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Divergence in urinary 8-*iso*-$PGF_{2\alpha}$ ($iPF_{2\alpha}$-III, 15-F_{2t}-IsoP) levels from gas chromatography–tandem mass spectrometry quantification after thin-layer chromatography and immunoaffinity column chromatography reveals heterogeneity of 8-*iso*-$PGF_{2\alpha}$ Possible methodological, mechanistic and clinical implications

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Abstract

Free radical-catalysed oxidation of arachidonic acid esterified to lipids leads to the formation of the F_2-isoprostane family which may theoretically comprise up to 64 isomers. We have previously shown that the combination of TLC and GC–tandem MS (referred to as method A) allows for the accurate and highly specific quantification of 8-*iso*-$PGF_{2\alpha}$ ($iPF_{2\alpha}$-III, 15-F_{2t}-IsoP) in human urine. Immunoaffinity column chromatography (IAC) with immobilized antibodies raised against 8-*iso*-$PGF_{2\alpha}$ (i.e. 15(*S*)-8-*iso*-$PGF_{2\alpha}$) has been shown by others to be highly selective and specific for this 8-*iso*-$PGF_{2\alpha}$ isomer when quantified by GC–MS. In the present study we established IAC for urinary 8-*iso*-$PGF_{2\alpha}$ for subsequent quantification by GC–tandem MS (referred to as method B). This method was fully validated and found to be highly accurate and precise for urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$. 8-*iso*-$PGF_{2\alpha}$ was measured in urine of 10 young healthy humans by both methods. 8-*iso*-$PGF_{2\alpha}$ was determined to be 291±102 pg/mg creatinine by method A and 141±41 pg/mg creatinine by method B. Analysis of the combined *through* and *wash* phases of the IAC step, i.e. of the unretained compounds, by method A showed the presence of non-immunoreactive 8-*iso*-$PGF_{2\alpha}$ at 128±55 pg/mg creatinine. This finding suggests that urinary 8-*iso*-$PGF_{2\alpha}$ is heterogenous, with 15(*S*)-8-*iso*-$PGF_{2\alpha}$ contributing by ~50%. $PGF_{2\alpha}$ and other 8-*iso*-$PGF_{2\alpha}$ isomers including 15(*R*)-8-*iso*-$PGF_{2\alpha}$ are not IAC-immunoreactive and are chromatographically separated from 15(*S*)-8-*iso*-$PGF_{2\alpha}$. We assume that *ent*-15(*S*)-8-*iso*-$PGF_{2\alpha}$ is also contributing by ~50% to urinary 8-*iso*-$PGF_{2\alpha}$. This finding may have methodological, mechanistic and clinical implications.
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1. Introduction

Nonenzymatic, free radical-catalysed peroxidation of arachidonic acid esterified to lipids results in the formation of isoprostanes (reviewed in Ref. [1]), F_2-isoprostanes being the most abundant among these isoeicosanoids [1–4]. 8-*iso*-Prostaglandin $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$), also known as $iPF_{2\alpha}$-III and 15-F_{2t}-IsoP, is one abundant endogenous F_2-isoprostane which is excreted into the urine of humans [5]. 8-*iso*-$PGF_{2\alpha}$ is at present the most thoroughly investigated F_2-isoprostane. Measurement of 8-*iso*-$PGF_{2\alpha}$ in human urine, sometimes complicated by incomplete separation from other F_2-isoprostanes, has been established as a reliable method to assess non-invasively lipid peroxidation in vivo [6–8].

Gas chromatography–mass spectrometry (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 isoprostane formation in vivo in humans [8]. Mainly two strategies have been followed up in this context so far. Quantification either of a certain F_2-isoprostane, usually 8-*iso*-$PGF_{2\alpha}$, or of several unknown F_2-isoprostanes in toto is performed. The first strategy yields results which can be more closely compared among various laboratories, but it requires highly specific techniques. The results from various laboratories following up the second strategy cannot be unconditionally compared to each other.

We have shown that GC–tandem MS, but not simple GC–MS, enables specific quantification of 8-*iso*-$PGF_{2\alpha}$ in human urine with a minimum of labour requiring a single thin-layer chromatographic (TLC) step for sample purification [9,10]. An alternative way to specifically quantitate 8-*iso*-$PGF_{2\alpha}$ is the combination of immunoaffinity chromatography (IAC) columns carrying immobilized antibodies raised against 8-*iso*-$PGF_{2\alpha}$, i.e. actually the isomer 15(*S*)-8-*iso*-$PGF_{2\alpha}$, with GC–MS [11]. This method has been largely restricted to a very small number of laboratories capable of preparing specific antibodies and immobilizing them on gels, but today such IAC columns are accessible to every laboratory due to commercial availability. To date, no other 8-*iso*-$PGF_{2\alpha}$ isomers except for 15(*S*)-8-*iso*-$PGF_{2\alpha}$ have been unequivocally identified in human urine, although theoretically seven additional 8-*iso*-$PGF_{2\alpha}$ stereoisomers may exist. The aim of the present study was to search for the expected but not yet shown heterogeneity of urinary 8-*iso*-$PGF_{2\alpha}$. For this purpose we quantitated 8-*iso*-$PGF_{2\alpha}$ in human urine by GC–tandem MS after use of two sample treatment procedures of distinctly different selectivity, with method B being by nature clearly more selective than method A. Heterogeneity of urinary 8-*iso*-$PGF_{2\alpha}$ would be evident when method A, i.e. TLC separation, would result in higher 8-*iso*-$PGF_{2\alpha}$ levels than method B, i.e. IAC extraction of 15(*S*)-8-*iso*-$PGF_{2\alpha}$. In order to achieve this goal we established and validated the IAC-based method for urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$.

2. Experimental

2.1. Materials

[3,3′,4,4′-$^2H_4$]-PGE_2 {9-oxo-11α,15(*S*)-dihydroxy-prosta-5*Z*,13*E*-dien-1-oic-3,3,4,4-$^2H_4$ acid, ≥98 at.% ^{2}H}, 8-*iso*-$PGF_{2\alpha}$ {9α,11α,15(*S*)-trihydroxy-(8β,12β)-prosta-5*Z*,13*E*-dien-1-oic acid; 15(*S*)-8-*iso*-$PGF_{2\alpha}$}, [3,3′,4,4′-$^2H_4$]-8-*iso*-$PGF_{2\alpha}$ {9α,11α,15(*S*)-trihydroxy-(8β,12β)-prosta-5*Z*,13*E*-dien-1-oic-3,3,4,4-$^2H_4$ acid, ≥98 at.% ^{2}H; [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$}, 12α,14α,8(*S*)-trihydroxy-(11β,15β)-prosta-5*Z*,9*E*-dien-1-oic-17,18,19,20-$^2H_4$ acid (≥98 at.% ^{2}H), 15(*R*)-8-*iso*-$PGF_{2\alpha}$ {9α,11α,15(*R*)-trihydroxy-(8β,12β)-prosta-5*Z*,13*E*-dien-1-oic acid}, [3,3′,4,4′-$^2H_4$]-2,3-dinor-6-oxo-$PGF_{1\alpha}$, other prostaglandins, arachidonic acid, 4-ml (1-ml gel resin) IAC columns (8-Isoprostane Affinity Column, catalog no. 416358) with immobilized antibody against 8-*iso*-$PGF_{2\alpha}$ {presumably 9α,11α,15(*S*)-trihydroxy-(8β,12β)-prosta-5*Z*,13*E*-dien-1-oic acid; 15(*S*)-8-*iso*-$PGF_{2\alpha}$}, and 20-ml (1-ml gel resin) IAC columns (Prostaglandin E_2 Affinity Column, catalog no. 414018) were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Unused and reused IAC columns were stored at 4 °C. Due to the manufacturer's product specifications,[2] the binding capacity of the IAC column is 10 ng of

[2] www.caymanchem.com:80/neptune/pdfs/416358.pdf.

15(*S*)-8-*iso*-$PGF_{2\alpha}$ per ml of gel resin. It is recommended that the sample should contain an amount of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ equal to 20 to 50% of the column capacity, i.e. 2 to 5 ng of 15(*S*)-8-*iso*-$PGF_{2\alpha}$. Also, the specificity (cross-reactivity) of the 8-Isoprostane Affinity Column is reported as 100% for 15(*S*)-8-*iso*-$PGF_{2\alpha}$, 7.6% for 8-*iso*-$PGF_{3\alpha}$, 2.85% for $PGF_{1\alpha}$, 0.88% for $PGF_{2\alpha}$, 0.83% for 11β-$PGF_{2\alpha}$, and less than 0.34% for other eicosanoids. Isolated ovine COX 1 was also obtained from Cayman Chemicals. Pentafluorobenzyl (PFB) bromide was obtained from Aldrich (Steinheim, Germany). *N*,*O*-bis(Trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce (Rockford, IL, USA). Phospholipase A_2 (PLA_2, phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4) from porcine pancreas obtained as ammonium sulfate suspension (≥600 units per mg protein), diclofenac and acetylsalicylic acid were purchased from Sigma (Deisenhofen, Germany).

2.2. Sample collection, IAC column extraction and derivatization procedures

Urine from spontaneous micturition by healthy adults was collected in polypropylene bottles, containing 1 m*M* each of 4-hydroxy-tempo and EDTA. Urine samples were divided into 1-ml aliquots and stored at −20 °C until further use.

The internal standard [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ was added to 1-ml aliquots of human urine samples resulting in a final concentration of 250 or 1000 pg/ml, if not otherwise specified. IAC column extraction of urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ was performed as recommended by the manufacturer with minor modifications.[2] Briefly, centrifuged urine samples (1 ml) were applied directly to 4-ml IAC columns. Columns were washed with 2 ml of column buffer (0.1 *M* potassium phosphate buffer, pH 7.4; 7.7 m*M* NaN_3; 0.5 *M* NaCl), followed by 2 ml of distilled water. Compounds were eluted by allowing 2 ml of the elution solution consisting of absolute ethanol–distilled water (95:5, v/v) to pass. IAC columns were regenerated by washing with 5 ml of distilled water and 5 ml of the column buffer, and stored at 4 °C in 2 ml of column buffer until the next use. The same procedure was used to extract PGE_2 from aqueous buffered solutions by means of PGE_2-IAC columns. Solvents were removed under a stream of nitrogen, and the PFB ester trimethylsilyl (TMS) ether derivatives were prepared using PFB bromide and BSTFA (50 μl), respectively, by standard derivatization procedures [9]. In the case of PGE_2, the PFB ester methoxyamine TMS derivatives were prepared.

