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LC–MS/MS methods for the detection of isoprostanes ($iPF_{2\alpha}$-III and 8,12-*iso*-$iPF_{2\alpha}$-VI) as biomarkers of CCl_4-induced oxidative damage to hepatic tissue

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Abstract

Isoprostanes are formed after peroxidation of arachidonic acid and are promising biomarkers for reactive oxygen species. A LC–MS/MS based method was developed for the quantitation of two isoprostanes ($iPF_{2\alpha}$-III and 8,12-*iso*-$iPF_{2\alpha}$-VI) in hepatocytes, tissue and urine samples of rats. A column switching method was used to reduce sample preparation to a minimum. Precision was 9.4% and accuracy was between 96 and 114% for free $iPF_{2\alpha}$-III in tissue at concentrations from 1.9 to 6.1 ng/g. Treatment of rats with CCl_4 to induce oxidative stress resulted in a dose-dependent increase (two- to three-fold) of $iPF_{2\alpha}$-III and 8,12-*iso*-$iPF_{2\alpha}$-VI in liver and kidney. For both isoprostanes an increase of four- to five-fold was observed in CCl_4 treated hepatocytes and six- to eight-fold in CCl_4 treated and glutathione depleted hepatocytes. In conclusion, the presented method is sensitive, specific and precise to be applied for the quantitation of $iPF_{2\alpha}$-III and 8,12-*iso*-$iPF_{2\alpha}$-VI which are shown to increase by CCl_4 treatment *in vitro* and *in vivo*.
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Keywords: Isoprostanes; 8-*iso* $PGF_{2\alpha}$; $iPF_{2\alpha}$-III; 8,12-*iso*-$iPF_{2\alpha}$-VI; Oxidative stress; Carbon tetrachloride; LC–MS/MS; Column switching; Electrospray

1. Introduction

Free radical-induced oxidation of biomolecules has been linked to the pathogenesis of different diseases including cancer, cardiovascular and neurodegenerative diseases [1–3]. Thus, increasing research is focused to assess the status of oxidative stress. A model compound to induce oxidative stress is CCl_4. It is metabolised to different radicals forming directly or via inflammation reactive oxygen species (ROS) known as oxidative stress.

Due to their high reactivity, free radicals and other ROS have a short half-life and are difficult to detect. Various methods have been developed to determine their reaction products or further degradation products such as oxidised DNA bases and proteins, aldehydes or isoprostanes. Some of these products, however, are not stable enough, are nonspecific or artefactually formed during the analytical process as previously summarised [4]. Increasing evidence suggests that isoprostanes, a class of prostaglandin-like compounds formed by free radical-catalysed peroxidation of arachidonic acid, may present promising markers of oxidative stress [5]. Moreover, increased formation of isoprostanes has been shown to occur in response to oxidant injury, can be modulated by antioxidants, but is not influenced by the content of dietary lipids [6].

Abbreviations: ROS, reactive oxygen species; GC–MS, gas chromatography/mass spectrometry; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; LC–MS/MS, liquid chromatography/tandem mass spectrometry; RIA, radio immunoassay; EIA, enzyme immunoassay; IAC, immunoaffinity chromatography; SPE, solid phase extraction; CAD, collision-activated dissociation; MRM, multiple reaction monitoring; Rt, retention time; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; MDA, malondialdehyde; GSH, glutathione; BHT, 2,6-di-*tert*-butyl-*p*-cresol; BSO, buthionine sulfoximine; DEM, maleic acid diethylester; bd wt, body weight; FCS, foetal calf serum.

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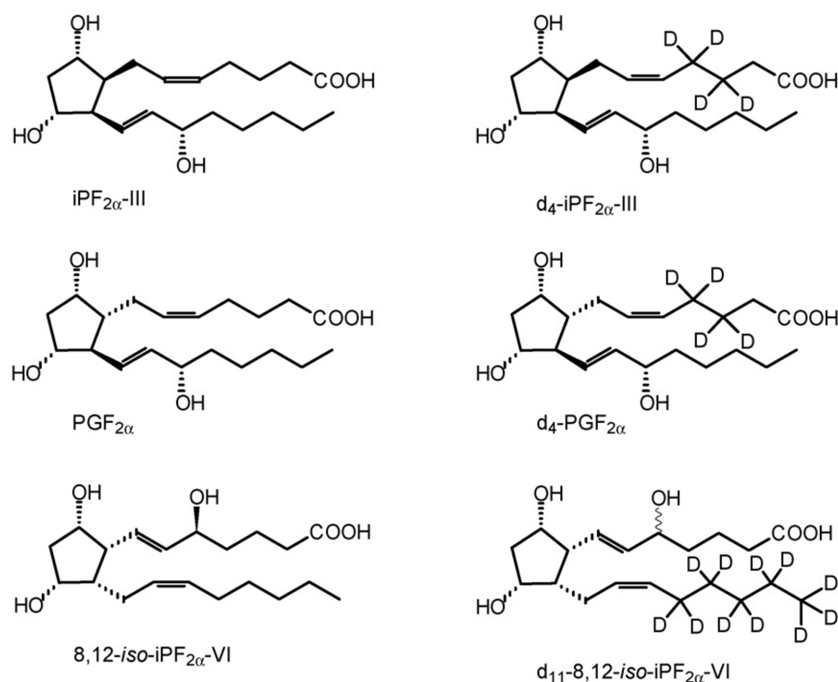

Fig. 1. Chemical structures of isoprostanes and the corresponding deuterated standards.

