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Determination of endothelin-1 in rats using a high-performance liquid chromatography coupled to electrospray tandem mass spectrometry

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ABSTRACT

A sensitive and specific liquid chromatography tandem mass spectrometry method with electrospray ionization for the determination of endothelin-1 in rat plasma and lung effluents has been developed and validated. Detection was achieved by an Applied Biosystems MDS Sciex API 2000 triple quadrupole mass spectrometer coupled to an Agilent 1100 LC system. The limit of detection and the limit of the quantification of ET-1 in matrix buffer was estimated at 40 pM and 1 nM, respectively. The precision and accuracy for both intra- and inter-day determination of the analyte ranged from 2.5% to 14.7% and from 104.2% to 113.3%, respectively. No significant relative matrix effect was observed. Stability of ET-1 established in a bench-top, autosampler, long-term storage stability as well as freeze/thaw cycles shown no significant degradation products in the samples. The results of the method validation indicated that this method is applicable for the determination of the ET-1 concentration in an effluent from the isolated lung preparation as well as *in vivo* in plasma samples to evaluate ET-1 as a potential biomarker of the progression of pulmonary endothelial dysfunction and pulmonary hypertension in rats induced by a monocrotaline injection.

1. Introduction

Endothelin-1 is an endogenous peptide with a potent vasoconstrictive, growth-promoting and profibrotic action. ET-1 (plasma half-life approximately 4–7 min) is synthesized from big-endothelin (big-ET-1) by endothelin converting enzymes (ECE) mainly in endothelial cells. There are equally two other endothelins (ET-2 and ET-3) though it is ET-1 that is the most widely expressed [1]. ET-1 exerts effects via two identified G-protein-coupled receptor subtypes: the endothelin type A (ETA; located on vascular smooth muscle cells, fibroblast, etc.) and the endothelin type B (ETB; located on endothelial cell, smooth muscle cell of the small pulmonary arteries, etc.) [2]. ET-1 causes the activation of short-term and long-term pathways in cells. Both consist in an increase in intracellular calcium and the activation of the calcium-sensitive proteins responsible for the constriction, secretion or releasing of prostaglandins and thromboxane A_2 (specific for ETB receptor) and stimulation of the calcium-calmodulin dependent pathways of mitogenesis, hypertrophy and differentiation [3–5].

Although ET-1 stimulates (in ETB-dependent manner) a healthy endothelial cell to release nitric oxide (NO) and prostacyclin (PGI_2) [6,7] ET-1 overactivation is closely linked to vascular pathology. In particular, ET-1 is considered to be an important mediator of vascular pathology in pulmonary circulation as well as in the systemic circulation. In fact, ET-1 stimulates fibroblasts for synthesis of the extracellular matrix, enhances monocyte–endothelial cell interactions [8,9], impairs the biological activity of NO, and PGI_2 by increasing superoxide and peroxynitrate production, promoting the BH4 deficiency and the uncoupling of the eNOS [10,11]. In addition, ET-1 enhances endothelial inflammation by the releasing of IL-6, and exerts a synergistic effect with TNFα and CRP on the expression of adhesion molecules on the endothelial cell surface [12,13]. All the above effects could contribute to the pathogenesis of pulmonary hypertension and it is proposed that ET-1 measurements in plasma represents a reliable marker of the progression of pulmonary hypertension and may have a prognostic significance in pulmonary hypertension in humans [14–18].

Reliable quantification of endogenous ET-1 in specific biological media requires the use of highly sensitive analytical methods. Most of them used for the determination of the tissue concentration of endothelins involve reverse phase mini columns which allow to separate proteins from peptides, after which the levels of endothelins are directly determined by immunological assays, which are able to achieve detection limits in the fentomole range.

The presence of specific endothelin isoforms (endothelin precursors, proendothelin-1, proendothelin-2 and proendothelin-

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3) in normal human endometrium was determined using a high-performance liquid chromatography combined with radioimmunoassay and immunocytochemistry. The use of HPLC in the reported study has permitted the separation of the endothelin peptides, which were subsequently measured in the appropriate fractions by radioimmunoassay. One of the main raises disadvantages of immunological assays is cross reactivity of ET-1 with isotopes, its degradation products or endothelin metabolites in tissues, and therefore the reported data are sometimes diverse [19].

The implementation of the LC/MS/MS method coupled to an atmospheric pressure electrospray ionization has proven to be a powerful tool for the analysis of macromolecules in complex biological matrices. Moreover, electrospray is known as a soft ionization technique produces mainly protonated ions without causing their fragmentation complicating analysis.

The implementation of column-switching nanoLC combined with mass spectrometry has given similar detection limits to those in immunoassays. In the study of Oosterkamp et al. [20] the developed on-line preconcentration nanoLC/μESI-MS method was used to analyze the endothelins in the supernatants of human umbilical vein endothelial cell (HUVEC) cultures. The sample preparation was reduced to protein precipitation and the peptides were preconcentrated using an on-line column-switching procedure based on a strong anion-exchange preconcentration column. Thereafter, samples were separated in a nanocolumn and analyzed using an ion trap mass spectrometer. The LOD obtained by this method was 30 fM and was of the same order as those in immunoassays.

The presence of ET-1 in HUVEC cultures was also measured by Ashby et al. [21]. They have used an immunoaffinity column combined with a solid phase extraction procedure to preconcentrate up to 500 mL of HUVEC cells and analyzed ET-1 by LC/ESI-MS technique. Finally, they have achieved a limit of detection of 6 pM.

Moreover, an on-line LC/ESI-MS method for the quantitative analysis of endothelins in HUVEC culture supernatants was reported by Carrascal et al. [22]. The analysis was isoform specific, employed solid phase extraction and subsequent HPLC fractionation followed by LC/ESI-MS analysis. The limit of detection of the method in a buffer matrix was estimated at 50 fM.

The aim of the study was therefore to investigate and validate an analytical quantitative method using a high-performance liquid chromatography coupled to a tandem mass spectrometry technique for the determination of the ET-1 in plasma of rats injected with monocrotaline, which represents a commonly used model of pulmonary hypertension. Furthermore, we analyzed whether the elevation of ET-1 coincides with the development of endothelial dysfunction in pulmonary circulation and the progression of pulmonary hypertension pathology in this experimental model of this disease.

2. Experimental

2.1. Materials and reagents

Standard compounds endothelin-1 (Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp) and N-Acetyl-[$_D$-Trp^{16}] Endothelin-1 (fragment 16–21) were purchased from Sigma (St. Louis, MO, USA).