2.3. Formation of 8-iso-$PGF_{2\alpha}$ and PGE_2 from arachidonic acid by isolated cyclooxygenase 1

Arachidonic acid (10 μ*M*) and COX 1 (5 units for PGE_2; 100 units for 8-*iso*-$PGF_{2\alpha}$) were incubated in 100 m*M* Tris buffer (1 ml), pH 8, containing phenol (2 m*M*), haematin (1 μ*M*) and EDTA (5 m*M*) for 3 min at 37 °C in the presence or absence of diclofenac (1 μ*M*) or acetylsalicylic acid (1 m*M*). Reaction was started by adding arachidonic acid and stopped by acidifying the sample to pH 3 with formic acid (10 *M*). The internal standards were added, samples were diluted with the IAC column buffer, retained and unretained reaction products present in the eluate (2 ml) and the ''passed through+wash'' phase (5 ml), respectively, were extracted and derivatized as described above.

2.4. Determination of free and esterified serum 15(S)-8-iso-$PGF_{2\alpha}$

Two experiments were performed by incubating serum aliquots (1 ml) at 37 °C without and with external addition of PLA_2. To investigate the effect of incubation time on PLA_2-catalysed release of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ from lipids, 40 units of PLA_2 were added and samples were incubated for up to 120 min. A second set of serum samples serving as control was incubated without addition of PLA_2. The effect of added PLA_2 activity (up to 100 units) to serum samples was investigated by incubating the samples for a fixed incubation time of 45 min. After addition of the internal standard [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ at a final concentration of 100 pg/ml, samples were subjected to 15(*S*)-8-*iso*-$PGF_{2\alpha}$-IAC column extraction and further analysis as described above for urine samples.

For comparison, unspiked serum samples and serum samples spiked with 160 pg/ml of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ were treated with PLA_2 (40 units) or 1 *M*

KOH (400 μl of a 4.46 *M* solution per ml of serum) for 45 min each in triplicate. After neutralisation and centrifugation of the KOH-treated samples, all samples were treated further as described for urine samples.

2.5. Urinary 15(S)-8-iso-$PGF_{2\alpha}$ in healthy smokers and non-smokers

15(*S*)-8-*iso*-$PGF_{2\alpha}$ was determined in urine and serum of 12 healthy non-smoking volunteers (seven females and five males, aged 30.7±9.2 years) and 10 healthy persons (three females and seven males, aged 25.1±4.4 years) with a smoking history of 14.9±13.8 packyears. The internal standard [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ was added to urine and serum samples (each 1 ml) at final concentrations of 1000 and 100 pg/ml, respectively. Urines and KOH-treated serum samples were subjected to 15(*S*)-8-*iso*-$PGF_{2\alpha}$-IAC column extraction and further analysis as described above (i.e. by method B). The study was approved by the Institutional Review Board of Hannover Medical School.

2.6. Isoprostane and prostacyclin synthesis in essential hypertension and health

Urinary excretion of 8-*iso*-$PGF_{2\alpha}$ and 2,3-dinor-6-oxo-$PGF_{1\alpha}$, the major urinary metabolite of prostacyclin, was determined in 17 newly diagnosed, untreated male subjects (39±10 years) with mild uncomplicated essential hypertension (EH; systolic blood pressure, 149±15 mmHg; diastolic blood pressure, 97±12 mmHg) and a positive family history of EH, and in 11 healthy male volunteers (41.5±9 years; systolic blood pressure, 125±14 mmHg; diastolic blood pressure, 82±8 mmHg) and a negative family history of EH. After an overnight fast of 12 to 14 h, participants were asked to empty their bladders at 06:00 h and subsequently remained at rest for 3 h. Then they passed urine which was frozen immediately at −20 °C until analysis for 8-*iso*-$PGF_{2\alpha}$ (by method A [9]) and 2,3-dinor-6-oxo-$PGF_{1\alpha}$ [12] by GC–tandem MS, and creatinine by the alkaline picric acid method. The study protocol was approved by the local Ethic Committee of the Silesian University School of Medicine, and informed consent was obtained from all subjects studied.

2.7. GC–MS and GC–tandem MS conditions

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). Fused silica capillary columns Optima 17 (15 m or 30 m×0.25 mm I.D., 0.25 μm film thickness) from Macherey–Nagel (Düren, Germany) were 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 30 eV. Electron energy was 200 eV and the emission current 600 μA. Injector, interface and ion source were kept at 280, 290 and 180 °C, respectively. The column was held at 70 °C for 2 min, then programmed to 280 °C at 25°/min followed to 320 °C at 4°/min. Quantification by GC–MS was performed by selected ion monitoring (SIM) of the parent ions $[M-PFB]^-$ at *m/z* 569.4 for 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and *m/z* 573.4 for [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ with a dwell time of 400 ms for each ion. In GC–tandem MS, the product ions $[M-PFB-3\times TMSOH]^-$ at *m/z* 299.1 for 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and *m/z* 303.1 for [$^2H_4$]-15(S)-8-*iso*-$PGF_{2\alpha}$ generated by CAD of the parent ions $[M-PFB]^-$ at *m/z* 569.4 and 573.4, respectively, were monitored in the selected reaction monitoring (SRM) mode with a dwell time of 400 ms for each ion. The corresponding ions were 524.4 and 268.1 for PGE_2, and 528.4 and 272.1 for [$^2H_4$]-PGE_2. The corresponding ions were 586.4 and 240.1 for 2,3-dinor-6-oxo-$PGF_{1\alpha}$, and 590.4 and 244.1 for the internal standard [$^2H_4$]-2,3-dinor-6-oxo-$PGF_{1\alpha}$. Aliquots (1 μl) were injected into the GC–tandem MS instrument in the splitless mode.

3. Results

3.1. Validation of method B combining IAC with GC–tandem MS for the quantitative determination of urinary 15(S)-8-iso-$PGF_{2\alpha}$

The results from the experiments on the validation of method B for urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$ are

Table 1
Data on the validation[a] of method B (IAC[b] plus GC–tandem MS) for a narrow concentration range of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in human urine (1 ml)

15(*S*)-8-*iso*-$PGF_{2\alpha}$ (pg/ml)			Recovery (%)	Precision (RSD, %)
Added	Measured mean	SD		
0	98.8	1.71	N.A.	1.73
40	141.9	3.88	107.8	2.74
80	178.4	2.14	99.5	1.20
160	271.4	2.72	107.9	1.00
400	504.9	18.4	101.5	3.79

[a] Analyses were performed in quadruplicate. The concentration of the internal standard [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ was 250 pg/ml in all samples.

[b] The IAC columns had been used before for other urine samples at least six times. N.A., not applicable.

shown in Tables 1 and 2. In the first experiment, the urine used contained endogenous 15(*S*)-8-*iso*-$PGF_{2\alpha}$ at 98.8±1.7 pg/ml (RSD, 1.73%). Externally added 15(*S*)-8-*iso*-$PGF_{2\alpha}$ was recovered from urine quantitatively with high accuracy (recovery, 104.2±4.3%) and low imprecision (RSD, 2.1±1.2%) at each added concentration, indicating the validity of the method in the whole concentration range tested (Table 1). The lowest concentration of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ added to urine, i.e. 40 pg/ml, was accurately (107.8%) and precisely (2.7%) discriminated ($P=9.2\times10^{-7}$) from the concentration of endogenous 15(*S*)-8-*iso*-$PGF_{2\alpha}$. Linear regression analysis between measured (*Y*) and added (*X*) 15(*S*)-8-*iso*-$PGF_{2\alpha}$ concentrations resulted in the regression equation $Y = 97.9 + 1.06X$, $r=0.9989$.

Table 2
Data on the validation[a] of method B (IAC[b] plus GC–tandem MS) for an extended concentration range of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in human urine (1 ml)

15(*S*)-8-*iso*-$PGF_{2\alpha}$ (pg/ml)			Recovery (%)	Precision (RSD, %)
Added	Measured mean	SD		
0	329	15.7	N.A.	4.77
360	661	25.3	92.2	3.83
719	959	87.5	87.6	12.2
1438	1420	18.5	75.9	1.30
3600	2894	164	71.3	5.67

[a] Analyses were performed in sixfold. The concentration of the internal standard [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ was 250 pg/ml in all samples.

[b] The IAC columns used in this experiment had been used at least four times before for other urine samples. N.A., not applicable.

In the second experiment, the urine used contained endogenous 15(*S*)-8-*iso*-$PGF_{2\alpha}$ at 329 pg/ml. Endogenous and externally added 15(*S*)-8-*iso*-$PGF_{2\alpha}$ were determined precisely in all urine samples (Table 2). However, no linear relationship was obtained from linear regression analysis between measured (*Y*) and added (*X*) 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in the whole range tested, i.e. up to 3600 pg/ml (Table 2). Recovery values gradually decreased with increasing 15(*S*)-8-*iso*-$PGF_{2\alpha}$ concentration from 92 to 71%. Polynomial regression analysis between measured (*Y*) and added (*X*) 15(*S*)-8-*iso*-$PGF_{2\alpha}$ concentrations resulted in the regression equation $Y = 339 + 0.8X - 3.4\times10^{-5}X^2$, $r=0.99924$.

The six unspiked urine samples from the second experiment were also analyzed by GC–MS. Urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$ was quantitated by GC–tandem MS and GC–MS with comparable precision (RSD, 4.8 vs. 3.7%). GC–MS revealed slightly (1.019-fold) but not significantly ($P=0.485$) higher levels (335.7±12.5 pg/ml) than GC–tandem MS (329.7±15.9 pg/ml), suggesting that urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$ can be precisely and accurately quantitated by simple GC–MS.

Use of IAC columns for 15(*S*)-8-*iso*-$PGF_{2\alpha}$ is associated with high initial costs. This factor may be a reason for using alternative procedures such as solid-phase extraction (SPE) on octadecylsilica (ODS or C_{18}) SPE cartridges for the extraction of 8-*iso*-$PGF_{2\alpha}$ from urine and other biological samples and subsequent purification by chromatographic procedures such as TLC, e.g. in method A. The ratio of the costs for the purchase of ODS and IAC columns for 15(*S*)-8-*iso*-$PGF_{2\alpha}$ is currently about 1:8. However, the final cost ratio may be shifted clearly in favor of IAC provided that the IAC columns can be regenerated and reused several times without the appearance of ‘‘memory effects’’ and loss of efficiency. By using a pooled urine sample of a healthy volunteer we investigated the effect of the reuse (1–7 preceding uses) of 21 IAC columns with the same lot number on the concentration of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in the urine as measured by GC–tandem MS. Table 3 shows that the frequency of the reuse of IAC columns did not significantly influence the recovery of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ from the urine for at least six cycles.

