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HPLC determination of arginases inhibitor N-(ω)-hydroxy-nor-L-arginine using core–shell particle column and LC–MS/MS identification of principal metabolite in rat plasma

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ABSTRACT

For the purpose of in vivo pharmacokinetic studies, an HPLC method was developed and validated for the quantification of *N*-(ω)-hydroxy-nor-L-arginine, L-arginine and *N*-(ω)-ethyl-L-arginine (internal standard) in rat plasma. Sample processing involved a solid-phase extraction on the Waters MCX cartridges and on-line pre-column derivatization of the analytes with o-phthaldialdehyde and 3-mercaptopropionic acid. Separation of the derivatives was carried out on a core–shell Kinetex C18 column in a gradient elution mode with a mobile phase consisting of methanol and water (pH = 3.00 adjusted with formic acid). Fluorimetric detection with the excitation/emission wavelengths of 235/450 nm was used. The method was validated according to the FDA guidelines and applied to pilot pharmacokinetic experiments. An unknown metabolite was extracted from the plasma of Wistar rats after a single bolus of *N*-(ω)-hydroxy-nor-L-arginine (*i.v.* 10 mg kg^{-1}). The metabolite was identified as nor-L-arginine using mass spectrometry. Validated method was successfully used for pilot pharmacokinetic experiment on rats.

1. Introduction

Arginases compete with nitric oxide synthases (NOS) for a common substrate L-arginine (ARG). Overexpression of arginases reduces the synthesis of nitric oxide (NO), while inhibition results in an increased NO formation [1]. An increased expression and activity of arginases have been found in many pathological states [2–5]. Arginase inhibitors decrease blood pressure and improve the reactivity of resistance vessels in adult spontaneously hypertensive rats [6]. In animal models of asthma, arginase inhibition protects against allergen-induced airway obstruction, hyperresponsiveness and inflammation [7]. Moreover, airway remodelling in chronic allergic asthma is attenuated [8]. If compared to healthy subjects, patients with asthma have significantly higher arginase expression in bronchoalveolar lavage cells and in the airway epithelium [9]. Patients who present with acute asthma exacerbation have higher serum arginase activity and lower levels of arginine as compared with healthy subjects [10]. Moreover, arginase activity is related to airflow abnormalities in severe asthma [11]. Arginase inhibitors thus have therapeutic potential in several NO-dependent smooth muscle disorders, including asthma and hypertension. *N*-(ω)-Hydroxy-nor-L-arginine (nor-NOHA), a more potent analogue of *N*-(ω)-hydroxy-L-arginine (NOHA), is an inhibitor of arginases with no affinity towards NOS [12]. An increasing number of published studies address the positive effects of nor-NOHA in various diseases [6,13–15] which strongly contrasts with the absence of analytical methods and pharmacokinetic data in the literature, unlike methods for arginine and its other derivatives [16–19]. The aim of this work was to develop and validate a liquid chromatographic method for nor-NOHA assessment in the rat plasma. Stationary phase Kinetex C18 with the core–shell particle technology was chosen for the method development. Recently, this kind of columns has become popular in pharmaceutical analysis and bioanalysis [20–25], owing to the fast separation of analytes with excellent efficiency [26–32]. Sensitive and selective fluorescence method of amino acid analysis was utilized with o-phthaldialdehyde (OPA) on-line derivatization and separation of isoindoles derivatives [33–37]. Fluorimetry is the primary detection technique. However, chromatographic conditions are compatible with mass-spectrometry (MS). The method also enables simultaneous quantification of ARG. The LC–MS mode was used for the identification of a putative metabolite of nor-NOHA found in the plasma of Wistar rats after *i.v.* administration of the compound. The metabolite was identified as nor-L-arginine (nor-ARG).

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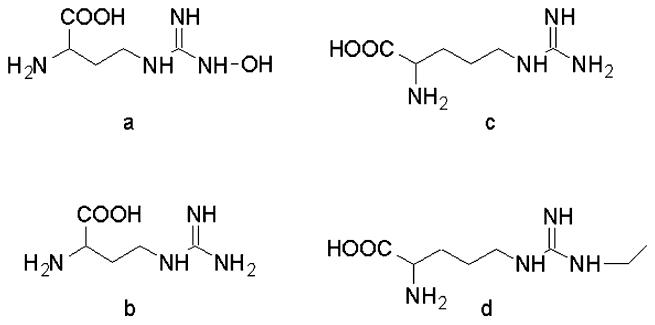

Fig. 1. Chemical structures of *N*-(ω)-hydroxy-nor-L-arginine (a), nor-L-arginine (b), L-arginine (c) and *N*-(ω)-ethyl-L-arginine (d).

2. Experimental

2.1. Reagents

N-(ω)-Hydroxy-nor-L-arginine was purchased from Bachem (Basel, Switzerland), *N*-(ω)-ethyl-L-arginine (NMEA, an internal standard) was purchased from Enzo Life Sciences (Farmingdale, NY, USA) and nor-L-arginine (nor-ARG) was obtained from IS Chemical Technology (Shanghai, China). Chemical structures of compounds under the study are presented in Fig. 1. Hydrochloric acid and sodium hydroxide were obtained from Lach-Ner (Neratovice, Czech Republic). L-Arginine, boric acid, potassium acetate, formic acid, 3-mercaptopropionic acid (3-MPA) and o-phthaldialdehyde were purchased from Sigma–Aldrich (Prague, Czech Republic) and gradient grade methanol from Fisher Scientific (Pardubice, Czech Republic). Water was purified with the MilliQ system (Millipore, Milford, MA, USA). OPA reagent was prepared by dissolving of 20.1 mg o-phthaldialdehyde in 1 ml of methanol. The solution was transferred to a 10 ml volumetric flask, filled with borate buffer (0.2 mol l^{-1}, pH = 9.5) and 10 μl of 3-MPA was added. Before use, reagent was held in a dark at room temperature for 90 min [34,38], then was transferred to the fridge (4 °C) and used at longest for two days [39]. All chemicals used in the study were of analytical-reagent grade or best available purity. Drug-free rat plasma for the method development and validation was acquired from sacrificed animals.

2.2. Instrumentation and chromatographic conditions

All analyses were performed on a 1100 series Agilent liquid chromatograph (Palo Alto, CA, USA) composed of a degasser, quaternary pump, light-tight autosampler unit set at 7 °C, thermostated column compartment held at 50 °C and a fluorescence detector set at the excitation/emission wavelengths of 235/450 nm, with photomultiplier gain 11. Chromatographic separation was carried out on a Kinetex C18 core–shell column 100 mm × 3 mm ID, 2.6 μm (Phenomenex, Torrance, CA, USA), protected with disposable in-line filter CrudCatcher (Phenomenex, Torrance, CA, USA). The mobile phase flowing at a rate of 0.85 ml min^{-1} consisted of water, adjusted with formic acid at pH = 3.00 (solvent A) and methanol (solvent B). The gradient elution program was as follows: from 5% to 39% (v/v) of solvent B in 9.4 min; 39% to 5% (v/v) of solvent B in 0.1 min, equilibration at 5% (v/v) of solvent B for 3.5 min. During the equilibration, on-line derivatization of the next sample was accomplished simultaneously. Injection program used Agilent ChemStation (Palo Alto, CA, USA) software statements in the following order: DRAW 1.5 μl from sample, DRAW 1.5 μl from vial 1 (OPA reagent), MIX 3.0 μl (in air, max. speed, 8 times), WAIT 0.4 min, INJECT. The time of sample analysis was 13 min including column re-equilibration. The mobile phase was filtered through a 0.22 μm Durapore filter (Millipore, Milford, MA, USA) and vacuum degassed prior to use. Data processing was handled by means of Agilent ChemStation software (Palo Alto, CA, USA). For the identification of an unknown metabolite, a quadrupole ion trap LCQ Fleet mass spectrometric detector (Thermo Scientific, San Jose, CA, USA) equipped with an electrospray (ESI) interface was used. Detector was coupled with 1200 Series Agilent HPLC (Palo Alto, CA, USA) composed of degasser, quaternary pump, cooled autosampler and column compartment. Chromatographic conditions were identical to that described above for fluorescence detection.