Numerous methods for the quantitation of isoprostanes in biological samples have already been published including gas chromatography/(tandem)–mass spectrometry (GC–MS), liquid chromatography/(tandem)–mass spectrometry (LC–MS), radio immunoassay (RIA) and enzyme immunoassay (EIA) [7–9]. While only one isoprostane can be detected per immunoassay, it is possible to detect several isoprostanes in the same analysis by mass spectrometry. LC–MS/MS is specific and very sensitive and has the advantage that less sample cleaning and no derivatisation are required compared to GC–MS. By using LC–MS/MS sample cleaning of human urine and plasma mostly includes one extraction step by solid phase extraction (SPE), organic solvents or immunoaffinity chromatography (IAC) [8,10–13]. Sample work up for rat tissue is more elaborate. There is one method described in literature for the quantitation of esterified isoprostane fraction including homogenisation, extraction of esterified isoprostanes with Folch solution, saponification and finally cleaning by SPE [14,15]. Isoprostanes, however, do not only occur esterified to phospholipids but also to a lower extent in their free form in rat tissue. Thus, the objective of this study was to develop two methods for quantitation of free and total isoprostanes in rat liver and kidney tissue and hepatocyte cultures by LC–MS/MS requiring fewer sample cleaning steps. The methods for quantitation of total and free isoprostanes include tissue homogenisation, saponification (only for the analysis of total isoprostanes), protein precipitation and extraction. They were validated by the example of rat liver samples. A column switching technique using a trap column similar to the one recently described by Haschke et al. [16] replaces sample cleaning by SPE. It is also shown in the following study that rat urine can directly be injected onto the HPLC for quantitation of 8,12-*iso*-iPF$_{2\alpha}$-VI without any sample cleaning by using the column switching technique.

Quantitation of iPF$_{2\alpha}$-III (also known as 8-*iso* PGF$_{2\alpha}$) and 8,12-*iso*-iPF$_{2\alpha}$-VI was performed in hepatocytes, liver, kidney and urine samples of rats treated with CCl_4 at doses known to induce lipid peroxidation to show the applicability of the method to different biological matrices in an *in vivo* and *in vitro* experiment.

2. Experimental

2.1. Chemicals

iPF$_{2\alpha}$-III, PGF$_{2\alpha}$, d$_{11}$-8,12-*iso*-iPF$_{2\alpha}$-VI, d$_4$-iPF$_{2\alpha}$-III, d$_4$-PGF$_{2\alpha}$ (see Fig. 1) and 8-isoprostane affinity columns (4 ml) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Solvents for the LC–MS/MS analysis were all of HPLC grade or better and supplied by Carl Roth (Karlsruhe, Germany). All other chemicals were reagent grade or better and obtained from several commercial suppliers. Filter units Millex-GV$_{13}$ (0.22 μm, 13 mm) used for the isolation of free isoprostanes from rat tissue were from Millipore (Schwalbach, Germany).

2.2. Animal study

Nine male F344/N rats (∼200 g) were purchased from Charles River (Sulzfeld, Germany) and maintained at 22 °C on a 12-h light:12-h dark cycle with food and water ad libitum. Following 1 week of acclimatisation, animals were adjusted to metabolic cages 3 days prior to treatment. Animals were divided into three groups ($n = 3$) and administered a single dose of carbon tetrachloride in a volume of corn oil (3 ml/kg bd wt) by oral

gavage. The control group (C) received corn oil only, the low dose group (L) 0.25 ml CCl_4/kg bd wt and the high dose group (H) 1 ml CCl_4/kg bd wt. Urine samples were collected on ice in tubes containing 1 ml of 1% sodium azide in water from −24 to 0 h (predose) and from 0 to 24 h (postdose). Urine samples were centrifuged (15,000 × *g*, 4 °C, 5 min), divided into aliquots of 0.5 ml and stored at −20 °C until analysis.

Animals were sacrificed 24 h after treatment by CO_2 asphyxiation and cervical dislocation. Liver and kidney were removed, divided into 2 or 3 aliquots, frozen in liquid nitrogen and stored at −80 °C until analysis.

2.3. Isolation of total isoprostanes from rat liver and kidney tissue

Isoprostanes are isolated from 0.2 g tissue which was first homogenised with 2 ml of Soerensen buffer (15 mM Na_2HPO_4 and KH_2PO_4, pH 6.8, with 0.001% 2,6-di-*tert*-butyl-*p*-cresol (BHT)) by a dispersing instrument (Ultra Turrax®). Then, d_4-$iPF_{2\alpha}$-III (2 ng) was added as an internal standard and 500 μl of 15% KOH for saponification. After 1 h at 37 °C, homogenates were acidified with 200 μl of glacial acetic acid and precipitated proteins separated by centrifugation (3345 × *g*, 10 min). The supernatant was extracted with 1.5 ml of ethyl acetate and the upper organic layer removed and evaporated under a gentle stream of nitrogen. The residue was dissolved in 1 ml of 10% methanol for LC–MS/MS analysis.

2.4. Isolation of free isoprostanes from rat liver and kidney tissue

Free isoprostanes were isolated from 0.4 g tissue which was first homogenised with 2 ml of Soerensen buffer as described above. The internal standard d_4-$iPF_{2\alpha}$-III (2 ng) was then added to the homogenate and 1 ml of ice cold ethanol to precipitate the proteins 15 min at −20 °C. Proteins were separated by centrifugation (3345 × *g*, 10 min). Ethanol was evaporated from the supernatant in an evaporation centrifuge. The supernatant was acidified with 200 μl of glacial acetic acid and extracted with 1 ml of ethyl acetate. The organic layer was removed and evaporated under a gentle stream of nitrogen. The residue was dissolved in 1 ml of 10% methanol for LC–MS/MS analysis and filtered.