Table 3
Endogenous 15(*S*)-8-*iso*-$PGF_{2\alpha}$ levels (pg/ml) in a pooled urine sample of a healthy volunteer measured by GC–tandem MS after IAC column extraction by using reused IAC columns[a]

Number of preceding repeated uses of IAC columns						
1	2	3	4	5	6	7
94.5	92.9	125.4	110.5	105.8	87.5	99.3
105.8	132.5	90.5	101.4	131.2	118.9	99.7
122.4	87.5	116.8	98.9	104.6	101.0	84.4
Mean±SD						
108±14	104±25	111±18	104±6	114±15	103±16	95±9

[a] Three columns each with the same frequency of reuse (1 to 7) were applied. Aliquots of 1-ml urine spiked with 250 pg/ml of the internal standard [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ were subjected to IAC column extraction.

Finally, we investigated the effect of urine volume subjected to IAC column extraction on the level of urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$. Table 4 indicates that the level of endogenous 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in human urine may slightly decrease with increasing volume of the urine subjected to IAC column extraction, i.e. by 1.9, 8.6, and 14.7% from 100 to 200, 500, and 1000 μl, respectively.

Representative chromatograms from the GC–MS and GC–tandem MS analysis of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ after IAC extraction from an unspiked and a spiked urine sample are shown in Fig. 1. GC–tandem MS analysis resulted in two peaks eluting with the retention times of synthetic 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (upper panel) and [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ in the unspiked and spiked urine sample (Fig. 1B and D, lower panels). On the other hand, GC–MS analysis resulted in many peaks from which one pair eluted with the retention times of synthetic 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (upper panel) and [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ in the unspiked and spiked urine sample (Fig. 1A and C, lower panels). Both GC–tandem MS and GC–MS yielded comparable values for 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in the unspiked (83.4 vs. 79.3 pg/ml) and spiked urine sample (171.5 vs. 160.6 pg/ml). It is worth mentioning, that despite higher peak areas in the GC–MS analyses, the signal-to-noise ratio (i.e. SN) for 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ is clearly in favor of GC–tandem MS (Fig. 1).

Table 4
Dependence of the level of endogenous 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in a pooled urine sample[a] of a healthy volunteer measured by GC–tandem MS after IAC column extraction by using reused 15(*S*)-8-*iso*-$PGF_{2\alpha}$-IAC columns[b] upon the urine volume extracted

Urine volume (μl)	15(*S*)-8-*iso*-$PGF_{2\alpha}$ levels (pg/ml)		
	Mean	SD	Precision (RSD, %)
100	395	21	5.32
200	388	39	10.1
500	361	13	3.60
1000	337	18	5.34
Mean±SD	370	31	8.35

[a] Urine volume was adjusted to 1000 μl by buffer. The internal standard [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ was added at a final concentration of 250 pg/ml.
[b] Two columns each with the same frequency of reuse (1 to 3) were applied.

3.2. Comparison of method A with method B

The results described in the section above underline the validity of method B (IAC+GC–tandem MS). The validity of method A (TLC+GC–tandem MS) for urinary 8-*iso*-$PGF_{2\alpha}$ has been shown previously in detail [9]. In the present study we compared these methods directly with each other.

Analysis of five identical aliquots from a pooled urine sample with method A resulted in an 8-*iso*-$PGF_{2\alpha}$ concentration of 403±26 pg/ml (RSD, 6.4%), whereas, by contrast, method B yielded a concentration of 167±5 pg/ml (RSD, 3.2%). Thus, both methods are comparably precise, but they yield statistically significantly ($P<0.0001$) different values for urinary 8-*iso*-$PGF_{2\alpha}$.

We quantified 8-*iso*-$PGF_{2\alpha}$ in urine of 15 healthy, young, non-smoking volunteers by both methods (Fig. 2A and B). Method A yielded significantly ($P=0.0037$) higher levels for endogenous 8-*iso*-

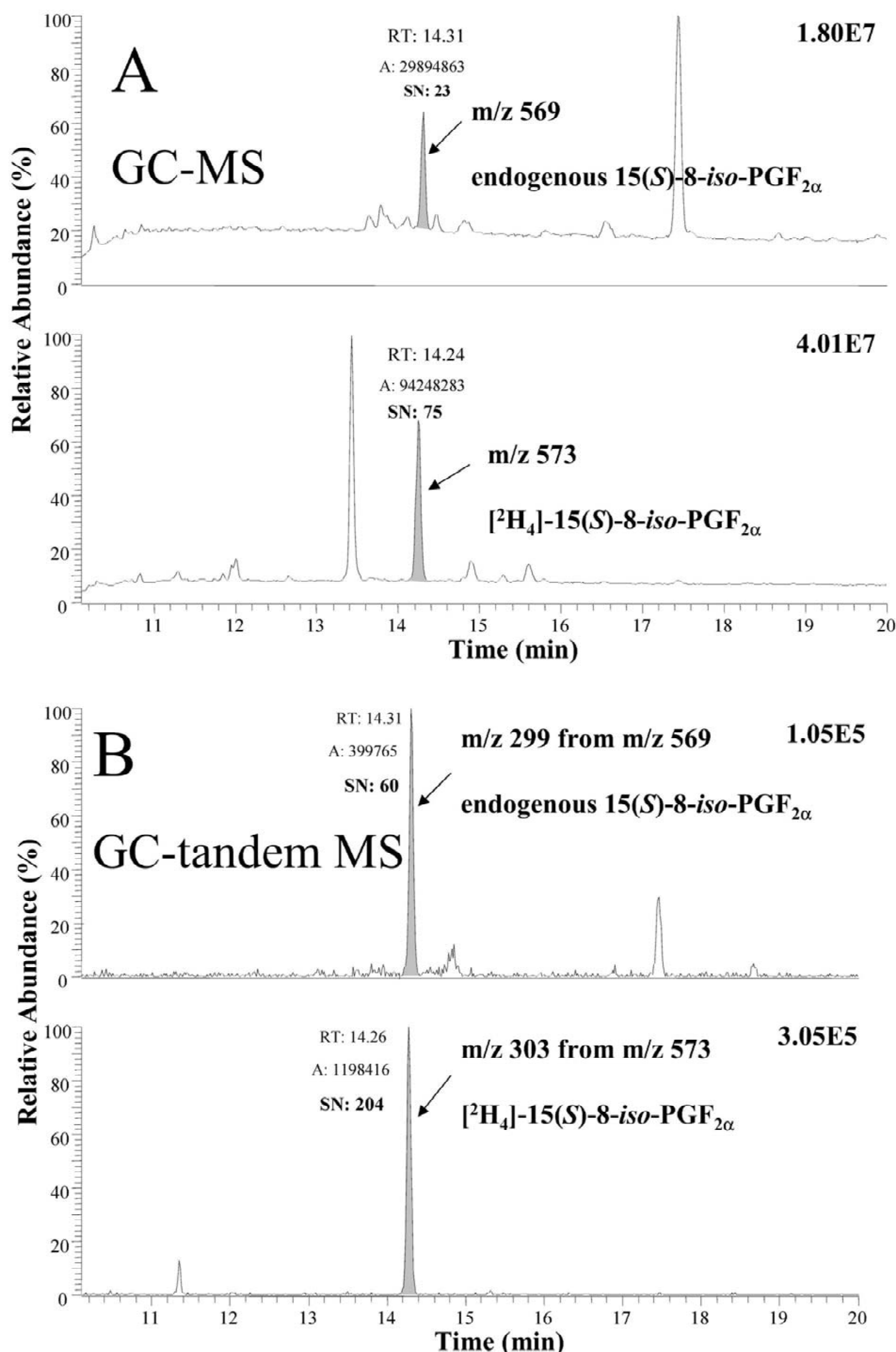

Fig. 1. Partial chromatograms from the GC–MS (A, C) and GC–tandem MS (B, D) analysis of an unspiked urine sample (1 ml) (A, B) and the same urine sample (1 ml) spiked with 80 pg of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (C, D) after IAC extraction (method B). The concentration of the internal standard [$^{2}H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ in the urine samples was 250 pg/ml. GC column, Optima 17, 15 m×0.25 mm I.D., 0.25 μm film thickness. RT, retention time (min); A, peak area (arbitrary units); SN, signal-to-noise ratio. The peaks of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and [$^{2}H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ are underlaid and indicated by arrows.