Gradient grade acetonitrile, water for chromatography and ethanol were obtained from Merck (Darmstadt, Germany) and reagent grade formic acid, sodium chloride, potassium phosphate monobasic, sodium hydrogen carbonate, magnesium sulfate heptahydrate, potassium chloride, calcium chloride dehydrate, monocrotaline, aprotinin from bovine lung, HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) and L-NAME (N^G-nitro-L-arginine methyl ester) were obtained from Sigma (St. Louis, MO, USA). Nadroparine (Fraxiparine) were purchased from GlaxoSmithKline (GlaxoSmithKline Pharma, Poland). Glucose anhydrous was obtained from Polish Chemical Reagents (POCH Joint-Stock Company, Poland), albumin bovine was obtained from Serva (Serva Electrophoresis GmbH, Germany).

Krebs-Henseleit buffer (KH buffer) with added albumin used in the experiment in an isolated perfused rat lung was prepared *ex tempore* and the KH buffer was of the following composition: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 12.5 mM $NaHCO_3$, 0.6 mM albumin, 5.6 mM glucose and 12.6 mM HEPES.

Control plasma was obtained from adult male Wistar rats Krf:(WI)WV (Charles River Laboratory, Germany). The plasma samples were separated by centrifugation (12,000 rpm, 10 min) and stored at −80 °C until used.

All animal procedures conformed with the European Community Guidelines for Animal Care and Treatment and the experimental procedures used in the present study were approved by the Local Jagiellonian University Ethics Committee on Animal Experiments.

2.2. High-performance liquid chromatography

Liquid chromatography was performed using an Agilent 1100 (Agilent Technologies, Waldbronn, Germany) LC system. Chromatographic separation was carried out with an XTerra C18 analytical column (30 mm × 2.1 mm, 3.5 μm, Waters, Ireland) set at 20 °C.

Two solvent mixtures were used: solvent A: acetonitrile–formic acid (1.8 mM) and solvent B: H_2O–formic acid (1.8 nM). The following gradient was used: 0–5 min, 0–100% A; 5–7 min, 100% A; 7–8 min, 100–0% A; 8–15 min, 100% B. The flow rate was set at 300 μL min^{-1}.

The autosampler temperature was set at 4 °C, and a sample volume of 25 μL was injected into the analytical column for peptide analysis.

2.3. Mass spectrometry

Mass spectrometric analyses were accomplished on an Applied Biosystems MDS Sciex (Concord, Ontario, Canada) API 2000 triple quadrupole mass spectrometer equipped with an electrospray ionization interface.

A standard solution of polypropylene glycols was used for instrument tuning and mass calibration according to the Applied Biosystems manual. The mass spectrometer was operated with a dwell time of 200 ms, and a 5 ms delay between scans for each transition in the selected reaction monitoring mode (SRM).

To find the optimal parameters for the ion source and ion path the mass spectrometer was tuned individually by direct infusion of each compound at a concentration of 40 nM with a flow rate of 10 μL min^{-1} using a Hamilton syringe pump (Hamilton, Reno, Nevada).

The ion source parameters were as follows: ion spray voltage (IS): 5500 V; nebulizer gas (gas 1): 45 psi; turbo gas (gas 2): 45 psi; temperature of the heated nebulizer (TEM): 300 °C; curtain gas (CUR): 10 psi, and collision gas (CAD): 12 psi. Nitrogen (99.9%) from Peak NM20ZA was used as the curtain and collision gas. The most favorable parameters of ion path for analyzed compounds are presented in Table 1.

The tandem mass spectrometer was operated in the positive ionization mode, at unit mass resolution, monitoring the transition of the protonated molecular ions $[M+3H]^{3+}$ m/z 832 to the product ion m/z 318 for ET-1, and m/z 887 to m/z 228 for the internal standard. The peak widths of the precursor and product ions were set to 0.7 full width half-height.

Table 1
Optimized parameters for SRM analysis of ET-1 and internal standard.

Parameters	ET-1	IS
Declustering potential (DP)	10	35
Focusing potential (FP)	400	350
Entrance potential (EP)	4	10
Collision cell entrance potential (CEP)	8	44
Collision cell exit potential (CXP)	25	5
Collision energy (CE)	35	60

The quantification analysis was performed via a peak area ratio of ET-1 to IS. Data acquisition and processing were accomplished using the Applied Biosystems Analyst version 1.4.2 software.

2.4. Preparation of stock and working solutions

The stock solution of ET-1 was prepared at a concentration of 10 μM in ethanol and was further diluted with ethanol to make working solutions at concentrations of 2000, 1200, 800, 400, 200, 100 and 10 nM. An internal standard working solution was prepared by diluting the stock solution at a concentration of 56.3 μM with ethanol to give a 5.63 μM working solution. All stock and working standards were stored at −80 °C.

2.5. Preparation of calibration standards and quality control samples

Quality control (QC) samples were prepared by spiking the blank plasma and KH buffer with ET-1 at three different concentrations along the calibration range. For the KH buffer, the QC samples were: low at 1.2 nM, medium at 12 nM and high at 160 nM, and for the plasma QC, concentrations were: 21.2, 32 and 180 nM.

2.6. Sample preparation

The precipitation of proteins to ethanol was a successful tool for ET-1 isolation [23,24]. A 100 μL aliquot of the studied matrices were pipetted out into centrifuge tubes placed on ice. The working internal standard solution (10 μL) and 200 μL of ethanol were added and then shaken for 2 min. After centrifugation at 15,000 rpm for 10 min (4 °C), the supernatants (100 μL) were transferred to other tubes and evaporated to dryness under a stream of nitrogen in a TurboVap LV (Caliper Life Science, Germany) and the sec residue was dissolved in 50 μL of ethanol, thereafter transferred to the inserts, and 25 μL was injected into the analytical column.

2.7. Animals and blood sampling

To induce the pulmonary hypertension monocrotalin injection (MCT, 60 mg kg^{-1} s.c., Sigma, St. Louis, MO, USA) was administered to male rats (180–200 g). After 1, 2, 3 and 4 weeks rats were anaesthetized with tiopenthal. The ET-1 concentration was assessed in the plasma and lung effluents of the rats collected after 30-min perfusion with the KH buffer of the isolated rat lung.

After the opening of the chest wall the blood was collected from the right ventricle to a syringe containing cold (5 °C) calcium citrate (9:1, v/v). The blood was moved to the eppendorf probes with aprotinin (650 j.m. mL^{-1} of blood) and was centrifuged (12,000 rpm, 10 min). The plasma was collected, instantly frozen and stored at −80 °C until used. The ET-1 concentration was measured in the plasma and lung effluents instantly after unfreezing.