2.5. Assay performance

For assay performance, liver homogenate was prepared by homogenising 2 g of liver in Soerensen buffer adjusting a concentration of 0.2 g of liver per g of homogenate. 1 ml of the homogenate was used for analysis.

Linearity was demonstrated by preparing dilutions of $iPF_{2\alpha}$-III in liver extract and distilled water (0.1–5 ng/ml of $iPF_{2\alpha}$-III). The average of three samples of each concentration was used. Intra-day assay precision was performed on two different liver tissues ($n = 5$). Inter-day assay precision was determined by analysing each of three samples of liver homogenate at four time points (fresh; after 1 day, 1 and 4 weeks of storage at −80 °C).

For the determination of the recovery, 10 samples of liver homogenate mixed with d_4-$iPF_{2\alpha}$-III were analysed and compared to five samples where the deuterated compound was added just before injection.

Accuracy was assessed by analysing samples of liver homogenate spiked with three different amounts of analyte (0.5, 1.0 and 1.5 ng/ml of $iPF_{2\alpha}$-III). The average of three samples of each concentration was used. Concentration of endogenous $iPF_{2\alpha}$-III was determined by triplicate analysis of unspiked liver homogenate and was 1.0 ng/ml.

When several liver samples are worked up at the same time, the first samples have to wait for the next step after weighing and after homogenisation up to 1 h on ice. To test the stability during this procedure, liver samples were analysed as described above but stored on ice for 1 h after weighing and for 1 h after homogenisation. The results were compared to samples which were rapidly worked up.

2.6. Isolation of hepatocytes and treatment

Rat hepatocytes were isolated by a two step liver perfusion with collagenase [17] purified by Percoll® centrifugation, and plated in collagen coated (0.006% collagen, w/v, for 30 min) 6-well Falcon Primaria® plates (Winiger AG, Wohlen, Switzerland) at a density of 9×10^5 cells/well for primary culture. Briefly, cells in 2 ml of culture medium I (William's E medium supplemented with penicillin, streptomycin (0.01%, w/v), insulin (10^{-7} M), dexamethasone (10^{-7} M) and foetal calf serum (FCS) (10%, v/v)) were seeded. After attachment of the cells to the culture dishes (2 h) in a humidified atmosphere (5% CO_2/95% air) at 37 °C, medium I was replaced prior to treatment (overnight conditioning) by 1 ml FCS free medium I. Alternatively, cells were pre-treated overnight with FCS free medium I containing 100 μM maleic acid diethylester (DEM) and 500 μM buthionine sulfoximine (BSO) to deplete intracellular glutathione (GSH) and inhibit GSH synthesis. After overnight culture, medium was replaced by 1 ml of FCS free medium I containing CCl_4 (1 mM) or vehicle (DMSO). 3 h after treatment, cells were scratched off in culture media and stored at −80 °C until further analysis.

2.7. Isolation of isoprostanes from rat hepatocytes

About 1.8 millions rat hepatocytes suspended in 2 ml of cell culture medium were mixed with 1 ng of d_4-$iPF_{2\alpha}$-III. Saponification and extraction of isoprostanes were performed as described for tissue.

2.8. Isolation of isoprostanes from rat urine

For quantitation of 8,12-*iso*-$iPF_{2\alpha}$-VI rat urine was only diluted (1:4), mixed with 2 ng of d_4-$iPF_{2\alpha}$-III and directly injected into the LC–MS/MS.

$iPF_{2\alpha}$-III was isolated from rat urine by IAC. The specificity of IAC columns is 100% for $iPF_{2\alpha}$-III but they also bind some other prostaglandins and isoprostanes (specificity < 8%). As recommended by the manufacturer, 0.5 ml of centrifuged rat urine

was mixed with 0.5 ml of column buffer (0.1 M potassium phosphate buffer, pH 7.4; 7.7 mM NaN_3; 0.5 M NaCl) and 1 ng of d_4-$iPF_{2\alpha}$-III and applied directly onto the column. The column was washed with 2 ml of column buffer and 2 ml of distilled water. $iPF_{2\alpha}$-III and the internal standard were eluted with 2 ml of ethanol/distilled water (95:5, v/v) and the eluate evaporated to dryness in an evaporation centrifuge. The column was regenerated with 5 ml of distilled water and 5 ml of column buffer. It was stored in about 2 ml of column buffer at 4 °C until the next use. The residue was dissolved in 1 ml of 10% methanol for LC–MS/MS analysis.

2.9. LC–MS/MS analysis

HPLC analysis was performed on an HP1100 system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler and two HPLC pumps. Samples were injected onto a trap column for further sample cleaning. After washing the trap column the compounds were then eluted onto the analytical column (see Fig. 2). In the loading position, 450 μl of the sample were injected onto a Strata-X trap column (20 mm × 20 mm, 25 μm; Phenomenex, Aschaffenburg, Germany) isocratically. Mobile phase consisted of 40% methanol and 60% 10 mM ammonium acetate containing 5% of methanol, pH 4.3, at a flow rate of 1 ml/min. After 5 min washing, the valco valve was switched to the elution position where the compounds were now eluted from the trap column onto the analytical column (Reprosil-Pur ODS-3, 150 mm × 2 mm, 3.5 μm; Maisch, Ammerbuch, Germany). The mobile phase now consisted of 0.005% of glacial acetic acid, pH 5.7 (A) and acetonitrile/methanol (19:1, v/v) (B) at a flow rate of 0.3 ml/min. Separation was carried out by gradient elution from 75% A to 58% A within 10 min.