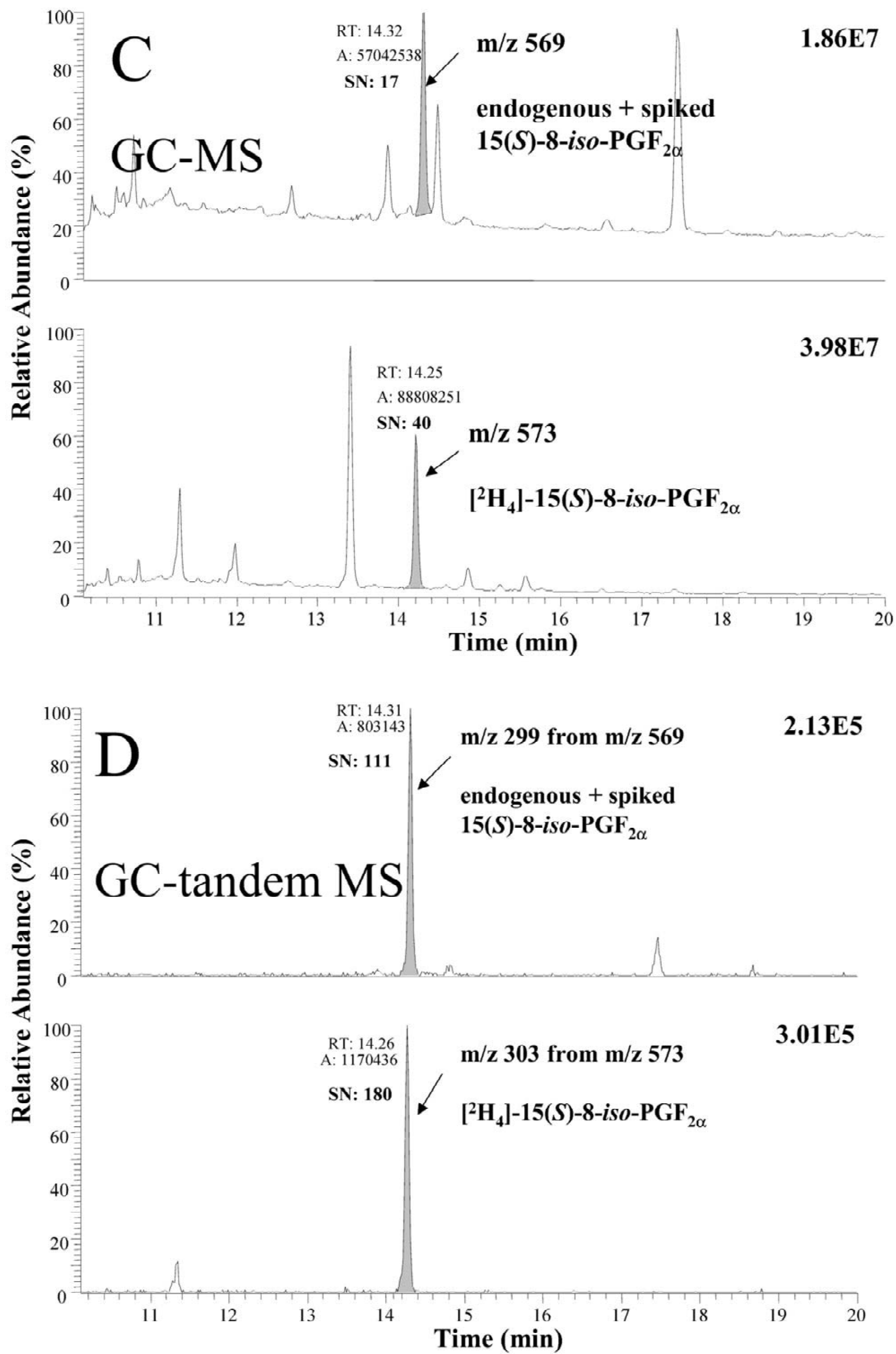

Fig. 1. (*continued*)

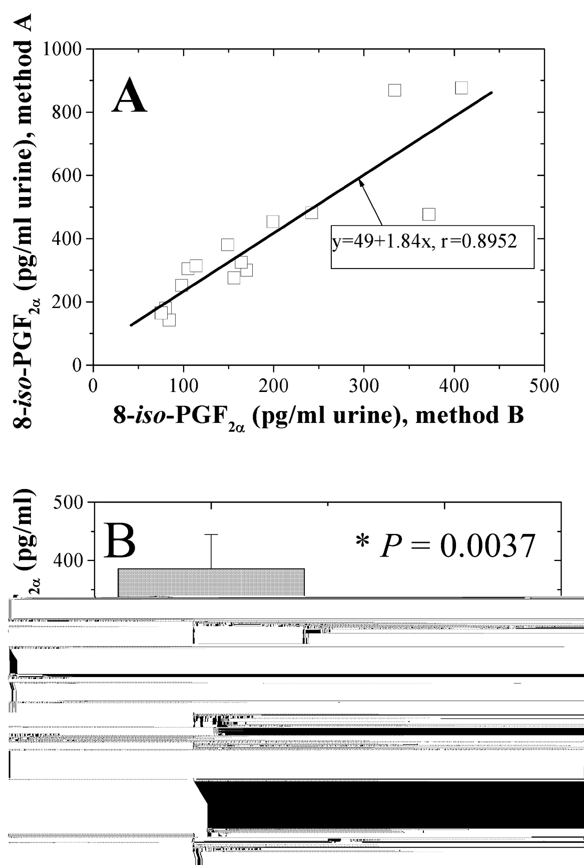

Fig. 2. Measurement of 8-*iso*-$PGF_{2\alpha}$ by method A and method B in urine of 15 healthy, non-smoking, young volunteers. (A) Correlation between the urinary levels of 8-*iso*-$PGF_{2\alpha}$ measured by method A (TLC+GC–tandem MS) and those by method B (IAC+GC–tandem MS). (B) Urinary levels of 8-*iso*-$PGF_{2\alpha}$ measured by method A (left column) and method B (right column).

$PGF_{2\alpha}$ than method B, i.e. 387±224 vs. 183±109 pg/ml (Fig. 2B). The ratio of the concentrations measured by method A to those measured by method B was 2.22±0.51. A good correlation (r=0.8952) between these concentrations was found (Fig. 2A).

That method A yielded reproducibly higher 8-*iso*-$PGF_{2\alpha}$ concentrations in urine than method B, which is specific for the isomer 15(S)-8-*iso*-$PGF_{2\alpha}$, led us to assume that method A measures further isomers in addition to 15(S)-8-*iso*-$PGF_{2\alpha}$, which do not bind to the antibody immobilized on the IAC column, but coelute with 15(S)-8-*iso*-$PGF_{2\alpha}$ in TLC as well as in GC. To address this issue we quantified 8-*iso*-$PGF_{2\alpha}$ in urine of 10 healthy non-smoking volunteers (age 31±5 years, body weight 72±14 kg) by both methods in parallel in identical urine specimens. Urinary 8-*iso*-$PGF_{2\alpha}$ was determined to be 291±102 pg/mg creatinine by method A, but 141±41 pg/mg creatinine by method B. Urinary excretion of 8-*iso*-$PGF_{2\alpha}$ was not significantly different in the female subpopulation (five subjects, age 30±4 years, body weight 63±2 kg) compared with the male subpopulation (five subjects, age 32±6 years, body weight 82±15 kg), i.e. 299±85 (females) vs. 282±115 pg/mg creatinine (males) by method A, and 158±39 (females) vs. 124±37 pg/mg creatinine (males) by method B. Urine samples passed through the IAC columns and the corresponding wash phases were combined, the internal standard [$^2H_4$]-15(S)-8-*iso*-$PGF_{2\alpha}$ was added, and samples were analyzed according to method A, i.e. purified by TLC and quantitated by GC–tandem MS. These analyses revealed that 8-*iso*-$PGF_{2\alpha}$ isomers not retained on the 15(S)-8-*iso*-$PGF_{2\alpha}$-specific IAC column were present in the urine at 128±55 pg/mg creatinine, i.e. at comparable concentrations to those of 15(S)-8-*iso*-$PGF_{2\alpha}$ (141±41 pg/mg creatinine). Linear regression analysis between the levels of 15(S)-8-*iso*-$PGF_{2\alpha}$ measured by method B plus those of other isomers non-binding to the IAC column measured by method A in the passed through and wash phases (Y) and the levels of 8-*iso*-$PGF_{2\alpha}$ measured by method A (X) in the urine of 10 volunteers resulted in the regression equation $Y = 36.2 + 0.8X$ and the correlation coefficient R=0.92241, indicating a good correlation (Fig. 3). The mean ratio of the levels obtained by method A to the sum of the levels measured by method B and method A amounts to 1.082±0.134, indicating that method A actually measures 15(S)-8-*iso*-$PGF_{2\alpha}$ plus other non-IAC-binding isomers.

Representative chromatograms from the GC–tandem MS analysis of 8-*iso*-$PGF_{2\alpha}$ in the same human urine samples by method A and method B are shown in Fig. 4. Method B usually results in two peaks eluting with the retention time of the PFB-TMS derivatives of synthetic 15(S)-8-*iso*-$PGF_{2\alpha}$ (upper panel) and [$^2H_4$]-15(S)-8-*iso*-$PGF_{2\alpha}$ (lower panel), which elutes a few seconds in front of 15(S)-8-*iso*-$PGF_{2\alpha}$ (Fig. 4B). On the other hand, method A yields one peak corresponding to the internal standard (lower panel), but four major peaks with m/z of 299 from which the middle peak coelutes with

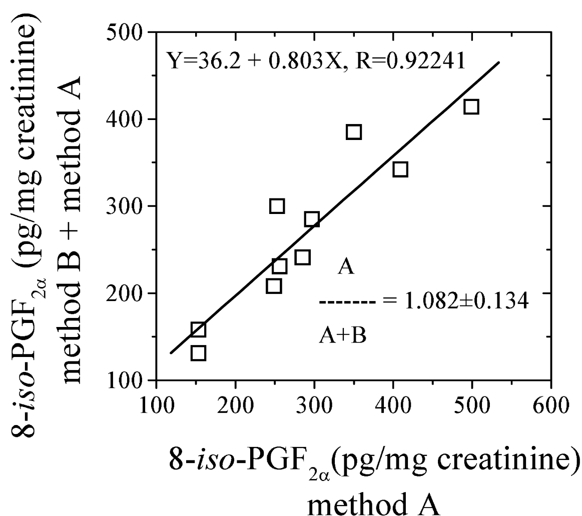

Fig. 3. Measurement of 8-*iso*-$PGF_{2\alpha}$ by method A and method B in urine of 10 healthy, non-smoking, young volunteers. Correlation between the urinary levels measured by method B + method A and those by method A. For more details see the text (Section 3.2).

synthetic 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and is assigned to endogenous 8-*iso*-$PGF_{2\alpha}$ (Fig. 4A).

Fig. 5 shows partial chromatograms from the analysis by method B of a urine sample spiked with the internal standard [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ before (Fig. 5A) and after (Fig. 5B) addition of equal amounts of the PFB-TMS derivatives of synthetic 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and 15(*R*)-8-*iso*-$PGF_{2\alpha}$. The left chromatogram shows partial chromatographic resolution of the PFB-TMS derivatives of these compounds, with the PFB-TMS derivative of 15(*R*)-8-*iso*-$PGF_{2\alpha}$ eluting in front of that of 15(*S*)-8-*iso*-$PGF_{2\alpha}$. Analysis of urinary 8-*iso*-$PGF_{2\alpha}$ by method A as well as by method B resulted in chromatographic peaks of the same peak width with those of the PFB-TMS derivatives of [$^2H_4$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$, 15(*R*)-8-*iso*-$PGF_{2\alpha}$ and 15(*S*)-8-*iso*-$PGF_{2\alpha}$, strongly suggesting that 15(*R*)-8-*iso*-$PGF_{2\alpha}$ is not physiologically present in human urine (Figs. 1, 4 and 5).

3.3. Formation of 15(S)-8-iso-$PGF_{2\alpha}$ and PGE_2 from arachidonic acid by isolated ovine cyclooxygenase 1

The major COX 1-dependent reaction product of arachidonic acid was found to be PGE_2. In the absence of any COX inhibitor, COX 1 (5 units) converted ~20% of arachidonic acid to PGE_2 and ~10% to $PGF_{2\alpha}$ within 3 min. Diclofenac (1 μ*M*) and acetylsalicylic acid (1 m*M*) inhibited COX 1-dependent PGE_2 formation by 88 and 26%, respectively. Only very small amounts of PGE_2 were found not to retain on the PGE_2-IAC column (data not shown), most likely because of exceeding the binding capacity of the column.