2.8. The isolated perfused rat lung set-up

To assess the physiological parameters of the pulmonary endothelial function an isolated rat lung perfused with Krebs-Henseleit buffer (IPL) was used as described previously [18]. Shortly after anesthetization with tiopenthal rats were placed on a surgical table. Artificial respiration (80 breaths min^{-1}) with positive pressures was initiated followed by tracheostomy. Afterwards laparothomy and sternoctomy nadroparine was injected into the right ventricle to protect from clots in the pulmonary circulation. Next, the pulmonary artery was cannulated via the right ventricle, the effluent's flow was initiated and the apex of the heart was removed. After cannulation of the left atrium through the left ventricle the lung–heart block was carefully removed from the chest of the animal and placed in the artificial chest of the IPL set-up. Respiration with negative pressures was initiated followed by a progressive increase in the flow of the effluent until 15 mL min^{-1}. Alongside ventilatory pressures were set at 2 mL for expiratory pressure and 6–10 mL for the inspiratory pressure to protect the tidal volume of 2.0–2.2 mL. Pulmonary circulation in IPL was washed to remove any residual blood with 100 mL of KH buffer followed by a 10-min period of stabilization with the recirculated KH buffer. After stabilization the isolated lung was perfused with the recirculated fresh KH buffer. After 30 min of recirculation the effluent was collected to the eppendorf probes containing aprotinin (650 j.m. mL^{-1} of effluent) and was frozen and stored at −80 °C until used. The ET-1 concentration was measured in the effluent samples instantly after unfreezing. The effluent samples were collected from the lungs of the control rats as well as of the rats injected with monocrotaline, 1, 2, 3 and 4 weeks after the MCT injection.

To analyze the endothelial function and NO-dependent function an isolated lung preparation was perfused with blood. A perfusion with blood began instantly after the initiation of respiration with negative pressure, without the washing of the pulmonary circulation with the KH buffer. A progressive increase of flow of the perfused blood was stopped at 14 mL min^{-1}. Respiratory pressures and the tidal volume were the same as in the IPL perfused with KH buffer. After 10 min of stabilization the normoxic mixture used for the ventilation of the IPL was switched to a hypoxic mixture. 10-min hypoxic ventilation was followed by 15-min periods of normoxia ventilation. After the second hypoxic ventilation L-NAME was added to the recirculated blood and incubated for 15 min and the procedure of hypoxic/normoxic ventilation was repeated two times. Changes of pulmonary arterial pressure (ΔPAP) between normoxic and hypoxic phases were noted and the effects of L-NAME on the magnitude of the HPV was considered as an assay for the functional activity of NO in pulmonary circulation.

2.9. Method validation

2.9.1. Selectivity and matrix effect

The absence of "cross-talk" between channels used for monitoring ET-1 and IS was confirmed by injecting separately samples (pure solutions) containing an IS at the concentration used in the assay and monitoring the response in the MS/MS channels used for detecting the analyzed substance, and by injecting sample of the compound of interest at the highest concentration on the standard line and monitoring the response in the IS channel.

An assessment of matrix effect was done by comparing the peak areas of analyte in extracted samples of blank plasma spiked with the studied compound with the corresponding peak areas obtained by injection of standard solutions at appropriate concentration. Since the compound of interest has an endogenous nature, samples of blank plasma were earlier analyzed to obtain the basic levels, which were subtracted in all following calculations.

For evaluation of the relative matrix effect five different sources of rat plasma were used and five samples of low QC concentration was assessed. Moreover, the absolute matrix effect was calculated by using pooled plasma coming from six different sources through

repeated analysis of the plasma samples spiked with ET-1 at the concentration of low QC level.

The matrix effect was also verified in the KH buffer spiked with ET-1 at the low concentration of the QC sample and comparing the peak areas of analyte in KH buffer samples with the corresponding peak areas obtained by injection of standard solution at appropriate concentration.

2.9.2. Linearity, LLOD, LLOQ and rLLOQ

A seven-points of standard curve were done to establish the calibration range. Calibration curves were prepared by spiking 90 μL of pooled blank plasma or the KH buffer with 10 μL of working solution of ET-1 to produce the calibration curve points equivalent to 200, 120, 80, 40, 20, 10 and 1 nM. Each sample also contained a 10 μL of the IS working solution. Before sample pretreatment, the mixture was vortex-mixed briefly.

Due to the levels of endogenous ET-1 in rat plasma, the quantitative determination of ET-1 in the pooled rat plasma samples was done beforehand and after spiking with different concentrations of ET-1 on the same and different days.

The best calibration curve fit was attained by subtracting the mean blank plasma response of the analyte from the calibration standards response. The results (peak area of analyte/peak area of IS) versus concentration were fitted to the linear equation $y = ax + b$, in which y is the peak area ratio and x is the concentration of the analyte by weighted ($1/x$) least-square regression forced to the zero. The quality of fit was evaluated by comparing back-calculated concentrations to the nominal ones.

The limit of the detection (LLOD) of ET-1 was estimated in the KH buffer as a signal to noise ratio (S/N > 3). The limit of quantification (LLOQ) also estimated in the matrix buffer was defined as the lowest macromolecule concentration which can be determined with RSD ≤ 20% and an accuracy of 100 ± 20%.

Because an endogenous existence of ET-1 was found in the rat plasma, the verification of LLOD and LLOQ in this matrix was not attempted. According to Tsikas [25] we estimated the relative lower limit of quantification (rLLOQ) defined as the percentage ratio of the LLOQ value using the equation (Eq. (1)):

$$\text{rLLOQ} = \left(\frac{C_{\text{LLOQ}}}{C_{0,\text{LN}}}\right) \times 100 \quad (1)$$

where C_{LLOQ} is a lowest added ET-1 concentration to rat plasma and $C_{0,\text{LN}}$ is a basal concentration of ET-1 in rat plasma.

2.9.3. Accuracy, precision and recovery

Accuracy (percent of recovery, Rec%) from the matrix buffer was calculated from the equation (Eq. (2)):

$$\text{Rec\%} = 100 - \text{relative bias} \quad (2)$$

Relative bias was calculated from the equation (Eq. (3)):

$$\text{Relative bias} = \frac{(C_m - C_n)}{C_n} \times 100 \quad (3)$$

where C_m is a measured concentration of ET-1 and C_n is a nominal concentration of ET-1.

