Talanta 82 (2010) 1434–1438

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Fast quantification of the exhaled breath condensate of oxidative stress 8-iso-prostaglandin F2α using on-line solid-phase extraction coupled with liquid chromatography/electrospray ionization mass spectrometry

Chien-Jen Wang[a], Ning-Hsiang Yang[b], Saou-Hsing Liou[a], Hui-Ling Lee[b,*]

[a] Division of Environmental Health and Occupational Medicine, National Health Research Institutes, Zhunan, Miaoli County, Taiwan, ROC
[b] Department of Chemistry, Fu Jen Catholic University, 510 Chung Cheng Road, Hsinchuang, Taipei County 24205, Taiwan, ROC

ARTICLE INFO

Article history:
Received 11 May 2010
Received in revised form 5 July 2010
Accepted 5 July 2010
Available online 13 July 2010

Keywords:
On-line SPE
EBC
8-iso-PGF2α
LC-MS/MS

ABSTRACT

A method using automated on-line solid-phase extraction (SPE) liquid chromatography–electrospray tandem mass spectrometry (LC–ESI-MS/MS) for the determination of 8-iso-PGF2α in human exhaled breath condensate (EBC) was developed and validated. A C18 SPE column with an affinity sorbent was used for on-line extraction. A C18 column was employed for LC separation and ESI-MS/MS was utilized for detection. 8-iso-PGF2α-d_4 was used as an internal standard for quantitative determination. The extraction, cleanup and analysis procedures were controlled by a fully automated six-port switch valve. Identification and quantification were based on the following transitions: m/z 353 → 193 for 8-iso-PGF2α and m/z 357 → 197 for 8-iso-PGF2α-d_4, respectively. Good recoveries from 98.94 to 99.86% were measured and satisfactory linear ranges for these analytical compounds were determined. Intra-day and inter-day precision showed that coefficients of variance (CV) ranged from 6.5 to 8.0% and 5.2 to 6.3%, respectively. The applicability of this newly developed method was demonstrated by analyzing human EBC samples for an evaluation of the future risk of human exposure to nanoparticles.

1. Introduction

Quantification of 8-iso-prostaglandin F2α (8-iso-PGF2α) has been suggested as a reliable indicator of lipid peroxidation [1,2]. Lipid peroxidation may be related to *in vivo* free radical generation, oxidative damage, and antioxidant deficiency [3]. Oxidative stress has been associated with several diseases such as lung cancer [4], asthma [5] and cardiovascular disease [6]. Particular attention has focused on 8-iso-PGF2α, which is stable, specific for lipid peroxidation, produced *in vivo*, and relatively abundant in biological fluids including plasma [7,8], serum [9], urine [8,10,11], expired breath condensate (EBC) [4], and tissues [12] as well as in lipid-rich foods. Exhaled breath condensate (EBC) is a safe, convenient, non-invasive medium for sampling the airway fluid lining the respiratory tract [4,13–15], airway surface liquid, and mediators of airway inflammation and oxidative stress [16,17].

Various traditional analytical approaches with different sensitivities and specificities can be used to measure 8-iso-PGF2α in biological fluids. These include immunological methods (ELISA and RIA) [5,18,19] that are simple and inexpensive, but requires validation with reference analytical techniques. A RIA for 8-isoprostane in EBC has been qualitatively validated with reverse phase-high performance liquid chromatography (RP-HPLC) [20]. Gas chromatography–mass spectrometry (GC/MS) [21,22] has high sensitivity and specificity for individual isoprostanes, but the sample preparation procedures are extensive, complex and time-consuming. Recently developed liquid chromatography tandem mass spectrometric (LC-MS/MS) [23,24] methods have good specificity, good sensitivity, have the capacity to simultaneously quantify multiple analytes, and are compatible with the additional cleanup and sample preparation methodologies required for the testing of urine, plasma and possibly EBC [25]. However, while repeating this procedure, there also was contamination of the curtain plate, which resulted in signal attenuation and reduced sensitivity. In the present study, we developed a new on-line SPE-LC-MS/MS method, that is rapid, automated, and provides accurate and precise measurements of 8-iso-PGF2α in human EBC samples. The aim of the present study was to present and compare different methods currently developed for the extraction, purification and analysis of 8-iso-PGF2α in various biological samples.