2.3. On-line derivatization

With the aim to achieve the optimum response of the fluorescence signal a special attention was paid to the optimization of the injector program used for the on-line derivatization as well as to the composition of the OPA reagent. The parameters under evaluation were the concentrations of OPA (the range of 1.9–30 mmol l^{-1}), 3-MPA (the range of 5.75–184 mmol l^{-1}) and, of borate buffer (the range of 50–400 mmol l^{-1}), the number of mixing cycles in the injection loop and, the time delay after mixing of the sample with the OPA reagent.

2.4. Development of separation conditions

Chromatographic conditions were selected to allow a rapid and efficient separation of the OPA derivatives of nor-NOHA, nor-ARG, ARG and NMEA (internal standard—IS) from plasma components. A critical pair of nor-NOHA and nor-ARG derivatives was the most difficult task. The use of non-volatile buffers was avoided during the method development in order to enable mass spectrometric detection. Several chromatographic parameters were optimized including the type of organic modifier (methanol, ACN), the flow rate (0.6–0.85 ml min^{-1}), the gradient program, the column length (150 vs. 100 mm) and, the temperature of the column compartment (35–50 °C). The decision criteria included selectivity of the assay, sufficient chromatographic resolution of the critical pair (R_s > 1.5), short runtime (<15 min) and, the back-pressure <400 bar due to the limitations of the Agilent 1100 HPLC system.

2.5. Preparation of calibration standards and quality control samples

Stock standard solutions of nor-NOHA (40 and 30 mmol l^{-1}), ARG (20 and 15 mmol l^{-1}) and NMEA (1 mmol l^{-1}) were prepared by dissolving of each pure substance in 0.01 mol l^{-1} hydrochloric acid. From the standard solution 40 mmol l^{-1} were prepared auxiliary stock solutions by consecutive dilution with hydrochloric acid (0.01 mol l^{-1}) to concentrations 0.20–0.50–1.0–2.0–10–20–40 mmol l^{-1}. The calibration standards of nor-NOHA were prepared from auxiliary stock solutions by a 20-fold dilution with blank rat plasma (standards L1–L7). Calibration standards of ARG were obtained using standard additions of 5–12.5–25–50–250–500–1000 μmol l^{-1} to rat plasma containing 122.4 μmol l^{-1} of endogenous ARG (standards L1–L7). ARG endogenous concentration was calculated from linear regression equations of calibration curves (n = 7) with standard additions of ARG as mentioned above. Calibration curves were extrapolated to zero response (y = 0) and concentration of ARG was ascertained from absolute value of intercept on the concentration axis (x). Quality control samples of nor-NOHA in blank rat plasma were prepared at concentrations of 30, 300 and 1500 μmol l^{-1} (QC1, QC2 and QC3, respectively). Standard additions of 15, 150 and 750 μmol l^{-1} ARG to rat plasma with a basal concentration of 122.4 μmol l^{-1} were performed to prepare QC1, QC2 and QC3, respectively. The calibration standards and QC samples were divided to 200 μl aliquots and kept frozen at −80 °C for a maximum of two weeks.

2.6. Solid-phase extraction of analytes from rat plasma

Samples of frozen plasma were thawed at room temperature and subjected to a solid-phase extraction (SPE) on the Oasis MCX cartridges (30 mg, 1 ml, Waters, Milford, MA, USA) filled with a mixed mode sorbent (reversed phase and strong cation exchanger). The sorbent was wetted twice with 1 ml of methanol and washed twice with 1 ml of ultrapure water. Two hundred microliters of plasma was mixed with 600 μl of phosphate buffer (0.05 mol l^{-1}, pH = 7.4) and 50 μl of IS (c = 1 mmol l^{-1}) and the mixture was loaded on the SPE cartridge. The cartridge was washed once with 1 ml of hydrochloric acid (0.1 mol l^{-1}) and 1 ml of methanol. The analytes were eluted from the air-dried sorbent using 1 ml of the elution agent composed of potassium acetate (0.5 M, pH = 7.4) and methanol (40:60, v/v). The eluates were evaporated to dryness in a thermoblock (30 min, 35 °C) under a gentle stream of nitrogen and reconstituted in 200 μl of ultrapure water. After vortex mixing for 15 s and centrifugation (14,000 × g, 5 min), the samples were transferred into vials with glass inserts and placed to the autosampler for on-line derivatization and analysis. Total volume of the sample taken for on-line derivatization was 1.5 μl.

2.7. Method validation

The optimal chromatographic conditions acquired in the course of method development were applied throughout the validation process. The method was evaluated for selectivity, linearity, the lower limit of quantification (LLOQ), precision, accuracy, extraction recovery and sample stability, using recommendations of the FDA Guidance for Industry – Bioanalytical Method Validation [40]. For all tests performed during the method validation, pooled blank plasma from fourteen rats was used.

2.7.1. Selectivity

The selectivity of the HPLC method was tested by comparing the blank, drug-free rat plasma from 14 different animals to the samples of calibration standard L3 and standard fortified with nor-NOHA, ARG and NMEA (IS) to the final concentration of 5 μmol l^{-1}, prepared in ultrapure water. Peak purity test was also performed for ARG because the amino acid is a common constituent of many biological matrices and blank plasma is unavailable. This test was based on the continuous measurement of the excitation or emission spectra during a chromatographic run. For this purpose, two runs for each blank plasma (n = 14) were carried out. First, the excitation spectra were recorded over the range of 220–400 nm and second, scanning of the emission spectra was performed in the range of 360–550 nm. Evaluation of the tests was undertaken with a "*Peak purity test*" function available in ChemStation software with a threshold value set at 990 [41,42]. Mass spectrometric data across ARG peak in blank samples were also acquired for confirmation of fluorimetric peak purity measurement. Peak purity function supported by ChemStation software utilizes statistical approach for computation of the spectra similarity within a chromatographic peak. Two exactly identical spectra have similarity factor equal to 1000. Values of similarity factor higher than 995 indicate that the spectra are very similar and can be assumed as identical. Detailed description of aforementioned peak purity function is in Refs. [41,42].

2.7.2. Linearity and LLOQ

Calibration curves were constructed using a weighted ($1/y^2$) least-square linear regression of the relationship between the peak areas ratios (the analyte to IS) and the concentrations of calibration standards. Each of the seven concentration levels was analyzed seven-times. The calibration ranged from 10 to 2000 μmol l^{-1} (nor-NOHA) and from 127.4 to 1127.4 μmol l^{-1} (ARG – final concentration). LLOQ was defined as the lowest concentration determined with acceptable precision and accuracy, i.e. with the relative standard deviation (RSD) below 20% and the relative error (RE) within ±20% of the true value. For the purpose of LLOQ determination, seven parallel samples were spiked with nor-NOHA to the final concentration of 5 μmol l^{-1} and with standard addition of ARG 5 μmol l^{-1} to the plasma with ARG basal concentration 122.4 mmol l^{-1}.

