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Determination of 8-*iso*-prostaglandin $F_{2\alpha}$ in exhaled breath condensate using combination of immunoseparation and LC–ESI-MS/MS

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ABSTRACT

Rapid and precise method for the determination of 8-*iso*-prostaglandin $F_{2\alpha}$, an essential marker of the oxidative stress, in exhaled breath condensate (EBC) was developed. The protocol consisted of stable isotope dilution, immunoseparation combined with selective and sensitive LC–ESI-MS/MS operated in multiple reaction monitoring (MRM) mode. The imprecision of the developed method was below 8.8%, the parameter of mean inaccuracy was determined as <9.6% (0–250 pg of 8-*iso*-prostaglandin $F_{2\alpha}$/ml EBC). The limit of detection (LOD) was 1 pg/ml EBC and limit of quantification (LOQ) 5 pg/ml EBC. A significant difference in 8-*iso*-prostaglandin $F_{2\alpha}$ content between the group of asbestosis patients and healthy volunteers was found.

1. Introduction

8-*iso*-Prostaglandin $F_{2\alpha}$ (=8-isoprostane) is produced *in vivo* by non-enzymatic direct oxidation of arachidonic acid on the cell surface by oxygen radicals. It is generally accepted as a significant biomarker of oxidative stress [1]. Recently, 8-*iso*-prostaglandin $F_{2\alpha}$ was also found in exhaled breath condensate (EBC)—a body aerosol, which well-reflects the composition of the airway surface liquid. EBC contains many biomarkers of oxidative and nitrative stress (8-*iso*-prostaglandin $F_{2\alpha}$, thiobarbituric acid, 3-nitrotyrosine, *S*-nitrosothiols, etc.) as well as mediators of inflammation processes (leukotriene B_4: LTB_4, cysteinyl leukotrienes: LTC_4, LTD_4, LTE_4, prostaglandins, histamine, adenosine, interleukins, etc.) [2,3]. Contemporarily, there is a growing evidence showing that measurements of biomarkers in EBC directly depict pulmonary processes, diseases, respectively (chronic obstructive pulmonary disease [4], *Asthma bronchiale* [5], lung cancer [6]). The collection of EBC is a simple, entirely noninvasive and inexpensive procedure. Moreover, its sampling directly from lungs allows distinguishing the processes occurring particularly in the respiratory system from the other systemic processes. However, the quantification of extraordinarily low concentrations of 8-*iso*-prostaglandin $F_{2\alpha}$ in EBC requires highly sensitive analytical methods. In the past, RIA, ELISA and GC–MS analytical techniques had been used for this task [7–10]. During the last decade, LC–MS/MS has become the technique of choice for the trace analysis of various compounds contained in complex biological matrices (plasma, urine, cerebrospinal fluid, bronchoalveolar lavage fluid, etc.). It has been successfully applied for the detection and quantification of EBC markers [10–13]. 8-*iso*-Prostaglandin $F_{2\alpha}$ as well as the other biomarkers are often detected in EBC at levels as low as picograms per milliliter. In order to achieve such a relatively low detection limit, it is often necessary to incorporate concentration steps (solid phase extraction, lyophilisation, etc.).

Currently, numerous publications can be found on markers contained in EBC, however the published data substantially vary in the mediator levels [10]. Absolute concentrations of markers in EBC reported in different studies are difficult to compare due to (1) significant diversity in EBC collection procedures and samples handling; (2) utilization of different analytical techniques (RIA, ELISA, GC–MS, HPLC); (3) incomplete characterization of factors that affect EBC analysis; (4) dissimilarities in clinical characteristics of study groups (diagnostic criteria, disease severity, treatment); (5) inter-individual biological variability [10].

In this study, a highly selective and sensitive method is presented for the quantification of 8-*iso*-prostaglandin $F_{2\alpha}$ in EBC, which respects all these requirements. The method combines a pretreatment part, serving the purpose of a rapid and effective isolation of 8-*iso*-prostaglandin $F_{2\alpha}$ from EBC an immunoaffinity separation, with detection by LC–ESI-MS/MS. The multiple reaction-monitoring (MRM) mode is used for its extremely high

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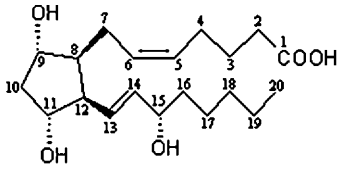
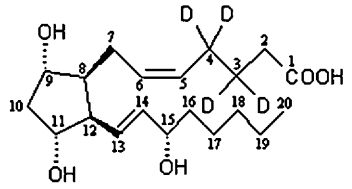


9α,11α 15S-trihydroxy-(8β)-prosta-5Z, 13E-dien-1-oic acid
8-*iso*-Prostaglandin $F_{2\alpha}$



9α,11α 15S-trihydroxy-(8β)-prosta-5Z, 13E-dien-1-oic 3,3,4,4-d_4-acid
8-*iso*-Prostaglandin $F_{2\alpha}$–d_4

Fig. 1. 8-*iso*-Prostaglandin $F_{2\alpha}$ and [3,3′,4,4′ $^2H_4$] 8-*iso*-prostaglandin $F_{2\alpha}$.

degree of selectivity and the stable isotope dilution assay for its high precision of quantification. The analytical procedure was optimized, validated and analytically tested on real EBC samples collected from patients diagnosed with asbestosis and on the control group of healthy subjects.

