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Specific and rapid quantification of 8-iso-prostaglandin $F_{2\alpha}$ in urine of healthy humans and patients with Zellweger syndrome by gas chromatography–tandem mass spectrometry

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Abstract

8-iso-Prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) is currently discussed as a potential index parameter of oxidative stress in vivo. We describe in this article a fully validated gas chromatographic–tandem mass spectrometric method for the quantitative determination of 8-iso-$PGF_{2\alpha}$ in human urine. The method is highly specific and requires a single thin-layer chromatographic step for sample purification. Inter- and intraday imprecision were below 8%. Mean inaccuracy was 5.3% for added levels of 8-iso-$PGF_{2\alpha}$ up to 2000 pg/ml of urine. We measured highly elevated excretion of 8-iso-$PGF_{2\alpha}$ in the urine of children with peroxisomal β-oxidation deficiency, i.e. Zellweger syndrome, (63.3±16.6 ng/mg creatinine) compared to that of healthy children (0.51±0.16 ng/mg creatinine) (mean±S.D., both $n=5$). The method could be useful for diagnosing Zellweger syndrome and for investigating the utility of 8-iso-$PGF_{2\alpha}$ as a novel marker for oxidative stress in vivo in man.

Keywords: 8-iso-Prostaglandin $F_{2\alpha}$; Prostaglandins

1. Introduction

Isoprostanes are prostaglandin (PG)-like compounds that are thought to be produced by non-enzymatic free radical-catalyzed peroxidation of arachidonic acid [1–5]. It has therefore been suggested that measurement of isoprostanes in biological fluids may provide a means to assess oxidative stress in vivo [3]. F_2-Isoprostanes are among the most abundant endogenous isoprostanes found in various tissues and biological fluids of humans and animals; they were found to be involved in many diseases (reviewed in [4,5]).

8-iso-$PGF_{2\alpha}$ has been shown to be formed in vivo in the rat [6]. This isoprostane exerts potent biological activities [7,8]. 8-iso-$PGF_{2\alpha}$ has been detected in human plasma by gas chromatography–mass spectrometry (GC–MS) following hydrolysis by alkali [9]. Urinary 8-iso-$PGF_{2\alpha}$ excretion in man has been immunologically characterized by Wang et al. [10]. A GC–MS method using immunoaffinity extraction has been described by Bachi et al. [11] for the quantitative determination of 8-iso-$PGF_{2\alpha}$ in urine of humans. Catella et al. [12] have also

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quantitated 8-iso-$PGF_{2\alpha}$ in human urine by a GC–negative-ion chemical ionization MS (GC–NICI-MS) method. Delanty et al. [13] have reported enhanced urinary excretion of 8-iso-$PGF_{2\alpha}$ in humans with paracetamol and paraquat poisoning, as measured by GC–NICI-MS. Recently, Ferretti and Flanagan [14] have described a GC–NICI-MS method for the measurement of 8-iso-$PGF_{2\alpha}$ in human urine using reversed-phase high-performance liquid chromatography (RP-HPLC) and thin-layer chromatography (TLC) for sample purification. Also, Schweer et al. [15] have reported a GC–tandem MS method in the NICI mode (GC–NICI-MS–MS) for quantification of 8-iso-$PGF_{2\alpha}$ in human urine that requires TLC and normal-phase HPLC for sample purification. Thus, with the sole exception of immunoaffinity extraction, specific quantitation of 8-iso-$PGF_{2\alpha}$ in human urine by GC–NICI-MS or GC–NICI-MS–MS seems to require extensive sample purification by various time-consuming chromatographic procedures, such as TLC and HPLC.

The aim of this work was to develop a GC–NICI-MS–MS method for specific but more simple and rapid measurement of 8-iso-$PGF_{2\alpha}$ in human urine. Because of the lack in stable isotope-labeled 8-iso-$PGF_{2\alpha}$ standards we began with the preparation of [1,1-$^{18}O_2$]8-iso-$PGF_{2\alpha}$ for use as an internal standard [16]. In the meantime, a tetradeuterated 8-iso-$PGF_{2\alpha}$ analog, i.e. [3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$, of higher isotopic purity, became available commercially. Using this internal standard, we developed and fully validated a GC–NICI-MS–MS method that involves a single TLC step for sample purification. In this article, we describe this method and demonstrate its applicability to the non-invasive assessment of 8-iso-$PGF_{2\alpha}$ production in healthy humans and patients. We report here for the first time elevated urinary excretion rates of 8-iso-$PGF_{2\alpha}$ by children with the inborn Zellweger syndrome (ZS). This disease is characterized by impaired peroxisomal β-oxidation of various compounds, including prostaglandins and leukotrienes [17–20].

2. Experimental

2.1. Chemicals

$PGF_{2\alpha}$, 9β,11α-$PGF_{2\alpha}$, 9α,11β-$PGF_{2\alpha}$ and [3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$ (≥98% at ^2H) were obtained from Cayman Chemicals (Ann Arbor, MI, USA). 8-iso-$PGF_{2\alpha}$ was purchased from Biomol (Hamburg, Germany). [3,3′,4,4′-$^2H_4$]-$PGF_{2\alpha}$ was bought from Merck Frosst (Montreal, Canada). Pentafluorobenzyl (PFB) bromide was obtained from Aldrich (Steinheim, Germany). *N*,*N*-Diisopropylethylamine and 1-butaneboronic acid were purchased from Sigma (Deisenhofen, Germany). *N*,*O*-bis-(Trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce (Rockford, IL, USA). Acetonitrile and 2-propanol, both of HPLC grade, and all other chemicals and solvents were obtained from Merck (Darmstadt, Germany).

2.2. Biological samples

Urine from spontaneous micturition by apparently healthy adults was collected in polypropylene bottles, containing 1 m*M* each of 5-hydroxy-tempo (HTMP) and ethylenediamine tetraacetic acid (EDTA). Urine was collected in a similar manner for 24 h. Urine samples were divided into 5 ml aliquots and stored at −20°C until use. They were used for validation of the method.