Precision was expressed as the percent relative standard deviations (coefficient of variation, RSD%). Within-day and between-day precision and accuracy of the assay were assessed by performing replicate analyses at different QC levels in the plasma and matrix buffer. The criteria for the acceptability of the data included accuracy within a ±15% deviation from the nominal values and precision within 15% RSD, except for LLOQ, where it should not exceed ±20%.

Recovery (%) from pooled rat plasma was estimated from the equation (Eq. (4)):

$$\text{Recovery}\,(\%) = \left(\frac{C_m - C_{0,\text{LN}}}{C_n}\right) \times 100 \quad (4)$$

where C_m is the ET-1 concentration measured in plasma, C_n is the nominal concentration of ET-1 added to the sample, $C_{0,\text{LN}}$ is the basal concentration of ET-1 in rat plasma.

For the extraction recovery of ET-1 in the plasma and KH buffer, extracted samples of pooled plasma and matrix buffer ($n = 6$) were spiked with ET-1 quality control levels and were compared with extracted samples at the same levels ($n = 6$). The extracts of the spiked plasma samples simulating a 100% theoretical recovery. The recovery value of the IS was determined by a single concentration of the working solution of 5.63 μM.

2.9.4. Stability study

The short-term temperature stability of ET-1 in the studied matrices during 2 h (bench-top) was determined on ice at concentrations of the QC samples. Long-term storage stability (freezer stability) of ET-1 in rat plasma and the matrix buffer was assessed by analyzing the QC samples stored both at −30 and −80 °C for three weeks. Similarly, freeze–thaw stability was evaluated both at −30 and −80 °C. Moreover, the stability of ET-1 was determined periodically by injecting replicate preparations of processed samples for up to 12 h (in the autosampler batch at 4 °C) after the initial injection.

The peak areas of the analyte obtained in the initial cycle were used as the reference to determine the stability of ET-1 at subsequent points. Samples were considered to be stable if the assay values of ET-1 were within the acceptable limits of accuracy and precision.

3. Results and discussion

3.1. Method development

To establish a quantitative method with higher sensitivity and specificity for the analysis of ET-1 in lung effluents and plasma we applied a LC/ESI-MS/MS technique. To our knowledge, this is the first report using a simple reversed-phase LC/ESI-/MS/MS method to determine the ET-1 concentration in the plasma and lung effluents of rats with pulmonary hypertension induced by a monocrotaline injection.

The electrospray mass spectrum of ET-1 (2491.9 amu) reveals the presence of a doubly charged $[M+2H]^{2+}$ ion at m/z 1246.9 and also a triply charged $[M+3H]^{3+}$ ion at m/z 832.1 (Fig. 1A). Mass spectra were acquired in Q1 mode from 100 to 1400 amu in a scan time of 200 ms with a 5 ms delay between scans. For quantitative experiments only a triply charged ion (m/z 832) and its fragment (m/z 318) were analyzed by the SRM mode (Fig. 1B).

In our study the peptide N-Acetyl-[$_D$-Trp^{16}] Endothelin-1 (fragment 16–21) was used as an internal standard. Its similar structure relative to ET-1 indicates that the adsorption and elution behavior of IS was comparable to those of ET-1 under the sample preparation conditions. Fig. 2A shows the mass spectrum of the internal standard acquired in the Q1 mode, and its parent (m/z 887.7) to product (m/z 228.7) spectrum is presented in Fig. 2B.

Different chromatographic conditions were investigated to optimize sensitivity, speed and peak shape. Various HPLC columns were tested and the Waters XTerra C18, 30 mm × 2.1 mm, 3.5 μm analytical column gave good retention and baseline separation of the analytes. A flow rate of 300 mL min^{-1} and a mobile phase composition of acetonitrile/water/formic acid with gradient elution provided a total analysis time of less than 4 min. The retention times

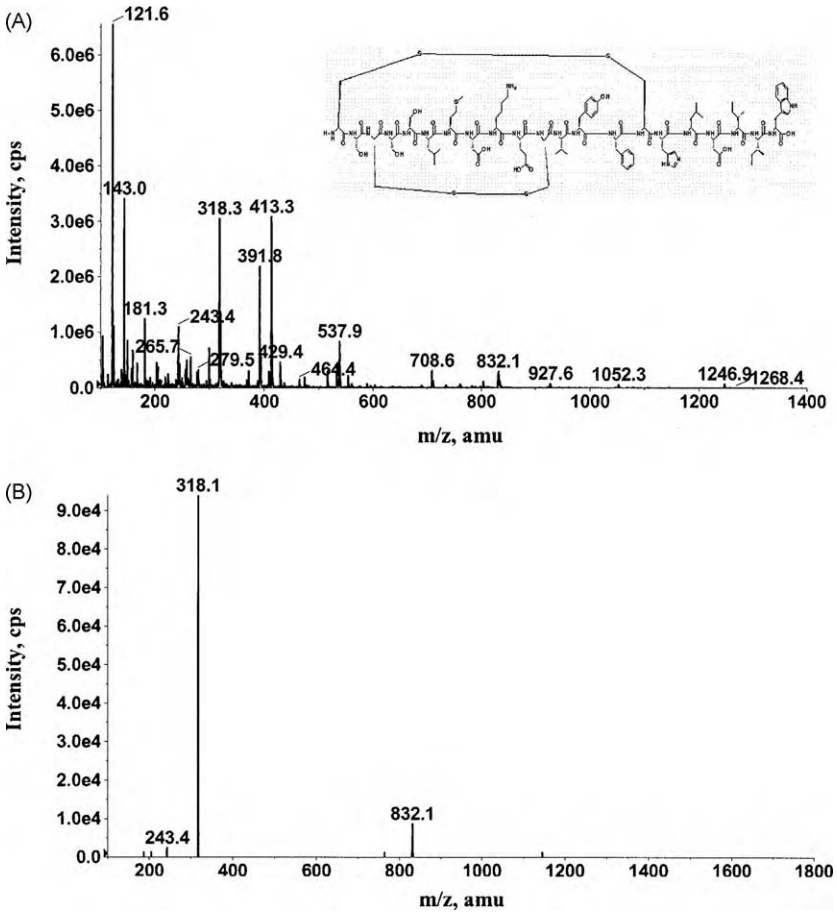

Fig. 1. ESI mass spectrum of ET-1 (2491.9 amu) at a concentration of 10 nM obtained by flow injection analysis in a Q1 mode (A) and the possible product ions formed after collision MS/MS of triple charged ion, *m*/*z* 832 (B).

of ET-1 and IS were 2.4 and 3 min, respectively and were stable during analysis.