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents were of commercial origin: 8-*iso*-prostaglandin $F_{2\alpha}$; [3,3′,4,4′ $^2H_4$] 8-*iso*-prostaglandin $F_{2\alpha}$ (Fig. 1); 8-isoprostane affinity sorbent (Cayman Chemical, USA); triethylamine (99.5%); ammonium hydroxide (28% NH_3 solution in water) (Aldrich, USA); acetonitrile; water; methanol (LC–MS grade); acetic acid (99.9%); formic acid (98–100%) (Riedel de Haën, Germany).

2.2. EBC sample collection

The commercially available condenser EcoScreen (Jaeger, Germany) was used for the EBC sample collection. The subjects (patients diagnosed with asbestosis and a control group of healthy non-smokers) were encouraged to perform tidal breath through a mouthpiece connected to the condenser (−20 °C) for 15 min, while wearing a nose-clip. The acquired EBC volumes of samples ranged from 1 to 2 ml. The samples were immediately frozen and stored at −80 °C for a period shorter than 1 month. All clinical samples were collected between 8 and 12 a.m. The detailed description of the EBC collection is available in a separate publication [10].

2.3. Amylase assay

Saliva typically contains high levels of eicosanoids [10]. To exclude saliva as a source of 8-*iso*-prostaglandin $F_{2\alpha}$, the concentration of α-amylase was monitored by α-amylase Biolatest Kit (Pliva-Lachema, Czech Republic). Maximal amylase activity in all samples did not exceed 0.1% of the saliva activity and thus the absence of the saliva contamination in EBC samples was sufficiently proved.

2.4. LC–ESI–MS/MS analysis—apparatus and conditions

A HPLC system ProStar equipped with the dual pump ProStar 210, degasser and Varian 410 autosampler (Varian, USA) with a Hypercarb Thermo 100 mm × 2.1 mm × 5 μm column connected to Hypercarb pre-column (Thermo Electron Corporation, USA) was used. The mobile phase acetonitrile:water 70:30 (v/v) adjusted to pH 11 by triethylamine was used for isocratic elution at the flow rate of 250 μl/min. The injection volume was 20 μl. The LC system was directly coupled to the triple quadrupole mass spectrometer Varian 1200 L (Varian, USA) equipped with an electrospray ion source operated in the negative ion mode (ESI^-). Full scan mass spectra were acquired using a continual infusion of the standard solutions (concentration 1 ng/ml, flow 50 μl/min). For the measurement of 8-*iso*-prostaglandin $F_{2\alpha}$ and its deuterated analogue [3,3′,4,4′ $^2H_4$] 8-*iso*-prostaglandin $F_{2\alpha}$, the MRM was used. The product ion mass spectra were obtained by isolating the molecular ion $[M-H]^-$ in the first quadrupole (Q1), followed by collision-induced dissociation (CID) in the second quadrupole (Q2), and final ion scanning in the third quadrupole (Q3). Selected product ion fragments corresponded to the maximum intensities for both the analyte and the deuterated internal standard ensuring maximum of sensitivity. The scan monitoring reaction used for analyses were 353 → 193 for 8-*iso*-prostaglandin $F_{2\alpha}$, and 357 → 197 for [3,3′,4,4′ $^2H_4$] 8-*iso*-prostaglandin $F_{2\alpha}$. CID was performed at 29 eV under 2.2 mTorr of argon pressure. The capillary voltage was −70 V. The needle voltage was −4500 V. The temperature of ESI ion source was 300 °C. Air (Linde, Czech Republic) was used as the nebulizing gas (50 psi) and nitrogen (Linde, Czech Republic) as the drying gas (17 psi). Data were acquired and evaluated using Varian MS Workstation software version 6.52 (Varian, USA).

2.5. Pretreatment method—immunoaffinity separation

The immunoseparation with 8-isoprostane affinity sorbent was executed as follows: 250 pg of deuterium-labeled standard [3,3′,4,4′ $^2H_4$] 8-*iso*-prostaglandin $F_{2\alpha}$ and 50 μl of the immunoaffinity sorbent were added to 1 ml of EBC and the resulting suspension was vortexed, followed by a continuous shaking for 60 min at 20 °C (laboratory shaker IKA KS 130 basic; 480 rpm [IKA-Werke GmbH & Co.KG, Germany]). Subsequently, the mixture was centrifuged (3000 rpm, i.e. 500 × *g*; time of centrifugation 5 min) and the supernatant separated. The immunoaffinity sorbent was washed two times with water—"washing step" (2 × 1 ml; vortexing; centrifugation; supernatant separation). After the "washing step", methanol was added to elute the substrate (8-*iso*-prostaglandin $F_{2\alpha}$ and [3,3′,4,4′ $^2H_4$] 8-*iso*-prostaglandin $F_{2\alpha}$) from the antibody–substrate complex—"elution step" (2 × 0.5 ml, 5 min shaking, centrifugation, supernatant separation). After the elution, methanol was dried by flushing with nitrogen. The dry rest was dissolved in the mobile phase (50 μl) prior to LC–ESI-MS/MS analysis.