Five children with ZS and five age- and sex-matched healthy infants were included in this study. All patients exhibited the characteristic clinical and biochemical abnormalities described for ZS [19]. The biochemical characteristics of the children with ZS have been published elsewhere [19]. Mitochondrial β-oxidation activity was found to be in the range of normal infants [19]. Convulsions were reported for all patients. None of the children with ZS had signs of cholestasis, liver failure or impaired renal function. Urine was obtained from spontaneous micturition and stored at −80°C until analysis. Creatinine was determined spectrophotometrically by the alkaline picric acid reaction with an automatic analyser (Beckman, Galway, Ireland) [21].

2.3. Solid-phase extraction and derivatization procedures

Aliquots (5 ml) of urine samples from healthy adults or healthy children and 0.6-ml aliquots from patients were spiked with 5 ng of [3,3′,4,4′-$^2H_4$]-8-

iso-$PGF_{2\alpha}$. The urine samples from patients were diluted with 4.4 ml of distilled water prior to extraction. The samples were acidified to pH 3.0 by the addition of 5 *M* HCOOH and allowed to stand on ice for 30 min. The samples were then applied to 500 mg octadecylsilica solid-phase extraction cartridges from Varian (Habour City, CA, USA), which were attached to a solid-phase extraction device, model SPE-21, from J.T. Baker (Phillipsburg, NJ, USA). The cartridges were preconditioned with 10 ml of methanol and 3 ml of 0.05 *M* HCOOH, washed with 20 ml of water and 2.5 ml of hexane and then air-dried. Compounds were eluted with 2 ml of ethyl acetate.

PFB esterification was performed by treatment of prostanoids with 10 μl of methanol, 100 μl of acetonitrile, 10 μl of *N*,*N*-diisopropylethylamine and 10 μl of PFB bromide solution in acetonitrile (30 wt.%) and by heating at 30°C for 1 h. In some experiments, PFB esters of synthetic and endogenous 8-iso-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ were converted to their cyclic butylboronate (BuB) derivatives, e.g. PFB–BuB, by incubation with 100 μl of a 10% (w/v) solution of 1-butaneboronic acid in pyridine and heating at 60°C for 1 h. After cooling to room temperature, the solvent was removed under nitrogen. The residue was treated with a 200-μl volume of water and extracted twice with a 1-ml volume of diethylether. The organic phases were decanted, combined and the solvent was evaporated. As a rule, trimethylsilyl (TMS) derivatization was carried out by reacting the PFB or PFB–BuB derivatives with a 20-μl volume of BSTFA for 1 h at 60°C.

2.4. Thin-layer chromatography

TLC on 20×20 cm silica gel 60 plates from Merck was carried out with a TLC-Applicator AS 30 and a DC-MAT, both of which were from Desaga (Wiesloch, Germany). Ethyl acetate, present in eluates following solid-phase extraction, was evaporated to dryness under nitrogen and PFB esterification was performed. Reagents and solvents from the PFB esterification mixtures were evaporated under nitrogen, and residues were reconstituted in 15 μl volumes of ethanol. Aliquots (10 μl) of these solutions were subjected to TLC using ethyl acetate–hexane (90:10, v/v) for elution. A reference plate that was spotted with the PFB derivatives of a mixture of 8-iso-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ (each at 1 μg) was developed in tandem. Each 0.6 cm band, centered around the reference compound [R_f=0.25 for $PGF_{2\alpha}$ and [3,3′,4,4′-$^2H_4$]-$PGF_{2\alpha}$; R_f=0.21 for 8-iso-$PGF_{2\alpha}$ (R_f 0.19 to 0.23), [3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$ and 9α,11β-$PGF_{2\alpha}$, and R_f=0.13 for 9β,11α-$PGF_{2\alpha}$; all R_f values were determined at coefficients of variation below 2%], was scraped off the TLC plate, compounds were extracted with 500 μl of ethanol and suspensions were centrifuged (4000 *g*, 10 min). Supernatants were decanted, ethanol was removed under nitrogen and PFB esters were converted to their TMS ether derivatives. Volumes of 1 μl were injected into the GC–MS–MS apparatus.

2.5. RP-HPLC

RP-HPLC of prostanoids 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 from Macherey-Nagel (Düren, Germany). Free acids were analysed isocratically using a mobile phase consisting of 10 m*M* NaH_2PO_4–acetonitrile–2-propanol (67:21:12, v/v/v), the pH of which was adjusted to 3.5 using *o*-phosphoric acid. The flow-rate was 1.8 ml/min and the effluent was detected at 205 nm. The following retention times (in min) were obtained from separate analyses of 1-μg amounts of each prostanoid: 11.54±0.21 for 8-iso-$PGF_{2\alpha}$ and 15.79±0.19 for $PGF_{2\alpha}$ (mean±SD, n=5). Deuterated compounds coeluted with their unlabeled analogs in this system. For quantitative measurements using RP-HPLC for sample purification, the following procedure was used: solvent present in the eluate from solid-phase extraction was evaporated to dryness. The residue was diluted in 200 μl of the mobile phase and injected into the RP-HPLC system. A 1.8-ml volume with the retention time of 8-iso-$PGF_{2\alpha}$ was collected, diluted with water (1:4, v/v), acidified to pH 3.5 using 2.5 *M* HCOOH, and the 8-iso-$PGF_{2\alpha}$ was solid-phase extracted as described above. Solvent was evaporated to dryness under a stream of nitrogen, the residue was derivatised by PFB bromide and PFB esters were subjected to TLC as described above.