2.7.3. Precision and accuracy

The intra- and inter-day accuracy and precision were assessed by conducting a replicate analysis of the QC samples at three concentration levels. Intra-day precision was measured at three concentration levels, each level in septuplicate. To determine the inter-day precision, the QC samples were analyzed over three different days at three concentration levels, each level in quintuplicate. The precision and the accuracy were expressed as the relative standard deviation (RSD) and relative error (RE), respectively.

2.7.4. Recovery

Relative recoveries (R%) of analytes were evaluated by comparing the results of replicate analyses (n = 5) of the plasma QC samples prepared at three concentration levels with those prepared by spiking the post-extracted plasma samples to the same concentrations. Recovery for NMEA (IS) was evaluated at the concentrations of 100, 200 and 400 μmol l^{-1} in the same manner as for the QC samples. Relative recovery of analytes was calculated according to the following equation [43]:

$$R\% = \frac{\text{response of analytes spiked to plasma before extraction}}{\text{response of analytes spiked to plasma after extraction}} \times 100$$

2.7.5. Stability

Long-term and short-term stability, post processing stability and freeze-thaw stability were assessed by replicate analyses (n = 5) of the low and high QC samples prepared in rat plasma. Long-term stability samples were stored at −80 °C, measured after 4 weeks and compared with freshly prepared samples. Short-term stability of the plasma at 25 °C was assessed after standing over 1 h before sample processing and analysis. Post-processing stability was measured by analyzing the low and high QC samples immediately after processing as well as after 24 h standing in the autosampler thermostated at 7 °C. The freeze–thaw stability was determined after each of three freezing–thawing cycles.

2.8. Pilot pharmacokinetic experiment

Male Wistar rats (n = 5) weighing from 310 to 410 g (Biotest, Konárovice, Czech Republic) were used in a preliminary pharmacokinetic experiment to verify the performance of the new chromatographic method. Animals were housed under controlled environmental conditions (12-h light–dark cycle, temperature 22 ± 1 °C) with a commercial food diet and water freely available. All experiments were approved in accordance with Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication, 1996) and under the supervision of the Ethical Committee of the Faculty of Medicine in Hradec Kralove, Czech Republic. A bolus dose of 10 mg kg^{-1} nor-NOHA was administered intravenously under the anesthesia induced by pentobarbital (50 mg kg^{-1}, i.p.) and samples of blood were collected into the EDTA KE Monovette tubes (Sarstedt, Nümbrecht, Germany) at various time intervals during the 2 h post-dosing. Blood samples were immediately cooled in a water bath (5 min, 10 °C) and plasma was separated by centrifugation (1000 × g, 10 min, 4 °C). Samples were stored at −80 °C until analysis.

2.9. Identification of the metabolite using mass spectrometry

In the course of preliminary pharmacokinetic experiments with nor-NOHA in rats, an unknown peak was found in the OPA-derivatized plasma extracts, with the kinetics related to that of nor-NOHA. We assumed that the peak belongs to a metabolite or degradation product of nor-NOHA emerging in vivo. Therefore, it was tested whether or not the origin of the compound is artificial, i.e. caused by nor-NOHA decomposition in vitro during sample processing. Within the framework of this testing, short-term stability was also evaluated (see Section 2.7.5). Blank rat blood was spiked with nor-NOHA and ARG to final concentrations of 301.8 and 150.5 μmol l^{-1}, respectively, and the volume was divided into five aliquots. The first aliquot was immediately processed and analyzed. From the second and third aliquots, plasma was prepared immediately and held at 25 °C and 4 °C for 1 and 2 h, before sample processing and analysis. The fourth and fifth aliquots were held at 25 °C and 4 °C for 1 and 2 h, respectively, and then plasma was separated, processed and analyzed. After this preliminary test, the compound was characterized by means of mass spectrometry. Full scan MS^1-ESI spectra and MS^n-ESI in the positive mode were recorded with the ion source and ion optics settings as follows: spray voltage 4 kV, capilary voltage 35 V, capilary temperature 250 °C, sheat gas 50 AU (arbitrary units), auxillary gas 30 AU, RF lens offset −7.5 V, lens(0) −4.5 V, multipole(0) offset −5 V, gate lens −52 V, multipole(1) offset −13.5 V, multipole RF 465 V_{p-p}, front lens −86 V, trap offset −10 V. Confirmation of the proposed structure was carried out with authentic standard of nor-ARG.

3. Results and discussion

3.1. Development of chromatographic conditions

Preliminary experiments were done using an Agilent Zorbax XDB-C18 column (150 mm × 4.6 mm, 5 μm) with optimized mobile phase consisting of acetonitrile–methanol–phosphate buffer (0.05 mol l^{-1}, pH = 6.5) in a gradient mode. Despite the fact that validation was successful, the runtime >35 min was found unsatisfactory. Such long runtime caused problems with post-processing instability of the analytes, especially during the analysis of large analytical batches. Another limitation was the phosphate buffer used in the mobile phase and impossibility to use the method in LC–MS mode for nor-NOHA metabolite identification, as this demand rose throughout the study.

During the last two years, columns with shell-type stationary phases have become popular owing to a fast and efficient separation with excellent efficiency [26–28,31]. Therefore, this new stationary phase technology was chosen in the present study also. Isocratic separation on a Kinetex C18 (150 mm × 3 mm ID, 2.6 μm) column gave disappointing results. The critical pair of OPA derivatives of nor-NOHA and nor-ARG showed insufficient resolution and NMEA (IS) eluted in the runtime exceeding 60 min. For a gradient elution, acetonitrile and methanol were tested as organic modifiers. Acetonitrile gave a low back-pressure profile but separation of the aforementioned critical pair remained problematic. Optimum chromatographic separation of all analytes and interferents was achieved with methanol. A 150 mm long Kinetex C18 column offered good separation, however with a runtime exceeding 17 min. A shorter, 100 mm column was therefore tested and was found to be sufficient for baseline separation of all OPA derivatives with the significantly shorter runtime of 13 min. Column temperature and flow rate were conformed to an acceptable value of the back-pressure and sufficient resolution of the critical pair. Kinetex C18 (100 mm × 3 mm ID, 2.6 μm) column was finally used in the present study. Parameters of the resulting method are presented in Section 2.2. Chromatographic performance data were computed by means of Agilent ChemStation software and are summarized in Table 1. Resolution was computed for consecutive peaks. Data for observed interferents are also included.

Table 1
Chromatographic performance data valid for standard unmodified Agilent 1100 series liquid chromatograph equipped with Kinetex C18 (100 mm × 3 mm ID, 2.6 μm) column.

Compound	Reduced ret. time $t_{R'}$ (min)	Peak width at half height $W_{1/2}$ (min)	Resolution R_s
Unknown peak	6.033	0.0499	–
nor-NOHA	6.345	0.0643	3.3
nor-ARG	6.522	0.0589	2.0
Unknown peak	7.053	0.0765	4.6
ARG	7.483	0.0594	4.8
NMEA (IS)	8.975	0.0599	16.2

3.2. Derivatization and injector program

OPA and 3-MPA in borate buffer (0.2 mol l^{-1}, pH = 9.5) were used for derivatization. OPA reagent composition used through the study was as follows: OPA 15 mmol l^{-1}, 3-MPA 11.5 mmol l^{-1}. Injector program settings are described in Section 2.2. 3-MPA was a preferred donor of a thiol group given the reported higher stability of the derivatives [44,45]. Data acquired during the optimization of the injector program are presented in Fig. 2. Peak areas of the analytes to IS were used for computation and results were normalized to a highest value in the dataset.

