day precision of the methods for 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ was assessed by analysing in duplicate four identical, unspiked urine samples (5 ml) on consecutive days. Intra-day precision was determined by analysing in duplicate urine samples (5 ml) collected unspiked from two volunteers. Similarly performed studies on 8-iso-$PGF_{2\alpha}$ confirmed previous data [16]. Instrumental precision was determined by repeated GC–tandem MS analysis (n=5) of a routinely worked-up unspiked urine sample.

Validation was carried out as follows. Pooled urine from a healthy volunteer was spiked with [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ (2 ng/ml), which served as the internal standard, and divided into 5-ml aliquots. To each 5-ml aliquot different amounts of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ were added (0, 0.1, 0.2, 0.5, 1 and 2 ng/ml). Urine samples were subjected to SPE as described above and PFB esterification was performed. Samples were subjected to TLC (method A) or to HPLC (method B) analysis. Quantification was performed by GC–tandem MS.

2.9. GC–MS and GC–tandem MS

GC–MS and GC–tandem MS analyses were performed on a Thermoquest TSQ 7000 triple-stage quadrupole mass spectrometer interfaced with a Thermoquest gas chromatograph Model Trace 2000 (Egelsbach, Germany). A fused-silica capillary column Optima 17 (30 m×0.25 mm I.D., 0.25 μm film thickness) from Macherey–Nagel was used. Helium was used as a carrier gas at a constant pressure of 55 kPa. For NICI methane was used as a reagent gas at a pressure of 65 Pa. Argon was used for collisionally-activated dissociation (CAD) at a pressure of 0.15 Pa. The collision energy was set to 25 eV. Electron energy was 200 eV and the emission current 600 μA. Injector, interface and ion source were kept at 280°C, 290°C and 180°C, respectively. The column was held at 70°C for 2 min, then programmed to 280°C at 25°C/min followed to 320°C at 4°C/min. The product ions $[M\text{-}PFB\text{-}3\times TMSOH]^-$ at m/z 273 for endogenous 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and m/z 277 for the ^{18}O-labelled analogue (see Results) were monitored in the selected reaction monitoring (SRM) mode; they were generated by CAD of the parent ions $[M\text{-}PFB]^-$ at m/z 543 and 547, respectively. 8-iso-$PGF_{2\alpha}$ and its tetradeuterated analogue were quantified by SRM as described previously [16]. Electron impact (EI) ionization was performed at 70 eV. Aliquots (1 μl) were injected into the GC–tandem MS instrument in the splitless mode.

3. Results

3.1. GC–MS and GC–tandem MS analyses

Structure elucidation of ^{18}O-labelled *ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ was performed by GC–MS analysis of the methyl ester (Me)-TMS derivatives of the labelled and unlabelled compounds in the EI mode (Fig. 2). From the GC peak with the retention time of authentic 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ we recorded the mass spectrum of ^{18}O-