The HPLC system was directly coupled to a triple-stage quadrupole mass spectrometer (API 3000; Applied Biosystems, Darmstadt, Germany) equipped with a TurboIonSpray source. Analytes were detected in the negative ion mode at a vaporizer temperature of 500 °C and an ion spray voltage of −4 kV. Spectral data were recorded with N_2 (CAD = 4) as collision gas and a declustering potential of −61 V. Data acquisition was performed in the MRM mode monitoring the transition of $[M–H]^-$ *m/z* 353 in Q1 to $[M–H–CO_2]^-$ *m/z* 309 in Q3 as qualifier and to *m/z* 193 in Q3 as quantifier for the isomers of the 15-series ($iPF_{2\alpha}$-III). The collision offset energies were −28 and −36 V, respectively. The corresponding transitions *m/z* 357 → *m/z* 313 and *m/z* 357 → *m/z* 197 were monitored for the deuterated standard compounds at a collision offset energy of −30 V. For the

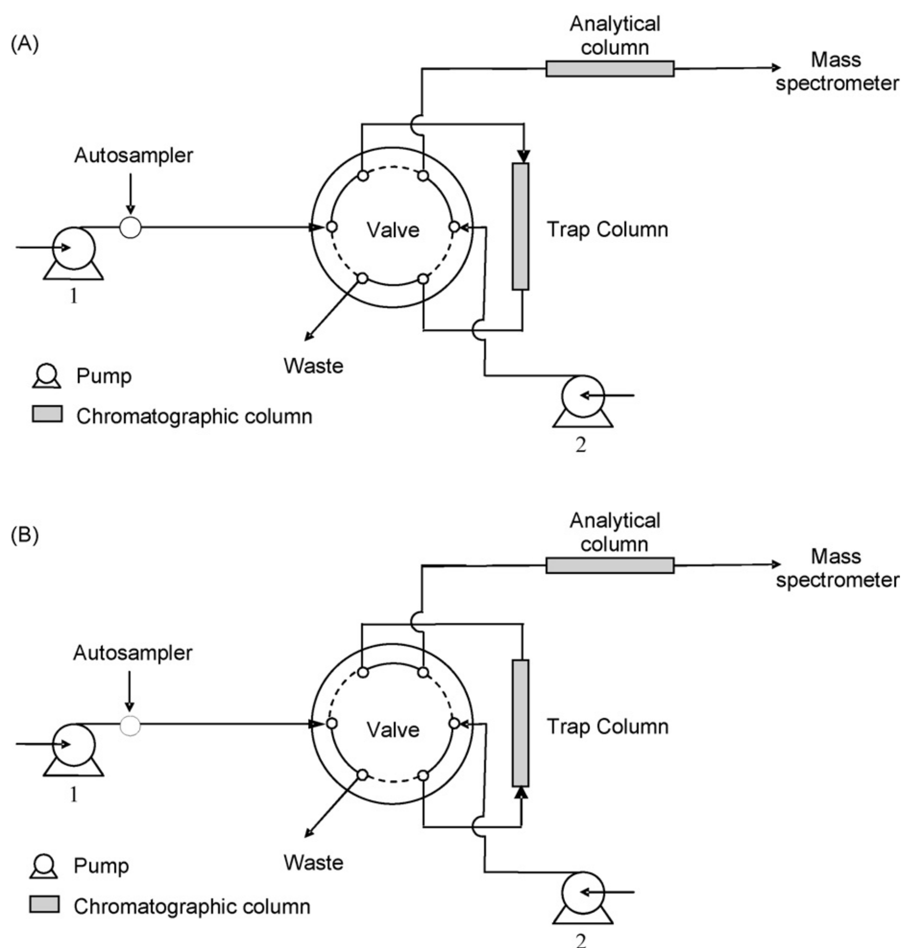

Fig. 2. Scheme of the LC–MS system with trap column, analytical column and six port valve in loading position (A) and elution position (B). Pump 1 delivers mobile phase for loading isoprostanes onto the trap column (A). Pump 2 delivers mobile phase for eluting isoprostanes off the trap column onto the analytical column and off the analytical column by gradient elution (B).

isomers of the 5-series (iPF$_{2\alpha}$-VI) the transition m/z 353 → m/z 115 was monitored at a collision offset energy of −30 V.

Quantitation of iPF$_{2\alpha}$-III was performed by internal calibration with dilution series of standard compound. Since only d$_{11}$-8,12-*iso*-iPF$_{2\alpha}$-VI was commercially available, 8,12-*iso*-iPF$_{2\alpha}$-VI was identified by its MS spectra and retention time (Rt) compared to the d$_{11}$-standard. It was quantified by the calibration curve of iPF$_{2\alpha}$-III.

2.10. Statistics

All statistical calculations were performed using StatView 5.0 (SAS Institute Inc.). Treatment effects in tissues, urine and hepatocytes were determined by ANOVA. They were regarded as significant at a P-value of <0.05.

3. Results

3.1. Assay performance for the analysis of total isoprostanes

For liver extract spiked with different amounts of analytes, the linear regression analysis of the ratios of areas (analyte divided by internal standard) plotted versus the ratios of concentrations resulted in r = 0.999 and an accuracy of 95–107% at concentrations from 0.3 to 5.3 ng/ml. Similar values were obtained from the linear regression analysis for water spiked with the same amounts of analytes as in liver (r = 0.999, accuracy 95–115%). The regression factor and accuracy show that the assay is linear from 0.1 to 5 ng/ml of iPF$_{2\alpha}$-III. Since the slopes of the curves obtained in water and in matrix were nearly identical, it was concluded that the matrix has no impact on ionisation. Thus, quantitation in tissue samples was performed with standard solutions in 10% methanol.