2.6. Standard preparation and calibration procedure

The stock solution (concentration of 20 ng/ml) was prepared by adding 8-*iso*-prostaglandin $F_{2\alpha}$ to acetonitrile:water = 70:30 solution. This stock solution was aliquoted and stored at −80 °C in 1.5 ml Eppendorf reaction tubes. The stock solution was later used for preparation of other standard concentrations used for the calibration (1, 2, 5, 10, 25, 50, 100, 250, 500, 1000 pg/50 μl in acetonitrile:water). For the quantification, the peak area of 8-*iso*-prostaglandin $F_{2\alpha}$ with its assigned internal standard of [3,3′,4,4′ $^2H_4$] 8-*iso*-prostaglandin $F_{2\alpha}$ (250 pg/50 μl) was used as the function of the concentrations.

2.7. Experiments on precision, accuracy and validation of methods

The validation of the method was carried out as follows: first, 8-*iso*-prostaglandin $F_{2\alpha}$-free EBC sample was prepared. Clinical EBC samples from ten healthy subjects were combined and endogenous 8-*iso*-prostaglandin $F_{2\alpha}$ was removed by a repeated immunoaffinity extraction. The residual 8-*iso*-prostaglandin $F_{2\alpha}$ content determined by LC–ESI-MS/MS was below 1 pg/ml. An aliquot (1 ml) of 8-*iso*-prostaglandin $F_{2\alpha}$-free EBC sample was spiked with the internal standard of [3,3′,4,4′ $^2H_4$] 8-*iso*-prostaglandin $F_{2\alpha}$ (250 pg). Subsequently, various amounts of 8-*iso*-prostaglandin $F_{2\alpha}$ (0, 2, 5, 10, 20, 50, 100, 250, 500, 1000 pg) were added to each 1 ml aliquot. These samples were submitted to the immunoseparation pretreatment procedure and quantified by LC–ESI-MS/MS. The limits of detection (LOD) and quantification (LOQ) were determined by using the assay of blank matrix ($n = 5$). The values of LOD and LOQ were calculated as a mean value of blank matrix signal plus three times standard deviation (LOD), ten times standard deviation (LOQ), respectively.

The intra-day physiological variations were determined by analyzing in duplicate EBC samples (2×1 ml) collected from five asbestosis-diagnosed patients. EBC samples were obtained in different day times (8, 12 and 20 h). The inter-day physiological variations of the method were assessed by analyzing in duplicate samples (5 asbestosis-diagnosed and 5 healthy volunteers) obtained during the five consecutive days from each subject.

2.8. Stability

Experiments on stability of 8-*iso*-prostaglandin $F_{2\alpha}$ in EBC were assessed by identical analysis of pooled EBC from ten volunteers. Parallely, artificially prepared samples of 8-*iso*-prostaglandin $F_{2\alpha}$ in acetonitrile were evaluated. Temperature parameters (25, 4, −20 and −80 °C) and a number of freeze–thaw cycles (0–7 cycles) were examined.

2.9. Human study

The objective of the clinical study was to determine oxidative stress in airways in subjects with previous exposure to asbestos dust. EBC samples were collected from 44 persons with previous exposure to asbestos of 24 years in average. The control group was represented by subjects of the identical average age and gender without occupational history of asbestos exposure. The study details are described in a different publication [14].

2.10. Statistic analysis

Acquired data were statistically analyzed using the software Statistica, version 6.0 (StatSoft.cz, Czech Republic). The statistical analyses were materialized using Student's *t*-test, where *P* values were considered significant if <0.05.

3. Results and discussion

3.1. LC–ESI-MS/MS optimization

Recent progress in liquid chromatography separation and mass spectrometry detection has markedly improved the detection sensitivity and enabled a simultaneous detection of multiple analytes in complex matrix samples. For this reason as well as its high sensitivity and specificity, LC–MS has recently attracted a great attention and has been recognized as the first choice for assaying biological samples. To benefit from the above advantages, this combination of liquid chromatography with mass spectrometry was also selected for the quantification of 8-*iso*-prostaglandin $F_{2\alpha}$ in EBC.