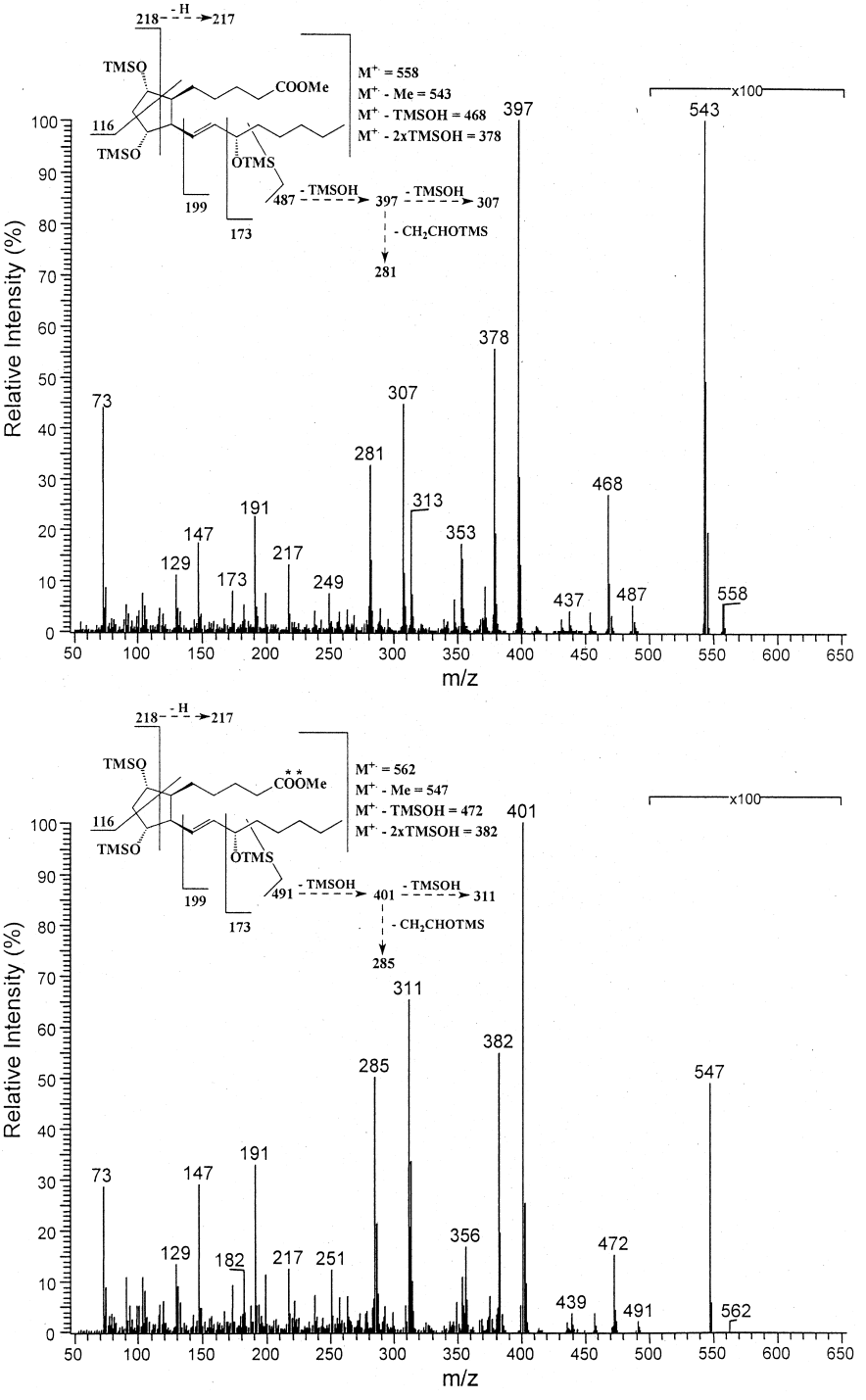

Fig. 2. EI mass spectra of the Me-TMS derivatives of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ (upper panel) and [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ (lower panel). Asterisks indicate the ^{18}O-label. The m/z range above 500 is amplified by a factor of 100.

labelled *ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ shown in Fig. 2 (lower panel). Differences each of 4 Da were observed for the molecule cations M^+ (*m/z* 558 and 562) and for the mass fragments $[M\text{-}90]^+$ (*m/z* 468 and 472), $[M\text{-}90\text{-}71]^+$ (*m/z* 397 and 401), $[M\text{-}2\times90]^+$ (*m/z* 378 and 382), and $[M\text{-}2\times90\text{-}71]^+$ (*m/z* 307 and 311) as well as for the ions at *m/z* 281 and 285 (see insertions in Fig. 2). These mass spectra clearly identify the ^{18}O-labelled *ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ as [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ produced by enzymatic incorporation of two ^{18}O atoms from ^{18}O-labelled water into the carboxylic group of the unlabelled molecule.

Sensitive quantification of prostanoids and isoprostanes in biological fluids by GC–MS and GC–tandem MS is accomplished in the NICI mode [20]. The GC–MS and GC–tandem MS properties of the PFB-TMS derivatives of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$, *ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$ and their stable-isotope labelled analogs were therefore investigated under NICI conditions (Table 1). In accordance with previously published data, NICI mass spectra of the PFB-TMS derivatives of 8-iso-$PGF_{2\alpha}$ and [$^2H_4$]-8-iso-$PGF_{2\alpha}$ showed prominent ions at *m/z* 569 and 573, respectively, which correspond to the $[M\text{-}PFB]^-$ ions [21]. The PFB-TMS derivative of [1,1′-$^{18}O_2$]-8-iso-$PGF_{2\alpha}$ ionised similarly yielding a prominent ion at *m/z* 573 ($[M\text{-}PFB]^-$) which is increased by 4 Da compared to that of 8-iso-$PGF_{2\alpha}$ [18]. The PFB-TMS derivatives of the dinor-dihydro compounds showed similar fragmentation patterns, i.e., *m/z* 543 for (*ent*-)2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and *m/z* 547 for [1,1-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$, both corresponding to the $[M\text{-}PFB]^-$ ions. The PFB-TMS derivatives of *ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ coeluted on the GC capillary column and generated virtually identical NICI mass spectra.