Intra-day assay precision determined for two different liver samples (n = 5) was 7.1 and 9.9% for iPF$_{2\alpha}$-III at concentrations of 4.7 and 9.0 ng/g and 6.2 and 11.9% for 8,12-*iso*-iPF$_{2\alpha}$-VI at concentrations of 32.9 and 72.1 ng/g. Inter-day assay precision was 10.2% for iPF$_{2\alpha}$-III and 17.4% for 8,12-*iso*-iPF$_{2\alpha}$-VI for liver homogenate which was prepared from fresh liver and then stored at −80 °C subsequent to homogenisation (Table 1). During analysis of inter-day assay precision it was observed that contents of isoprostanes increased up to 40% in homogenates stored for 4 weeks at −80 °C in comparison to homogenates which were analysed directly after preparation.

Table 1
Inter-day assay precision in liver homogenate

	iPF$_{2\alpha}$-III		8,12-*iso*-iPF$_{2\alpha}$-VI	
	ng/g	CV (%)	ng/g	CV (%)
Fresh	9.5 ± 0.7	6.9	32.9 ± 2.9	8.7
1 day	10.1 ± 1.3	13.1	37.1 ± 5.1	13.8
1 week	10.2 ± 0.6	6.2	40.0 ± 4.5	11.2
4 weeks	11.3 ± 0.9	8.2	46.3 ± 7.6	16.4
Average	10.3 ± 1.0	10.2	39.1 ± 6.8	17.4

Values are means ± S.D., n = 3.

Recovery was 56% for d$_4$-iPF$_{2\alpha}$-III. Accuracy determined by the analysis of samples spiked with 0.5, 1.0 or 1.5 ng of iPF$_{2\alpha}$-III was 101, 126 and 110%, respectively.

Liver samples which were analysed to check the stability of isoprostanes showed that 8,12-*iso*-iPF$_{2\alpha}$-VI was stable during the sample preparation procedure even when samples were kept on ice for 2 h prior to further preparation steps. In contrast, amounts of iPF$_{2\alpha}$-III increased slightly during the prolonged sample preparation time from 13.2 ± 0.7 to 18.8 ± 1.1 ng/g. For this reason a constant preparation time should be aspired to ensure comparability of data.

3.2. Assay performance for the analysis of free isoprostanes

Intra-day assay precision determined by analysing five samples from the same liver homogenate was 9.4% for iPF$_{2\alpha}$-III and 13.6% for 8,12-*iso*-iPF$_{2\alpha}$-VI. Inter-day assay precision was 11.3% for iPF$_{2\alpha}$-III and 15.0% for 8,12-*iso*-iPF$_{2\alpha}$-VI with contents of 2.0 ± 0.2 and 2.3 ± 0.1 and 0.8 ± 0.1 and 1.2 ± 0.1 ng/g, respectively. Recovery was 52% for d$_4$-iPF$_{2\alpha}$-III. Accuracy determined by the analysis of samples each spiked with 0.5, 1.0 or 1.5 ng of iPF$_{2\alpha}$-III was 102, 96 and 114%, respectively.

The validation of the method for iPF$_{2\alpha}$-III isolation in urine samples by IAC has already been described in detail by Tsikas et al. [18].

3.3. Determination of isoprostanes in biological samples in response to oxidative stress

3.3.1. Animal study

A typical chromatogram of a liver extract after saponification is shown in Fig. 3. iPF$_{2\alpha}$-III (Rt 10.36 min) and PGF$_{2\alpha}$ (Rt 11.30) were assigned according to the corresponding d$_4$-standards. 8,12-*iso*-iPF$_{2\alpha}$-VI (Rt 12.70 min) was assigned due

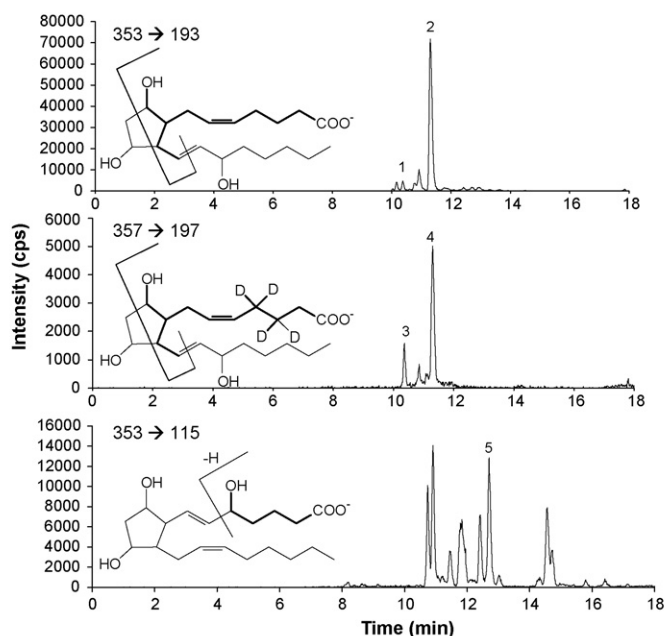

Fig. 3. Chromatogram of a liver extract from a control rat. 1, iPF$_{2\alpha}$-III; 2, PGF$_{2\alpha}$; 3, d$_4$-iPF$_{2\alpha}$-III; 4, d$_4$-PGF$_{2\alpha}$; 5, 8,12-*iso*-iPF$_{2\alpha}$-VI.