2. Experimental

2.1. Chemicals and reagents

8-iso-PGF2α, 8-iso-PGF2α-d_4 and 8-isoprostane affinity sorbent were purchased from Cayman Chemicals Co. (Ann Arbor, MI, USA).

* Corresponding author. Tel.: +886 2 29053573; fax: +886 2 29052472.
E-mail address: 076308@mail.fju.edu.tw (H.-L. Lee).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.07.015

Table 1
Timetable for the column-switching procedure.

Time (min)	Flow rate (mL/min)	Eluent I (trap)[a]		Flow rate (mL/min)	Eluent II (analysis)[b]		Time (min)	Valve	Remarkers
		Solvent A	Solvent B		Solvent A	Solvent B		Position	
0	1	85	15	0.5	75	25	0	A	Loading and washing samples on trap column
5.5	1	85	15	0.5	75	25	4	B	Inject samples to analytical column
9.5	1	0	100	0.5	0	100	5.5	A	End of injection; cleanup and reconditioning trap column
9.6	1	85	15	0.5	75	25	12	A	
12	1	85	15	0.5	75	25			

[a] Eluent I: solvent A: 1% ACN (v/v) with 0.1% FA; solvent B: 90% ACN (v/v) with 0.1% FA.
[b] Eluent II: solvent A: 100% H_2O (v/v) with 0.1% FA; solvent B: 100% ACN.

All HPLC grade solvents were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Stock solutions and working solutions

A stock standard solution of 8-iso-PGF2α (1000 pg/mL) was prepared in 1% acetonitrile (v/v) with 0.1% formic acid, and it was then stored in polypropylene screw-top tubes at −80 °C. The standard solutions were diluted to 9 and 900 pg/mL with 1% ACN (v/v) with 0.1% formic acid and spiked with a fixed amount of 100 pg/mL 8-iso-PGF2α-d_4 as internal standards (IS).

2.3. Sample preparation

EBC was collected using a commercially available condenser (ECoScreen turbo, Viasys GmbH, Höchberg, Germany) that allows non-invasive sampling of condensable parts of expired air when cooled to about −10 °C, which was performed according to the manufacturer's specified method, and as described in previous publications [9,13,26,27]. All subjects breathed in a relaxed manner (tidal breathing) for 15 min without wearing a noseclip [28]. The EBC produced during a 15-min period (2–3 mL) was collected in polypropylene tubes and stored at −80 °C until analysis. For EBC analysis, each 0.6 mL EBC sample was spiked with 60 μL of the 8-iso-PGF2α-d_4 internal standard and 60 μL of affinity sorbent, vortexed, kept in an ultrasonic bath for 60 min at 4 °C, then centrifuged (12,000 × *g* for 10 min at 4 °C) and the supernatant separated. The affinity sorbent was washed with 0.3 mL water, centrifuged (12,000 × *g* for 10 min at 4 °C), and then the analytes were eluted with 0.6 mL ACN. The supernatants were collected and filtered using a 0.22-μm filter membrane, followed by drying under nitrogen. Finally, 30 μL of solvent (1% ACN, v/v with 0.1% formic acid) were added and the solution was mixed by vortexing for subsequent LC-MS/MS analysis.