The parent ions $[P]^-$, i.e., $[M\text{-}PFB]^-$, of the compounds investigated were subjected to CAD. Table 1 summarises the most prominent product ions observed. The most intense product ions of the PFB-TMS derivatives of 8-iso-$PGF_{2\alpha}$ and [$^2H_4$]-8-iso-$PGF_{2\alpha}$ were *m/z* 299 and *m/z* 303, respectively, both corresponding to $[P\text{-}3\times TMSOH]^-$. [1,1′-$^{18}O_2$]-8-iso-$PGF_{2\alpha}$ exhibited a different CAD fragmentation pattern in comparison with [$^2H_4$]-8-iso-$PGF_{2\alpha}$: there were observed product ions from the consecu-

Table 1
Major mass fragments in the NICI mass spectra of the PFB-TMS derivatives of unlabelled and labelled isoprostanes[a]

Ion assignment\compound	8-Iso-$PGF_{2\alpha}$	[$^2H_4$]-8-Iso-$PGF_{2\alpha}$	[$^{18}O_2$]-8-Iso-$PGF_{2\alpha}$	2,3-Dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$	*ent*-2,3-Dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$	[$^{18}O_2$]-*ent*-2,3-Dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$
GC–MS mass spectra						
$[M\text{-}PFB]^-$	569 (100)	573 (100)	573 (100)	543 (100)	543 (100)	547 (100)
$[M\text{-}PFB\text{-}TMSOH]^-$	479 (15)	483 (15)	483 (14)	453 (4)	453 (3)	457 (5)
$[M\text{-}PFB\text{-}TMSOH\text{-}(CH_3)_2Si{=}CH_2]^-$	407 (12)	411 (13)	411 (12)	381 (6)	381 (5)	385 (5)
GC–tandem MS mass spectra						
$[P]^-$	569 (53)	573 (44)	573 (80)	543 (8)	543 (10)	547 (14)
$[P\text{-}TMSOH]^-$	479 (8)	483 (11)	483 (6)	453 (5)	453 (4)	457 (1)
$[P\text{-}TMS^{18}OH]^-$			481 (8)			455 (7)
$[P\text{-}2\times TMSOH]^-$	389 (12)	393 (12)	393 (11)	363 (4)	363 (5)	367 (5)
$[P\text{-}TMSOH\text{-}TMS^{18}OH]^-$			391 (6)			365 (3)
$[P\text{-}2\times TMSOH\text{-}(CH_3)_2Si{=}CH_2]^-$	317 (14)	321 (17)	321 (19)	291 (12)	291 (14)	295 (15)
$[P\text{-}TMSOH\text{-}TMS^{18}OH\text{-}(CH_3)_2Si{=}CH_2]^-$			319 (5)			293 (6)
$[P\text{-}3\times TMSOH]^-$	299 (100)	303 (100)	303 (68)	273 (100)	273 (100)	277 (45)
$[P\text{-}2\times TMSOH\text{-}TMS^{18}OH]^-$			301 (100)			275 (100)
$[P\text{-}2\times TMSOH\text{-}(CH_3)_2Si{=}CH_2\text{-}CO_2/C^{18}O_2]^-$	273 (37)	277 (20)	273 (52)	247 (22)	247 (21)	247 (25)
$[P\text{-}2\times TMSOH\text{-}CO_2/C^{18}O_2]^-$	255 (62)	259 (52)	255 (100)	229 (35)	229 (31)	229 (50)

[a] Major mass fragments (intensity is given in parentheses) in the GC–NICI mass spectra of the PFB-TMS derivatives of unlabelled and labelled isoprostanes. The ions $[M\text{-}PFB]^-$, i.e., $[P]^-$, were subjected to CAD.

tive loss both of TMSOH (90 Da) and TMS^{18}OH (92 Da). The most intense product ions of [1,1′-$^{18}O_2$]-8-iso-$PGF_{2\alpha}$ were observed at m/z 303 [P-3×TMSOH]$^-$ and 301 [P-2×TMSOH-1×TMS^{18}OH]$^-$. CAD of the ions [M-PFB]$^-$ of the unlabelled and labelled dinor-dihydro compounds showed similar CAD fragmentation pattern. The most intense product ions were observed at m/z 273 [P-3×TMSOH]$^-$ for unlabelled (*ent*-)2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ but at m/z 275 [P-2×TMSOH-1×TMS^{18}OH]$^-$ and 277 [P-3×TMSOH]$^-$ for [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. These observations suggest that a rearrangement of the TMS group from the hydroxyl groups to the carboxylic group occurred during the CAD process. Similar results were also observed from the CAD of the parent ions [M-PFB]$^-$ of the PFB-TMS derivatives of other ^{18}O-labelled eicosanoids (unpublished data). The PFB-TMS derivatives of *ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ generated virtually identical daughter mass spectra and were co-chromatographed under the GC conditions used.