2.6. GC–NICI-MS–MS

GC–NICI-MS and GC–NICI-MS–MS analyses were carried out on a triple-stage quadrupole mass spectrometer (Finnigan MAT TSQ 45) interfaced with a Finnigan MAT gas chromatograph 9611 (San Jose, CA, USA). The gas chromatograph was equipped with a fused-silica capillary column SPB-1701 (30 m×0.25 mm I.D., 0.25 μm film thickness) from Supelco (Bellefonte, PA, USA) or with a fused-silica capillary column DB-5MS (30 m×0.25 mm I.D., 0.25 μm film thickness) from J&W Scientific (Rancho Cordova, CA, USA). In some analyses, 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 (70 kPa) was used as the carrier gas. The following oven temperature program was used for the SPB-1701 column: 2 min at 80°C, then increased to 250°C at a rate of 25°C/min, followed by an increase to 280°C at a rate of 2°C/min, where the temperature was kept constant for 5 min. For the DB-5MS and Optima-17 columns, the following oven temperature program was used: 2 min at 80°C, then increased to 250°C at a rate of 25°C/min, followed by increases to 280 and 320°C at rates of 2 and 4°C/min, respectively. The SPB-1701 and Optima-17 columns were used for quantitative analysis, while the DB-5MS was used mainly for the analysis of butylboronate derivatives. The columns were inserted directly into the ion source of the mass spectrometer. The interface, injector and ion source were kept at 280, 280 and 140°C, respectively. Electron energy and electron current were 90 eV and 220 μA, respectively. Methane (65 Pa) and argon (0.2 Pa) were used as the reagent and collision gases, respectively. A collision energy of 18 eV was found to be optimal for the PFB–TMS derivatives of the prostanoids investigated in this study.

3. Results

3.1. Gas chromatographic–mass spectrometric analyses

GC–NICI-MS from the PFB–TMS and PFB–BuB–TMS derivatives of 8-iso-$PGF_{2\alpha}$, $PGF_{2\alpha}$, their tetradeuterated analogs and other F_2-isomers generated virtually identical mass spectra that show prominent ions at m/z 569 and 573 for the PFB–TMS derivatives and at m/z 491 and 495 for the PFB–BuB–TMS derivatives of the unlabeled and labeled compounds, respectively (data not shown). These ions correspond to the anions $[M-PFB]^-$ and they were used for quantification by selected ion monitoring (SIM). Subjection of these ions to collisionally activated dissociation (CAD) resulted in the generation of daughter ions, as summarized in Table 1. For quantitative measurements, selected reaction monitoring (SRM) of the most intense daughter ions at m/z 299 and 303 for unlabeled and labeled prostanoids, respectively, was performed.

The gas chromatographic retention times of the derivatives of the prostanoids prepared in this study were determined by SIM of the $[M-PFB]^-$ ions (Table 2). For quantitative analysis, urinary prostanoids were converted to their PFB–TMS derivatives and separated using the SPB-1701 because of its better resolution with respect to the isomeric F_2-prostanoids investigated in this study (Table 2). The PFB–BuB–TMS derivatives of 8-iso-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ did not emerge from the SPB-1701 column. Using five mixtures, each containing 100 ng of 8-iso-$PGF_{2\alpha}$ and $PGF_{2\alpha}$, derivatization was performed with PFB bromide followed by 1-butaneboronic acid and BSTFA. The derivatives were then analysed on the DB-5MS column by SIM on m/z 491 and 569. The peak area ratios of the PFB–BuB–TMS over PFB–TMS derivatives were determined as 203±90 (mean±S.D.) for $PGF_{2\alpha}$, but only as 4±2 for 8-iso-$PGF_{2\alpha}$, indicating almost complete conversion of $PGF_{2\alpha}$ to the PFB–BuB–TMS derivative, in contrast to 8-iso-$PGF_{2\alpha}$.

3.2. Linearity, precision and accuracy

Standard curves were generated by GC–NICI-MS–MS analysis of the PFB–TMS derivatives of mixtures, each containing 50 pg/μl of [3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$ and various amounts of 8-iso-$PGF_{2\alpha}$ (0–200 pg/μl, $n=7$). Linear regression analysis of the peak area ratio of m/z 299 to 303 (y) and the amount ratio of 8-iso-$PGF_{2\alpha}$ to [3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$ (x) resulted in the regression equation $y=0.006+0.985x$ ($r=0.999$).

Table 1
Major mass fragments (intensity >5% is given in parentheses) in the GC–NICI-MS–MS mass spectra of the PFB–TMS and PFB–BuB–TMS derivatives of the prostaglandins

Ion assignment	8-iso-$PGF_{2\alpha}$	$^2H_4$-8-iso-$PGF_{2\alpha}$	$PGF_{2\alpha}$	$^2H_4$-$PGF_{2\alpha}$
PFB–TMS derivatives				
$[P]^-$	569 (26)	573 (15)	569 (10)	573 (17)
$[P-TMSOH]^-$	479 (17)	483 (15)	479 (12)	483 (30)
$[P-2\times TMSOH]^-$	389 (32)	393 (37)	389 (32)	393 (40)
$[P-2\times TMSOH-(CH_3)_2Si=CH_2]^-$	317 (23)	321 (33)	317 (46)	321 (28)
$[P-3\times TMSOH]^-$	299 (100)	303 (100)	299 (100)	303 (100)
$[P-2\times TMSOH-(CH_3)_2Si=CH_2-CO_2]^-$	273 (33)	273 (60)	277 (50)	277 (37)
$[P-3\times TMSOH-CO_2]^-$	255 (76)	255 (62)	259 (50)	259 (33)
PFB–BuB–TMS derivatives				
$[P]^-$	491 (100)	n.d.[a]	491 (30)	495 (32)
$[P-C_4H_9BO]^-$	407 (10)	n.d.	407 (40)	411 (80)
$[P-TMSOH]^-$	401 (37)	n.d.	401 (16)	405 (45)
$[P-C_4H_9BO-TMSOH]^-$	317 (56)	n.d.	317 (100)	321 (100)
$[P-TMSOH-BuB]^-$	299 (28)	n.d.	299 (82)	303 (73)
$[P-C_4H_9BO-TMSOH-CO_2]^-$	273 (3)	n.d.	273 (32)	277 (24)
$[P-TMSOH-BuB-CO_2]^-$	255 (50)	n.d.	255 (72)	259 (48)

The ions $[M-PFB]^-$, i.e. $[P]^-$, were subjected to CAD.
[a]n.d.=not determined.