2.4. Inter-day and intra-day analysis

Experiments on the intra-day and inter-day variation in 8-iso-PGF2α in EBC were assessed by identical analyses of pooled EBC from 3 volunteers. The intra-day accuracy and recovey of the assay were determined by analyzing replicates ($n = 5$) at spiked 20 and 100 pg/mL in pooled EBC from 3 volunteers and calculating the CV (coefficient of variation). The inter-day was assessed by analyzing replicates of pooled EBC from 3 volunteers ($n = 5$) obatined on different days from the same subject, as described below. Aliquots (1 mL) of mixed EBC sample were transferred to eppendorf tubes. There were a total of 15 samples, which were separated into 3 groups: group I was spiked with 100 μL 8-iso-PGF2α-d_4 internal standard (500 pg/mL), 50 μL solvent (1% ACN, v/v with 0.1% FA) and 110 μL adsorbent; group II was spiked with 100 μL 8-iso-PGF2α-d_4 internal standard (500 pg/mL), 50 μL 8-iso-PGF2α (20 pg/mL) and 110 μL of adsorbent; and group III was spiked with 100 μL 8-iso-PGF2α-d_4 internal standard (500 pg/mL), 50 μL 8-iso-PGF2α (100 pg/mL) and 110 μL of adsorbent. The solutions were mixed in an ultrasonic bath for 60 min at 4 °C, followed by centrifugation at 12,000 × *g* for 10 min at 4 °C. The affinity sorbent was washed with 0.5 mL water, centrifuged (12,000 × *g* for 10 min at 4 °C), and then the analytes were eluted with 1.0 mL ACN. The supernatants were collected and filtered using a 0.22-μm filter membrane followed by drying under nitrogen. Finally, 50 μL of solvent (1% ACN, v/v with 0.1% formic acid) was added and the solution mixed by vortexing for LC-MS/MS analysis.

2.5. Automated on-line SPE

The column-switching system used in the present study is described in detail elsewhere. This system consisted of a switching valve (10-port, 2-position microelectric actuator from Valco Instrument Co., Ltd.) and an Inertsil ODS (33.3 mm × 4.6 mm × 5 μm) column. The switching valve function was controlled by Analyst1.4.2™ software (AB SCIEX, Canada). The column-switching operation, including the LC gradients, that was used during the on-line cleanup and the analytical procedures are summarized in Table 1. When the switching valve was at position A, 30 μL of prepared EBC sample was loaded on the cartridge by an autosampler (Agilent 1200SL; Agilent Technology, U.S.A.), and a binary pump (Agilent 1200; Agilent Technology) delivered the 85% solvent A (1% ACN, v/v with 0.1% formic acid) and 15% solvent B (90% ACN, v/v with 0.1% formic acid) at a flow rate of 1 mL/min as the loading and washing buffer (Eluent I). After the column was flushed with the loading buffer for 4 min, the valve was switched to the injection position (position B) to inject the sample into the LC system. At 5.5 min after injection, the valve was switched back to position A, and the column was eluted with solvent A (Eluent I) using a linear gradient from 85% solvent A to 100% solvent B (90% ACN, v/v with 0.1% formic acid) for 4.0 min, followed by 85% solvent A for 2.4 min for equilibration of the trap column and preparation for the next analysis. The total run time was 12 min.

2.6. Liquid chromatography

The HPLC system consisted of a quaternary pump, an autosampler (Agilent 1200SL, Agilent Technology, U.S.A.), and an Inertsil 5 μm, ODS-80A, 150 mm × 2.1 mm. The chromatography elution using eluent II was used to separate the analytes. After automatic sample cleanup for 4 min, the sample was automatically eluted from the trap column into the analytical column. The mobile phase A (solvent A) was 100% H_2O (v/v) with 0.1% FA, solvent B was 100% ACN, and each was delivered at a flow rate of 0.5 mL/min. The column was incubated at 60 °C.

Table 2
MRM transitions and analyte-specific mass spectrometry parameters.

Compounds monitored	8-iso-PGF2α	8-iso-PGF2α-d_4
MRM (quantitative pair)	353/193	357/197
MRM (qualitative pair)	353/309	357/313
DP (declustering potential)	−80 V	−75 V
CE (collision energy)	−34 V	−36 V
CXP (cell exit potential)	−11 V	−13 V
GS1 (nebulizer gas)	50 psi	50 psi
GS2 (heater gas)	65 psi	65 psi
Curtain gas	10 psi	10 psi
IS (Ion spray voltage)	−4500	−4500 V
TEM (temperature)	500 °C	500 °C

2.7. Electrospray ionization MS/MS

The sample eluting from the HPLC system was introduced into a Turbo V™ source using an ESI probe installed on an API 4000™ triple-quadrupole mass spectrometer (AB SCIEX, Canada), operated in negative mode with a needle voltage of −4.5 kV. Nitrogen gas was used as the nebulizing, heating, curtain and collision gas, and the heater gas temperature was set at 500 °C. Data acquisition and quantitative processing were accomplished using Analyst1.4.2™ software (AB SCIEX, Canada). The optimized source parameters MRM mode is shown in Table 2.