3.2. Method validation

3.2.1. Selectivity

The high selectivity of the SRM mode avoids the need for a complete chromatographic separation between the target compounds and the interferences from the matrix, allowing for a quantitative analysis.

Fig. 3A shows the extracted ion chromatogram of ET-1 in lung effluents due to the endogenous presence of ET-1 in control rats. Fig. 3B shows the chromatogram of internal standard at concentration of 5.63 μM spiked with lung effluents of control rats. Fig. 3C shows the chromatogram of the matrix buffer spiked with 20 nM of ET-1, and Fig. 3D shows the chromatogram of ET-1 in the lung effluents of rats with induced pulmonary hypertension.

3.2.2. Calibration

Calibration curves of ET-1 in the KH buffer and plasma were linear over a concentration range from 1 to 200 nM and from 21 to 220 nM, respectively. The correlation coefficients for ET-1 in the plasma and matrix buffer were 0.9962 and 0.9978, respectively.

For each point of the calibration standards, the concentrations were back-calculated from the equation of the linear regression curves, and the relative standard deviations were calculated. A linear regression of the back-calculated concentrations versus the nominal ones provided a unit slope and an intercept equal to zero (Student's *t*-test). The distribution of the residuals (the difference between nominal and back-calculated concentrations) shows random variations, the number of positive and negative values being approximately equal.

The measured basal ET-1 concentration ($C_{0,LN}$) in rat plasma and lung effluents was 20.02 ± 1.04 and 16.79 ± 1.39 nM, respectively.

The LLOD of ET-1 in the KH buffer was equal to 40 pM. The limit of quantification of ET-1 in the matrix buffer has proven to be 1 nM. At the LLOQ, the precision and accuracy of the method in the matrix buffer did not exceed 20%. According to Tsikas [25] the relative lower limit of quantification calculated from rat plasma was equal to 5 nM.

3.2.3. Matrix effect

Co-eluting, undetected matrix components may reduce or enhance the ion intensity of the analytes and affect the reproducibility and accuracy of the assay. The majority of matrix effects occur in the solvent front of the chromatographic run therefore if the analytes can be retained to some degree chromatographically then matrix effect can be minimized.

The absolute matrix effect for ET-1 (ion suppression) was observed for five different plasma pools and the matrix buffer. The results did not show a good repeatability of the samples coming from the pooled matrices. This fact confirms the existence of an absolute matrix effect, but the calibration curve quantification approach according to Matuszewski et al. [26] could be applied. According to these authors when the absolute matrix effect is below 100%, we observe an ion suppression effect, whereas when the value is above 100%, there is an ion enhancement effect. In a LC/MS/MS bioanalysis, the presence of the absolute matrix effect is not very critical [27].

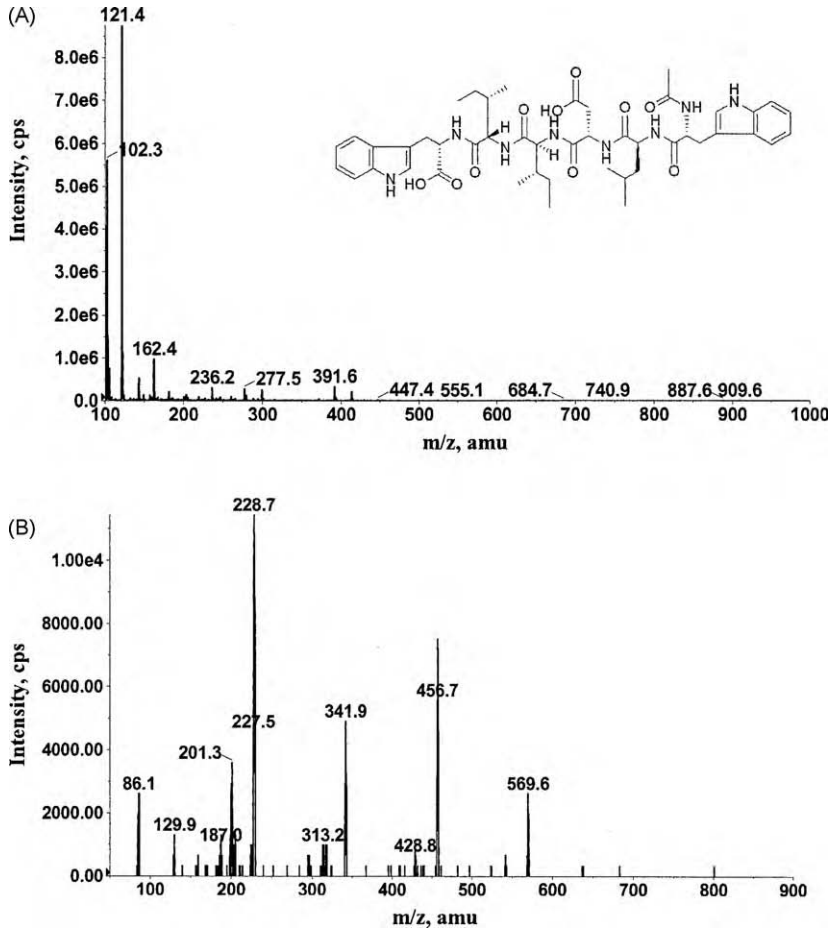

Fig. 2. ESI mass spectrum of the internal standard (m/z 887.7) at a concentration of 56 nM (A) and tandem mass spectrum of the internal standard (B).