Precision and accuracy were investigated by spiking, in triplicate, 5 ml volumes of pooled urine samples from three healthy volunteers with 1 ng/ml of [3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$, which served as the internal standard, and various amounts of 8-iso-$PGF_{2\alpha}$ (0.1, 0.2, 0.5, 1.0, 1.5 and 2 ng/ml). Linear regression analysis between the peak area ratio of m/z 299 over m/z 303 (y) and the amount ratio of 8-iso-$PGF_{2\alpha}$ over [3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$ (x) resulted in straight lines with the following regression equations for the three volunteers: $y=0.368+0.958x$ ($r=0.997$), $y=0.358+0.936x$ ($r=0.992$) and $y=0.750+1.149x$ ($r=0.997$). Mean concentrations, S.D., R.S.D. and inaccuracy were calculated as a measure of intraassay reproducibility and validity of the method. Table 3 shows that the method is characterized by good intraassay reproducibility, precision and accuracy. In addition, the intraday reproducibilities for basal 8-iso-$PGF_{2\alpha}$, which were measured in four 5-ml urine samples from three healthy volunteers was determined as 7.8, 6.5 and 5.2%, respectively. Interday reproducibility was determined by analysing 5 ml aliquots of a pooled urine sample from a healthy volunteer on five consecutive days. Basal 8-iso-$PGF_{2\alpha}$ was determined to be 130±8.8 pg/ml (mean±S.D.) at a R.S.D. of 6.8%.

Instrumental precision was determined as follows: A 5-ml urine sample spiked with 1 ng/ml of [3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$ was extracted, purified by TLC, derivatized and analysed by GC–NICI-MS–MS, as described in Section 2.6. Aliquots (1 μl) of this sample were injected five times into the GC–MS–MS system and the peak area ratio of unlabeled to labeled 8-iso-$PGF_{2\alpha}$ was calculated. Endogenous 8-iso-$PGF_{2\alpha}$ was measured at an instrumental impre-

Table 2
Gas chromatographic retention times of PFB–TMS and PFB–BuB–TMS derivatives of the prostanoids investigated in this work using two different capillary columns

F_2-Prostaglandin derivative	Retention time (min)/relative retention time	
	DB-5MS	SPB-1701
9β,11α-$PGF_{2\alpha}$–PFB–TMS	22.88/1.0000	22.05/1.0000
$^2H_4$-8-iso-$PGF_{2\alpha}$–PFB–TMS	22.90/1.0009	22.19/1.0063
8-iso-$PGF_{2\alpha}$–PFB–TMS	22.97/1.0039	22.25/1.0091
9α,11β-$PGF_{2\alpha}$–PFB–TMS	23.48/1.0262	22.62/1.0258
$^2H_4$-$PGF_{2\alpha}$–PFB–TMS	23.70/1.0358	23.05/1.0454
$PGF_{2\alpha}$-PFB–TMS	23.77/1.0389	23.11/1.0481
$^2H_4$-$PGF_{2\alpha}$–PFB–BuB–TMS	28.43/1.2426	n.a.[a]
$PGF_{2\alpha}$–PFB–BuB–TMS	28.52/1.2465	n.a.
$^2H_4$-8-iso-$PGF_{2\alpha}$–BuB–TMS	29.68/1.2972	n.a.
8-iso-$PGF_{2\alpha}$–PFB–BuB–TMS	29.75/1.3003	n.a.

[a]n.a.=not applicable.

Table 3
Imprecision and inaccuracy of the method

8-iso-$PGF_{2\alpha}$ spiked (pg/ml)	8-iso-$PGF_{2\alpha}$ measured minus the basal level[a] (pg/ml)			R.S.D./inaccuracy (%)			Mean±S.D. of R.S.D/inaccuracy (%)
	Subject			Subject			
	1	2	3	1	2	3	
0	0	0	0	4.2/n.a.	5.1/n.a.	9.8/n.a.	6.4±3.0/n.a.
100	86	92	90	7.7/14	7.1/8.0	7.8/10	7.5±0.3/10.7±3.1
200	202	204	188	8.1/1.0	1.2/2.0	7.8/6.0	5.7±3.9/3.0±2.6
500	474	529	504	8.8/5.2	4.6/5.8	4.5/0.8	5.9±2.4/3.9±2.7
1000	1010	989	1142	8.3/1.0	6.6/1.1	9.7/14	8.2±1.5/5.4±7.6
1500	1496	1571	1543	3.9/0.3	3.9/4.7	8.1/2.8	5.3±2.4/2.6±2.2
2000	2072	2050	2238	6.1/3.6	1.1/2.5	5.7/12	4.3±2.8/6.0±5.1
mean±S.D. of R.S.D.				6.7±2.0	4.2±2.4	7.6±1.9	6.2±1.3
mean±S.D. of inaccuracy				4.2±5.2	4.0±2.6	7.6±5.3	5.3±3.0

[a]Mean basal levels were 350, 440 and 730 pg/ml, respectively.
n.a., not applicable.

cision of 2.1%. The limits of detection of the method were determined to be about 5 pg/ml of urine for [3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$.

3.3. Urinary excretion of 8-iso-$PGF_{2\alpha}$ in healthy subjects and in patients with Zellweger syndrome

Fig. 1 shows GC–NICI-MS–MS chromatograms that were recorded in the SRM from a urine sample of a healthy child (upper panel) and a Zellweger patient (lower panel). In the trace of the internal standard at m/z 303, a single peak corresponding to the PFB–TMS derivative of [3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$ is present. In the trace of endogenous 8-iso-$PGF_{2\alpha}$, there were a few peaks, of which, the peak eluting a few seconds later than [3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$ corresponds to endogenous 8-iso-$PGF_{2\alpha}$. The other peaks from unidentified products do not interfere with 8-iso-$PGF_{2\alpha}$, so that specific quantification of endogenous 8-iso-$PGF_{2\alpha}$ can be performed. This was confirmed by quantifying 8-iso-$PGF_{2\alpha}$ in human urine by GC–NICI-MS–MS following consecutive sample clean-up by RP-HPLC and TLC. Fig. 2 shows that, with the exception of 8-iso-$PGF_{2\alpha}$, the combination of RP-HPLC and TLC completely eliminates all compounds that appear in the chromatogram when TLC alone is used. Fivefold analysis of 5 ml volume of a pooled urine sample from a healthy volunteer, using a single TLC step and a combination of RP-HPLC with TLC, resulted in basal 8-iso-$PGF_{2\alpha}$ levels of (mean±S.D.) 148±12 pg/ml (R.S.D., 8%) and 152±13 (R.S.D., 8.5%), respectively. This finding is strong evidence that our method involving a single TLC step for sample purification specifically measures 8-iso-$PGF_{2\alpha}$ in human urine.