3. Results and discussion

3.1. Development of the extraction procedure for the on-line SPE system

Our goal in the present study was to develop a simple and rapid method with demonstrated validity for the detection/identification/quantification of 8-iso-PGF2α in EBC samples. Many variables affect separation and extraction performance and signal intensities, such as the type of column, organic solvents, pH, and buffer and wash times. To achieve maximum sensitivity and optimize the peak shape, we evaluated different types of solvents, including methanol and acetonitrile—both containing ammonium acetate and formic acid. Different organic solvents were found to significantly affect the 8-iso-PGF2α detection sensitivity [24]. The C18 SPE column was utilized as a trapping column. The loading solution, washing solution and elution solution were evaluated for on-line SPE LC separation. The purpose of the washing solution was to remove endogenous components in the EBC and to minimize their interference with the measurement.

However, it is well known that EBC contains large amounts of endogenous matrix [26] components that can decrease sensitivity and specificity. Extraction and purification procedures are often critical and time-consuming, requiring successive chromatographic steps, which can lead to a substantial loss of target compounds. In the present study, two main analytical approaches were developed for 8-iso-PGF2α measurement: the 8-isoprostane affinity sorbent step, and the on-line C18 SPE column-switch technique. Thus, a combination of affinity sorbent sample preparation and the column-switch technique reduced the contamination of the mass spectrometer interface and made this method more robust and specific. The composition of the mobile phase, which included an on-line SPE trap column and an analysis column, enhanced chromatographic selectivity and reproducibility, as shown in Table 1. Under optimal conditions, the separation of the 8-iso-PGF2α and 8-iso-PGF2α-d_4 had well-defined peak shapes and maximal sensitivity, as shown in Fig. 1. We compared different sample preparation methods. In particular, we compared solvent extraction (methyl acetate, ethyl acetate and ACN), evaporation to dryness under a stream of N_2, and an affinity sorbent step. Using

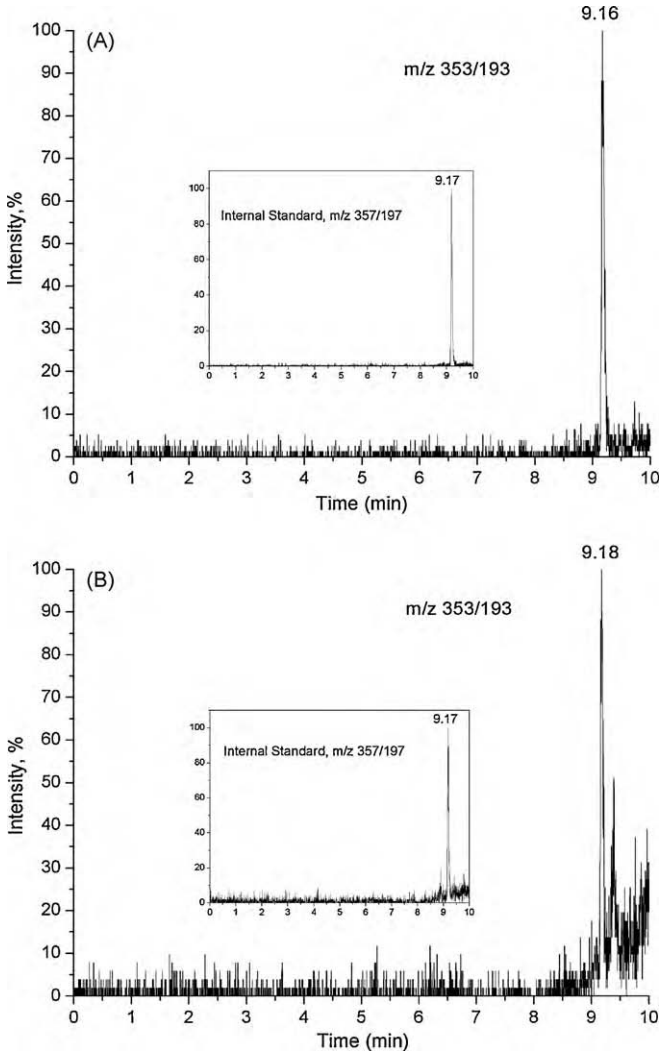

Fig. 1. LC-MS/MS chromatogram of reference compounds using MRM (multiple reaction monitor) mode. (A) Spiked with 100 pg/mL 8-iso-PGF2α and 100 pg/mL 8-iso-PGF2α-d_4 and (B) spiked with 100 pg/mL 8-iso-PGF2α and 100 pg/mL 8-iso-PGF2α-d_4 in EBC sample. All compounds were dissolved in [1% ACN + 0.1% FA].