Within 3 min COX 1 (100 units) converted ~0.04% of arachidonic acid to a compound eluting with the retention time of synthetic 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in the absence of any COX inhibitor (Fig. 6). In the control experiment as well as in the presence of diclofenac or acetylsalicylic acid the molar ratio of unretained (“passed through + wash” phase) 8-*iso*-$PGF_{2\alpha}$ to 8-*iso*-$PGF_{2\alpha}$ retained on the 15(*S*)-8-*iso*-$PGF_{2\alpha}$-IAC column was relatively constant (6.8±1.7, 7.1±2.6, 7.0±1.3, respectively), indicating that not more than ~13% of the 8-*iso*-$PGF_{2\alpha}$-like material was 15(*S*)-8-*iso*-$PGF_{2\alpha}$. Diclofenac (1 μ*M*) and acetylsalicylic acid (1 m*M*) inhibited 15(*S*)-8-*iso*-$PGF_{2\alpha}$ formation by 12.4 and 57%, respectively (Fig. 6). Acetylsalicylic acid inhibited formation of 8-*iso*-$PGF_{2\alpha}$-like material by the same extent (by 59%). By contrast, diclofenac inhibited the formation of this 8-*iso*-$PGF_{2\alpha}$-like material stronger (by 21.5%) than that of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (by 12.4%).

3.4. PLA_2-catalysed release of 15(S)-8-iso-$PGF_{2\alpha}$ from serum lipids

In native serum and plasma samples from three healthy volunteers free 15(*S*)-8-*iso*-$PGF_{2\alpha}$ concentration was measured to be 7.4±3.1 pg/ml. Addition of PLA_2 resulted in increases in 15(*S*)-8-*iso*-$PGF_{2\alpha}$ concentrations in a manner depending upon incubation time and PLA_2 activity added yielding concentrations of up to 40 pg/ml (Fig. 7). In the absence of external PLA_2, serum concentrations of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ also increased with incubation time, but they were lower than in the presence of exogenous PLA_2 (Fig. 7A). Methods precision (RSD) for serum 15(*S*)-8-*iso*-$PGF_{2\alpha}$ analysed in duplicate (Fig. 7B) was 9.8±4.4%. In the serum sample, unesterified endogenous 15(*S*)-8-*iso*-$PGF_{2\alpha}$ was determined to be 13.4±5.3 pg/ml. In the serum sample spiked with 160 pg/ml of 15(*S*)-8-*iso*-$PGF_{2\alpha}$, 15(*S*)-8-*iso*-$PGF_{2\alpha}$ was determined to be 175±26 pg/ml which corre-

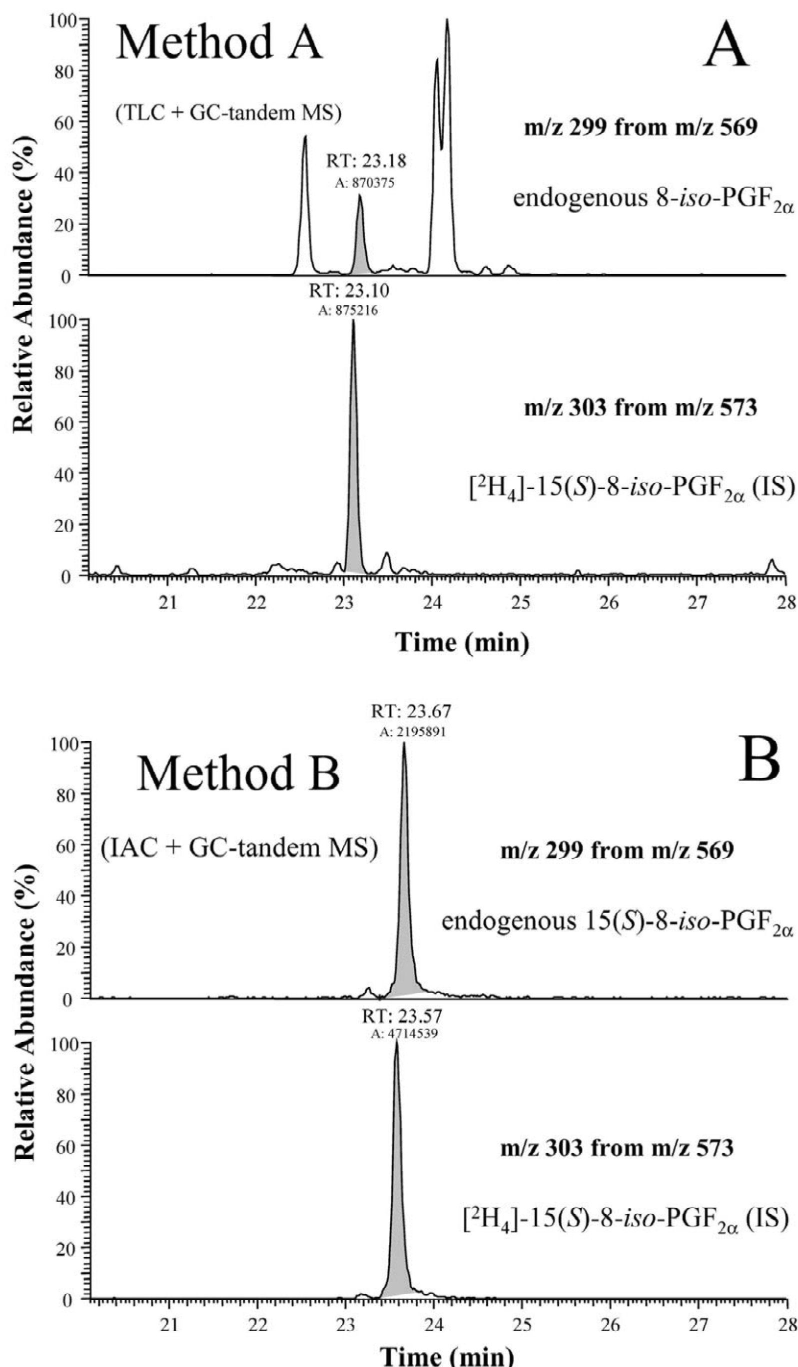

Fig. 4. Partial chromatograms from the GC–tandem MS analysis of 8-*iso*-$PGF_{2\alpha}$ in unspiked urine samples (1 ml) after sample treatment by (A) method A and (B) method B. The concentration of the internal standard [$^{2}H_{4}$]-15(*S*)-8-*iso*-$PGF_{2\alpha}$ in the urine samples was 250 pg/ml each. In this urine sample, 8-*iso*-$PGF_{2\alpha}$ is determined to be 248 pg/ml by method A and 116 pg/ml by method B. GC column, Optima 17, 30 m×0.25 mm I.D., 0.25 μm film thickness. RT, retention time (min); A, peak area (arbitrary units).

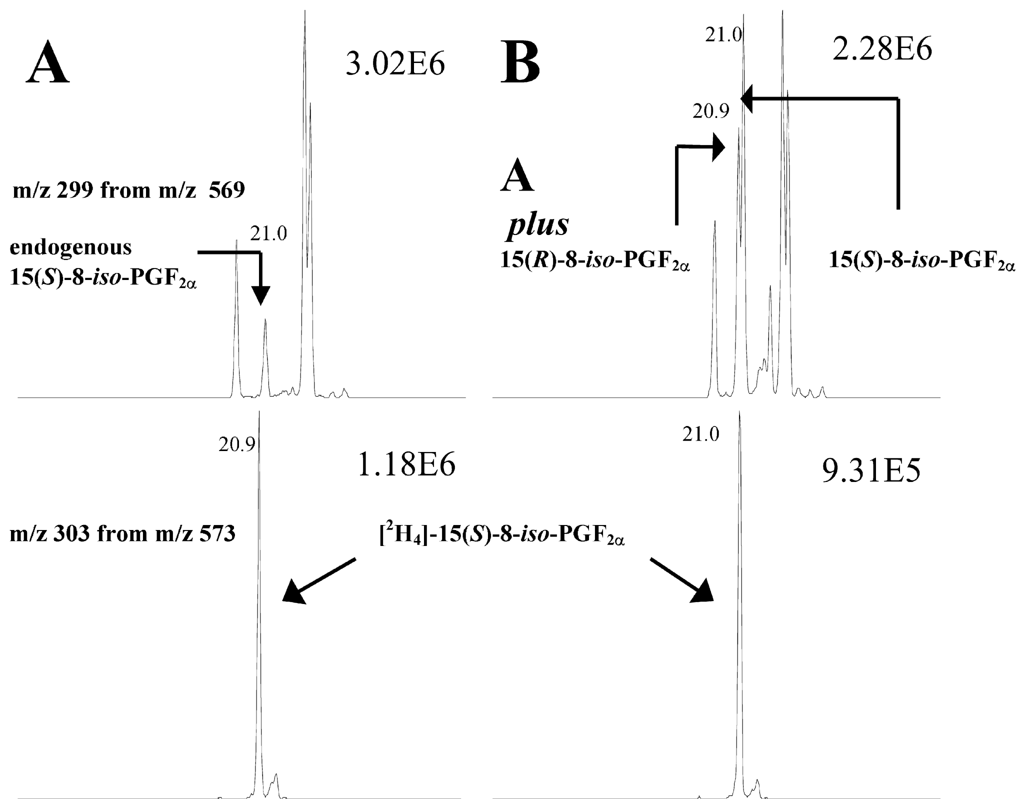

Fig. 5. Partial chromatograms from the GC–tandem MS analysis of 15(*S*)-8-*iso*-PGF$_{2\alpha}$ in a urine sample (1 ml) by method B (IAC+GC–tandem MS) before (A) and after (B) addition of an equimolar mixture containing the PFB-TMS derivatives of synthetic 15(*S*)-8-*iso*-PGF$_{2\alpha}$ and 15(*R*)-8-*iso*-PGF$_{2\alpha}$ (~100 pg each).