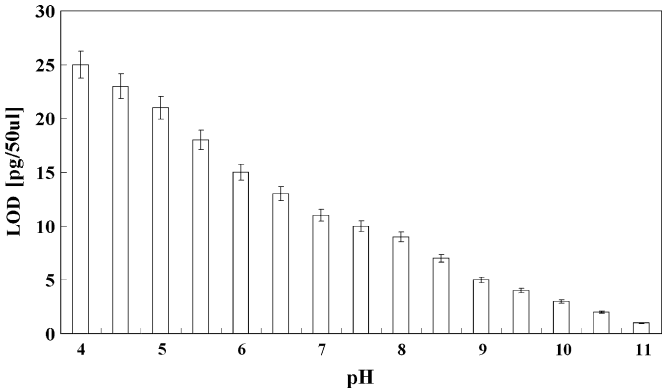


Fig. 2. pH dependence of the LC–ESI-MS/MS detection limit.

Liquid chromatography separation was performed on a Hypercarb column and mobile phase acetonitrile:water = 70:30 with adjustment of pH to the value of 11 by triethylamine (isocratic elution) at the flow rate of 250 μl/min. LC conditions allowed to simply retain 8-*iso*-prostaglandin $F_{2\alpha}$ from the solvent front avoiding signal suppression effects during ionization due to co-elution of salts and endogenous matrix components including other prostanoids detected in EBC (leukotriene B_4: $LTB_4 - R_t = 2.8$ min; cysteinyl leukotrienes C_4, D_4, E_4: $LTC_4 - R_t = 1.4$ min, $LTD_4 - R_t = 1.8$ min, $LTE_4 - R_t = 2.0$ min) [15]. Under the applied analytical conditions, the retention time of both 8-*iso*-prostaglandins was 2.20 min (dead time of the column was 0.8 min). LC conditions were optimized to achieve maximal sensitivity of the mass spectrometry detection. The results of optimizing pH of the mobile phase is depicted in Fig. 2 (pH was adjusted by formic acid or triethylamine, acetic acid or hydroxylamine alternatively) where the maximal response of the mass spectrometer is apparent at pH 11 (higher values of pH exceeded the operating pH range of column).

High sensitivity is required for the determination of endogenous 8-*iso*-prostaglandin $F_{2\alpha}$, since it rapidly metabolizes and is swiftly eliminated from the human body, leaving only trace and instantaneous concentrations (pg/ml). ESI^-–MS/MS in MRM mode represents a highly sensitive and selective tool for trace analyses of metabolites contained in body fluids. This method was used and optimized for the determination and quantification of 8-*iso*-prostaglandin $F_{2\alpha}$ in EBC. In the ESI^- spectra of 8-*iso*-prostaglandin $F_{2\alpha}$ and [3,3′,4,4′ $^2H_4$] 8-*iso*-prostaglandin $F_{2\alpha}$, their quasi-molecular ions (deprotonated molecular ions) $[M-H]^-$ were observed at $m/z = 353$, $m/z = 357$, respectively. These were used as precursor ions (parent ions) isolated on the first quadrupole for the application of MRM mode selected for the quantification of 8-*iso*-prostaglandin $F_{2\alpha}$ contained in picogram levels per milliliter in EBC. Fig. 3 depicts ESI^--MS/MS spectra of 8-*iso*-prostaglandin $F_{2\alpha}$ and its deuterium-labeled analogue. In both spectra, the base ions ($m/z = 193$, $m/z = 197$) were selected as the daughter ions for MRM. The effects of instrument parameters (capillary voltage, needle voltage and temperature of ESI ion source) on the sensitivity were investigated. Maximal intensities of $[M-H]^-$ were obtained under the analytical conditions on LC as mentioned above and ion source parameters described in Section 2. The product ion mass spectra were obtained by CID in a collision cell—the second quadrupole (Q2), of molecular ion $[M-H]^-$ previously isolated on the first quadrupole (Q1), and by scanning of daughter spectra on the third quadrupole (Q3). Fig. 4 shows the dependence of the collision energy on the yields of the parent and daughter ions.

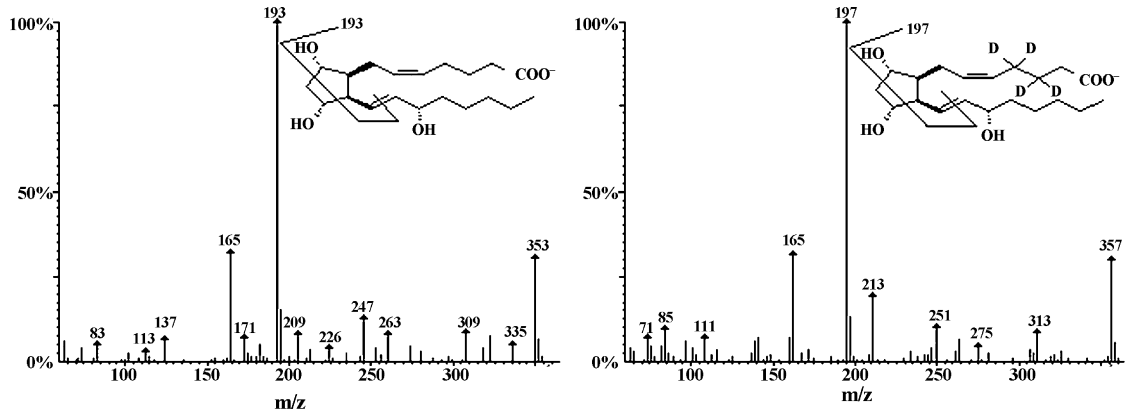


Fig. 3. ESI⁻–MS/MS spectra of 8-*iso*-prostaglandin $F_{2\alpha}$ and 8-*iso*-prostaglandin $F_{2\alpha}$–d_4.