3.2. Standardisation of [1,1′-$^{18}O_2$]-ent-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$

The concentration of [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ in the stock solution was determined by using an accurately weighed amount of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. After complete derivatization, selected ion monitoring (SIM) of m/z 547 and m/z 543 revealed a final yield of 68% for the enzymatic labelling. Isotopic purity of [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ was determined by SIM of m/z 543 for the unlabelled compound, m/z 545 for the simply ^{18}O-labelled compound, and m/z 547 for the double ^{18}O-labelled compound. Only 0.1% of the starting material was found to remain unlabelled. Thus, the internal standard contributed to endogenous 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ by only 2 pg/ml of urine. 5.3% and 94.6% of the labelled compound were found to contain one and two ^{18}O atoms, respectively.

The usefulness of [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ as an internal standard in GC–MS and GC–tandem MS was investigated as follows. Various amounts of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ were mixed with a fixed amount of [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and SIM of m/z 543 and 547 or SRM of m/z 273 and 277 for the unlabelled and labelled compound was performed. Linear regression analysis of the peak area ratio of m/z 543 to 547 (y) and the amount ratio of unlabelled to labelled compound added (x) revealed the regression equation $y=0.001+0.977x$ ($r=0.999$). Linear regression analysis of the peak area ratio of m/z 273 to 277 (y) and the amount ratio of unlabelled to labelled compound added (x) revealed the regression equation $y=0.005+3.984x$ ($r=0.999$). The slope of the regression equation from SRM is increased by a factor of 4.08 compared with the slope of the regression equation from SIM. The slope of 0.977 from SIM proves the expected concentration of [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ in the stock solution to be true. In quantitative measurements, the peak area ratio of m/z 543 to 547 measured was multiplied with the concentration of [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ added to urine to obtain the concentration of the endogenous compound. Loss of TMS^{18}OH from [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ during the CAD process (Table 1) resulted in a higher slope from SRM of m/z 273 to 277. In quantitative measurements, the peak area ratio of m/z 273 to 277 measured was multiplied with the concentration of [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ added and divided by 4.08 to obtain the concentration of the endogenous compound.

3.3. Precision, accuracy and validation of the method

Precision and accuracy were determined for methods A and B using the same pooled urine by adding different amounts of exogenous 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ ($n=2$). Basal concentrations were 235 pg/ml for 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ (method A) and 681 pg/ml for F_2-isoprostane metabolites (method B). The data of Table 2 indicate that both methods are characterised by good precision and accuracy. Mean accuracy was higher for method A that involves TLC for sample purification.

Table 2
Precision and accuracy of the methods[a]

2,3-Dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ added[b]	Method A		Method B	
	2,3-Dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ measured minus the basal level[c]	RSD/accuracy (%)	2,3-Dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ measured minus the basal level[c]	RSD/accuracy (%)
0	0	1.0/n.a.[d]	0	1.7/n.a.
100	108	6.0/92	136	3.7/64
200	179	3.9/89	168	0.6/84
500	504	4.8/99	553	6.6/89
1000	1074	2.4/93	1005	1.9/99
2000	2086	1.7/96	2129	0.4/94
Mean±SD of RSD (%)		3.3±1.8		2.5±2.2
Mean±SD of accuracy (%)		94±3		86±12

[a] Mean concentrations, SD, RSD and accuracy were calculated as a measure of intra-assay reproducibility and validity of the method after subtraction of the respective basal concentrations.
[b] All concentrations are given in pg/ml.
[c] Mean basal levels were 235 pg/ml in method A for 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and 681 pg/ml in method B for F_2-isoprostane metabolites.
[d] n.a., not applicable.

Intra- and inter-day reproducibility were found to be 4 and 6% for method A and 3 and 6% for method B, respectively. Instrumental precision was 1.2% for endogenous urinary 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. The limit of detection (LOD) was determined as 50 fg of authentic 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ at a signal-to-noise ratio of 20:1 by injecting 1 μl of a solution containing 50 fg/μl of this compound as the PFB-TMS derivative in BSTFA. On the basis of quantitative recovery this LOD corresponds to a theoretical concentration of 1 pg/ml of urine for endogenous 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$.

The validity of the methods for urine samples was further tested quantifying 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ by GC–tandem MS in samples from 14 healthy humans by method A (only TLC), method B (only HPLC) and by combination of HPLC with TLC. Fig. 3 shows the respective chromatograms obtained from the GC–tandem MS analysis of one urine sample. The peak area ratio of *m/z* 273 to 277 was 0.928 for method A (Fig. 3A). The peak area ratio of *m/z* 273 to 277 remained unchanged for the same urine sample when HPLC was combined with TLC, i.e., 0.924 (Fig. 3C). Similar results have been obtained for all other urine samples (data not shown). Equal chromatographic properties have been reported for 8-iso-$PGF_{2\alpha}$ [16]. Fig. 3B shows that the GC peak with the retention time of endogenous 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ is considerably broader than that of Fig. 3A and C. The peak area ratio of *m/z* 273 to 277 was significantly higher, i.e., 2.894. Urinary excretion rates of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ determined by methods A and B are summarised in Table 3. These data indicate that method A allows for the specific quantification of urinary 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ by GC–tandem MS. In contrast, method B, i.e., the use of HPLC alone, revealed values which are three-fold higher than those obtained by method A or by a combination of HPLC and TLC. These data strongly suggest that dinor-dihydro metabolites of other F_2-isoprostanes coelute both in HPLC and GC with 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. Thus, method A suffices for the accurate quantification of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and method B allows for quantification of several dinor-dihydro metabolites of F_2-isoprostanes.