Based on the results presented in Fig. 2, an attention should be paid to OPA and 3-MPA concentrations. On the other hand, the concentration of borate buffer influences the fluorescence response of OPA derivatives only slightly. An optimum composition was as follows: 15 mmol l^{-1} OPA and 11.5 mmol l^{-1} 3-MPA in borate buffer (0.2 mol l^{-1}, pH 9.5). The count of mixing cycles during the on-line derivatization is the most important instrumental parameter to optimize while the delay time in the injector loop after mixing has only a marginal influence on the fluorescence intensity.

3.3. Solid-phase extraction of analytes from rat plasma

Solid phase extraction on the Waters MCX cartridges resulted in clean extracts and a high recovery of the analytes. The procedure recommended in the Oasis MCX cartridge brochure [46] was tested first, but alkaline ammonia solution used in the elution step completely decomposed nor-NOHA. Similar behavior was described by others for NOHA [47]. To prevent the decomposition of nor-NOHA during the sample processing, alkaline solutions (pH > 8) has to be avoided. Therefore, various elution agents were investigated each composed of methanol and organic or inorganic salt (potassium acetate, potassium phosphate, potassium chloride, ammonium chloride). The best results were achieved with a solution containing potassium acetate (c = 0.5 mol l^{-1}, pH = 7.4) and methanol (40:60, v/v). This solution was used through the study.

3.4. Results of validation

3.4.1. Selectivity

Selectivity was investigated using blank and spiked samples of rat plasma (nor-NOHA, nor-ARG and NMEA) and by means of a peak purity test and LC–MS (ARG). Baseline resolution was achieved for all analytes. Samples of blank rat plasma (n = 14) were found free of interfering peaks of endogenous compounds at the retention times of nor-NOHA, nor-ARG and NMEA (IS) (Fig. 3).

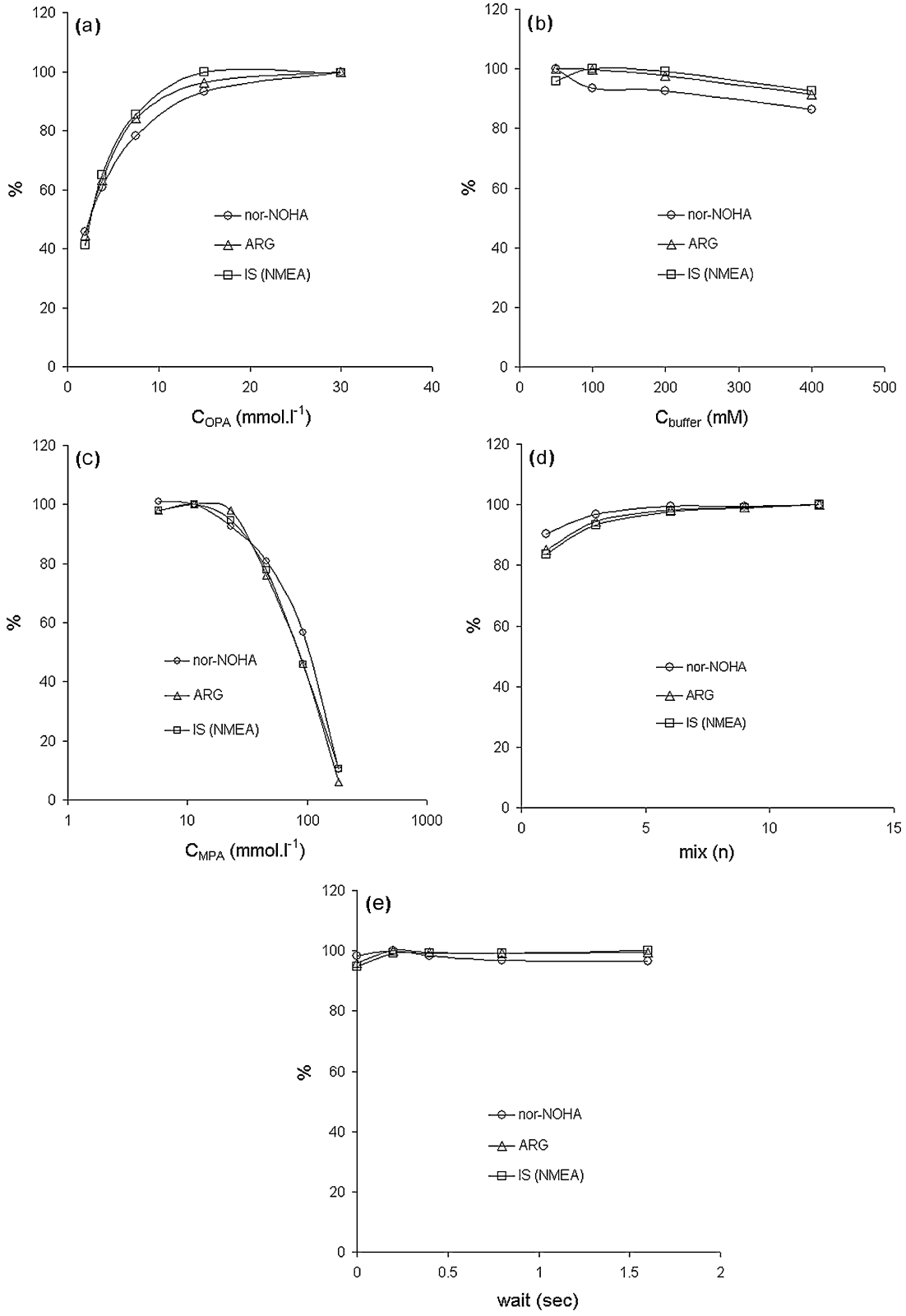

Fig. 2. Influence of derivatization parameters on the fluorescence response. Concentrations of the components of the derivatization reagent: o-phthaldialdehyde (a), borate buffer (b) and 3-mercaptopropionic acid (c). Instrumental parameters: the count of mixing cycles after aspiration of a sample and OPA reagent (d), the time interval between mixing of a sample with OPA reagent and injection (e).

The peak purity test demonstrated that similarity factors of the ARG OPA spectra of all samples of rat plasma ($n = 14$) were higher than 997.2 and 996.2 with the mean (SD) values of 998.4 (0.5129) and 999.0 (0.6111) for excitation end emission spectra, respectively. Inasmuch as the aforementioned peak purity test is not able to detect interference with exactly the same retention time and peak shape as that of ARG OPA, results of the peak purity test were also confirmed with the help of the mass spectra recorded

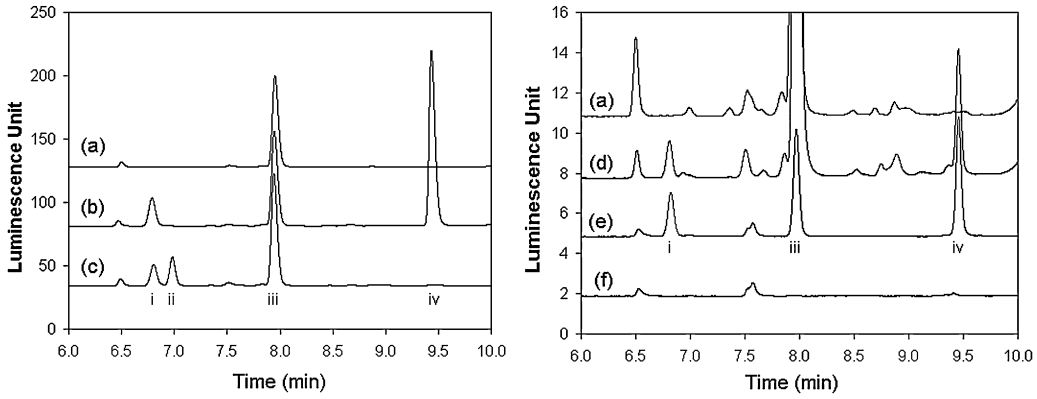

Fig. 3. Representative chromatograms of blank rat plasma (a), plasma fortified with 50 μmol l^{-1} nor-NOHA, 147 μmol l^{-1} ARG (final concentration) and 200 μmol l^{-1} NMEA (b), rat plasma withdrawn 5 min after nor-NOHA bolus (10 mg kg^{-1} *i.v.*) (c), plasma fortified with nor-NOHA and NMEA (5 μmol l^{-1} each compound) (d), water fortified with nor-NOHA, ARG and NMEA (5 μmol l^{-1} each compound) (e), water used for sample reconstitution (f). Peak identification: nor-NOHA t_R = 6.8 min (i), nor-ARG t_R = 7.0 min (ii), ARG t_R = 7.9 min (iii), NMEA t_R = 9.4 min (iv).

across the ARG OPA peak in all samples of rat plasma (n = 14). Results demonstrated the ability of the method to unequivocally assess the analytes of interest in a complex matrix of rat plasma.