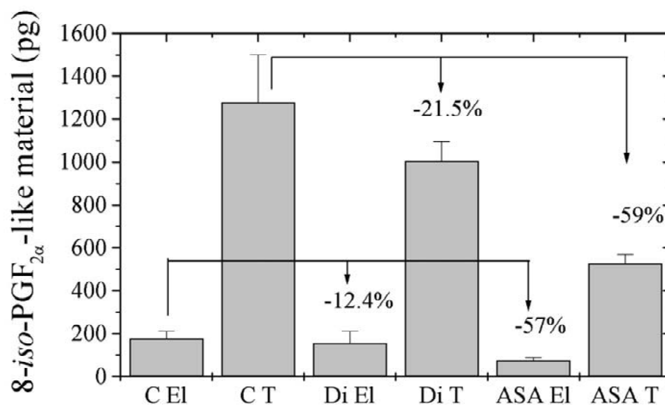

Fig. 6. Distribution of cyclooxygenase 1 (100 units)/arachidonic acid (10 μ*M*)-derived 8-*iso*-PGF$_{2\alpha}$-like material in the eluate (El, 2 ml) and in the ''passed through+wash'' phase (T, 5 ml) of IAC in the absence of any inhibitor (C, control) and in the presence of diclofenac (Di, 1 μ*M*) or acetylsalicylic acid (ASA, 1 m*M*). The ''passed through+wash'' phase was spiked with the internal standard (1250 pg of [$^2H_4$]-15(*S*)-8-*iso*-PGF$_{2\alpha}$), acidified to pH 3, reaction products were extracted twice with ethylacetate (2.5 ml) and derivatized. Data are presented as mean±SD from triplicate incubations. Numbers over the columns indicate the extent of inhibition for retained and unretained reaction products on 15(*S*)-8-*iso*-PGF$_{2\alpha}$-IAC columns with respect to the respective phase of the control experiment.

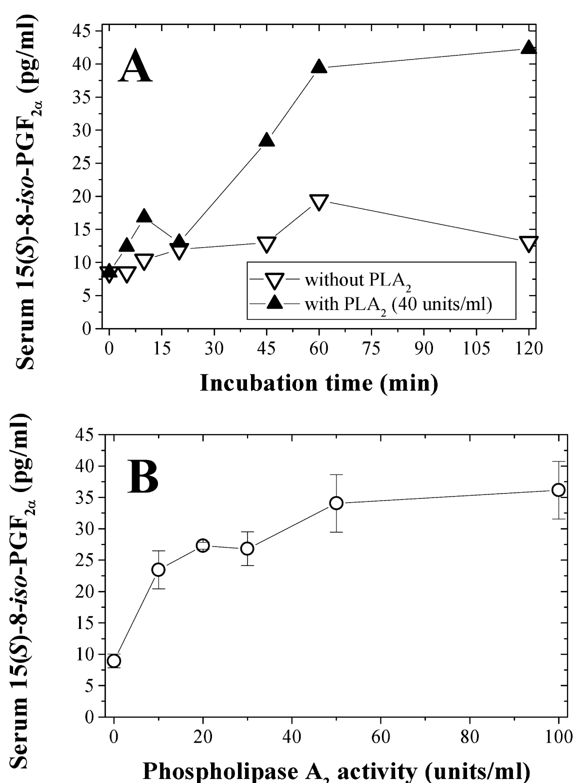

Fig. 7. Serum 15(S)-8-*iso*-$PGF_{2\alpha}$ concentration profiles as a function of incubation time (A) and phospholipase A_2 (PLA_2) activity added externally (B). In experiment A, PLA_2 activity was 40 units per ml serum. In experiment B, incubation time was 45 min, and data are shown as mean±SD from duplicate incubations. In both experiments 1-ml serum aliquots from a healthy human were incubated at 37 °C.

sponds to a recovery of 101%. In the unspiked serum sample treated with PLA_2 or KOH, 15(S)-8-*iso*-$PGF_{2\alpha}$ levels were determined to be 44±12 and 94±14 pg/ml, respectively, suggesting that the majority of 15(S)-8-*iso*-$PGF_{2\alpha}$ is esterified to serum lipids.

3.5. Urinary and circulating 15(S)-8-iso-$PGF_{2\alpha}$ in healthy smokers and non-smokers

No statistically significant differences were found for urinary creatinine in smokers and non-smokers (14.2±6.1 vs. 15.3 mM). Urinary creatinine-corrected 15(S)-8-*iso*-$PGF_{2\alpha}$ values as well as serum 15(S)-8-*iso*-$PGF_{2\alpha}$ levels were insignificantly different in smokers compared with non-smokers (100±14 vs. 78±11 nmol/mol creatinine for urine and 81±10 vs. 73±5 pg/ml for serum, respectively) (Fig. 8). The ratios of the urinary excretion rates and the circulating serum levels of 15(S)-8-*iso*-$PGF_{2\alpha}$ in smokers and non-smokers were 1.287 and 1.109, respectively. Females and males of both groups were found to excrete similar amounts of 15(S)-8-*iso*-$PGF_{2\alpha}$ into the urine (98±55 and 101±42 nmol/mol creatinine in smokers, and 68±14 and 53±7 nmol creatinine in non-smokers, respectively). Linear regression analysis between urinary creatinine-corrected 15(S)-8-*iso*-$PGF_{2\alpha}$ levels (Y) and number of cigarettes reportedly smoked (X) revealed the linear regression equation $Y = 69 + 2.1X$ and the regression coefficient $R=0.67$. This suggests a weak dependence of urinary excretion of 15(S)-8-*iso*-$PGF_{2\alpha}$ in

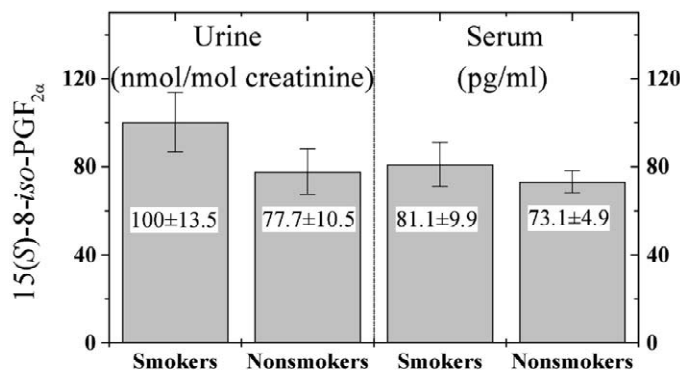

Fig. 8. Urinary excretion and serum levels of 15(S)-8-*iso*-$PGF_{2\alpha}$ in 12 healthy non-smoking subjects and 10 healthy smoking persons. 15(S)-8-*iso*-$PGF_{2\alpha}$ serum levels represent free plus esterified 15(S)-8-*iso*-$PGF_{2\alpha}$ released from serum lipids by alkaline hydrolysis. Data are shown as mean±SE. Method B (IAC+GC–tandem MS) was used.

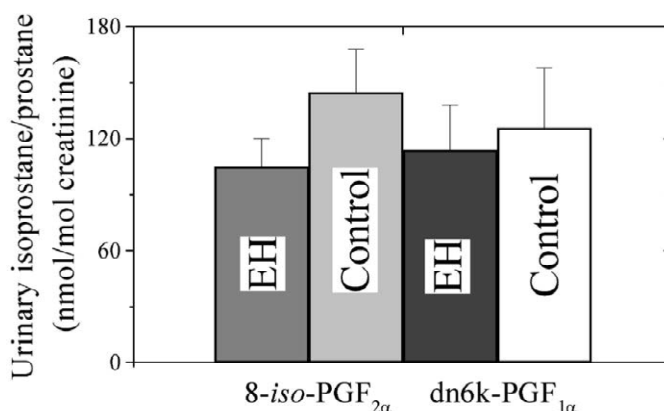

Fig. 9. Creatinine-corrected urinary levels of 8-*iso*-PGF$_{2\alpha}$ and 2,3-dinor-6-oxo-PGF$_{1\alpha}$ (dn6k-PGF$_{1\alpha}$), the major urinary metabolite of prostacyclin in man, in 17 male subjects suffering from essential hypertension (EH), and in 11 healthy male volunteers serving as control. Data are shown as mean±SE. Student's *t*-test of log-transformed data revealed no statistically significant differences between both groups for 8-*iso*-PGF$_{2\alpha}$ ($P=0.13$) and 2,3-dinor-6-oxo-PGF$_{1\alpha}$ ($P=0.65$). Method A (TLC+GC–tandem MS) was used.

smokers on their smoking habit with an increase in the urinary excretion of 15(*S*)-8-*iso*-PGF$_{2\alpha}$ of 2 nmol/mol creatinine per packyear. The *Y*-axis intercept value of 69 nmol/mol creatinine is comparable to the mean value of non-smokers. No correlation existed between circulating and urinary 15(*S*)-8-*iso*-PGF$_{2\alpha}$ in both groups.

3.6. Isoprostane and prostacyclin synthesis in essential hypertension

Patients suffering from essential hypertension (EH) and healthy normotensive volunteers showed similar, statistically (Student's *t*-test for log-transformed data) not significantly different urinary excretion rates of 8-*iso*-PGF$_{2\alpha}$ (measured by method A) and 2,3-dinor-6-oxo-PGF$_{1\alpha}$ (Fig. 9), and of creatinine clearance (119±15 vs. 118±19 ml/min), indicating similar systemic production of 8-*iso*-PGF$_{2\alpha}$ and prostacyclin in essential hypertension and health.

4. Discussion

4.1. Evidence for the heterogeneity of urinary 8-iso-PGF$_{2\alpha}$ in humans

Mass spectrometry (MS)-based analytical approaches such as GC–MS and GC–tandem MS are widely and routinely used for the quantitative determination of numerous synthetic and natural compounds including the eicosanoids, i.e. the prostanes and isoprostanes, the oxidative metabolites of arachidonic acid from enzymically and nonenzymically catalysed reactions, respectively [13]. In general, unequivocal identification and accurate quantification of prostanes, isoprostanes and their metabolites, which commonly occur at very low concentrations in biological fluids such as plasma and urine, require use of chromatographic techniques, such as HPLC, TLC and IAC for sample purification [13]. In recent years, affinity chromatography including immunoaffinity column chromatography (IAC) has been developed to a superior technique for sample preparation of numerous classes of compounds in MS-based methods [14]. IAC as a sample preparation in GC–MS and GC–tandem MS has been developed and applied to various eicosanoids [14], including the isoprostane 8-*iso*-PGF$_{2\alpha}$ [11,15]. Comparison regarding levels in biological fluids of 8-*iso*-PGF$_{2\alpha}$ in particular and F_2-isoprostanes in general between different research groups, who apply, as a rule, different analytical methods, is very important due to the generally accepted validity of these eicosanoids as reliable quantitative markers of oxidative stress in vivo in man.