In the five children with ZS investigated in this study, we determined the urinary excretion rate of 8-iso-$PGF_{2\alpha}$. Children with ZS were found to excrete 124-fold higher amounts of 8-iso-$PGF_{2\alpha}$ into the urine than healthy children (Fig. 3). Identification of 8-iso-$PGF_{2\alpha}$ in the urine of a child with ZS was achieved by generating a daughter ion mass spectrum by CAD of the m/z 569 of the peak with the retention time of the PFB–TMS derivative of 8-iso-$PGF_{2\alpha}$. The mass spectrum obtained was virtually identical with that of the PFB–TMS derivative of synthetic 8-iso-$PGF_{2\alpha}$ (data not shown). In addition, the identity of 8-iso-$PGF_{2\alpha}$ in a pooled urine sample (from diseased children) that was spiked with [3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$ was accomplished by SIM of the ions m/z 491 and 495 of the PFB–BuB–TMS derivatives using the DB-5MS column. At both m/z values, we were able to detect peaks with the retention times of the PFB–BuB–TMS derivatives of synthetic 8-iso-$PGF_{2\alpha}$ and [3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$ (not shown). Also, SRM of the ions at m/z 299 and 303 from the same sample showed the appearance of peaks with the retention times of the PFB–TMS derivatives of synthetic 8-iso-$PGF_{2\alpha}$ and

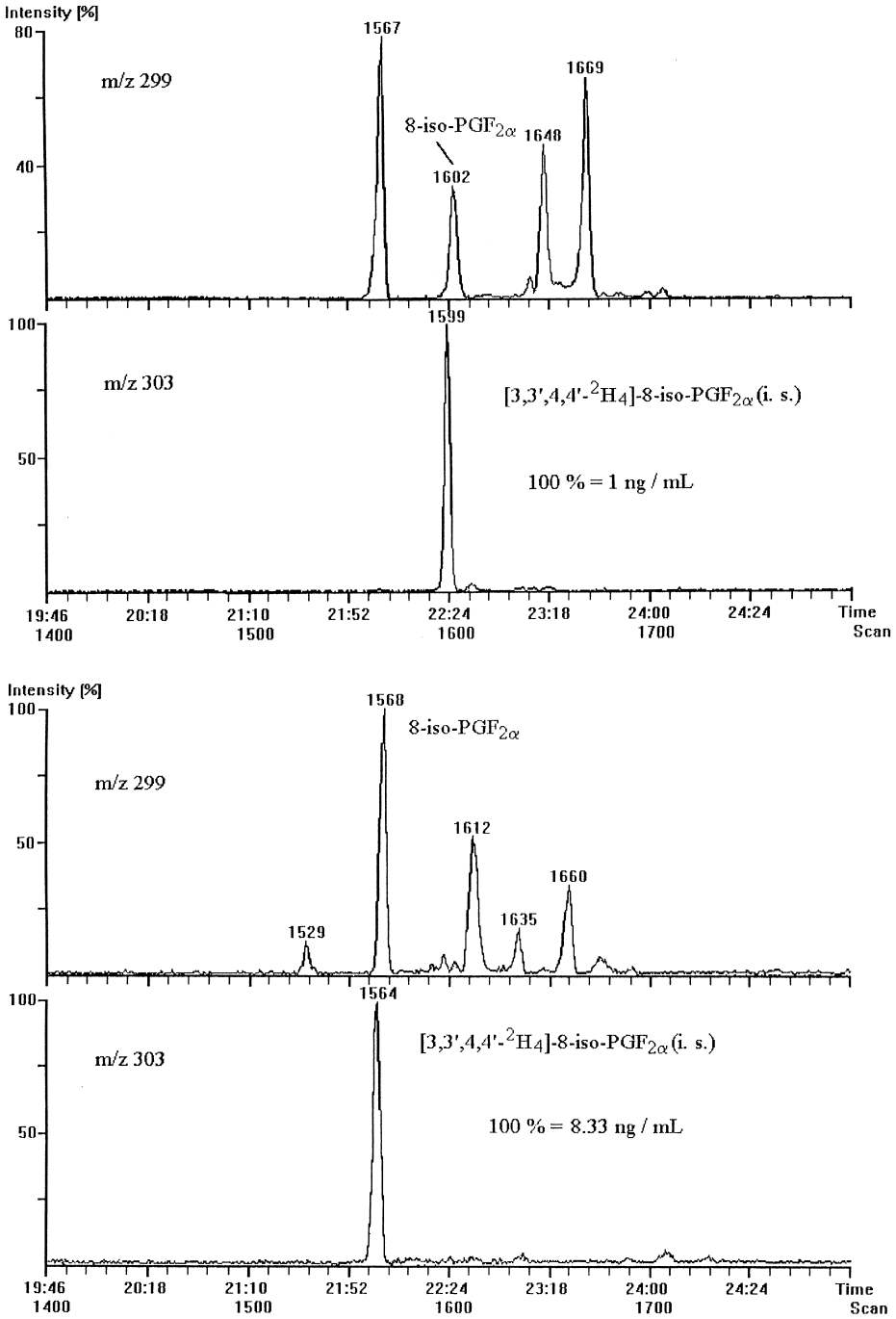

Fig. 1. Partial chromatograms from the GC–NICI-MS–MS analysis of urine samples from a healthy child (upper panel) and from a child with Zellweger syndrome (lower panel). The TLC fraction containing the PFB derivatives of 8-iso-$PGF_{2\alpha}$ and [3,3′,4,4′-$^{2}H_{4}$]-8-iso-$PGF_{2\alpha}$ was derivatized with BSTFA and subjected to GC–NICI-MS–MS analysis. Capillary column, SPB-1701.

