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Short Communication

SIMULTANEOUS DETERMINATION OF NITRITE AND NITRATE BY ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY IN RAT PLASMA

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ABSTRACT

Objective: To develop an ultra- performance liquid chromatography (Acquity™ UPLC) method for simple, fast, accurate and simultaneous determination of nitrite and nitrate ions as stable forms of nitric oxide (NO) production in rat plasma.

Methods: The ion-pairing Acquity™ UPLC method with reverse phase BEH C18 column was used along with photodiode array (PDA) UV-Vis detector to separate and detect nitrite and nitrate ions at an absorbance of 212 and 208 nm, respectively. Due to small particle size (1.7 µm) of the BEHC18 column, a narrow peak was achieved with an improved sensitivity and separation at the cost of pressure. The anions were analyzed in ultra-filtered deproteinized rat plasma sample (10 kDa, cut-off).

Results: The UPLC bioanalytical method was linear (r=0.9998, 0.9988) over wide ranges of 4-500 µM (nitrite) and 6-400 µM (nitrate) concentration. The most important advantages of the present method are; short time run time, no sample pre-treatment (unlike enzymatic reaction and pre-column treatment), low sample volume and simultaneous estimation of nitrite and nitrate in rat plasma.

Conclusion: It was concluded that the developed UPLC method for determination of simultaneous nitrite and nitrate in rat plasma was rapid, sensitive, accurate and linear over the wide ranges of concentration. This method can be used for simultaneous determination of nitrite and nitrate in rat plasma with various advantages over the reported methods.

Keywords: UPLC, PDA, Nitrite, Nitrate, NO

Nitric oxide (NO), a free radical and a signaling molecule, plays an important role in the regulation of vascular tone, neurotransmission, host immunity, nutrient metabolism and whole-body homeostasis. The biochemistry of NO and its redox-activated forms have been reviewed in the past. In aqueous phase, free of biological material, NO is exclusively and quantitatively converted to nitrite, which is rapidly oxidized to nitrate. However, because of its short half-life 10-30 seconds in the biological system, the direct measurement of NO is extremely difficult, particularly in the complex physiological milieu [1-3].

Typically, these ions have been determined by the Griess assay, in which nitrite is diazotized with sulfanilamide and then reacted with *N*-1-naphthyl-ethylenediamine to form a colored product. It is, thus necessary to reduce the nitrate to nitrite either chemically by a reduction of cadmium or by enzymatic reduction steps. This method has few disadvantages as it requires a long and demanding pre-treatment of the sample, which makes it quite expensive and time-consuming. Apart from this, the sample volume requirement is very high and it is challenging to get the large samples volume from small animals viz mice and rats. The Griess assay is not a direct method for estimation of nitrate and using this assay the samples need to be first estimated for nitrite followed by conversion of nitrate in the samples to nitrite and again estimation using the deproteinized samples. This process will double the efforts, samples requirement and need to fit the samples in the limited range of calibration curve provided in the kit either by dilution of the samples or by preliminary identifying the range finding of the nitrite and nitrate levels followed by individual sample analysis, which is very complex, time consuming and laborious[4]. Hence, for this reason, various analytical techniques have been developed to determine nitrite and nitrate in biological samples. Some automated and batch methods include the fluorometry, flow or sequential injection analysis with visible absorbance, chemiluminescence and electrochemical detection (ECD). Separation based method includes gas chromatography-mass spectrometry (GC-MS), capillary electro-phoresis (CE), and high-performance liquid chromatography (HPLC) with a variety of detection system that is also used in the batch methods. Even after a huge list of the available methods, none of the methods is easy, completely fulfilling the requirement and associated with a lot of limitations viz time-consuming, long run time, sample pre-treatment and more efforts with low sensitivity [5-7].

Thus considering the limitations of the various existing methods described, the present study was performed with the aim and objective to develop a simple, rapid, accurate, low cost and simultaneous measurement of nitrite and nitrate in rat plasma samples using UPLC equipped with a PDA UV-Vis detector. Till recently there were no reports or methods available predominantly with rapid, simple, accurate, short run time for the quantification of nitrite and nitrate rat plasma samples.