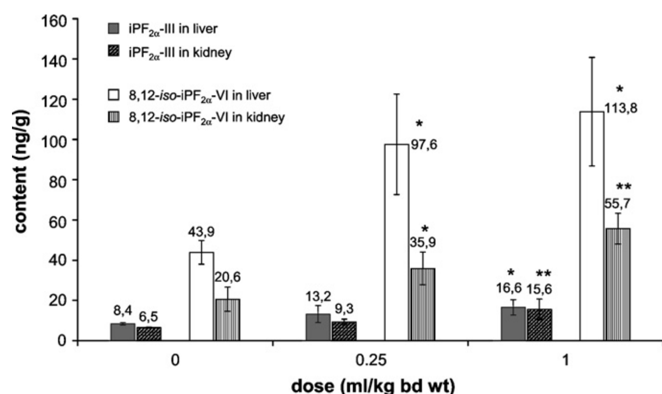

Fig. 4. Isoprostane contents in rat liver and kidney after administration of CCl_4 (0 (control), 0.25 (low dose) and 1 ml/kg bw wt (high dose)). Data are presented as means ± S.D. of three individual animals. Mean values are given. Differs from control, $^{*}P < 0.05$ and differs from control and low dose, $^{**}P < 0.05$.

to the d_{11}-standard.

Treatment of CCl_4 resulted in a dose-dependent increase of $iPF_{2\alpha}$-III and 8,12-*iso*-$iPF_{2\alpha}$-VI in liver and kidney tissue as shown in Fig. 4. Increase was statistically significant in high dose group for both $iPF_{2\alpha}$-III and 8,12-*iso*-$iPF_{2\alpha}$-VI and in low dose group for 8,12-*iso*-$iPF_{2\alpha}$-VI in liver and kidney tissue. Regarding isoprostane contents in kidney tissue, high dose group even differed from low dose group.

8,12-*iso*-$iPF_{2\alpha}$-VI also increased dose dependently in rat urine by a factor of 2 (low dose) and 5.7 (high dose). Increase, however, was not significant in either of the groups. In contrast to 8,12-*iso*-$iPF_{2\alpha}$-VI, concentration of $iPF_{2\alpha}$-III was not impacted by CCl_4 treatment and remained nearly unchanged (Table 2).

3.3.2. Hepatocyte cultures

Background concentrations of $iPF_{2\alpha}$-III and 8,12-*iso*-$iPF_{2\alpha}$-VI were nearly identical in both control samples obtained from untreated and pre-treated primary hepatocyte cultures. Treatment of hepatocytes with CCl_4 resulted in significant increases of $iPF_{2\alpha}$-III and 8,12-*iso*-$iPF_{2\alpha}$-VI (4.1- and 4.7-fold, respectively), which were even more pronounced in cells depleted of GSH (7.9- and 6.3-fold increase, respectively, compared to controls) (Table 3). Pre-treatment of hepatocytes for GSH depletion significantly enhanced treatment effects of CCl_4.

4. Discussion

4.1. Assay performance

Different methods for the isolation and detection of F_2-isoprostanes from biological samples have been described in the literature. One possibility for quantitation of isoprostanes is by RIA or EIA. So far, one EIA is commercially available for the detection of $iPF_{2\alpha}$-III which is used in most clinical laboratories. EIA, however, is expensive and may overestimate levels of $iPF_{2\alpha}$-III compared to GC–MS measurement [19,20]. Analysis by GC-(tandem) MS with negative chemical ionisation is another possibility to separate and detect isoprostanes. However, this method requires extensive, time-consuming sample cleaning including two SPE steps, TLC or even HPLC and derivatisation [7,21,22]. By using IAC, sample cleaning can be reduced but then it is possible to detect only $iPF_{2\alpha}$-III as it is in EIA [18]. Analysis of isoprostanes by LC–MS/MS is also described with the advantage that no derivatisation step is necessary and sample clean up can be reduced to a single extraction step by SPE, organic solvents or online extraction [8,11,12,16]. For the isolation of esterified isoprostanes from rat tissue, however, in addition to homogenisation liquid–liquid extraction with Folch solution (chloroform/methanol, 4:1) is required [14,15].

By using the column switching technique comprised of a trap column and an analytical column, sample preparation can be significantly reduced before LC–MS/MS analysis, as manual extraction steps are no longer necessary. For injection of samples

Table 2
Isoprostane concentrations in urine samples of rats after administration of CCl_4

	C		L		H	
	Predose	Postdose	Predose	Postdose	Predose	Postdose
$iPF_{2\alpha}$-III (ng/mg creatinine)	n.a.	n.a.	0.79 ± 0.08	0.81 ± 0.53	0.85 ± 0.16	0.86 ± 0.40
8,12-*iso*-$iPF_{2\alpha}$-VI (ng/mg creatinine)	5.8 ± 1.3	7.2 ± 1.6	6.1 ± 0.5	12.2 ± 1.2	4.2 ± 0.3	24.1 ± 15.2

Values are means ± S.D., $n = 3$. C, Control 0 ml CCl_4/kg bd wt; L, low dose 0.25 ml CCl_4/kg bd wt; H, high dose 1 ml CCl_4/kg bd wt. n.a., Not analysed. Changes are not statistically significant ($P > 0.05$).