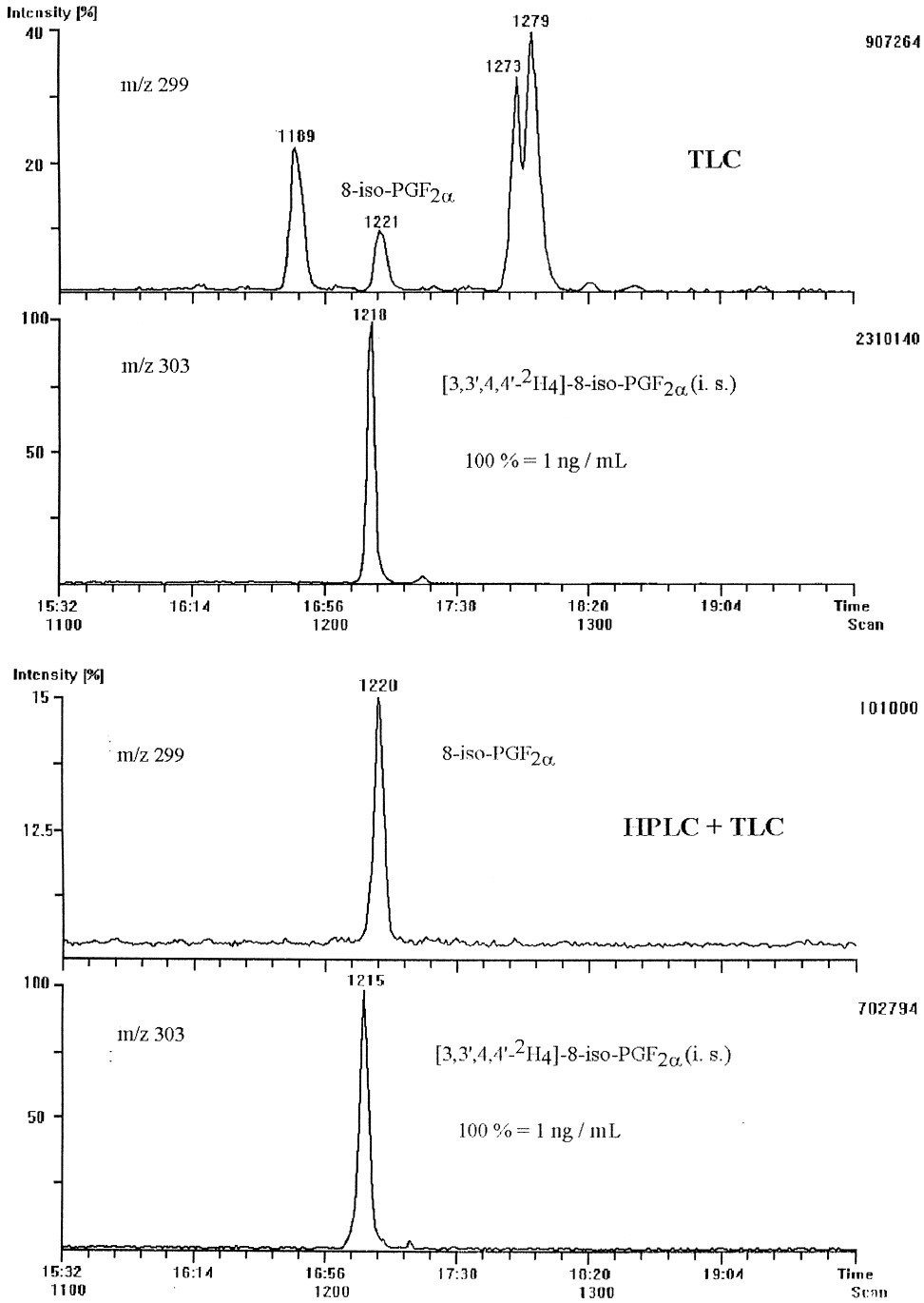

Fig. 2. Partial chromatograms from the GC–NICI-MS–MS analysis of a urine sample from a healthy volunteer using TLC alone (upper panel) and in combination with RP-HPLC preceding TLC (lower panel). Capillary column, Optima-17.

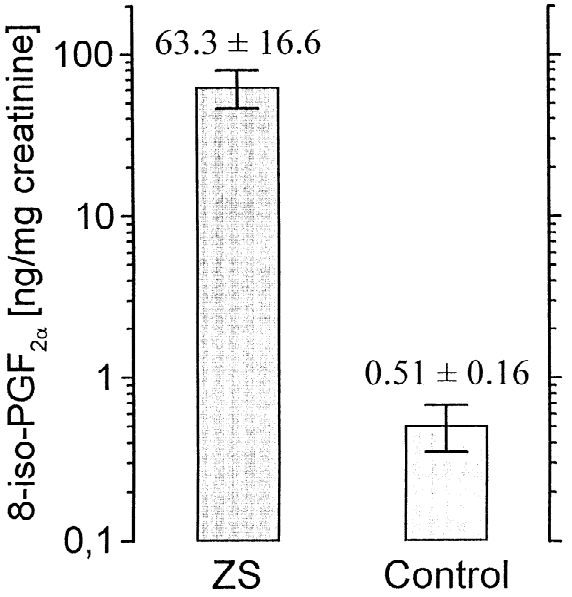

Fig. 3. Urinary excretion rates of 8-iso-$PGF_{2\alpha}$ in five children with Zellweger syndrome (ZS), and five age-matched healthy children serving as the control group. Values are presented as mean±S.D. Capillary column, SPB-1701.

[3,3′,4,4′-$^2H_4$]-8-iso-$PGF_{2\alpha}$, indicating incomplete conversion to the PFB–BuB–TMS derivatives (not shown).