the affinity sorbent step, however, increased the sensitivity and specificity with lower noise compared with other methods. With our new method, the sample throughput is increased significantly (more than 100 samples can be processed daily) due to employment of rapid on-line SPE and LC-MS/MS procedures, optimal removal of the EBC matrix, and minimal contamination of the curtain plate. Moreover, by reducing the extent of manual operations, this procedure also reduces the potential for human error. Compared with the solvent extraction method, dry N_2 and affinity sorbent more effectively purified the EBC samples. This technique offers a diagnostic approach to identify biomarkers associated with oxidative stress, age-related diseases and evaluation of the future risk of human exposure to nanoparticles.

3.2. Calibration curve and limit of quantification

Two important considerations in the development of any analytical method are the linearity range and the sensitivity of the method. Calibration curves were constructed by plotting a peak-area ratio of the reference and internal standards against concentration, as shown in Fig. 2. Our calibration curves ranged from 9 to 900 pg/mL, and the 8-iso-PGF2α-d_4 standards were dissolved in 1% ACN (v/v) using 0.1% FA and an EBC (pooled EBC from

Table 3
Intra-assay and inter-assay precision of the QC samples prepared in EBC at low and medium concentration of 8-iso-PGF2 using different extraction methods.

Level (pg/mL)	Method (1)			Method (2)		
	Intra-day[a] ($n=5$)	Inter-day[a] ($n=5$)	Recovery (%)	Intra-day[a] ($n=5$)	Inter-day[a] ($n=5$)	Recovery (%)
20	6.5	5.2	99.86	11.4	7.2	220.0
100	8.0	6.3	98.94	4.4	6.8	113.0

Method (1) uses the affinity sorbent. Method (2) uses N_2 dry.
[a] CV: coefficient of variation, $n=5$.

Table 4
Literature basal level of 8-iso-PGF2α in EBC samples from healthy subjects.

Study	Method	Level[a] (pg/mL ± SD)	LOD (pg/mL)	Reference
Montuschi et al.	EIA[b]	10.8 ± 0.8 ($n=10$)	4	[29]
Battaglia et al.	ELISA[c]	3.6 (2.9–7.6) ($n=15$)	5	[18]
Carpenter et al.	GC–MS[b]	7.0 ± 4.0 ($n=10$)	0.02	[22]
Samitas et al.	EIA[b]	16.4 ± 1.6 ($n=19$)	5	[5]
Syslova et al.	LC-MS/MS	47.0 ± 7.8 ($n=46$)	1	[13,24]
Present method	On-line SPE–LC-MS/MS	4.44 ± 2.01 ($n=39$)	1	This study

[c]ELISA: enzyme linked immunosorbent assay.
[a] Healthy subjects.
[b] EIA: enzyme immunoassay.

3 volunteers). The calibration curves had correlation coefficients higher than 0.998. Comparison of the slopes and the intercepts showed no evidence of a significant matrix effect, as the slopes and intercetps were similar (slopes were 2.52 and 1.89, respectively). The limit of detection (LOD) and limit of quantification (LOQ) were defined by peak heights three and ten times, respectively, higher than the maximum baseline height of the blank. The LOD and LOQ for 8-iso-PGF2α were between 1 and 10 pg/mL. The accuracy of the procedure was demonstrated by spiking pooled EBC samples with two concentrations of 8-iso-PGF2α standard – 20 and 100 pg/mL – to evaluate inter-day and intra-day assay precision. These preparations were also used as quality control samples to monitor the day-to-day performance of the assay. The intra- ($n=5$) and inter- ($n=5$) assay variations were 6.5–8.0% and 5.2–6.3% for nonsmokers, respectively, as shown in Table 3. All validation parameters were within an acceptable range. In particular, more than 100 EBC samples have been assayed continuously without significant contamination of the curtain plate (data not shown). The variation of the signal intensities for the same standard, tested at the beginning and after 50 EBC samples, was less than 5%.

