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# Novel liquid chromatography method based on linear weighted regression for the fast determination of isoprostane isomers in plasma samples using sensitive tandem mass spectrometry detection

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ABSTRACT

A simple, fast, sensitive and accurate methodology based on a LLE followed by liquid chromatography–tandem mass spectrometry for simultaneous determination of four regioisomers (8-*iso* prostaglandin $F_{2\alpha}$, 8-*iso*-15(R)-prostaglandin $F_{2\alpha}$, 11β-prostaglandin $F_{2\alpha}$, 15(R)-prostaglandin $F_{2\alpha}$) in routine analysis of human plasma samples was developed. Isoprostanes are stable products of arachidonic acid peroxidation and are regarded as the most reliable markers of oxidative stress *in vivo*. Validation of method was performed by evaluation of the key analytical parameters such as: matrix effect, analytical curve, trueness, precision, limits of detection and limits of quantification. As a homoscedasticity was not met for analytical data, weighted linear regression was applied in order to improve the accuracy at the lower end points of calibration curve. The detection limits (LODs) ranged from 1.0 to 2.1 pg/mL. For plasma samples spiked with the isoprostanes at the level of 50 pg/mL, intra-and interday repeatability ranged from 2.1 to 3.5% and 0.1 to 5.1%, respectively. The applicability of the proposed approach has been verified by monitoring of isoprostane isomers level in plasma samples collected from young patients ($n = 8$) subjected to hyperbaric hyperoxia (100% oxygen at 280 kPa(a) for 30 min) in a multiplace hyperbaric chamber.



## 1. Introduction

Plasma $F_2$-isoprostanes ($F_2$-IsoPs) are isomers of prostaglandin $F_{2\alpha}$ generated from non-enzymatic free-radical per oxidation of arachidonic acid initiated *in vivo*, including arachidonic esters in phospholipids. They become biomarker of oxidative stress due to their chemical stability and wide availability in numerous biological samples, such as blood plasma, exhaled breath condensate, urine, cerebrospinal fluid or meconium [1–6]. Theoretically 64 different isomers can be generated during this oxidation among which 8-iso-prostaglandin $F_{2\alpha}$ is the most recognized isomer, therefore is used as a marker of oxidative stress. Formation of isoprostanes has been implicated in variety of human disorders including: cancer [7], neurodegenerative diseases [8], asthma [9–11], pulmonary sarcoidosis [12] or acute respiratory distress syndrome [13].

Recently, several analytical techniques have been applied for determination of isoprostanes in biological specimens. The isoprostanes are commonly measured in plasma or urine by well-established gas chromatography–mass spectrometry (GC–MS) [14–18] and enzyme-linked immunosorbent assay (ELISA) methods [19–21]. Generally immunoassay methods offer high-throughput analysis and require less expensive instrumentation, however it can be possible to measure only one isomer of isoprostane per immunoassay [22,23]. Despite high sensitivity and specificity of GC–MS based technique, it usually requires expensive and time-consuming sample preparation procedure due to the necessity of use of derivatisation and clean-up steps. Nowadays, a liquid chromatography–mass spectrometry (LC–MS) technique is a method of choice for analysis of isoprostanes in biological specimens and can be used as an alternative approach to GC–MS. This is mainly due to the fact that HPLC–MS can overcome above men-

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tioned problems. This strategy is often supplemented by the use of multiple stable isotope-labeled standards prior to extraction steps to compensate the ion suppression effects and to control the losses of the analytes and reduce contamination [24,25]. Several scientific groups have already reported the determination of isoprostanes with the use of LC-MS/MS [3,22,23,25,26].

Nowadays in bioanalytical approaches based on MS/MS detection more and more often weighted linear regression models are used. The use of OLSR for calibration of analytical method with negligence of heteroscedasticity (variance is increasing with concentration) can lead to significant impairment of accuracy, especially at the lower concentrations of the calibration curve [27–30]. It should be stressed, that isoprostanes are present in biological specimens at very low concentration levels, typically at pg/mL level [6], therefore precision, especially at the low end points of the calibration curve should be maintained for ensuring the reliability of the results. A simple and effective tool to compensate the strong heteroscedasticity in the data and loss in analytical method precision in chromatographic techniques is the use of weighted least squares linear regression (WLSLR), which is useful if the data random errors are not constant across all levels of the calibration curve [29–31].

The purpose of this study was to develop a sensitive, robust and fast method for the separation and determination of trace levels of four isomers of $F_2$-isoprostanes in plasma samples. The proposed method consisted of one step isolation and clean-up by simple liquid–liquid extraction (LLE) with ethyl acetate at micro scale followed by high performance liquid chromatography coupled to tandem mass spectrometry (HPLC–MS/MS). To ensure the reliability of the developed method, validation in terms of: matrix effect, linearity, limit of quantification (LOQ), limit of detection (LOD), accuracy, intra- and interday precision was performed. Matrix-induced suppression was avoided thank to the use of matrix-matched calibration curve. As a part of assay validation weighted least squares linear regression model (WLSLR) was used to construct calibration curve for determination of trace levels of isoprostanes in plasma samples. Optimized methodology may be valuable for evaluation of differences in concentration of free isoprostanes in human plasma between various groups of patients with different disease severity. We focused also on stressing the relevance and steps to be taken for application of weighting schemes for linear regression analysis for determination of trace levels of isoprostanes. To the best of our knowledge this is the first paper describing the separation of F2-isoprostanes in biological material within less than 8 min using weighted regression at calibration stage. The developed method was applied to plasma samples collected from patients subjected to hyperbaric hyperoxia in a multiplace hyperbaric chamber for quantification of local (*i.e.* pulmonary) oxidant stress by measurements of isoprostanes level. Our aim was also to investigate whether hyperbaric hyperoxia increases levels of isoprostanes in plasma of human.