3.4.2. Linearity of calibration and LLOQ

Least-squares linear regression with different weights (no weighting, $1/x$, $1/x^2$, $1/y$ and $1/y^2$) was tested. The best results were achieved using a weighted ($1/y^2$) least-squares linear regression. The calibration curve of nor-NOHA and ARG OPA derivatives were linear within the concentration ranges of 10–2000 and 5–1000 μmol l^{-1}, respectively. The mean (±SD) regression equations of calibration curves (n = 7) were: $y = 0.001047(\pm 0.00008624)x + 0.001086(\pm 0.0007810)$ ($r^2 > 0.998$) and $y = 0.002886(\pm 0.0003632)x + 0.3532(\pm 0.07832)$ ($r^2 > 0.997$) for nor-NOHA-OPA and ARG OPA, respectively.

Lower limit of quantification 5 μmol l^{-1} for nor-NOHA and ARG, respectively was confirmed with the measurement of accuracy and precision, where limits given by FDA [40] were fulfilled. The method proved to be linear over the range demanded for analysis of samples from pharmacokinetic experiment on rats (see Section 3.5) with sufficient quantification limits.

3.4.3. Precision and accuracy

The data of the intra and inter-day precision and accuracy are shown in Table 2. For all QC samples were the relative standard deviations better than 11% and assayed concentrations between 90 and 110% of the true values. Precision and accuracy of the present method met the criteria of the FDA Guidance for the Bioanalytical Method Validation [40].

Table 2
Precision, accuracy, LLOQ and recovery results of the method for nor-NOHA and ARG determination. Mean unspiked plasma (a), spike added (b), mean spiked plasma (c) and spike found (d).

Analyte	a (μmol l^{-1})	b (μmol l^{-1})	c (μmol l^{-1})	d (μmol l^{-1})	RSD (%)	RE (%)
Inter day n = 15						
nor-NOHA	–	30.18	–	31.55	7.20	4.54
nor-NOHA	–	301.8	–	302.4	10.80	0.21
nor-NOHA	–	1509	–	1644	6.00	8.98
ARG	122.4	15.05	138.1	15.73	7.71	4.52
ARG	122.4	150.5	279.3	156.9	2.21	4.25
ARG	122.4	752.3	913.3	790.9	5.70	5.13
Intra day n = 5						
nor-NOHA	–	30.18	–	29.88	5.67	−0.99
nor-NOHA	–	301.8	–	325.9	3.70	8.00
nor-NOHA	–	1509	–	1522	0.19	0.85
ARG	122.4	15.05	15.43	14.55	9.81	−3.32
ARG	122.4	150.5	136.8	135.9	5.06	−9.70
ARG	122.4	752.3	759.2	758.3	1.43	0.80
LLOQ n = 7						
nor-NOHA	–	5.03	–	5.56	12.35	10.54
ARG	122.4	5.02		5.49	15.86	9.44

Recovery n = 5	a (μmol l^{-1})	b (μmol l^{-1})	c (μmol l^{-1})	d (μmol l^{-1})	Recovery (%) (mean ± SD)
nor-NOHA	–	30.18	–	29.40	97.4 ± 3.7
nor-NOHA	–	301.8	–	309.0	102.4 ± 2.5
nor-NOHA	–	1509	–	1396	92.5 ± 4.8
ARG	122.4	15.05	136.3	13.84	91.98 ± 2.6
ARG	122.4	150.5	261.8	139.4	92.64 ± 3.4
ARG	122.4	752.3	888.6	766.1	101.84 ± 2.9
NMEA	–	103.1	–	90.62	87.90 ± 3.26
NMEA	–	206.2	–	177.8	86.24 ± 3.22
NMEA	–	412.4	–	363.0	88.02 ± 2.50

Table 3
Stability of nor-NOHA and ARG in rat plasma. Mean unspiked plasma (a), spike added (b), mean spiked plasma (c), and spike found (d).

Analyte	Matrix	Time	Temp.	a (μmol l^{-1})	b (μmol l^{-1})	c (μmol l^{-1})	d (μmol l^{-1})	RSD (%)	RE (%)
Short term stability (n = 5)									
nor-NOHA	Plasma	1 h	25 °C	–	30.18	–	29.12	4.07	−3.51
ARG	Plasma	1 h	25 °C	104.2	15.05	118.4	14.20	2.95	−5.65
nor-NOHA	–	0 h[a]	–	–	301.8	–	291.0	3.08	−3.57
nor-NOHA	Plasma	1 h	4 °C	–	301.8	–	319.9	0.54	6.01
nor-NOHA	Plasma	1 h	25 °C	–	301.8	–	304.6	2.57	0.91
nor-NOHA	Plasma	2 h	4 °C	–	301.8	–	243.2	6.81	−19.42
nor-NOHA	Plasma	2 h	25 °C	–	301.8	–	226.4	3.28	−24.99
nor-NOHA	Whole blood	1 h	4 °C	–	301.8	–	308.8	1.06	2.31
nor-NOHA	Whole blood	1 h	25 °C	–	301.8	–	307.8	1.01	1.98
nor-NOHA	Whole blood	2 h	4 °C	–	301.8	–	250.3	4.88	−17.07
nor-NOHA	Whole blood	2 h	25 °C	–	301.8	–	241.1	4.04	−20.11
ARG	–	0 h[a]	–	104.2	150.5	256.0	151.8	2.93	0.84
ARG	Plasma	1 h	4 °C	104.2	150.5	254.3	150.1	0.39	−0.30
ARG	Plasma	1 h	25 °C	104.2	150.5	251.7	147.5	0.55	−1.98
ARG	Plasma	2 h	4 °C	104.2	150.5	260.4	156.2	0.07	3.77
ARG	Plasma	2 h	25 °C	104.2	150.5	260.2	156.0	0.87	3.67
ARG	Whole blood	1 h	4 °C	104.2	150.5	249.5	145.3	1.22	−3.48
ARG	Whole blood	1 h	25 °C	104.2	150.5	248.9	144.7	0.53	−3.86
ARG	Whole blood	2 h	4 °C	104.2	150.5	257.5	153.3	0.59	1.85
ARG	Whole blood	2 h	25 °C	104.2	150.5	248.4	144.2	0.93	−4.17
Stability in the autosampler (n = 5)									
nor-NOHA		24 h	7 °C	–	30.18	–	28.23	5.47	−6.47
nor-NOHA		24 h	7 °C	–	1509	–	1517	0.55	0.53
ARG		24 h	7 °C	122.4	15.05	136.1	13.72	4.45	−8.81
ARG		24 h	7 °C	122.4	752.3	1518	765.9	0.14	1.81
Freze–thaw stability (3 cycles, −80 °C to 25 °C, n = 5)									
nor-NOHA	1 cycle	–	–	–	30.18	–	29.70	2.37	−1.59
nor-NOHA	1 cycle	–	–	–	1509	–	1440	4.14	−4.57
nor-NOHA	2 cycle	–	–	–	30.18	–	29.10	2.10	−3.58
nor-NOHA	2 cycle	–	–	–	1509	–	1254	5.85	−16.91
nor-NOHA	3 cycle	–	–	–	30.18	–	31.8	2.91	5.37
nor-NOHA	3 cycle	–	–	–	1509	–	1395	0.94	−7.55
ARG	1 cycle	–	–	122.4	15.05	137.41	15.00	1.74	−0.33
ARG	1 cycle	–	–	122.4	752.3	856.91	734.5	1.49	−2.37
ARG	2 cycle	–	–	122.4	15.05	137.91	15.50	1.41	2.99
ARG	2 cycle	–	–	122.4	752.3	837.51	715.1	0.97	−4.94
ARG	3 cycle	–	–	122.4	15.05	138.01	15.60	0.99	3.65
ARG	3 cycle	–	–	122.4	752.3	822.31	699.9	0.25	−6.97
Long term stability (n = 5)									
nor-NOHA		4 weeks	−80 °C	–	30.18	–	28.641	1.85	−5.10
nor-NOHA		4 weeks	−80 °C	–	1509	–	1460	2.95	−3.25
ARG		4 weeks	−80 °C	122.4	15.05	136.51	14.097	1.18	−6.33
ARG		4 weeks	−80 °C	122.4	752.3	805.42	683.01	3.24	−9.21