3.2. Immunoseparation

The immunoseparation was performed with commercial 8-isoprostane affinity sorbent, a mouse anti-8-isoprostane covalently bound to Sepharose 4B (binding capacity 100 μl of the immunoaffinity sorbent/1 ng of 8-*iso*-prostaglandin $F_{2\alpha}$; declared specificity for 8-*iso*-prostaglandin $F_{2\alpha}$ (100%), 8-*iso*-prostaglandin $F_{3\alpha}$ (7.6%), 8-*iso*-prostaglandin $F_{1\alpha}$ (2.8%) and all the other isoforms demonstrated lower specificity than 1%). Immunoaffinity extraction was optimized with the purpose to obtain conditions exhibiting a high reproducibility and precision. The experiments were performed with artificially added 8-*iso*-prostaglandin $F_{2\alpha}$ into 8-*iso*-prostaglandin $F_{2\alpha}$-free EBC sample and 50 μl of 8-isoprostane affinity sorbent. This amount was selected with respect to binding capacity and expected amounts of 8-*iso*-prostaglandin $F_{2\alpha}$ in EBC. The objective was to optimize the immunoaffinity sorbent:analyte ratio and the time of immunoseparation, taking into account a low stability of the analyte under the separation conditions. The dependence of the immunoseparated quantity of 8-*iso*-prostaglandin $F_{2\alpha}$ on the amount originally contained in EBC (Fig. 5) well correlated with an adsorption isotherm. Under a low ratio of analyte:sorbent, the separated quantity grew almost linearly, while significant changes occurred under high ratios as a result of a free antibody being absent in a sufficient amount for 8-*iso*-prostaglandin $F_{2\alpha}$ binding. In reference to the estimated levels of 8-*iso*-prostaglandin $F_{2\alpha}$ in clinical EBC samples (10–100 pg/1 ml EBC), the experimentally verified quantity of the immunoaffinity sorbent (50 μl) was determined as optimal, since under the given conditions, the immunoseparation was realized in the "linear" part of the adsorption isotherm, i.e. under a large excess of free antibody molecules. Fig. 6 shows the time progression of the immunoseparation at the ratio of the immunoaffinity sorbent:8-*iso*-prostaglandin $F_{2\alpha}$ = 50 μl:100 pg in 1 ml of EBC. The optimal period for immunoextraction was determined as 60 min. After

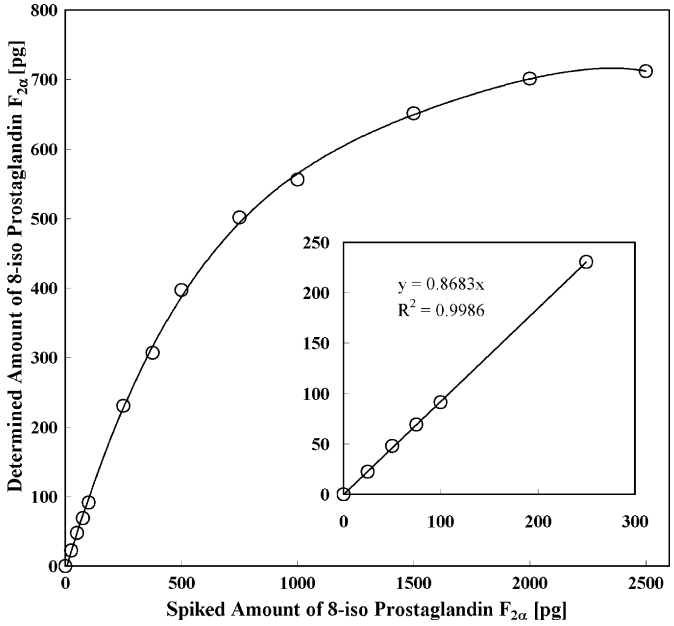


Fig. 5. Efficiency of immunoseparation of 8-*iso*-prostaglandin $F_{2\alpha}$.

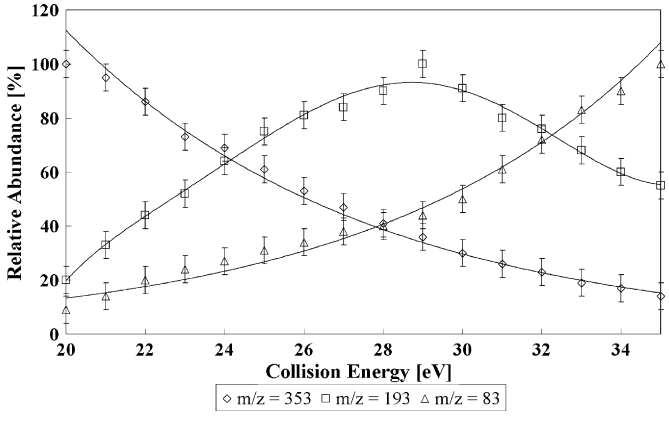


Fig. 4. Dependence of relative abundance of parent and daughter ions on collision energy.