In previous work [9], we reported on a fully validated GC–tandem MS method which allows specific and accurate quantitative determination of

8-*iso*-PGF$_{2\alpha}$ in human urine after purification of PFB derivatives by TLC. This method, i.e. method A, reproducibly yields four peaks with m/z 299 (from m/z 569) for the endogenous compounds and a single peak with m/z 303 (from m/z 573) for the internal standard [^{2}H$_4$]-8-*iso*-PGF$_{2\alpha}$ (Fig. 4A), suggesting that the TLC band scraped off contains at least three further compounds in addition to 8-*iso*-PGF$_{2\alpha}$. The GC peak eluting with almost identical retention time (t_R) with that of [^{2}H$_4$]-8-*iso*-PGF$_{2\alpha}$ was identified as 8-*iso*-PGF$_{2\alpha}$. The peaks eluting in front and behind 8-*iso*-PGF$_{2\alpha}$ had not been further investigated. HPLC separation of SPE extracts of urine samples containing the free acids of endogenous eicosanoids and of externally added [^{2}H$_4$]-8-*iso*-PGF$_{2\alpha}$ followed by TLC purification and GC–tandem MS quantification, i.e. by method A, resulted in a single peak each at m/z 299 due to 8-*iso*-PGF$_{2\alpha}$ and at m/z 303 due to [^{2}H$_4$]-8-*iso*-PGF$_{2\alpha}$ [9]. However, additional use of HPLC did not affect the concentration 8-*iso*-PGF$_{2\alpha}$ measured in human urine samples [9]. Similar results were obtained for the major urinary metabolite of 8-*iso*-PGF$_{2\alpha}$, i.e. 2,3-dinor-5,6-dihydro-8-*iso*-PGF$_{2\alpha}$ [10]. These findings and literature data on the t_R of F$_2$-isoprostanes in GC and LC suggest that the endogenous compound from human urine eluting with the t_R of synthetic 8-*iso*-PGF$_{2\alpha}$ is 9α,11α,15(*S*)-trihydroxy-(8β,12β)-prosta-5*Z*,13*E*-dien-1-oic acid, i.e. 15(*S*)-8-*iso*-PGF$_{2\alpha}$. It cannot, however, be excluded that other F$_2$-isoprostane diastereomers, such as 15(*R*)-8-*iso*-PGF$_{2\alpha}$, 15(*S*)-12-*iso*-PGF$_{2\alpha}$, 15(*R*)-12-*iso*-PGF$_{2\alpha}$, 15(*S*)-*ent*-12-*iso*-PGF$_{2\alpha}$, 15(*R*)-*ent*-12-*iso*-PGF$_{2\alpha}$, 15(*S*)-*ent*-8-*iso*-PGF$_{2\alpha}$ and 15(*R*)-*ent*-8-*iso*-PGF$_{2\alpha}$, coelute. This is supported by the finding that 15(*S*)-2,3-dinor-5,6-dihydro-8-*iso*-PGF$_{2\alpha}$ and *ent*-15(*S*)-2,3-dinor-5,6-dihydro-8-*iso*-PGF$_{2\alpha}$ cannot be discriminated by TLC and GC–tandem MS [10].

Chiabrando and co-workers have shown that IAC extraction with anti-8-*iso*-PGF$_{2\alpha}$ immunosorbents selectively bind their nominal antigenic ligand from urine and other biological media [11,15]. Although not all possible F$_2$-isoprostanes have been tested for cross-reactivity against anti-8-*iso*-PGF$_{2\alpha}$ immunosorbents, the data available so far suggest that the IAC columns used in the present study are highly selective for 15(*S*)-8-*iso*-PGF$_{2\alpha}$. The highest cross-reactivity towards anti-8-*iso*-PGF$_{2\alpha}$ immunosorbents has been reported for 8-*iso*-PGF$_{3\alpha}$ (7.6%), PGF$_{1\alpha}$ (2.85%), PGF$_{2\alpha}$ (0.88%), and 11β-PGF$_{2\alpha}$ (0.83%). As is well known, all these F-prostaglandins can be separated from 15(*S*)-8-*iso*-PGF$_{2\alpha}$ by HPLC, TLC and GC–tandem MS. It is, therefore, highly unlikely that these compounds have contributed to 15(*S*)-8-*iso*-PGF$_{2\alpha}$ in method B (IAC + GC–tandem MS) in the present study. Also, it can be excluded that the above mentioned compounds have contributed to 8-*iso*-PGF$_{2\alpha}$ in method A (TLC + GC–tandem MS) previously [9,10] and in the present study.

We have presumed that the most likely candidate for coelution with 15(*S*)-8-*iso*-PGF$_{2\alpha}$ in method A would be 15(*R*)-8-*iso*-PGF$_{2\alpha}$, *ent*-15(*R*)-8-*iso*-PGF$_{2\alpha}$ and *ent*-15(*S*)-8-*iso*-PGF$_{2\alpha}$. We found that less than 1.5% of the amount of 15(*R*)-8-*iso*-PGF$_{2\alpha}$ (5 ng in the column buffer) applied to the 15(*S*)-8-*iso*-PGF$_{2\alpha}$-IAC column was present in the eluate of the IAC column, whereas 15(*S*)-8-*iso*-PGF$_{2\alpha}$ and [^{2}H$_4$]-15(*S*)-8-*iso*-PGF$_{2\alpha}$ (5 ng of each in the column buffer) were quantitatively recovered. However, 15(*R*)-8-*iso*-PGF$_{2\alpha}$ can be largely excluded as the major contributor to 8-*iso*-PGF$_{2\alpha}$ in method A, because the PFB-TMS derivative of 15(*R*)-8-*iso*-PGF$_{2\alpha}$ emerges from the GC column clearly in front of that of 15(*S*)-8-*iso*-PGF$_{2\alpha}$, although no complete resolution was reached (Fig. 5). The relative t_R of 15(*R*)-8-*iso*-PGF$_{2\alpha}$ was 0.995 with respect to 15(*S*)-8-*iso*-PGF$_{2\alpha}$. The relative t_R of the PFB-TMS derivative of 12α,14α,8(*S*)-trihydroxy-(11β,15β)-prosta-5*Z*,9*E*-dien-1-oic-17,18,19,20-^{2}H$_4$ acid, the tetradeuterated analog of a physiological F$_2$-isoprostane (i.e. iPF$_{2\alpha}$-IV), was 1.013 with respect to that of [^{2}H$_4$]-8-*iso*-PGF$_{2\alpha}$. This disqualifies iPF$_{2\alpha}$-IV from being a contributor to 8-*iso*-PGF$_{2\alpha}$ in method A. Due to the lack of synthetic *ent*-15(*S*)-8-*iso*-PGF$_{2\alpha}$ we were not able to test the chromatographic, immunochromatographic and mass spectrometric properties of this isoprostane. Chiabrando et al. have shown that IAC extraction with anti-8-*iso*-PGF$_{2\alpha}$ immunosorbents do not efficiently bind six of eight possible F$_2$-isoprostanes of the 8-*iso*-PGF$_{2\alpha}$ family tested, including 15(*R*)-8-*iso*-PGF$_{2\alpha}$ and *ent*-15(*S*)-8-*iso*-PGF$_{2\alpha}$ [15]. This group has also shown that the 15(*S*)-2,3-dinor-5,6-dihydro-8-*iso*-PGF$_{2\alpha}$ immunosorbent cannot recognize its enantiomer, *ent*-15(*S*)-2,3-dinor-5,6-dihydro-8-*iso*-PGF$_{2\alpha}$ [15]. These findings together with the observation that 15(*S*)-2,3-

dinor-5,6-dihydro-8-*iso*-$PGF_{2\alpha}$ and *ent*-15(*S*)-2,3-dinor-5,6-dihydro-8-*iso*-$PGF_{2\alpha}$ cannot be discriminated by TLC and GC–tandem MS [10], strongly suggest that 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and *ent*-15(*S*)-8-*iso*-$PGF_{2\alpha}$ can also not be discriminated by TLC and GC–tandem MS. It is, therefore, likely that urinary 8-*iso*-$PGF_{2\alpha}$ consists of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and *ent*-15(*S*)-8-*iso*-$PGF_{2\alpha}$.

4.2. Methodological considerations

Most likely, our previously described GC–tandem MS method (method A) measures specifically 15(*S*)-8-*iso*-$PGF_{2\alpha}$ plus *ent*-15(*S*)-8-*iso*-$PGF_{2\alpha}$, while the present IAC-column extraction involving method, i.e. method B, assesses selectively 15(*S*)-8-*iso*-$PGF_{2\alpha}$. In general, it can be assumed that accurate immunoaffinity-based methods for 15(*S*)-8-*iso*-$PGF_{2\alpha}$, such as IAC extraction with subsequent quantification by GC–MS or immunoassays (EIA, RIA), should regularly yield lower values for urinary 8-*iso*-$PGF_{2\alpha}$ than other purely physicochemical methods. Consequently, caution should be exercised when comparing 8-*iso*-$PGF_{2\alpha}$ and/or other F_2-isoprostane levels which were obtained by using different analytical methods. Moreover, comparison should be performed and indicated not by reporting simply the coefficient of correlation from linear regression analysis, but also by reporting the accompanying regression equation [16–18].

4.3. Mechanistic considerations—8-iso-$PGF_{2\alpha}$ as a biomarker of oxidative stress in vivo

The results of the present study generated from the combination of both methods suggest that urinary 8-*iso*-$PGF_{2\alpha}$ comprises of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and most likely of its enantiomer, *ent*-15(*S*)-8-*iso*-$PGF_{2\alpha}$, each contributing by ~50% to 8-*iso*-$PGF_{2\alpha}$. Occurrence of these compounds in a stoichiometry of 1:1 could be explained by assuming a common intermediate which is formed COX-independently through an intermolecular reaction between two arachidonic acid molecules esterified to neighbouring lipids and three molecules of molecular oxygen being between these lipids. Reduction of the peroxy groups, e.g. by phospholipid hydroperoxy glutathione peroxidase in membranes and lipoproteins, and subsequent enzymic hydrolysis of the esterified 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and *ent*-15(*S*)-8-*iso*-$PGF_{2\alpha}$, e.g. by PLA_2, would yield the free acids 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and *ent*-15(*S*)-8-*iso*-$PGF_{2\alpha}$, which are then excreted into the urine with a molar ratio of 1:1.

To date, contradictory results have been reported on the mechanisms leading to 8-*iso*-$PGF_{2\alpha}$ formation in vitro and in vivo, in humans and in animals, suggesting both enzymatic and nonenzymatic formation [19–22]. Evidence for enzyme-catalyzed formation of 8-*iso*-$PGF_{2\alpha}$ is usually based on the inhibition of 8-*iso*-$PGF_{2\alpha}$ formation by COX inhibitors such as acetylsalicylic acid, indomethacin, and naproxen. Interestingly, Bachi et al. found by IAC extraction of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and its subsequent quantification by GC–MS that in rats indomethacin did not change esterified 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in liver and plasma lipids, but indomethacin and naproxen reduced significantly excretion into the urine both of thromboxane and prostacyclin metabolites and of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ [22]. In man, however, naproxen did not change urinary excretion of 15(*S*)-8-*iso*-$PGF_{2\alpha}$, unlike the major urinary metabolites of thromboxane and prostacyclin [22].