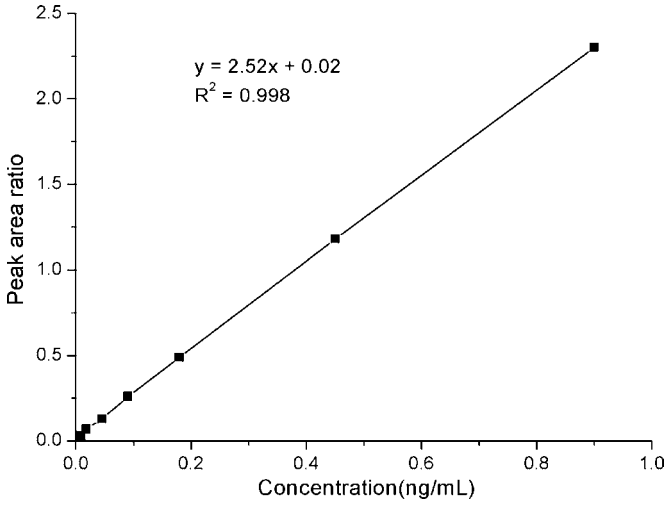

Fig. 2. Calibration curve for 8-iso-PGF2α in 1% ACN + 0.1% FA.

3.3. Recovery of metabolites in EBC

The recovery rates of 8-iso-PGF2α in 0.6 mL of human EBC determined for SPE using affinity sorbent, at two different concentrations (20 and 100 pg/mL) are shown in Table 3. The recovery rates using affinity sorbent were greater than those dried under a stream of N_2. The mean recovery rates of 8-iso-PGF2α using affinity sorbent at 20 and 100 pg/mL were 99.86 and 98.94% respectively. Thus, affinity sorbent was the cartridge of choice in the sample preparation process. The developed method was applied to the analysis of the EBC samples from human subjects. Our method compares favorably with known methods in terms of analytes applicable, LOD and detection levels, as shown in Table 4. Thus, the combination of affinity sorbent sample preparation and column-switch technique reduced the contamination of the mass spectrometer interface, and made this method more robust.

4. Conclusions

A rapid LC-MS/MS method combined with an on-line SPE step was developed for the detection, identification, and quantification of 8-iso-PGF2α in human EBC. Satisfactory recovery and precision was obtained and ranged from 98.94 to 99.86% at trace levels in all cases, with a coefficient of variance (CV) lower than 8.0%. The selectivity and sensitivity of this method to simultaneously measure 8-iso-PGF2α would greatly facilitate studies of the indicators of oxidative stress for 8-iso-PGF2α relative to the role of oxidative stress in human disease.

Acknowledgements

This study was supported by grants from the National Science Council (Grant no. NSC98-2113-M-030-001-MY2) and 99D9-EOIOSH01 from the Division of Environmental Health and Occupation Medicine, National Health Research Institutes, Taiwan, ROC.

References

[1] P. Montuschi, P.J. Barnes, L.J. Roberts 2nd., FASEB J. 18 (2004) 1791–1800.
[2] P. Montuschi, P.J. Barnes, L.J. Roberts 2nd., Curr. Med. Chem. 14 (2007) 703–717.
[3] B. Buszewski, M. Kesy, T. Ligor, A. Amann, Biomed. Chromatogr. 21 (2007) 553–566.
[4] H.P. Chan, C. Lewis, P.S. Thomas, Lung Cancer 63 (2009) 164–168.