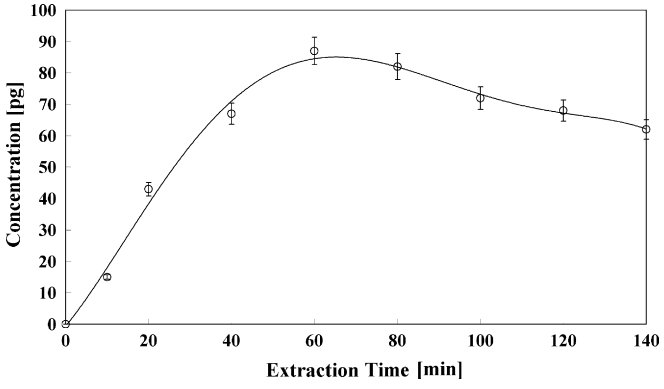


Fig. 6. Time dependence of bound 8-*iso*-prostaglandin $F_{2\alpha}$ in immunoseparation.

reaching the maximum, the separated quantity decreased with an increasing time of immunoseparation probably as a consequence of a low stability of 8-*iso*-prostaglandin $F_{2\alpha}$ in the matrix of EBC at room temperature.

3.3. Stability

At present, EBC analysis is generally considered more reliable for relative measurements (differential diagnostics) than for determining absolute levels of biomarkers [10]. The absence of standardization of EBC processing and analysis is currently the primary limitation of this technique rendering the comparison of data obtained in different laboratories difficult. One of the principal problems is the low stability of biomarkers contained in EBC and a relatively high number of parameters with crucial impact on the constant composition of EBC. Therefore, the optimization of sample handling should not be omitted. The most important parameters are: a low time of operation with EBC under laboratory conditions (especially temperature) and a reliable method used for isolation or enrichment of biomarkers. The stability of 8-*iso*-prostaglandin $F_{2\alpha}$ was experimentally evaluated and changes in concentration during operations with EBC clinical sample as well as the immunoseparation-based pretreatment method were monitored by adding deuterated internal standard to EBC immediately after its collection (250 pg in 1 ml of EBC). Similarly important—pH changed after the collection from an average value of 6.5–7.3 to a value of 6.0–5.5 in the course of 120 min, probably as a consequence of absorption of CO_2 from the surrounding air. Storage under inert atmosphere was found suitable keeping the pH value constant.

Effects of the storage temperature (−80, −20, 4 and 25 °C) and matrix type (EBC, acetonitrile) on the stability of 8-*iso*-prostaglandin $F_{2\alpha}$ were evaluated experimentally (Table 1). 8-*iso*-Prostaglandin $F_{2\alpha}$ is long stable in its crystalline form, whereas in acetonitrile solution used as the mobile phase, its stability was markedly reduced. Furthermore, the temperature sensitivity effect was significant especially at temperatures above 0 °C when the 8-*iso*-prostaglandin $F_{2\alpha}$ stability was fairly decreased. The matrix effect on 8-*iso*-prostaglandin $F_{2\alpha}$ stability was also significant at temperatures above 0 °C where the stability was lower in EBC than in acetonitrile, while at −20 °C the stability was comparable in both environments. At −80 °C, the substance was stable for the whole experimentally evaluated period (180 days) and the matrix effect under this temperature was negligible.

The number of thaw–freeze cycles was an important parameter (thawing–freeze cycle was realized between −80 °C and laboratory temperature). Each cycle leads to 8–9% decrease of the total amount of 8-*iso*-prostaglandin $F_{2\alpha}$ in EBC (Fig. 7).

Table 1
Stability of 8-*iso*-prostaglandin $F_{2\alpha}$ in EBC matrix and in acetonitrile

Time	Concentration (pg)	SD (pg)	RSD (%)
Stability in EBC matrix			
Temperature −80 °C			
0	101.3	4.5	4.4
7 days	98.9	4.6	4.7
30 days	95.2	3.2	3.3
90 days	89.3	3.8	4.3
180 days	87.6	3.5	4.5
Temperature −20 °C			
0	100.5	3.6	3.6
7 days	95.8	4.1	4.3
30 days	78.1	3.6	4.6
90 days	68.5	4.2	6.1
180 days	56.1	3.1	5.5
Temperature 4 °C			
0	101.3	3.6	3.6
1 h	94.6	4.1	4.3
3 h	76.8	3.9	5.1
6 h	67.2	3.3	4.9
12 h	59.3	3.1	5.2
24 h	43.8	3.0	6.8
Temperature 25 °C			
0	100.8	3.5	3.5
1 h	85.3	5.1	6.0
3 h	60.1	3.7	6.2
6 h	45.6	3.2	7.0
12 h	28.3	4.8	17.0
24 h	15.9	4.2	26.4
Stability in acetonitrile			
Temperature 4 °C			
0	99.2	4.6	4.6
1 h	96.8	4.1	4.2
3 h	83.1	4.0	4.8
6 h	75.1	3.5	4.7
12 h	68.4	3.1	4.5
24 h	55.7	3.3	5.9
Temperature 25 °C			
0	100.3	4.7	4.7
1 h	88.4	4.1	4.6
3 h	70.9	4.2	5.9
6 h	55.8	3.8	6.8
12 h	39.4	3.3	8.4
24 h	22.7	3.8	16.8