Using isolated ovine COX 1, we found that only a negligible portion of arachidonic acid is converted to 15(*S*)-8-*iso*-$PGF_{2\alpha}$-like material, and that this conversion was inhibited by 1 μM diclofenac weakly (by 12%), by 1 m*M* acetylsalicylic acid, however, strongly (by 57%). Similar results have been reported for the ex vivo formation of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ [21], 8-*iso*-$PGF_{2\alpha}$ [19], and TxB_2 [19,22] during spontaneous whole blood clotting in man as measured by GC–MS, with 15(*S*)-8-*iso*-$PGF_{2\alpha}$ serum concentrations being ~3000 times smaller than those of TxB_2 [22]. These results suggest a minor contribution of COX to 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and 8-*iso*-$PGF_{2\alpha}$. Ex vivo in human serum, activated neutrophils and platelets could contribute to 8-*iso*-$PGF_{2\alpha}$ due to free radical generation [19]. In vitro, using isolated COX 1, formation of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and 8-*iso*-$PGF_{2\alpha}$ from unesterified arachidonic acid could also be formed due to COX 1-dependent free radical generation on the basis of inhibition of COX activity by nonsteroidal antiphlogistic drugs. However, the extent of COX-dependent free radical-catalyzed generation leading to formation of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and 8-*iso*-$PGF_{2\alpha}$ is several orders of magnitude smaller than that leading to the regular COX-dependent arachidonic acid

metabolites, e.g. PGE_2, $PGF_{2\alpha}$, TxB_2 and PGI_2. With the reservation that the metabolism of endogenous 15(*S*)-8-*iso*-$PGF_{2\alpha}$ is insufficiently investigated [15,23], especially with regard to $PGF_{2\alpha}$ metabolism, urinary excretion rates of 8-*iso*-$PGF_{2\alpha}$ and its presently known metabolites are of the same order as those of $PGF_{2\alpha}$. On the basis of the knowledge available so far, it can be assumed that the origin of urinary and circulating 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and other F_2-isoprostanes in man is free radical-catalyzed peroxidation of arachidonic acid esterified to lipids, but not COX-dependent peroxidation of arachidonate [24].

Based on the observation that COX inhibition by naproxen and indomethacin caused significant reduction in urinary excretion of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in rats but not in man, Chiabrando et al. concluded that in rats, unlike in humans, a significant amount of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ originates from COX [22]. However, this conclusion is difficult to reconcile with the finding that esterified 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in liver and plasma remained unchanged after indomethacin [22]. A possible explanation for the unexpected finding might be that the reduction of the urinary excretion of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ is indirect, i.e. due to inhibition by these drugs of PLA_2 activity in the kidney and in other organs of the rat rather than due to inhibition of COX activity. This explanation is supported by previous findings showing that indomethacin inhibits basal and Ca^{2+}-stimulated PLA_2 activity in rabbits [25], whereas acetylsalicylic acid inhibits expression of the interleukin-1β-inducible group II PLA_2 in rats [26]. Because TxB_2 may stimulate both PLA_2 activity and COX activity in isolated glomeruli from rats [27], it can be assumed that inhibition of TxB_2 formation in platelets by naproxen [22] or acetylsalicylic acid [19] could have indirectly led to inhibition of PLA_2-catalysed release of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ from lipids. In the present study we have shown that external addition of PLA_2 to human serum increases 15(*S*)-8-*iso*-$PGF_{2\alpha}$ concentrations in a time- and PLA_2-activity-dependent manner (see Fig. 7).

4.4. Urinary and serum 15(S)-8-iso-$PGF_{2\alpha}$ in healthy smokers and non-smokers

By means of method B, we found that healthy young smokers excreted insignificantly more 15(*S*)-8-*iso*-$PGF_{2\alpha}$ into the urine than healthy non-smokers of the same age. A weak correlation ($R=0.67$) between urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$ excretion and number of reportedly consumed cigarettes existed. Using 15(*S*)-8-*iso*-$PGF_{2\alpha}$-IAC and GC–MS, Bachi et al. [11] found that healthy young smokers excreted 2.3 times more 15(*S*)-8-*iso*-$PGF_{2\alpha}$ into the urine than healthy young non-smokers. Ohashi and Yoshikawa [28] found by LC–MS that smokers excreted statistically insignificantly 1.5 times higher amounts of 8-*iso*-$PGF_{2\alpha}$ into the urine than non-smokers, while plasma concentrations of unesterified 8-*iso*-$PGF_{2\alpha}$ were similar in both groups [28]. This finding collaborates with our observation for 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (Fig. 8). Ohashi and Yoshikawa [28] reported basal levels for 8-*iso*-$PGF_{2\alpha}$ of 136 nmol/mol creatinine in urine and 42 pg/ml in plasma of non-smokers. Urinary basal levels for 8-*iso*-$PGF_{2\alpha}$ reported by Ohashi and Yoshikawa in non-smokers are comparable with those measured by us in the present study in the urine of non-smokers by method A (Fig. 8, control group) and approximately two times higher that those measured by method B in our non-smokers (Fig. 8). This suggests that in human urine both GC–tandem MS (i.e. method A) and LC–MS [28] measure 15(*S*)-8-*iso*-$PGF_{2\alpha}$ plus *ent*-15(*S*)-8-*iso*-$PGF_{2\alpha}$. Unlike urine, basal plasma concentrations of free 8-*iso*-$PGF_{2\alpha}$ measured by LC–MS [28] are approximately six times higher than those measured by method B in the present study (42 vs. 7 pg/ml), suggesting that the LC–MS method of Ohashi and Yoshikawa might suffer from coeluting interferences. By means of LC–tandem MS, Murai et al. [29] reported that healthy young non-smokers excreted into the urine 8-*iso*-$PGF_{2\alpha}$ at mean concentrations of 135 nmol/mol creatinine which are almost identical with those found by us previously and in the present study by method A as well as with those reported by Ohashi and Yoshikawa [28]. The applicability of Murai et al.'s LC–tandem MS method to determine plasma concentrations of free 8-*iso*-$PGF_{2\alpha}$ has not been reported thus far.

4.5. Isoprostane and prostacyclin synthesis in essential hypertension

In patients suffering from essential hypertension we found by method A an insignificant tendency to lower urinary excretion of 8-*iso*-$PGF_{2\alpha}$ as compared

with normotensive healthy volunteers, suggesting that oxidative stress is not enhanced in uncomplicated essential hypertension. Also, urinary excretion of 2,3-dinor-6-oxo-$PGF_{1\alpha}$, a reliable indicator of whole body synthesis of prostacyclin in man [30], was similar in essential hypertension and health, also suggesting that prostacyclin synthesis is not altered in essential hypertension. Interestingly, in essential hypertension urinary excretion of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ tended to lower levels in comparison with that of the control group, similar to that of 8-*iso*-$PGF_{2\alpha}$. Thus, the same tendency to reduced whole body synthesis of 8-*iso*-$PGF_{2\alpha}$ and prostacyclin argues against an increased oxidative stress in uncomplicated essential hypertension in man, which has been suggested by other groups on the basis of other biomarkers of oxidative stress such as plasma malondialdehyde, diene conjugates, and thiobarbituric acid reactive substances [31–33]. Our results are supported by recent findings by Minuz et al. [34], who found by RIA similar urinary levels of 8-*iso*-$PGF_{2\alpha}$ (most likely 15(*S*)-8-*iso*-$PGF_{2\alpha}$) in patients with essential hypertension and healthy subjects.

5. Conclusions

Use of commercially available IAC columns with immobilized antibodies raised against 15(*S*)-8-*iso*-$PGF_{2\alpha}$ allows for the highly selective and inexpensive extraction of this F_2-isoprostane from urine and plasma or serum samples. Quantification of urinary and circulating 15(*S*)-8-*iso*-$PGF_{2\alpha}$ is best accomplished by GC–tandem MS in the NICI mode. Combination of TLC for isolation of 8-*iso*-$PGF_{2\alpha}$ from urine and subsequent quantification by GC–tandem MS yields twice as high values for 8-*iso*-$PGF_{2\alpha}$ than the combination of IAC extraction with GC–tandem MS, revealing the heterogeneity of urinary 8-*iso*-$PGF_{2\alpha}$ in humans.

Compounds unable to bind to the 15(*S*)-8-*iso*-$PGF_{2\alpha}$-IAC column are not included with the 15(*R*)-8-*iso*-$PGF_{2\alpha}$ isomer, suggesting an intermolecular mechanism of peroxidation of arachidonic acid esterified to lipids, with *ent*-15(*S*)-8-*iso*-$PGF_{2\alpha}$ being the complementary partner of 15(*S*)-8-*iso*-$PGF_{2\alpha}$. In incubations of arachidonate and isolated ovine COX 1 in vitro, merely a very little portion of unesterified arachidonic acid is converted to 15(*S*)-8-*iso*-$PGF_{2\alpha}$, compared with 20% to PGE_2. Most likely, urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and *ent*-15(*S*)-8-*iso*-$PGF_{2\alpha}$ originate from oxidation of arachidonic acid esterified to lipids by a free radical-catalysed, COX-independent mechanism. It is also very likely that reduction of urinary excretion of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ by COX inhibitors seen in rats does not reflect COX-dependent formation, but it is rather an indirect effect resulting from inhibition of PLA_2 activity by non-steroidal antiphlogistic drugs (NSAIDs). Urinary 8-*iso*-$PGF_{2\alpha}$ is a reliable biomarker of oxidative stress in humans. However, decrease or increase in urinary excretion of 8-*iso*-$PGF_{2\alpha}$ by pharmacological and/or nutritional events might feign reduction or elevation of oxidative stress due to unrelated changes in PLA_2 activity. This possibility must be considered in clinical studies dealing with oxidative stress.

Young healthy smokers excrete into the urine insignificantly higher amounts of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ than young healthy non-smokers, whereas the difference between the groups with regard to nonesterified plus esterified 15(*S*)-8-*iso*-$PGF_{2\alpha}$ to plasma lipids is clearly lower than for urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$. In uncomplicated essential hypertension, whole body synthesis of 8-*iso*-$PGF_{2\alpha}$ and prostacyclin tends to the same direction, arguing against an elevated oxidative stress in this disease.

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