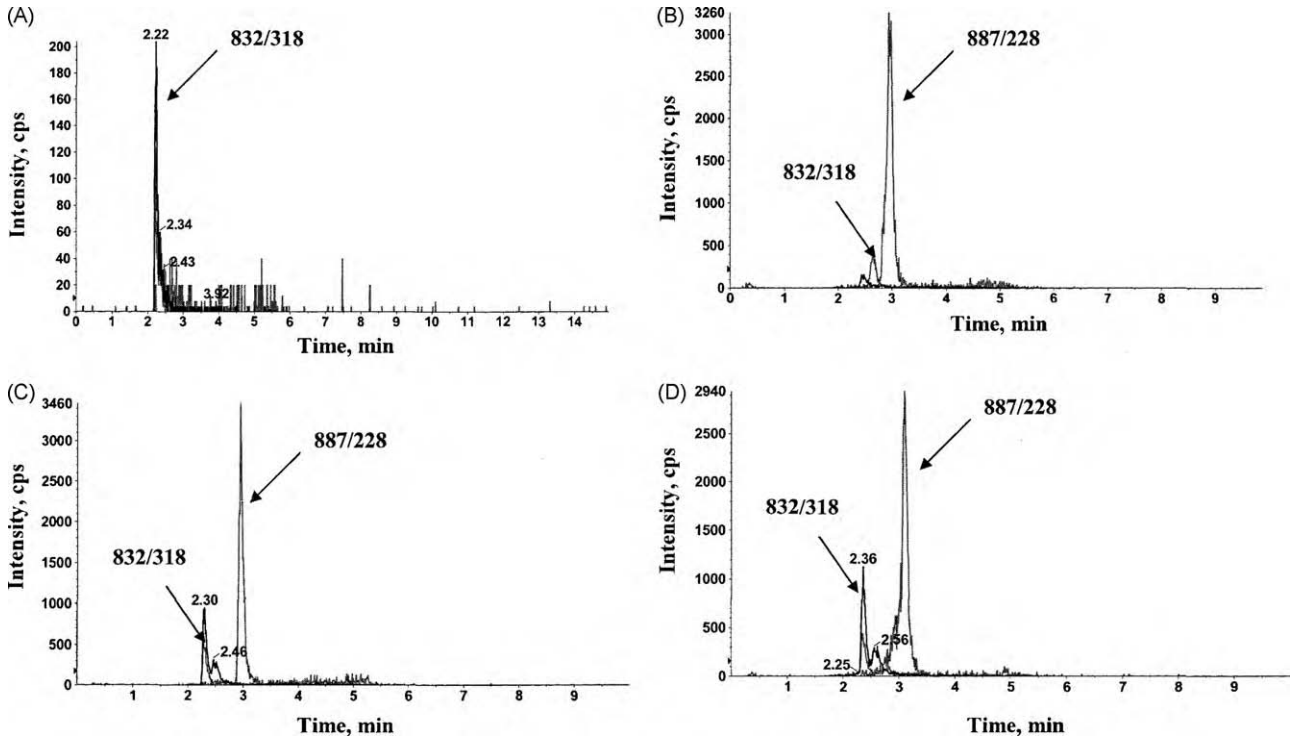

Fig. 3. Chromatogram of the endogenous ET-1 (16.2 nM) in lung effluents of control rats (A), chromatogram of the internal standard (5.63 μM) in lung effluents of control rats (B), chromatogram of ET-1 at concentration of 20 nM spiked with KH buffer (C), and chromatogram of ET-1 at a concentration of 35.2 nM in lung effluents of rats with induced pulmonary hypertension (D).

Table 2
Matrix effect from five low QC samples of ET-1 in rat plasma.

ET-1	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5
Mean found concentration [nM]	21.1 ± 2.5	21.2 ± 2.2	21.4 ± 3.1	21.4 ± 3.2	21.2 ± 2.2
RSD [%]	11.8	10.2	14.6	14.8	10.4
Accuracy [%]	89	93	111	113	90

Table 3
Matrix effect from five low QC samples of ET-1 in the Krebs-Henseleit buffer.

ET-1	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5
Mean found concentration[nM]	1 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	1.3 ± 0.2	1.1 ± 0.2
RSD [%]	10.8	12.2	13.4	14.1	14.3
Accuracy [%]	85	96	105	112	93

A more important parameter in the evaluation and validation of a bioanalytical method using the LC/MS/MS technique was the demonstration of the absence of the relative matrix effect. Therefore, five unique batches of rat plasma spiked with a low QC standard were compared and the data were listed in Tables 2 and 3. As can be seen, a relative matrix effect was not observed.

3.2.4. Precision and accuracy

Data for within-batch and between-batch precision and accuracy of the method for the determination of ET-1 are presented in Table 4. The precision of the method both for the intra- and inter-day determination of ET-1 in the matrix buffer ranged from 3.3–11.7% and 2.9–14.7% and in plasma 4–11.3% and 2.5–10.4%, respectively. The within-assay and between-assay accuracy of the method in the studied matrices were 105–111.7% and 104.2–113.3% in the matrix buffer and 100.5–107.4% and 100.9–105.5% in plasma. All values of precision and accuracy are generally accepted due to the guidance for bioanalytical method validation [28–30].

3.2.5. Recovery

The results of the comparison of the extracted samples versus the matrix extracts spiked with ET-1 at the concentration of QC samples show that the absolute recoveries in the studied matrices ranged from 78.3 to 90.2% (Table 4). The absolute recovery of the internal standard at a working concentration of 5.63 μM was equal to 83.4%.

3.2.6. Stability

Over a period of a short-term stability test (2 h) on ice, a long-term freezer stability at −80 °C, a freeze–thaw stability at −80 °C, and 12 h injection time in the autosampler batch at 4 °C, the predicted concentrations for ET-1 in QC samples deviated within 15% of the nominal ones and no significant degradation products were observed in the samples (Table 5), whereas ET-1 was unstable during storage at −30 °C.

3.2.7. Application of the method

The LC/ESI-MS/MS method developed and described in the present work was evaluated *in vivo* by measuring the changes of the ET-1 concentrations in plasma and in the effluent of an isolated lung preparation taking advantage of the model of rats with pulmonary hypertension induced by a monocrotaline injection [31].

In this model, approximately 4 weeks after an MCT injection there develops: a pulmonary hypertension associated with marked right ventricular hypertrophy, right ventricle heart failure, with increased right ventricle pressure, as well as lung pathology characterized by neutrophil infiltration, severe remodeling of the pulmonary arteries and endothelial injury was characterized by us and shown previously [32,33]. In lung effluents already in the first week after the MCT injection the ET-1 concentration was elevated (29.66 ± 3.15 nM vs. 16.79 ± 1.39 nM, MCT-injected and control rats, respectively) and remained elevated at the later stages of pulmonary hypertension development, Fig. 4A. Interestingly, in rat plasma there was a biphasic increase in the ET-1 concentration. At 2 weeks after the MCT injection a mild increase in ET-1 was noted (37.49 ± 3.13 nM) and then 4 weeks after the MCT injection nearly a 4-fold elevation of the ET-1 concentration was observed (88.16 ± 11.43 nM), Fig. 4B.