[a] Immediatelly processed sample.

3.4.4. Recovery

Extraction recoveries of nor-NOHA, ARG and NMEA from rat plasma are shown in Table 2. For all compounds under study, the extraction recoveries were high, reproducible and consistent over the tested concentration range.

3.4.5. Stability

Detailed data are presented in Table 3. The samples of rat plasma containing nor-NOHA and ARG were found to be stable at least for 30 days in a freezer at −80 °C. The stability of nor-NOHA in the plasma at higher temperatures is limited: the concentration decreases of 25% (at 25 °C) and 19% (at 4 °C) occurred after 2 h of standing. In whole blood, similar decreases of nor-NOHA concentration were observed after 2 h: 20% at 25 °C and 17% at 4 °C. Throughout the study samples of whole blood and plasma were, therefore, processed within 1 h of thawing or collection. Freeze–thaw stability was also lower for nor-NOHA. A significant concentration decrease was found after the second freeze–thaw cycle (Table 3). Post-processing stability in the autosampler was sufficient for at least 24 h if the samples were held at 7 °C (Table 3), giving the possibility to analyze large batches (up to 110 samples per day).

Interesting phenomenon of nor-NOHA instability was observed after repeated injections of the same sample from a single vial. A continuous decrease in the concentration down to 83% of the initial value was detected after 20 injections. This phenomenon was not observed if the same sample was injected once from each of 20 different vials. It was assumed that the effect was caused by nor-NOHA decomposition or adsorption with the metal of the injector needle. In order to verify this assumption, the aliquots of the same sample (QC1) were transferred into two vials and serial injections (n = 20) were carried out from the first vial and, afterwards, from the second one. Interestingly, two overlapping curves starting at 100% of the initial nor-NOHA concentration were obtained. Thus, prolonged contact of nor-NOHA-containing samples with metal surfaces has to be avoided. This kind of behavior was not observed for ARG and NMEA (IS).

3.5. Application of the method to a pilot pharmacokinetic experiment

The validated HPLC method was found to be suitable for nor-NOHA quantification in the samples of rat plasma collected from Wistar rats (n = 5) after a single dose *i.v.* administration of 10 mg kg^{-1} nor-NOHA. The plasma profiles of nor-NOHA observed are shown in Fig. 4a. As mentioned in Section 2.9, the peak of an unknown compound, which was later identified as nor-ARG, was found in the OPA-derivatized extracts of the plasma collected

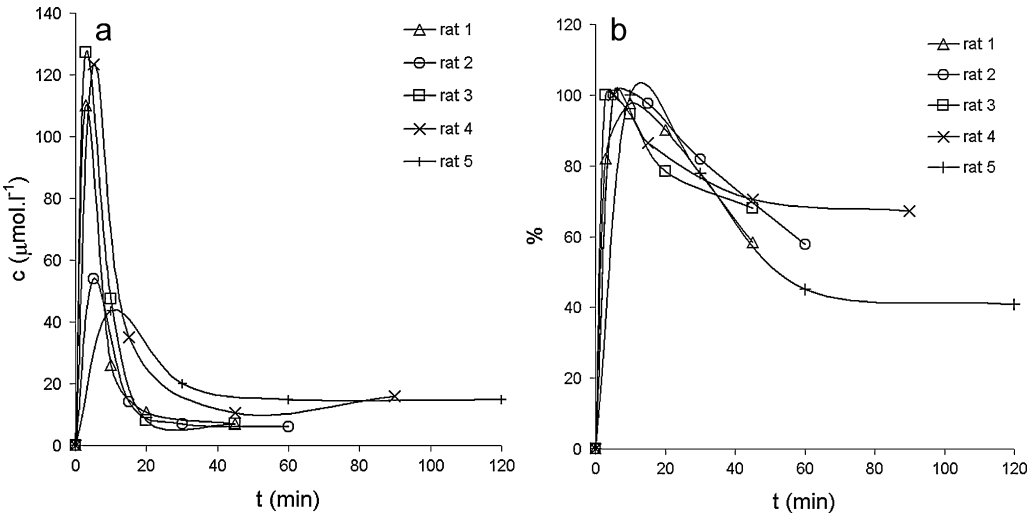

Fig. 4. Mean plasma concentration–time profiles of nor-NOHA (a) and nor-ARG in relative units (b) after an *i.v.* bolus of 10 mg kg^{-1} nor-NOHA to five Wistar rats.

during the pharmacokinetic experiment. As the authentic standard of nor-ARG of quality suitable for precise weighting was not available, the relative concentrations of nor-ARG are expressed in percent of the maximum observed concentration set to 100% (Fig. 4b). Plasmatic concentrations of the ARG were in the range of 81–226 µmol l^{-1}.

3.6. LC–MS identification of the unknown metabolite

All measured chromatograms of the plasma and blood extracts spiked with nor-NOHA and ARG were inspected for the peak of the metabolite at t_R = 7.0 min. This peak was not found in any chromatogram providing the evidence that the compound is not