3.4. Method reliability

The calibration graph was generated from artificially prepared samples of increasing amounts of 8-*iso*-prostaglandin $F_{2\alpha}$ in acetonitrile:water solution (70:30). The samples were immediately analyzed by LC–MS in MRM mode. A calibration graph was constructed using the least square regression of analyte amount versus the peak area ratio. Using the standard sample volume of 50 μl, a linear response was received from 1 to 1000 pg of 8-*iso*-prostaglandin $F_{2\alpha}$ (250 pg of internal standard [3,3′,4,4′ $^2H_4$] 8-*iso*-prostaglandin $F_{2\alpha}$ was added). The correlation coefficient of the regression line was 0.9994. The equal calibration graph was constructed for spiked pooled EBC (the residual amount of 8-*iso*-prostaglandin $F_{2\alpha}$ was determined lower than 1 pg/ml EBC). 8-*iso*-Prostaglandin $F_{2\alpha}$ was separated from EBC samples by immunoseparation with the corresponding amount of added sorbent. The correlation coefficient of the regression line was 0.9988. Having compared both calibrations, no matrix effect of EBC on the slope and intercepts of the calibration curve was found (Table 2). Both obtained calibration dependences were linear within the range of 1–1000 pg (Fig. 8) and produced correlation coefficients

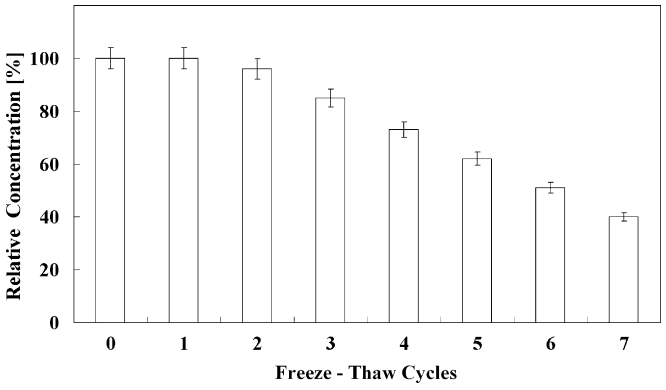


Fig. 7. Dependence of 8-*iso*-prostaglandin $F_{2\alpha}$ stability on number of thawing–freeze cycles ($n = 10$).

Table 2
Linear regression analysis of calibration curve of 8-*iso*-prostaglandin $F_{2\alpha}$ (8-*iso* $PGF_{2\alpha}$) ($n = 10$)

Analyte	Slope	Intercept	SD	RSD (%)
Matrix: acetonitrile:water solution (70:30)	383.32	−32.81	6.21	1.71
Matrix: pooled EBC (the residual 8-*iso*-prostaglandin $F_{2\alpha}$ < 1 pg/ml)	372.25	−48.40	12.02	3.23

Table 3
Recovery values of immunoaffinity separation obtained from repeated analysis ($n = 3$)

Added amount	Found amount (mean)	SD	Precision RSD (%)	Accuracy RE (%)	Recovery (%)
25	22.6	2.0	8.8	−9.6	90.4
50	47.8	4.1	8.6	−4.4	95.6
75	69.2	5.7	8.2	−7.7	92.3
100	91.4	6.3	6.9	−8.6	91.4
250	230.6	9.5	4.1	−7.7	92.2

higher than 0.998. The absence of matrix effect was caused by a low concentration of compounds in EBC that consisted mainly of water with low amount of non-volatile compounds.

The accuracy of the method was assessed by determining five concentrations in three independent series of spiked EBC samples as shown in Table 3. The imprecision of the developed method was determined to 8.8%. The mean inaccuracy was determined to lower than 9.6% for the added levels of 8-*iso*-prostaglandin $F_{2\alpha}$ up to 250 pg/ml EBC. The LOD for 8-*iso*-prostaglandin $F_{2\alpha}$ was determined to be 1 pg/ml EBC, the limit for the quantification (LOQ) 5 pg/ml EBC.

For the patients diagnosed with asbestosis ($n = 5$), the intra-day physiological variation was determined 58 ± 11 in the morning sampling (8 a.m.), 65 ± 8 in the noon sampling (12 a.m.) and 76 ± 9 pg/ml in the evening sampling (20 p.m.). Regarding the inter-day physiological variations for the group of 5 healthy persons and 5 patients diagnosed with asbestosis, the values did not differ over a period of 5 consecutive days by more than 7.9% for each individual.