Increased activity of endothelial ET-1 is associated with a decrease in the activity of endothelial NO [34]. Therefore for comparison we assayed NO activity in pulmonary circulation in an isolated lung preparation by measuring the magnitude of the NO-dependent attenuation of hypoxic pulmonary vasoconstriction (HPV). In the control blood-perfused lung in the presence of L-NAME, HPV was significantly potentiated (ΔPAP = 8.90 ± 1.82 and 31.45 ± 4.33 before and after L-NAME, respectively). L-NAME-induced potentiation was progressively diminished starting 1 week after the MCT injection (Table 6) suggesting the progressive development of endothelial dysfunction and impaired NO functional activity.

Accordingly, changes in the ET-1 concentration detected by the method described in the present work are parallel to the

Table 4
Assessment of intra- and inter-day precision, accuracy and recovery of the method for ET-1 in the studied matrices (n = 5–6).

Nominal concentration [nM]	Within-assay			Between-assay			Recovery [%]
	Mean found concentration [nM]	Precision [%]	Accuracy [%]	Mean found concentration [nM]	Precision [%]	Accuracy [%]	
Buffer							
1.2	1.3 ± 0.2	11.7	107	1.4 ± 0.2	14.7	113	86.3 ± 3.3
12	13.4 ± 1.1	8.2	112	13 ± 0.9	7	108	80.7 ± 4.5
160	168 ± 5.6	3.3	105	167 ± 5	2.9	104	78.3 ± 5.6
Plasma							
21.2	21.3 ± 2.4	11.3	101	21.4 ± 2.1	9.8	101	90.2 ± 4.6
32	33.8 ± 3.3	9.8	106	33.6 ± 3.5	10.4	105	86.3 ± 3.2
180	194 ± 7.8	4	107	191 ± 4.8	2.5	106	79.5 ± 5.8

Table 5
Stability data of ET-1 in rat matrices (n = 3–4).

Conditions	Nominal concentration [nM]					
	Buffer			Plasma		
	1.2	12	160	21.2	32	180
Bench-top on ice stability (2 h)	8.6	9.7	−6.4	−9.3	7.9	−3.6
Long-term at −80 °C stability	−5.8	8.3	−7.4	7.3	−6.4	−8.2
Freeze–thaw stability	7.8	−5.9	8.3	−6.8	5.7	9.2
Autosampler stability (12 h)	−8.3	6.2	7.3	−5.6	5.3	−6.1

Table 6
Progression of endothelial dysfunction in pulmonary circulation measured by the assessment of the participation of NO in hypoxic pulmonary vasoconstriction.

ΔPAP	Control group	1 week after MCT	2 weeks after MCT	4 weeks after MCT
HPV	8.9 ± 2	5.4 ± 1	7.7 ± 1.5	5.1 ± 1.4
L-NAME (300 μM) + HPV	31.5 ± 4.3	19.3 ± 2.6	9.8 ± 3	4.7 ± 3.3

development of the impairment of the NO-dependent function in pulmonary circulation. As such, the increased concentration of ET-1 detected in effluents from the isolated lung point to endothelial dysfunction in pulmonary endothelium. Importantly, the rise in ET-1 concentration in the lung effluent can be detected already in the first week after the MCT injection. It is then followed by an increased ET-1 concentration in the blood plasma, visible two weeks after the MCT injection. At the final stage of pulmonary hypertension at 4 weeks after the MCT injection the pronounced right ventricular hypertrophy and pulmonary artery remodeling—typical signs of advanced pulmonary hypertension were evident [32,33] as well as an approximately 4-fold increase in the ET-1 concentration in the blood plasma indicating advanced pathology of pulmonary hypertension and possibly also additional sources of ET-1 production in the kidneys and heart as suggested previously [35].

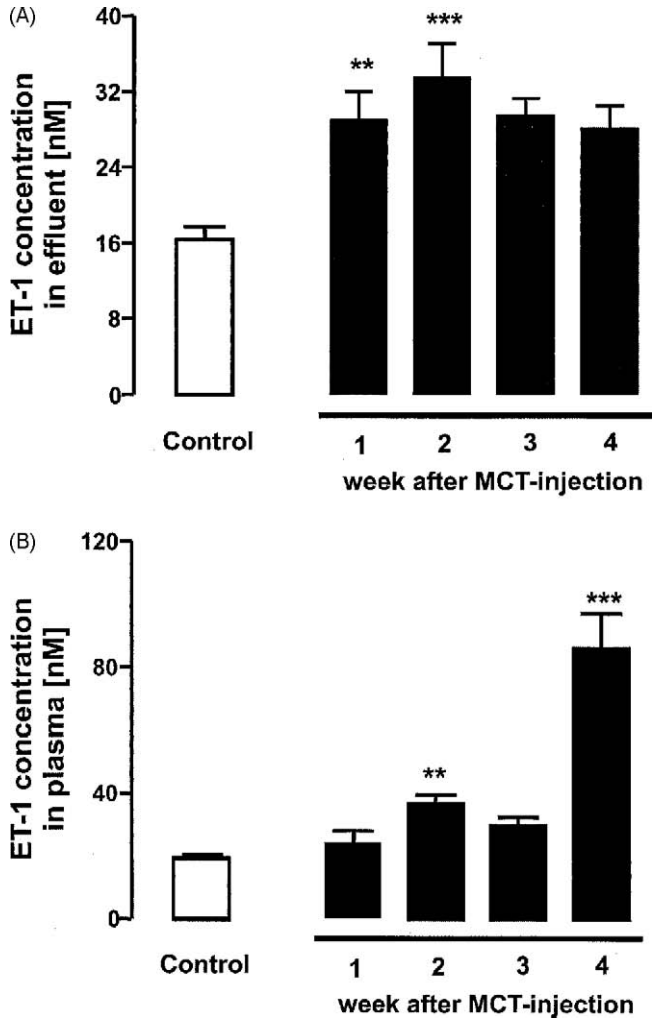

Fig. 4. Elevated ET-1 concentration in the MCT-induced pulmonary hypertension. Increased ET-1 concentration in effluents from the isolated perfused lung in the first and second week after MCT-injection (A). Enhanced ET-1 plasma concentration only in the second and fourth week after the MCT-injection (B).

Accordingly, a determination of the ET-1 concentration using a high-performance liquid chromatography coupled to an electrospray tandem mass spectrometry in effluents from the isolated rat lung and rat plasma from animals treated with MCT could describe the development of pulmonary endothelial dysfunction and the progression of pulmonary hypertension in this experimental model.