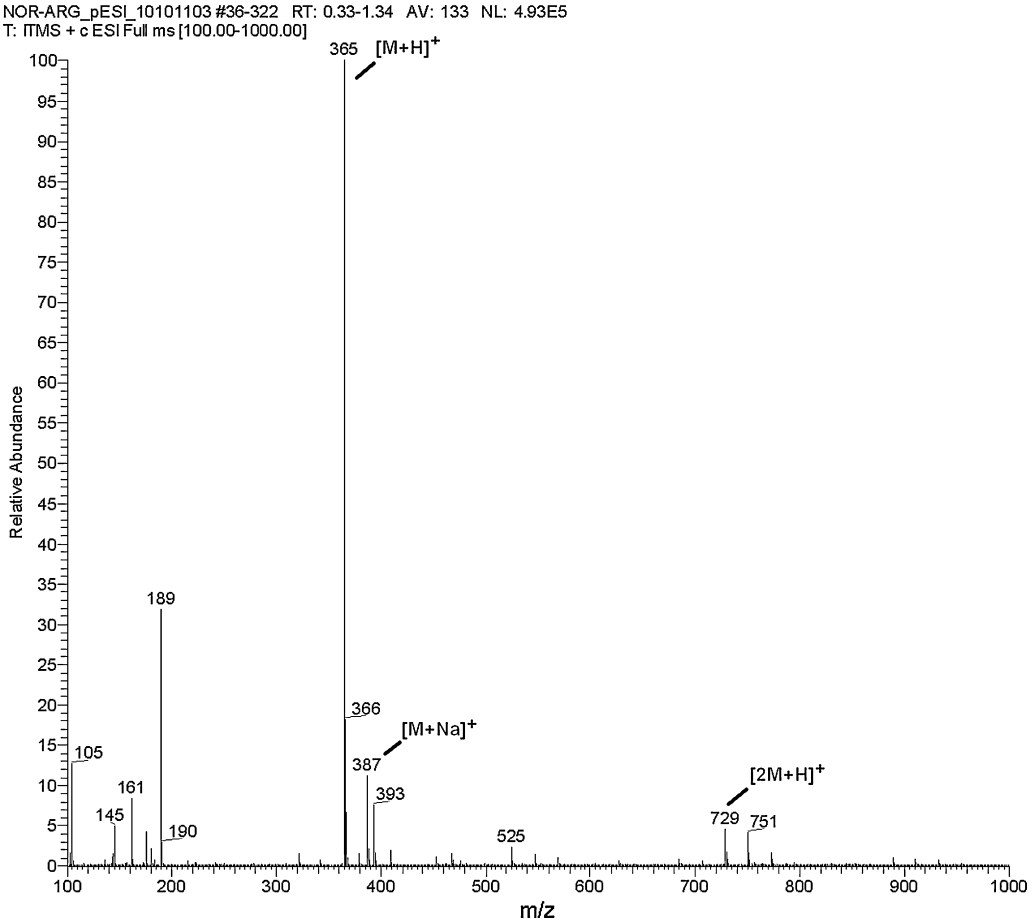

Fig. 5. Full scan MS spectra acquired during a continual infusion of *N*-(ω)-hydroxy-nor-L-arginine (1 mmol l^{-1}) to the mobile phase (A:B, 70:30, v/v). Parent mass $[M+H]^+$ at *m*/*z* 365, sodium adduct $[M+Na]^+$ at *m*/*z* 387 and dimer $[2M+H]^+$ at *m*/*z* 729. Peak at *m*/*z* 189 belongs to background contamination.

Table 4
Mass spectrometric data and retention times for an unknown metabolite and nor-ARG authentic standard. Rat plasma after 10 mg kg^{-1} nor-NOHA *i.v.* bolus (a), nor-ARG authentic standard prepared in blank rat plasma (b), a continual infusion of 8 μl min^{-1} nor-ARG (1 mmol l^{-1}) to the flow of the mobile phase consisting of water (pH = 3.00 adjusted with HCOOH) and methanol (70:30, v/v) (c).

	Compound	t_R[a] (min)	$[M+H]^+$ (m/z)	MS^2 (m/z)	MS^3 (m/z)	MS^4 (m/z)
(a)	Unknown	7.15	365	[365 → 321]	[365 → 259 → 217]	–
				[365 → 259]	–	–
				[365 → 215]	–	–
(b)	nor-L-Arginine	7.12	365	[365 → 321]	[365 → 259 → 217]	–
				[365 → 259]	–	–
				[365 → 215]	–	–
(c)	nor-L-Arginine	–	365	[365 → 321]	[365 → 321 → 215]	[365 → 259 → 217 → 160]
				[365 → 259]	[365 → 259 → 217]	[365 → 215 → 173 → 145]
				[365 → 215]	[365 → 215 → 173]	–

[a] Valid for Agilent 1200 Series liquid chromatograph connected to Thermo LCQ Fleet mass detector.

emerging artificially, i.e. during sample processing. Instead, its formation occurred in vivo, after nor-NOHA administration to rats. For the purpose of metabolite identification, LC–MS analysis of the biological sample was carried out in the ESI positive mode, with detector settings according to Section 2.9. MS^1 spectra were measured during chromatographic separations over the m/z ranges of 100–1000. For this purpose, plasma was prepared and processed from whole blood withdrawn at 5 min after *i.v.* administration of 10 mg kg^{-1} nor-NOHA. A full scan TIC profile was inspected for the peaks of nor-NOHA, the metabolite and ARG OPA derivatives. Expected masses derived from theoretical structure were found: nor-NOHA-OPA (m/z 381) and ARG OPA (m/z 379). Mass of the unknown metabolite was found at m/z 365. In the second stage, MS^n from the parent mass m/z 365 were carried out during the chromatography. The MS^2 product ion mass spectra showed predominant peaks at m/z 321, 259 and most intensive peak at m/z 215. MS^3 product ion spectra from the mass m/z 215 showed only one peak at m/z 173 (Table 4). Based on the measured mass of the metabolite-OPA derivative, its fragmentation behavior and, the well-known reaction mechanism of the derivatization reaction [33,36], we propose that the unknown compound matches nor-L-arginine (Fig. 1b). As the next step, an authentic standard of nor-ARG was used to spike rat plasma. After sample processing and derivatization with OPA, the derivative was characterized by its retention time under the given chromatographic conditions and by MS and MS^n data (Table 4). Parent mass $[M+H]^+$ at m/z 365 with sodium adduct $[M+Na]^+$ at m/z 387 and dimer $[2M+H]^+$ at m/z 729 were observed.

Mass spectra from MS^1 (Fig. 5) to the MS^4 were measured during an infusion of the nor-ARG OPA derivative (1 mmol l^{-1} in ultrapure water) at a volume rate of 8 μl min^{-1} to the mobile phase flowing at a rate of 0.85 ml min^{-1} with the following composition: 70:30, solvent A:solvent B (v/v). Detector settings are listed in Section 2.9. Measured data presented in Table 4 clearly demonstrate that the unknown compound found in rat plasma after nor-NOHA administration is nor-L-arginine.

4. Conclusion

In this study, the development and validation of a chromatographic method for the determination of arginase inhibitor nor-NOHA in rat plasma was described for the first time. The method enables simultaneous quantification of ARG. Effective baseline separation of the analytes was achieved. Other advantages of the method include short runtime and compatibility with both fluorimetric and mass-spectrometric detectors. On-line derivatization with OPA performed in the autosampler as a part of the injection sequence was beneficial for two reasons: a negative influence of post-processing instability of the OPA derivatives was avoided and a less laborious sample preparation was achieved. The method was also used in the LC–MS mode for determination of a putative metabolite of nor-NOHA, identified as nor-ARG. A room for improvement is seen in the use of small 4 μl detection flow cell instead of standard 8 μl cell for even better resolution and also downscaling of the sample volume, inasmuch as the injected volume is less than 1% of the sample volume available after SPE extraction.