[5] K. Samitas, D. Chorianopoulos, S. Vittorakis, E. Zervas, E. Economidou, G. Papatheodorou, S. Loukides, M. Gaga, Respir. Med. 103 (2009) 750–756.
[6] X. Wu, H. Cai, Y.B. Xiang, Q. Cai, G. Yang, D. Liu, S. Sanchez, W. Zheng, G. Milne, X.O. Shu, Cancer Epidemiol. Biomarkers Prev. 19 (2010) 947–952.
[7] N.E. Bastani, T.E. Gundersen, R. Blomhoff, Rapid Commun. Mass Spectrom. 23 (2009) 2885–2890.
[8] D. Sircar, P.V. Subbaiah, Clin. Chem. 53 (2007) 251–258.
[9] E. Dalaveris, T. Kerenidi, A. Katsabeki-Katsafli, T. Kiropoulos, K. Tanou, K.I. Gourgoulianis, K. Kostikas, Lung Cancer 64 (2009) 219–225.
[10] L.J. Roberts 2nd., K.P. Moore, W.E. Zackert, J.A. Oates, J.D. Morrow, J. Biol. Chem. 271 (1996) 20617–20620.
[11] B. Zhang, K. Saku, J. Lipid Res. 48 (2007) 733–744.
[12] G.L. Milne, H. Yin, J.D. Brooks, S. Sanchez, L.J Roberts, 2nd., J.D. Morrow, Methods Enzymol. 433 (2007) 113–126.
[13] D. Pelclova, Z. Fenclova, P. Kacer, M. Kuzma, T. Navratil, J. Lebedova, Ind. Health 46 (2008) 484–489.
[14] A. Koutsokera, S. Loukides, K.I. Gourgoulianis, K. Kostikas, Curr. Med. Chem. 15 (2008) 620–630.
[15] D.H. Conrad, J. Goyette, P.S. Thomas, J. Gen. Intern. Med. 23 (Suppl. 1) (2008) 78–84.
[16] P. Montuschi, P.J. Barnes, Trends Pharmacol. Sci. 23 (2002) 232–237.
[17] S.A. Kharitonov, P.J. Barnes, Biomarkers 7 (2002) 1–32.
[18] S. Battaglia, H. den Hertog, M.C. Timmers, S.P. Lazeroms, A.M. Vignola, K.F. Rabe, V. Bellia, P.S. Hiemstra, P.J. Sterk, Thorax 60 (2005) 639–644.
[19] P. Montuschi, C. Mondino, P. Koch, P.J. Barnes, G. Ciabattoni, J. Allergy Clin. Immunol. 118 (2006) 347–353.
[20] P. Montuschi, E. Ragazzoni, S. Valente, G. Corbo, C. Mondino, G. Ciappi, G. Ciabattoni, Inflamm. Res. 52 (2003) 502–507.
[21] D. Tsikas, J. Chromatogr. B: Biomed. Sci. Appl. 717 (1998) 201–245.
[22] C.T. Carpenter, P.V. Price, B.W. Christman, Chest 114 (1998) 1653–1659.
[23] P. Montuschi, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 1272–1280.
[24] K. Syslova, P. Kacer, M. Kuzma, P. Klusackova, Z. Fenclova, J. Lebedova, D. Pelclova, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 867 (2008) 8–14.
[25] D. Tsikas, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 878 (2010) 133–148.
[26] P. Montuschi, P.J. Barnes, G. Ciabattoni, Methods Mol. Biol. 594 (2010) 73–84.
[27] P. Montuschi, F. Macagno, P. Parente, S. Valente, L. Lauriola, G. Ciappi, S.A. Kharitonov, P.J. Barnes, G. Ciabattoni, Thorax 60 (2005) 827–833.
[28] I. Horvath, J. Hunt, P.J. Barnes, K. Alving, A. Antczak, E. Baraldi, G. Becher, W.J. van Beurden, M. Corradi, R. Dekhuijzen, R.A. Dweik, T. Dwyer, R. Effros, S. Erzurum, B. Gaston, C. Gessner, A. Greening, L.P. Ho, J. Hohlfeld, Q. Jobsis, D. Laskowski, S. Loukides, D. Marlin, P. Montuschi, A.C. Olin, A.E. Redington, P. Reinhold, E.L. van Rensen, I. Rubinstein, P. Silkoff, K. Toren, G. Vass, C. Vogelberg, H. Wirtz, Eur. Respir. J. 26 (2005) 523–548.
[29] P. Montuschi, J.V. Collins, G. Ciabattoni, N. Lazzeri, M. Corradi, S.A. Kharitonov, P.J. Barnes, Am. J. Respir. Crit. Care Med. 162 (2000) 1175–1177.