3.5. Human study

The developed analytical method was used for the clinical study evaluating the possibility to assess the extent of oxidative stress *in vivo*. In this study, the objective was to compare the concentration values of 8-*iso*-prostaglandin $F_{2\alpha}$ in EBC by group of subjects with a long-term exposure to asbestos *versus* the control group. Both groups were of a similar average age and an equal gender (71 *versus* 66 years, male subjects). The control group was further restricted to individuals without any apparent incidence of exposure to asbestos. In both groups, non-smoking status was verified by the determination of cotinine level in urine. All the samples were inspected for the potential presence of α-amylase. The verified α-amylase absence had in these samples refuted the underlying chance for the contamination of samples by saliva, where 8-*iso*-prostaglandin $F_{2\alpha}$ could also be contained. The potential contamination by saliva originated from the process of EBC sample collection would have resulted in positively false results. All the samples were collected between 8 and 12 a.m. Fig. 9 depicts the results of the above-mentioned clinical study. The variations observed in EBC matrix of both clinical groups were relatively tenuous, nevertheless the values of the mean concentrations demonstrated a statistical significance.

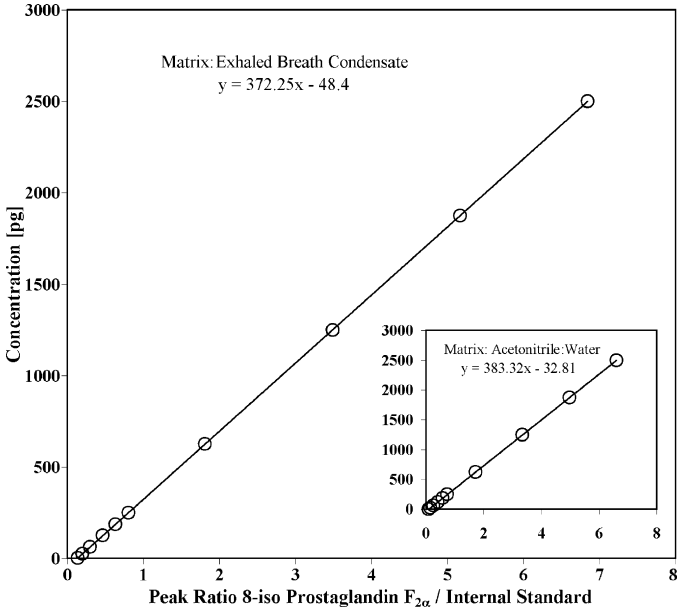


Fig. 8. Calibration curves for 8-*iso*-prostaglandin $F_{2\alpha}$ in EBC and mobile phase matrixes.

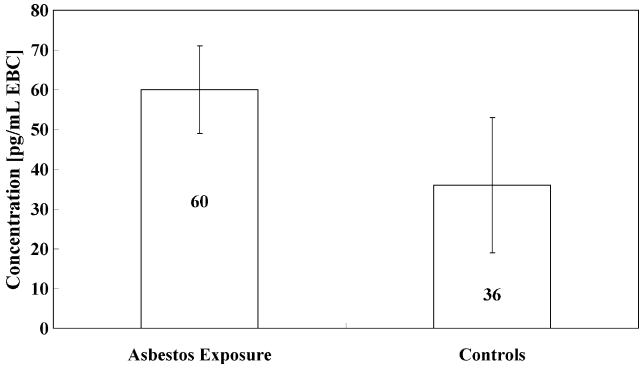


Fig. 9. Concentration of 8-*iso*-prostaglandin $F_{2\alpha}$ in EBC from patients with asbestos exposure and healthy subjects.

4. Conclusion

A method for the detection and quantification of 8-*iso*-prostaglandin $F_{2\alpha}$ in EBC was developed. The method took into account the majority of possible sources of errors associated with the handling, preparation, and analysis of EBC clinical samples. It incorporated a pretreatment part serving the purpose of rapid and effective isolation of 8-*iso*-prostaglandin $F_{2\alpha}$ from EBC by immunoaffinity separation (90.4% recovery). Considering the limited stability of 8-*iso*-prostaglandin $F_{2\alpha}$ both in EBC matrix and solution, this step fulfilled the requirements for rapid isolation prior to subsequent analysis. LC–ESI-MS/MS was used as the detection method, where the MRM mode was used for its extremely high

degree of sensitivity and selectivity, and the deuterated internal standard assay for its high precision in quantification well recognized as the technique of choice for trace analyses of various compounds in complex biological matrices. The combination of mass spectrometry detection with separation using liquid chromatography was selected to retain the analyte from the solvent front as well as to avoid the co-elution of salts and endogenous matrix components that could suppress the ionization of the analyte during the ESI. The analytical procedure was optimized and validated (imprecision 8.8%). The method could be easily modified for other biological matrices, e.g. plasma, urine, cerebrospinal fluid, etc.

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