Table 3
Isoprostane concentrations in rat hepatocytes under conditions of oxidative stress

	C[a]	T[a]	C[b]	T[b]
$iPF_{2\alpha}$-III (ng/1 m cells)	0.40 ± 0.03	1.65 ± 0.05*	0.37 ± 0.05	2.93 ± 0.39**
8,12-*iso*-$iPF_{2\alpha}$-VI (ng/1 m cells)	3.7 ± 0.3	17.5 ± 0.5*	4.3 ± 1.1	27.0 ± 3.7**

Values are means ± S.D., $n = 2$. C, Control cells; T, cells treated with 1 mM CCl_4 for 3 h.
[a] Cells pre-treated with FCS free medium over night.
[b] Cells pre-treated with 100 μM DEM and 500 μM BSO in FCS free medium over night for GSH depletion.
* Differs from C ($P < 0.05$).
** Differs from C and T[a] ($P < 0.05$).

and loading onto the trap column, the percentage of methanol in the eluent is set as high as possible, allowing isoprostanes to be adsorbed on the trap column, while the majority of unwanted matrix components is flushed directly into the waste.

Typically, extraction of liver homogenate with Folch solution is applied where only isoprostanes esterified to phospholipids are extracted, whereas free isoprostanes remain in the aqueous phase. Deuterated free isoprostanes used as internal standards are added after the extraction and thus, do not compensate extraction losses at this sample preparation step. In the presented method Folch extraction was omitted and after adding the internal standard liver homogenate was saponified. Subsequent to precipitation of proteins total isoprostanes were extracted by ethyl acetate.

It was shown that $iPF_{2\alpha}$-III slightly increased during a prolonged sample preparation time. When preparing different liver samples in parallel, samples have to wait after weighing and after homogenisation for further sample preparation. For this reason we recommend to first prepare a liver homogenate from different parts of the whole liver. The internal standards should be added into aliquots of the homogenate which either can be directly analysed or stored at −80 °C for further analysis. Using liver homogenate, a rapid sample cleaning can be ensured to improve assay precision. Since contents of both isoprostanes increased with storage time, we recommend analysing the liver homogenate within 1 week after its preparation. Increasing concentrations of F_2-isoprostanes were observed by Wu et al. in blood samples after being stored on ice for 36 h showing instability of isoprostanes in biological matrices [23].

The presented method is fast, sensitive, specific and reproducible for $iPF_{2\alpha}$-III and 8,12-*iso*-$iPF_{2\alpha}$-VI as shown by the validation parameters. To our knowledge, it is the first validated method for the quantitation of total isoprostanes in rat tissue by LC–MS/MS combined with online extraction.

Quantitation of free isoprostanes can also be performed by the method described before. Concentrations of $iPF_{2\alpha}$-III and 8,12-*iso*-$iPF_{2\alpha}$-VI are sufficiently high to allow accurate determination not only in rats treated with CCl_4 but also in controls. Intra-day and inter-day assay precision were comparable to values obtained for the analysis of total isoprostanes. Recovery for free and total isoprostanes was only about 54% which may be ascribed to protein precipitation. When recovery was determined in water instead of liver homogenate it was 100%.

Since urine contains much less proteins than tissue and isoprostanes present in their free form, protein precipitation and saponification is not necessary. After dilution of urine and addition of the internal standard, urine was directly injected onto the trap column and 8,12-*iso*-$iPF_{2\alpha}$-VI quantified. As shown in Fig. 5A the peak of $iPF_{2\alpha}$-III is very small in comparison to the background and not well separated from the other peaks. Thus, we preferred the quantitation of $iPF_{2\alpha}$-III after cleaning the urine by IAC prior to LC–MS/MS analysis (Fig. 5B). Since IAC is a very selective method to isolate $iPF_{2\alpha}$-III, these samples can directly be injected onto the analytical column without column switching. However, another peak, presumably $PGF_{2\alpha}$ was also detected showing that the affinity columns are not exclusively specific to $iPF_{2\alpha}$-III. Validation of the method for $iPF_{2\alpha}$-III isolation by IAC is described in detail by Tsikas et al. [18]. A separate validation for the quantitation 8,12-*iso*-$iPF_{2\alpha}$-VI in urine samples was not performed, since urine samples were only diluted as a single preparation step and isotope labelled internal standards compensate for variations concerning the matrix effect of urine. So assay precision should be comparable to instrument precision.

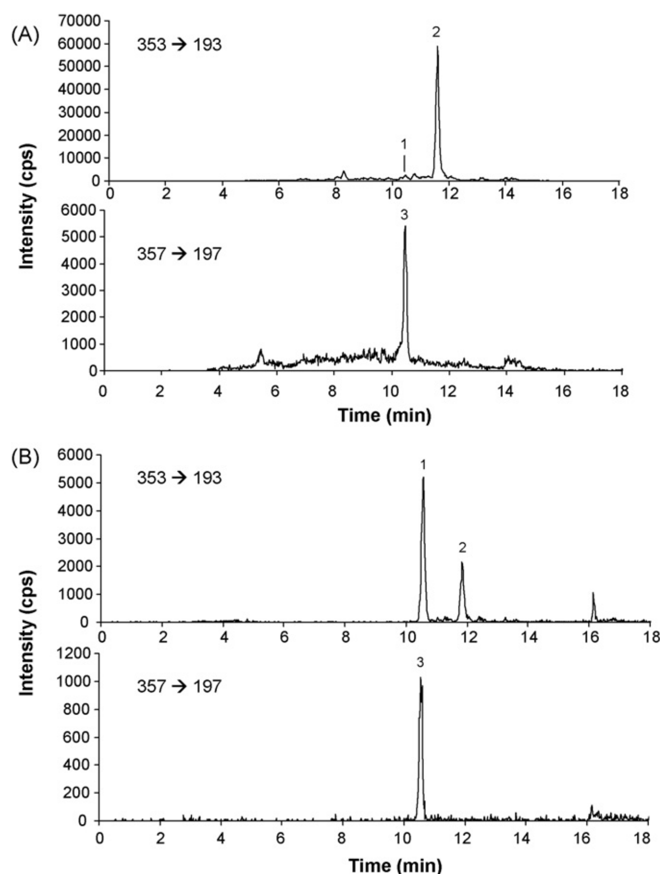

Fig. 5. Chromatogram of rat urine (A) injected without any sample cleaning, (B) injected after IAC. 1, $iPF_{2\alpha}$-III; 2, $PGF_{2\alpha}$; 3, d_4-$iPF_{2\alpha}$-III.