3.4. Simultaneous determination of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$

Our previous work [16] and the results described above show that TLC alone allows for the specific

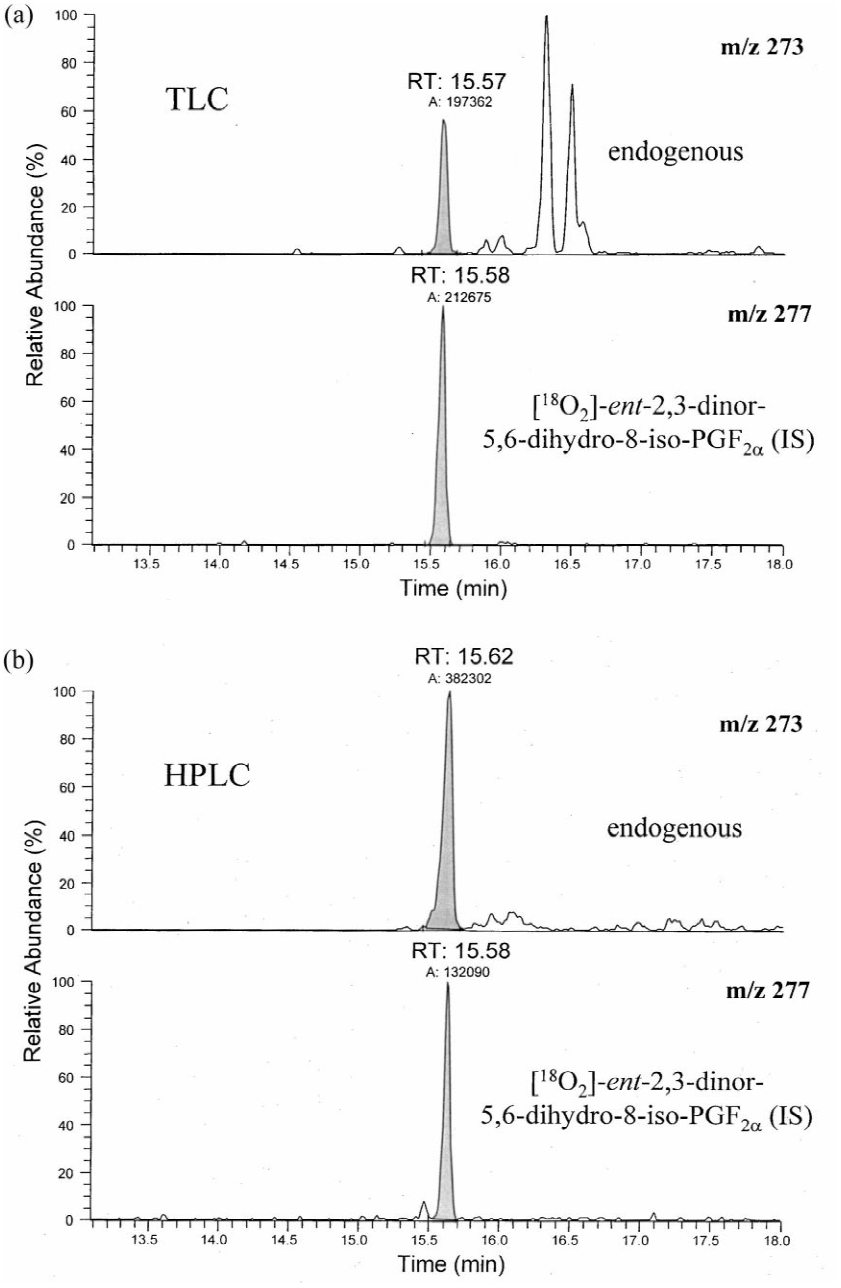

Fig. 3. Partial chromatograms from the GC–tandem MS analysis: typical chromatograms obtained in the SRM mode at m/z 273 for endogenous 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and at m/z 277 for the internal standard [1,1′-$^{18}O_2$]-*ent*-2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. Chromatograms from the GC–tandem MS analysis are shown (A) after TLC (upper panel), (B) after HPLC (middle panel), and (C) after HPLC followed by TLC (lower panel).