We found that urinary excretion of 8-iso-$PGF_{2\alpha}$ varied in three-hourly collected urine samples within 24 h in two healthy adult persons: the mean intrasubject R.S.D. was 17%. The excretion rate of 8-iso-$PGF_{2\alpha}$ was determined as 418±72 and 258±80 pg/mg creatinine (mean±S.D.) in these volunteers. In five healthy adults, we measured a urinary excretion rate of 22.8±6.8 ng/h for 8-iso-$PGF_{2\alpha}$. Similar excretion rates for 8-iso-$PGF_{2\alpha}$ in the urine of healthy adults have been reported using GC–NICI-MS, GC–NICI-MS–MS and immunoassays [10–15].

4. Discussion

F_2-Isoprostanes have been proposed as index-parameters of oxidative stress in vivo [3,13]. 8-iso-$PGF_{2\alpha}$ has been shown to be present in human plasma [9] and urine [10]. Measurement of 8-iso-$PGF_{2\alpha}$ in urine is non-invasive and avoids artefactual formation from lipids present in high amounts in plasma or tissues [22]. Therefore, several attempts have been undertaken to quantitate 8-iso-$PGF_{2\alpha}$ in human urine vicarious for F_2-isoprostanes. The analytical methods published on this issue until now are based on GC–NICI-MS or GC–NICI-MS–MS following various time-consuming extraction and purification procedures [11–15]. Ferretti and Flanagan [14] have reported the absolute need for a combination of RP-HPLC of free acids and TLC of PFB esters for the specific analysis of 8-iso-$PGF_{2\alpha}$ in human urine by GC–NICI-MS. This group has reported that 8-iso-$PGF_{2\alpha}$ could not be accurately measured even by using GC–NICI-MS–MS before the introduction of HPLC. Schweer et al. [15] have also reported that specific measurement of 8-iso-$PGF_{2\alpha}$ in human urine by GC–NICI-MS–MS requires the combination of normal-phase HPLC with TLC of the PFB esters. This group has suggested that a method without HPLC purification would overestimate the 8-iso-$PGF_{2\alpha}$ in human urine.

In the present work, we describe a more rapid and specific GC–NICI-MS–MS method for the measurement of 8-iso-$PGF_{2\alpha}$ in human urine. Accurate quantification of 8-iso-$PGF_{2\alpha}$ in human urine was achieved by scraping a narrow zone from the TLC plate, in combination with the GC separation of the PFB–TMS derivative on SPB-1701 or Optima-17 columns and tandem mass spectrometric detection. The use of TLC alone results in at least three other compounds apart from 8-iso-$PGF_{2\alpha}$. Additional RP-HPLC eliminates these compounds without, however, improving the specificity of the method. By contrast, the combination of normal-phase HPLC with TLC of PFB esters yields an additional peak that elutes immediately prior to 8-iso-$PGF_{2\alpha}$ on the capillary column [15]. This second GC peak most probably results from the use of a broad zone (R_f 0.02–0.16), scraped off the TLC plate, which was chosen in order to measure total F_2-isoprostanes [15]. Obviously, the compound corresponding to this GC peak could not be eliminated by normal-phase HPLC. The urinary levels of 8-iso-$PGF_{2\alpha}$ both of healthy adults and healthy children measured by us are of the same order as reported by Schweer et al. [15], who, however, used an additional normal-phase HPLC step. This agreement suggests that our method is as specific as that of Schweer et al. Thus, in contrast to previous reports, our results demonstrate

that the GC–NICI-MS–MS method described in this article allows for the accurate and specific quantification of 8-iso-$PGF_{2\alpha}$ in human urine with substantially less labor. We were not able to accurately quantitate 8-iso-$PGF_{2\alpha}$ in human urine by GC–NICI-MS when TLC alone was used for sample purification. However, a preceding RP-HPLC separation step for free acids allowed quantification of 8-iso-$PGF_{2\alpha}$ by GC–NICI-MS as well: a tight correlation ($r=0.972$) was found between 8-iso-$PGF_{2\alpha}$ concentrations in unspiked urine samples from four healthy subjects measured by these two techniques. This finding is in agreement with the data reported by Ferretti and Flanagan [14]. Thus, extensive sample purification by the combination of TLC with HPLC makes the use of GC–NICI-MS–MS superfluous for the quantification of 8-iso-$PGF_{2\alpha}$ in human urine.

We also tried to selectively extract urinary 8-iso-$PGF_{2\alpha}$ on phenylboronic acid cartridges. Unlike methoximated thromboxane B_2 (TxB_2) [23], solid-phase extraction of 8-iso-$PGF_{2\alpha}$ on phenylboronic acid cartridges was unsuccessful, despite the favorable geometry of the 1,3-diol of 8-iso-$PGF_{2\alpha}$. Also unexpectedly, we found that, despite the same geometry of the 9α,11β-diol of the cyclopentane ring, 8-iso-$PGF_{2\alpha}$ was more inert against 1-butaneboronic acid than $PGF_{2\alpha}$, indicating that the side-chain at C_8 of 8-iso-$PGF_{2\alpha}$ hinders the reaction with 1-butaneboronic acid more effectively than that of its isomer $PGF_{2\alpha}$. Furthermore, our results indicate that quantitative analysis of 8-iso-$PGF_{2\alpha}$ as its PFB–BuB–TMS derivative is not favourable because of the lower sensitivity as a result of incomplete derivatization and less volatility of the derivatives. However, conversion of 8-iso-$PGF_{2\alpha}$ to the boronate derivative is of valuable importance for the distinction of 8-iso-$PGF_{2\alpha}$ from 9α,11β- and 9β,11α-PGF_2 isomers. The usefulness of 1-butaneboronic acid for the identification of 8-iso-$PGF_{2\alpha}$ in urine of a child with ZS was demonstrated in the present work.