Since 8,12-*iso*-$iPF_{2\alpha}$-VI was not commercially available, quantitation of 8,12-*iso*-$iPF_{2\alpha}$-VI was performed by the calibration of $iPF_{2\alpha}$-III and its d_4-derivative as internal standard. Since the focus of this study was to assess the response of 8,12-*iso*-$iPF_{2\alpha}$-VI to the treatment of CCl_4 more than to determine the absolute concentrations of 8,12-*iso*-$iPF_{2\alpha}$-VI this procedure should be possible. In addition 8,12-*iso*-$iPF_{2\alpha}$-VI was characterised by its d_{11}-8,12-*iso*-$iPF_{2\alpha}$-VI derivative due to retention time and d_{11}-shifted fragmentation pattern. The product ion spectrum of 8,12-*iso*-$iPF_{2\alpha}$-VI is comparable to the spectrum previously shown by Lawson et al. and the MS–MS transitions described before are derived from this spectrum [24].

4.2. Animal study

In this study, a significant (high dose) increase in the concentration of both $iPF_{2\alpha}$-III and 8,12-*iso*-$iPF_{2\alpha}$-VI was evident in livers and kidneys of rats treated with CCl_4 compared to untreated controls. In urine, however, only 8,12-*iso*-$iPF_{2\alpha}$-VI

was increased, whereas iPF$_{2\alpha}$-III levels remained constant. This is in apparent contrast to a previous report by Kadiiska et al. [25], who measured iPF$_{2\alpha}$-III and 8,12-*iso*-iPF$_{2\alpha}$-VI in urine after a single dose of CCl_4 (0.75 ml/kg bd wt, i.p.), which might be comparable to the high dose of 1 ml/kg of CCl_4 (p.o.) in our study. In their study, a 6.6- and 6-fold increase of 8,12-*iso*-iPF$_{2\alpha}$-VI and iPF$_{2\alpha}$-III was observed 16 h after CCl_4 administration. While the increase in urinary 8,12-*iso*-iPF$_{2\alpha}$-VI in our study was only slightly lower (5.7-fold) as compared to the study by Kadiiska et al., iPF$_{2\alpha}$-III levels remained unchanged. A potential explanation for these differences would be the route of administration of CCl_4, suggesting that differences in urinary levels of iPF$_{2\alpha}$-III might be due to different kinetics after i.p. and oral application of CCl_4.

Morrow et al. [26] describe the time course of esterified F$_2$-isoprostane levels in liver after a single dose of 1 ml CCl_4/kg bd wt applied by oral gavage. The highest levels were detected 2 h after CCl_4 administration, but levels were still elevated (about 25 ng/g) after 24 h. Concentrations of iPF$_{2\alpha}$-III in liver were slightly lower in our study (16.6 ± 3.8 ng/g), which might be due to fact that we only quantified iPF$_{2\alpha}$-III and not total isoprostanes. In control rats, they describe levels of 6.0 ng/g in liver and 1.2 ng/g in kidney. We also found lower values in kidney compared to liver but the differences were smaller (8.4 ± 0.6 ng/g in liver and 6.5 ± 0.2 ng/g in kidney). In contrast to the values after CCl_4 administration our values of control rats were slightly higher than the values reported by Morrow et al.

4.3. Hepatocytes

Similar to the effects of CCl_4 treatment on isoprostane levels observed in the animal study, significantly increased concentrations of iPF$_{2\alpha}$-III and 8,12-*iso*-iPF$_{2\alpha}$-VI were also detected in rat hepatocytes treated with CCl_4 *in vitro*. As might be expected, depletion of GSH further enhanced these effects. Conjugation of GSH is thought to play an important role in the detoxification of CCl_4 and its corresponding primary and secondary metabolites. Johnston and Kroening also observed an increase in the iPF$_{2\alpha}$-III level after incubating rat hepatocytes with CCl_4 gas [27]. Thus, rat hepatocytes may serve as an *in vitro* model where compounds can easily be tested with respect to their potential to induce oxidative stress.

5. Conclusions

The LC–MS/MS method described in this work allows for the simultaneous determination of the isoprostanes 8,12-*iso*-iPF$_{2\alpha}$-VI and iPF$_{2\alpha}$-III in rat liver and kidney tissue, rat urine and in rat primary hepatocytes. 8,12-*iso*-iPF$_{2\alpha}$-VI and iPF$_{2\alpha}$-III proved as dose-dependent biomarkers of CCl_4-induced oxidative stress in liver and kidney of rats. 8,12-*iso*-iPF$_{2\alpha}$-VI was found to give the most sensitive response to CCl_4-induced lipid peroxidation under the conditions employed in this study. Increased renal elimination of 8,12-*iso*-iPF$_{2\alpha}$-VI suggests the suitability of this particular isoprostane as a noninvasive biomarker of oxidative stress *in vivo*. Finally, these biomarkers were demonstrated to reflect cellular oxidative stress conditions induced by CCl_4 in hepatocytes, supporting the use of rat primary hepatocytes as a potential *in vitro* model for this type of toxicity.

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