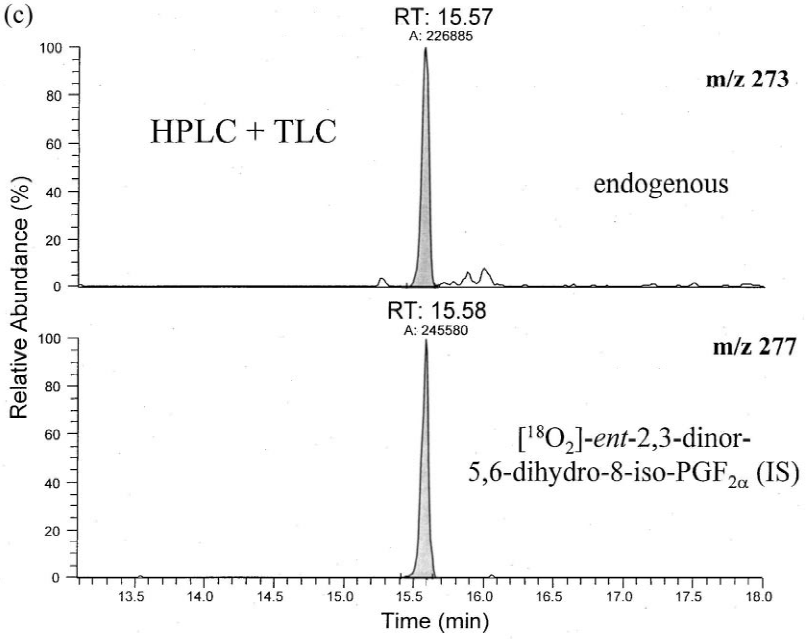

Fig. 3. (*continued*)

Table 3
Creatinine-corrected excretion rates of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and dinor-dihydro F_2-isoprostane metabolites in 14 healthy volunteers measured by GC–tandem MS succeeding method A or B

Subject		TLC 2,3-Dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ (pg/mg creatinine)	HPLC F_2-isoprostane metabolites (pg/mg creatinine)
Sex	Age (years)		
f	31	273	821
f	44	674	1760
f	35	451	1262
f	27	452	567
f	27	456	1421
f	23	409	2961
m	33	328	951
m	28	788	2920
m	35	152	696
m	38	438	2674
m	28	1166	1875
f	29	488	1244
m	28	759	1766
f	28	245	450
Mean	31	506	1526
SD	5	255	816
RSD	17	50	53

quantification of the 8-iso-$PGF_{2\alpha}$ and its major urinary metabolite 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ by GC–tandem MS. We therefore measured both compounds in a single human urine sample using TLC and GC–tandem MS. In 14 healthy volunteers, mean values of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ were higher than those of 8-iso-$PGF_{2\alpha}$, i.e., 506±255 vs. 223±135 pg/mg creatinine. A tight correlation (r=0.86) between the urinary excretion of these isoprostanes was observed (Fig. 4). Determination of 8-iso-$PGF_{2\alpha}$ and 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ was accompanied by quality control samples (n=3). Pooled unspiked urine (5 ml) was analysed in duplicate alongside with the individual urine samples. Relative standard deviation (RSD) was calculated as a measure of intra-assay reproducibility: 3.4 and 2% for 8-iso-$PGF_{2\alpha}$ and 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$, respectively.

3.5. Comparison of GC–MS with GC–tandem MS

Method A was applied to quantitate 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ by GC–MS (SIM of m/z 543 and 547) and GC–tandem MS (SRM of m/z 273 and 277). Urinary 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ was determined as 529±315 pg/mg creatinine by GC–MS and 506±255 pg/mg creatinine by GC–tandem MS (Fig. 5). A tight correlation (r=0.84) was found. However, some values from GC–MS analyses greatly differed from the respective GC–tandem MS data (Fig. 5). The ratio of urinary 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$levels measured by GC–MS to the urinary 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ levels measured by GC–tandem MS was calculated as 1.0745±0.5064 (RSD, 47%).

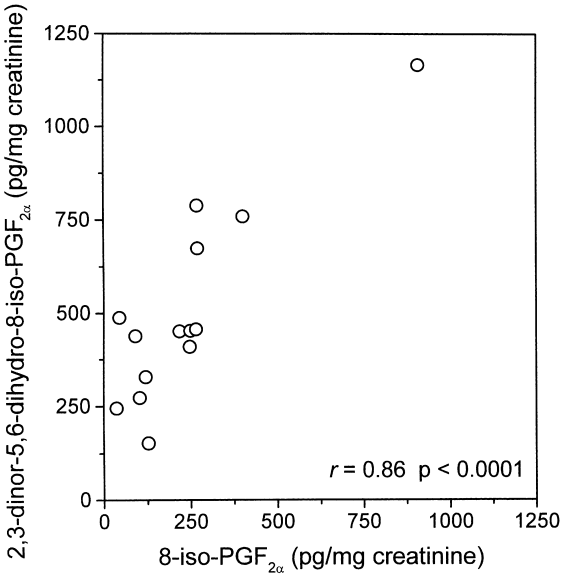

Fig. 4. Correlation between urinary excretion of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ determined in single urine samples of 14 healthy volunteers measured by method A.