## 2. Material and methods

### 2.1. Chemicals

HPLC-MS grade acetonitrile was purchased from Sigma–Aldrich (Poland) and formic acid was obtained from P.O.Ch (Gliwice, Poland). Ultrapure water was prepared using$HPLC_5$ system from Hydrolab (Poland). Standards of isoprostanes: 8-*iso* prostaglandin $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$, **8**-*iso*P), 8-*iso*-15(R)-prostaglandin $F_{2\alpha}$, (8-*iso*-15(R)-$PGF_{2\alpha}$, 8,15-*iso*P), 11β-prostaglandin $F_{2\alpha}$ (11β-$PGF_{2\alpha}$, 11-*iso*P), 15(R)-prostaglandin $F_{2\alpha}$ (15(R)-$PGF_{2\alpha}$, 15-*iso*P) and internal standard 8-*iso* prostaglandin $F_{2\alpha}$ – $d_4$ (8-*iso*-$PGF_{2\alpha}$ – $d_4$) were obtained from SPI-BIO (Montigny le Bretonneux, France).

### 2.2. Standard solutions

Standards of four isoprostanes (8-*iso*P, 8,15-*iso*P, 11-*iso*P and 15-*iso*P) and internal standard I.S. were diluted in mixture of methanol and water (80:20, v/v) in order to obtain stock solution at concentration 1 μg/mL. They were stored at −20 °C until analysis. The calibration solutions of isoprostanes were prepared by diluting the stock solutions of isoprostanes with mixture of acetonitrile and water (15:85, *v*/v) to obtain 5, 10, 25, 50, 100, 200 pg/mL of isoprostanes in plasma. The calibration curves were constructed freshly in triplicate by plotting peak area ratio of analytes to internal standard *vs* concentration of isoprostanes in plasma samples. Calibration samples were prepared by spiking plasma samples with four isoprostanes. To account for endogenous isoprostanes, the ratio of endogenous each isoprostane peak area divided by the IS peak area in unspiked plasma sample was subtracted from area ratios of calibration samples (corrected analyte area/IS area ratio). The concentration of internal standard in final extract was maintained at 100 pg/mL. All solutions were kept at 4 °C until analysis.

### 2.3. Subjects and specimens collection

Blood samples were collected from young (age from 21 to 35 years) and healthy humans ($n = 8$) subjected to hyperbaric hyperoxia (100% oxygen at 280 kPa(a) for 30 min) in a multiplace hyperbaric chamber.

Blood (typically 1 mL) obtained from volunteers was collected immediately before and after the exposure and centrifuged (5 min at 8000 rpm followed by 10 min at 1600 rpm, 4 °C). Separated plasma layers were immediately transferred to Eppendorf tubes and kept at −85 °C and stored at this temperature until LC–MS/MS analysis. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Medical University of Gdansk.

### 2.4. Extraction of isoprostanes from samples

An original, novel sample preparation procedure has been evaluated for four isoprostanes isolated from human plasma samples. Analytes were extracted using liquid–liquid extraction (LLE) with ethyl acetate.

Briefly, 400 μL of plasma sample was transferred to glass tubes and 4 mL of water acidified with hydrochloric acid (pH = 2) and 4 mL of ethyl acetate was added. Before extraction, deuterated internal standard, 8-isoP-$d_4$, was added to reach the final concentration of 100 pg/mL. Such mixture was shaken for 10 min using vortex. Subsequently, content of vessel was centrifuged for 4 min at 4400 rpm and the organic layer was collected. Extraction with 4 mL of ethyl acetate has been repeated (totally tree times). Then organic solvent layer was combined and evaporated under a gentle stream of nitrogen. The dry residue was redissolved in 400 μL of mobile phase ($H_2O$/ACN + 0.01% FA, 15/85, v/v). Finally, 50 μL was subjected for final LC–MS/MS analysis.

### 2.5. HPLC analysis

Isoprostane analyses were performed using LCMS-8050 system (Shimadzu) that consists of binary pump (NEXERA X2 LC-30 AC LIQUID CHROMATOGRAPHY), thermostat (CTO – 20 AC PROMINENCE COLUMN OVEN) and autosampler (NEXERA X2 SIL – 30 AC AUTOSAMPLER).

The separation was achieved using a Kinetex (100 × 2.1 mm, 2.6 μm) column (Phenomenex), maintained at 40 °C. The flow rate was kept at 0.8 mL/min and the injection volume was set to 50 μL. The selected mobile phase consists of $H_2O$ with 0.01% (v/v) of FA (component A) and ACN with 0.01% (v/v) of FA (component B). At