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References

[1] H. Maarsingh, T. Pera, H. Meurs, Naunyn Schmiedebergs Arch. Pharmacol. 378 (2008) 171.
[2] C.R. Morris, S.M. Morris Jr., W. Hagar, J. Van Warmerdam, S. Claster, D. Kepka-Lenhart, L. Machado, F.A. Kuypers, E.P. Vichinsky, Am. J. Respir. Crit. Care Med. 168 (2003) 63.
[3] T.W. Hein, C. Zhang, W. Wang, C.I. Chang, N. Thengchaisri, L. Kuo, FASEB J. 17 (2003) 2328.
[4] M.J. Romero, D.H. Platt, H.E. Tawfik, M. Labazi, A.B. El-Remessy, M. Bartoli, R.B. Caldwell, R.W. Caldwell, Circ. Res. 102 (2008) 95.
[5] S. Ryoo, G. Gupta, A. Benjo, H.K. Lim, A. Camara, G. Sikka, J. Sohi, L. Santhanam, K. Soucy, E. Tuday, E. Baraban, M. Ilies, G. Gerstenblith, D. Nyhan, A. Shoukas, D.W. Christianson, N.J. Alp, H.C. Champion, D. Huso, D.E. Berkowitz, Circ. Res. 102 (2008) 923.
[6] T. Bagnost, A. Berthelot, M. Bouhaddi, P. Laurant, C. Andre, Y. Guillaume, C. Demougeot, J. Hypertens. 26 (2008) 1110.
[7] H. Maarsingh, A.B. Zuidhof, I.S. Bos, M. van Duin, J.L. Boucher, J. Zaagsma, H. Meurs, Am. J. Respir. Crit. Care Med. 178 (2008) 565.
[8] H. Maarsingh, B.G. Dekkers, A.B. Zuidhof, I.S. Bos, M.H. Menzen, T. Klein, G. Flik, J. Zaagsma, H. Meurs, Eur. Respir. J. 38 (2011) 318.
[9] N. Zimmermann, N.E. King, J. Laporte, M. Yang, A. Mishra, S.M. Pope, E.E. Muntel, D.P. Witte, A.A. Pegg, P.S. Foster, Q. Hamid, M.E. Rothenberg, J. Clin. Invest. 111 (2003) 1863.
[10] C.R. Morris, M. Poljakovic, L. Lavrisha, L. Machado, F.A. Kuypers, S.M. Morris, Am. J. Respir. Crit. Care 170 (2004) 148.
[11] A. Lara, S.B. Khatri, Z. Wang, S.A.A. Comhair, W. Xu, R.A. Dweik, M. Bodine, B.S. Levison, J. Hammel, E. Bleecker, W. Busse, W.J. Calhoun, M. Castro, K.F. Chung, D. Curran-Everett, B. Gaston, E. Israel, N. Jarjour, W. Moore, S.P. Peters, W.G. Teague, S. Wenzel, S.L. Hazen, S.C. Erzurum, N.H.L.B.I. Sever, Am. J. Respir. Crit. Care 178 (2008) 673.
[12] J.P. Tenu, M. Lepoivre, C. Moali, M. Brollo, D. Mansuy, J.L. Boucher, Nitric Oxide-Biol. Ch. 3 (1999) 427.
[13] G. Jeyabalan, J.R. Klune, A. Nakao, N. Martik, G. Wu, A. Tsung, D.A. Geller, Nitric Oxide-Biol. Ch. 19 (2008) 29.
[14] K.M. Reid, A. Tsung, T. Kaizu, G. Jeyabalan, A. Ikeda, L. Shao, G. Wu, N. Murase, D.A. Geller, Am. J. Physiol. Gastrointest. Liver Physiol. 292 (2007) G512.
[15] P. Beranova, K. Chalupsky, A.L. Kleschyov, C. Schott, J.L. Boucher, D. Mansuy, T. Munzel, B. Muller, J.C. Stoclet, Eur. J. Pharmacol. 516 (2005) 260.
[16] P. Markowski, I. Baranowska, J. Baranowski, Anal. Chim. Acta 605 (2007) 205.
[17] H.M. Mao, W. Wei, W.J. Xiong, Y. Lu, B.G. Chen, Z. Liu, Clin. Biochem. 43 (2010) 1141.
[18] W.Z. Zhang, D.M. Kaye, Anal. Biochem. 326 (2004) 87.
[19] C.E. Jones, C.J. Darcy, T. Woodberry, N.M. Anstey, Y.R. McNeil, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 878 (2010) 8.
[20] E.G. Karageorgou, V.F. Samanidou, J. Sep. Sci. 34 (2011) 1893.
[21] P. Marhol, R. Gazak, P. Bednar, V. Kren, J. Sep. Sci. 34 (2011) 2206.

[22] V. Jimenez, R. Companyo, J. Guiteras, Talanta 85 (2011) 596.
[23] V.F. Samanidou, E.G. Karageorgou, Drug Test Anal. 3 (2011) 234.
[24] C.F. Clavijo, K.L. Hoffman, J.J. Thomas, B. Carvalho, L.F. Chu, D.R. Drover, G.B. Hammer, U. Christians, J.L. Galinkin, Anal. Bioanal. Chem. 400 (2011) 715.
[25] L.J. Cai, J. Zhang, X.M. Wang, R.H. Zhu, J. Yang, Q.Z. Zhang, W.X. Peng, Biomed. Chromatogr. 25 (2011) 1308.
[26] F. Gritti, C.A. Sanchez, T. Farkas, G. Guiochon, J. Chromatogr. A 1217 (2010) 3000.
[27] F. Gritti, I. Leonardis, D. Shock, P. Stevenson, A. Shalliker, G. Guiochon, J. Chromatogr. A 1217 (2010) 1589.
[28] F. Gritti, G. Guiochon, J. Chromatogr. A 1217 (2010) 1604.
[29] A. Fanigliulo, D. Cabooter, G. Bellazzi, D. Tramarin, B. Allieri, A. Rottigni, G. Desmet, J. Sep. Sci. 33 (2010) 3655.
[30] S. Fekete, K. Ganzler, J. Fekete, J. Pharm. Biomed. Anal. 54 (2011) 482.
[31] S. Fekete, J. Fekete, Talanta 84 (2011) 416.
[32] J.O. Omamogho, J.D. Glennon, Anal. Chem. 83 (2011) 1547.
[33] M. Roth, Anal. Chem. 43 (1971) 880.
[34] I. Molnar-Perl, I. Bozor, J. Chromatogr. A 798 (1998) 37.
[35] D. Kutlan, P. Presits, I. Molnar-Perl, J. Chromatogr. A 949 (2002) 235.
[36] Y. Mengerink, D. Kutlan, F. Toth, A. Csampai, I. Molnar-Perl, J. Chromatogr. A 949 (2002) 99.
[37] I. Molnar-Perl, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 879 (2011) 1241.
[38] I. Molnar-Perl, J. Chromatogr. A 913 (2001) 283.
[39] T. Teerlink, R.J. Nijveldt, S. de Jong, P.A. van Leeuwen, Anal. Biochem. 303 (2002) 131.
[40] Guidance for Industry, Bioanalytical Method Validation, Centre for Drug Evaluation and Research (CDER), 2001.
[41] Agilent Technologies Inc., <http://www.chem.agilent.com/Library/usermanuals/Public/G2180-90024_spectra_ebook.pdf> (accessed 09/2011).
[42] Agilent Technologies, Inc., <http://www.chem.agilent.com/Library/applications/5988-8647EN.pdf> (accessed 09/2011).
[43] S. Bansal, A. DeStefano, AAPS J. 9 (2007) E109.
[44] T. Teerlink, P.A. van Leeuwen, A. Houdijk, Clin. Chem. 40 (1994) 245.
[45] H. Godel, T. Graser, P. Foldi, P. Pfaender, P. Furst, J. Chromatogr. 297 (1984) 49.
[46] Waters Corporation, <http://www.younglin.com/brochure_pdf/waters/MCXbro98.pdf> (accessed 09/2011).
[47] P. Vetrovsky, J.C. Stoclet, G. Entlicher, Int. J. Biochem. Cell Biol. 28 (1996) 1311.