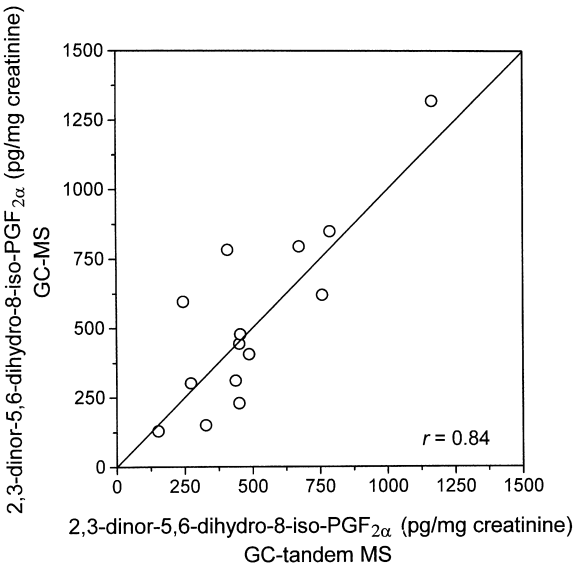

Fig. 5. Comparison of GC–MS with GC–tandem MS. Correlation of urinary 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ measured in single urine samples of 14 volunteers using GC–MS or GC–tandem MS and TLC for sample purification.

4. Discussion

Cyclooxygenase-dependent prostanoids, their specific circulating metabolites, and their major urinary metabolites have been shown to be best assessed by GC–MS and GC–tandem MS [20]. Since the discovery of the isoprostanes GC–MS and GC–tandem MS have been established in the analysis of these compounds in biological fluids [9,16,21,22]. These techniques have greatly contributed to show the usefulness of isoprostanes as markers of oxidative stress in vivo [7–9,23]. For several reasons, the measurement of metabolites instead of primary pros-

tanoids in human plasma and urine was found to be more reliable to assess whole body synthesis of these prostanoids [14]. Nevertheless, quantification of some primary prostanoids, e.g., PGE_2 and TxB_2, is useful to determine their production in the kidney [15]. Recently, 8-iso-$PGF_{2\alpha}$ was shown to undergo metabolism in vivo to 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and 2,3-dinor-8-iso-$PGF_{2\alpha}$ [10,11]. Analogous to the prostanoids, the metabolites of isoprostanes should be indicators of systemic isoprostane production. Thus, systemic lipid peroxidation would rather be accompanied by urinary isoprostane metabolites than by primary urinary isoprostanes. Therefore, we applied GC–tandem MS for the quantitative determination of urinary F_2-isoprostane metabolites, e.g., 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$.

In our previous work we showed that a combination of TLC for sample purification and GC–tandem MS for quantification enables specific determination of 8-iso-$PGF_{2\alpha}$ in human urine [16]. The usefulness of this method to assess oxidative stress in vivo has been recently demonstrated by our group [24]. The present study demonstrates that the same combination, i.e., TLC and GC–tandem MS, applies for the specific and accurate quantification of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ in human urine, too. Moreover, we show that this method enables specific and accurate quantification both of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ in a single urine sample (Fig. 6). We present data on the excretion of 8-iso-$PGF_{2\alpha}$ and its major urinary metabolite 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ in single urine samples of healthy humans using stable isotope dilution mass spectrometry. Basal urinary levels of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ were found to be higher than those of 8-iso-$PGF_{2\alpha}$ (506 ± 255 vs. 223 ± 135 pg/mg creatinine). Interestingly, urinary excretion rates of the precursor and its metabolite were found to be tightly correlated. Recently, γ-linolenic acid has been discussed as a potential additional source of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ [13]. The tight correlation between 8-iso-$PGF_{2\alpha}$ and 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ found in the present work may argue against this possibility.

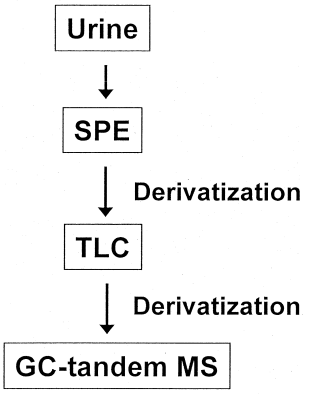

Fig. 6. Summary of the extraction and derivatization steps in method A for the analysis of 8-iso-$PGF_{2\alpha}$ and 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ in human urine.