Children with ZS excreted 124-fold higher amounts of 8-iso-$PGF_{2\alpha}$ into the urine than healthy children. We previously showed that these children also excreted elevated amounts of the cyclooxygenase-dependent prostanoids, TxB_2 (80-fold), 6-oxo-$PGF_{1\alpha}$ (30-fold) and PGE_2 (60-fold) into the urine, compared with healthy children [20]. At present, limited information is available about the metabolism of 8-iso-$PGF_{2\alpha}$ in healthy humans and no data exist for patients with Zellweger syndrome. β-Oxidation of TxB_2, 6-oxo-$PGF_{1\alpha}$ and PGE_2 is impaired in Zellweger patients [20], but ω-oxidation of PGE_2 [20] and $PGF_{2\alpha}$ [24] has been shown to be intact in this disease. Whether highly elevated urinary levels of 8-iso-$PGF_{2\alpha}$ in Zellweger patients are in part due to the formation of this isoprostane as a result of oxidative stress remains to be investigated. Oxidative stress has been shown to moderately (two- to threefold) elevate urinary excretion rates of 8-iso-$PGF_{2\alpha}$ and other F_2-isoprostane metabolites in humans in vivo [11,25]. The extremely highly elevated urinary levels of 8-iso-$PGF_{2\alpha}$ measured in Zellweger patients strongly suggest that quantitation of this isoprostane in urine could be a useful alternative method for diagnosing Zellweger syndrome.

5. Conclusions

The identification of 8-iso-$PGF_{2\alpha}$ as an abundant isoprostane in human urine led to the development of analytical methods based on GC–MS. The GC–NICI-MS–MS method for the quantitative measurement of 8-iso-$PGF_{2\alpha}$ in human urine, described in this article, involves a single TLC step for sample purification and it is, therefore, substantially more rapid than previously described related methods, and allows for the accurate and specific quantification of 8-iso-$PGF_{2\alpha}$ in urine of healthy and diseased humans. Patients with Zellweger syndrome excrete highly elevated amounts of 8-iso-$PGF_{2\alpha}$ into the urine. From the data available, it is unclear whether or not this disease is associated with oxidative stress. Measurement of urinary 8-iso-$PGF_{2\alpha}$ by this method could be useful for diagnosing Zellweger syndrome. The GC–NICI-MS–MS described here should be useful to prove the suitability of 8-iso-$PGF_{2\alpha}$ as a novel marker of oxidative stress in vivo in humans and also to investigate the mechanism(s) of formation of this isoprostane in man.

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References

[1] J.D. Morrow, T.A. Minto, C.R. Mukundan, M.D. Campbell, W.E. Zackert, V.C. Daniel, K.F. Badr, I.A. Blair, L.J. Roberts II, J. Biol. Chem. 269 (1994) 4317.
[2] J.D. Morrow, J.A. Award, A. Wu, W.E. Zackert, V.C. Daniel, L.J. Roberts II, J. Biol. Chem. 271 (1996) 23185.
[3] J.D. Morrow, L.J. Roberts II, Free Radic. Biol. Med. 10 (1991) 195.
[4] L.J. Roberts II, J.D. Morrow, Biochim. Biophys. Acta 1345 (1997) 121.
[5] J.D. Morrow, L.J. Roberts II, Biochem. Pharmacol. 51 (1996) 1.
[6] J.D. Morrow, T.A. Minto, K.L. Badr, L.J. Roberts II, Biochim. Biophys. Acta 1210 (1994) 244.
[7] K. Takahashi, T.M. Nammour, M. Fukunaga, J. Ebert, J.D. Morrow, L.J. Roberts II, R.L. Hoover, K.F. Badr, J. Clin. Invest. 90 (1992) 136.
[8] K.H. Kang, J.D. Morrow, L.J. Roberts II, J.H. Newman, M. Banerjee, J. Appl. Physiol. 74 (1993) 460.
[9] J. Nourooz-Zadeh, N.K. Gopaul, S. Barrow, A.I. Mallet, E.E. Anggard, J. Chromatogr. B 667 (1995) 199.
[10] Z. Wang, G. Ciabattoni, C. Creminon, J. Lawson, G.A. FitzGerald, C. Patrono, J. Maclouf, J. Pharmacol. Exp. Ther. 275 (1995) 94.
[11] A. Bachi, E. Zuccato, M. Baraldi, R. Fanelli, C. Chiabrando, Free Radic. Biol. Med. 20 (1996) 619.
[12] F. Catella, M.P. Reilly, N. Delanty, J.A. Lawson, N. Moran, E. Meagher, G.A. FitzGerald, Adv. Prostaglandin Thromboxane Leukot. Res. 23 (1995) 233.
[13] N. Delanty, M. Reilly, D. Praticò, D.J. FitzGerald, J.A. Lawson, G.A. FitzGerald, Br. J. Clin. Pharmacol. 42 (1996) 15.
[14] A. Ferretti, V.P. Flanagan, J. Chromatogr. B 694 (1997) 271.
[15] H. Schweer, B. Watzer, H.W. Seyberth, R.M. Nüsing, J. Mass Spectrom. 32 (1997) 1362.
[16] D. Tsikas, E. Schwedhelm, F.-M. Gutzki, O. Jahn, P. Fakistas, J.C. Frölich, J. Label. Compd. Radiopharm. 39 (1997) 531.
[17] P. Lazarow, H.W. Moser, in C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Editors), The Metabolic Basis of Inherited Disease, McGraw-Hill, New York, 1989, p. 1479.
[18] R.J. Wanders, R.B. Schutgens, H. van den Bosch, J.M. Tager, W.J. Kleijer, Prenatal Diagn. 11 (1991) 253.
[19] E. Mayatepek, W.D. Lehmann, J. Fauler, D. Tsikas, J.C. Frölich, R.B. Schutgens, R.J. Wanders, D. Keppler, J. Clin. Invest. 91 (1993) 881.
[20] J. Fauler, D. Tsikas, E. Mayatepek, D. Keppler, J.C. Frölich, Pediatr. Res. 36 (1994) 449.
[21] M.Z. Jaffe, Physiol. Chem. 10 (1889) 391.
[22] J.D. Morrow, T. Harris, L.J. Roberts II, Anal. Biochem. 184 (1990) 1.
[23] J.A. Lawson, A.R. Brash, J. Doran, G.A. FitzGerald, Anal. Biochem. 150 (1985) 463.
[24] U. Diczfalusy, B.F. Kase, S.E.H. Alexson, I. Björkhem, J. Clin. Invest. 88 (1991) 978.
[25] J.D. Morrow, L.J. Roberts, Prog. Lipid Res. 36 (1997) 1.