4. Conclusion

In this manuscript we have described a sensitive and selective high-performance liquid chromatography–tandem mass spectrometry method for the analysis of ET-1 in rat plasma and lung effluents. Validation of the method for ET-1 in selected conditions shows that the method is selective and precise with a linear response of mass spectrometer. The precipitation procedure to ethanol produced clean chromatograms and high and reproducible recovery was obtained for the studied compound. The method has been successfully applied to assess the progression of endothelial dysfunction in pulmonary circulation and the development of pulmonary hypertension in rats injected with monocrotaline.

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References

[1] N. Galié, A. Manes, A. Branzi, Cardiovasc. Res. 61 (2004) 227–237.
[2] A. Inoue, M. Yanagisawa, S. Kimura, Y. Kasuya, T. Miyauchi, K. Goto, T. Masaki, Proc. Natl. Acad. Sci. U.S.A. 89 (1989) 2863–2867.
[3] M. Yanagisawa, H. Kurihara, S. Kimura, K. Goto, T. Masaki, J. Hypertens. Suppl. 6 (1988) S188–S191.
[4] H. Takase, P. Moreau, T.F. Luscher, Hypertension 25 (1995) 739–743.
[5] H. Arai, S. Hori, I. Aramori, H. Ohkubo, S. Nakanishi, Nature 348 (1990) 730–732.
[6] G. deNucci, R. Thomas, P. D'Orleans-Juste, E. Antunes, C. Walder, T.D. Warner, J.R. Vane, Proc. Natl. Acad. Sci. U.S.A. (1988) 9797–9800.
[7] H. Lal, B. Woodward, K.I. Williams, Br. J. Pharmacol. 118 (1996) 1931–1938.
[8] A. Lerman, D.R. Holmes Jr., M.R. Bell, K.N. Garratt, R.A. Nishimura, J.C. Burnett Jr., Circulation 92 (1995) 2426–2431.

[9] S. Sartore, A. Chiavegato, E. Faggin, R. Franch, M. Puato, S. Ausoni, P. Pauletto, Circ. Res. 89 (2001) 1111–1121.
[10] R. Budhiraja, R.M. Tuder, P.M. Hassoun, Circulation 109 (2004) 159–165.
[11] G.E. Callera, R.C. Tostes, A. Yogi, A.C. Montezano, R.M. Touyz, Clin. Sci. 110 (2006) 243–253.
[12] S. Verma, S.H. Li, M.V. Badiwala, R.D. Weisel, P.W. Fedak, R.K. Li, B. Dhillon, D.A. Mickle, Circulation 105 (2002) 1890–1896.
[13] E.D. Loomis, J.C. Sullivan, D.A. Osmond, D.M. Pollock, J.S. Pollock, J. Pharmacol. Exp. Ther. 315 (2005) 1058–1064.
[14] V.V. McLaughlin, S.L. Archer, D.B. Badesch, R.J. Barst, H.W. Farber, J.R. Lindner, M.A. Mathier, M.D. McGoon, M.H. Park, R.S. Rosenson, L.J. Rubin, V.F. Tapson, J. Varga, Circulation 119 (2009) 2250–2294.
[15] P.L. Selvais, A. Robert, S. Ahn, F. van Linden, J.M. Ketelslegers, H. Pouleur, M.F. Rousseau, J. Card. Fail. 6 (2000) 201–207.
[16] B. Ruo, M.T. Tripputi, P.Y. Hsue, M. Saigo, P. Ouyang, D.D. Waters, Am. J. Cardiol. 96 (2005) 335–338.
[17] G. Warwick, P.S. Thomas, D.H. Yates, Eur. Respir. J. 32 (2008) 503–512.
[18] S. Chlopicki, J.B. Bartus, R.J. Gryglewski, Pol. J. Pharmacol. 54 (2002) 433–441.
[19] I.T. Cameron, C. Plumpton, R. Champeney, C. van Papendorp, M.J. Ashby, A.P. Davenport, J. Reprod. Fertil. 97 (1993) 251–255.
[20] A.J. Oosterkamp, E. Gelpi, J. Abian, J. Mass Spectrom. 33 (1988) 976–983.
[21] M.J. Ashby, C. Plumpton, P. Teale, R.E. Kuc, E. Houghton, A.P. Davenport, J. Cardiovasc. Pharmacol. 26 (1995) S247–S249.
[22] M. Carrascal, K. Schneider, C. Peralta, G. Escolar, E. Gelpi, J. Abian, Biomed. Chromatogr. 18 (2004) 388–395.
[23] M. Fountoulakis, J.F. Juranville, J.D. Avila, D. Roder, P. Jacob, P. Berndt, S. Evers, H. Langen, Amino Acids 27 (2004) 249–259.
[24] A. Bodzon-Kulakowska, A. Bierczynska-Krzysik, T. Dylag, A. Drabik, P. Suder, M. Noga, J. Jarzebinska, J. Silberring, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 849 (2007) 1–31.
[25] D. Tsikas, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877 (2009) 2244–2251.
[26] B.K. Matuszewski, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 830 (2006) 293–300.
[27] I. Fu, E.J. Woolf, B.K. Matuszewski, J. Pharm. Biomed. Anal. 18 (1998) 347–357.
[28] K.J. Miller, R.R. Bowsher, A. Celniker, J. Gibbons, S. Gupta, J.W. Lee, S.J. Swanson, W.C. Smith, Pharm. Res. 18 (2001) 1373–1383.
[29] J. Smolec, B. DeSilva, W. Smith, R. Weiner, M. Kelly, B. Lee, M. Khan, R. Tacey, Pharm. Res. 22 (2005) 1425–1431.
[30] W.A. Colburn, J.W. Lee, Clin. Pharmacokinet. 42 (2003) 997–1022.
[31] H. Nakazawa, M. Hori, H. Ozaki, H. Karaki, Br. J. Pharmacol. 128 (1999) 1098–1104.
[32] R.C. Tyler, M. Muramatsu, S.H. Abman, T.J. Stelzner, D.M. Rodman, K.D. Bloch, I.F. McMurtry, Am. J. Physiol. 276 (1999) L297–L303.
[33] Z. Rakotoniaina, P. Guerard, F. Lirussi, F. Goirand, L. Rochette, M. Dumas, M. Bardou, Naunyn Schmiedebergs Arch. Pharmacol. 374 (2006) 195–206.
[34] R. Mathew, G.A. Zeballos, H. Tun, M.H. Gewitz, Cardiovasc. Res. 30 (1995) 739–746.
[35] T. Miyauchi, R. Yorikane, S. Sakai, T. Sakurai, M. Okada, M. Nishikibe, M. Yano, I. Yamaguchi, Y. Sugishita, K. Goto, Circ. Res. 73 (1993) 887–897.