Basal urinary levels of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ in our study are in the same order of magnitude as first reported by Morrow et al. [12]. Despite the utility of 8-iso-$PGF_{2\alpha}$ as an index parameter of oxidative stress, some pitfalls may be circumvented by quantitating 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ instead. Artifactual formation of 8-iso-$PGF_{2\alpha}$ may occur ex vivo [25]. Nevertheless, further investigation is needed to establish 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ as a systemic indicator of oxidative stress in vivo. Also, additional work has to be performed to determine whether and to what extent the kidney is the origin of 8-iso-$PGF_{2\alpha}$ [26].

Isoprostanes have been shown to have distinctly different chromatographic properties from their enzymatically formed analogs [21,27]. However, it can be expected that some F_2-isoprostanes, e.g., 8-iso-15(*R*)-$PGF_{2\alpha}$, 9β-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$, exhibit similar liquid (HPLC) and gas chromatographic (GC) properties [28]. The measurement of a specific isoprostane in human urine, e.g., 8-iso-$PGF_{2\alpha}$, requires numerous chromatographic steps or specific immunoaffinity extraction [29–31]. Therefore, the preferred method has been the assessment of various F_2-isoprostanes by GC–MS [26]. Furthermore, it has been proposed that the quantification of various F_2-isoprostanes in total rather than a specific F_2-isoprostane such as 8-iso-$PGF_{2\alpha}$ may provide a more useful tool for the integrative impact of oxidative stress in vivo [32]. For this purpose, we developed a method involving HPLC for sample purification. This method allows for the quantification of dinor-dihydro metabolites of various not yet fully identified F_2-isoprostanes by GC–tandem MS. Urinary levels of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and dinor-

dihydro metabolites of various F_2-isoprostanes in the same group of healthy volunteers indicate that at least one dinor-dihydro F_2-isoprostane metabolite coelutes both on the HPLC and GC column with 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. 2,3-Dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ seems to represent about 30% of the dinor-dihydro F_2-isoprostanes metabolites which coelut with this metabolite. Obviously, TLC completely separates dinor-dihydro F_2-isoprostanes metabolites distinct from 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. Further separation of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ by preceding HPLC did not alter the values obtained by TLC alone. Previously, similar results have been obtained for 8-iso-$PGF_{2\alpha}$ [16]. These are strong evidences that sample purification by TLC and quantification by GC–tandem MS allows for simultaneous specific analysis of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and its parent molecule, i.e., 8-iso-$PGF_{2\alpha}$, in human urine.

Due to its inherent accuracy GC–tandem MS enables specific quantification of prostanoids [20]. The present work provides evidence that 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ is quantitated accurately by GC–tandem MS, too. Quantification of urinary 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ by analysing the same samples by GC–MS and GC–tandem MS revealed very similar mean levels, i.e., 506 ± 255 and 529 ± 315 pg/mg creatinine, respectively. However, individual values differed greatly (Fig. 5). The comparison of GC–MS with GC–tandem MS in more simple analytical problems such as the measurement of urinary nitrate revealed an RSD of only 2.8% for the ratio of concentrations measured by these techniques, i.e., 0.9855 ± 0.0275 [33]. In more difficult analyses such as the measurement of urinary 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$, the RSD for the ratio of concentrations measured by GC–MS to GC–tandem MS (1.0745 ± 0.5064) was considerably higher, i.e., 47%, underlining the superiority of GC–tandem MS in the analysis of isoprostanes in complex biological fluids.

5. Conclusions

Two chromatographic procedures were developed for the accurate quantification of dinor-dihydro F_2-isoprostane metabolites in human urine by GC–MS or GC–tandem MS. TLC in combination with GC–tandem MS is useful for the rapid and simultaneous quantification of 8-iso-$PGF_{2\alpha}$ and its major urinary metabolite 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ in human urine. HPLC in combination with GC–tandem MS allows for the assessment of several dinor-dihydro F_2-isoprostane metabolites in addition to 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. Healthy humans were found to excrete twice as much 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ as 8-iso-$PGF_{2\alpha}$ into the urine. Both chromatographic procedures in combination with GC–tandem MS should be useful in investigating the formation and metabolism of 8-iso-$PGF_{2\alpha}$ and other F_2-isoprostanes in vivo. The utility of urinary 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and other dinor-dihydro F_2-isoprostanes metabolites as indicators of oxidative stress in vivo in humans and the relationship to their parent molecules remain to be investigated in this context. The methods described in this work should be useful in such studies.

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