Multiscreen® filter plates with Ultracel®-10 membranes (Millipore Corporation, Ireland) were used for sample plasma purification. A solution of 40 weight % tetrabutylammonium hydroxide in methanol (Acro Organics, USA) was used as the anion-pairing agent for the UPLC separation of nitrite and nitrate. Potassium dihydrogen orthophosphate was procured from Qualigens Fine Chemicals, India. LiChrosolv® Methanol for chromatography was sourced from Merck, India. Analytical grade sodium nitrite and potassium nitrate were purchased from Thomas Baker, India. Mobile phases were filtered through 0.2 µm Ultipor® N_{66}® nylon 6, 6, the membrane filter (Pall Life Sciences, India). Nitrite and nitrate stock solutions were freshly prepared in Milli-Q® water to obtain the required concentrations.

A blank plasma sample from male sprague-dawley rats (200-220g) was procured from animal house facility, Piramal Life Sciences Limited, India. Plasma samples were deproteinized by passing through Multiscreen® filter plates with Ultracel®-10 membranes. The filtrate was collected and diluted with Milli-Q® water in a ratio of 1:5. The location of nitrite and nitrate peaks was determined from known standard solution in Milli-Q®water and was confirmed by spiking in rat plasma.

All analysis were performed on Acquity™ UPLC system (Waters, USA), including the binary solvent manager, the sample manager, column compartment and photo-diode array (PDA) detector, connected with Waters Empower Pro software. Further, an Acquity UPLC® BEH C18 column (1.7 µm, 2.1 x 50 mm) was used. The column temperature was maintained at 30±2 °C and auto-sampler at 10±2 °C. The standard and sample were separated using a gradient method with the following mobile phase composition: mobile phase A consisted of tetrabutylammonium hydroxide (12 mM), potassium dihydrogen phosphate (pH 7.0; 10 mM). Mobile phase B consisted of tetrabutylammonium hydroxide (2.8 mM), methanol (30% v/v), potassium dihydrogen phosphate (pH 5.5; 100 mM). The gradient elution was a stepwise linear method of 5% A for 1.10 min, followed by an increase to 95% A in 1.30 min, then maintained till 95% A in 2.60 min, and kept at 5% A at 3.25 min, and then equilibrated till 4.00 min. The mobile phases were filtered through 0.2 µm Ultipor® and degassed. The chromatographic column was conditioned for four min with the gradient flow of 0.1 ml/min and sample injection volume of 1.5 µl. Detection of nitrite and nitrate was carried out by maximum absorbance at 212 and 208 nm respectively using PDA UV-Vis detector.

In preparing the mobile phase, several combinations of an ion-pairing reagent and organic modifiers have been tried. It was found that the above-mentioned combination of mobile phase A and B produced good peaks of nitrite and nitrate with well-defined separation and devoid of plasma interferences. Fig. 1 shows chromatogram of nitrite and nitrate in rat plasma (spiked) following subtraction of the endogenous or intrinsic nitrite and nitrate present in blank plasma and fig. 2 depict chromatogram of standard nitrite and nitrate in the Milli-Q® water with maximum absorbance at 212 and 208 nm respectively.

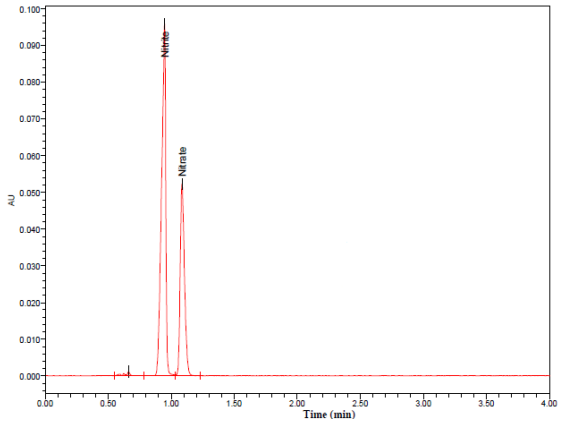

Fig. 1: Chromatogram of nitrite and nitrate in rat plasma

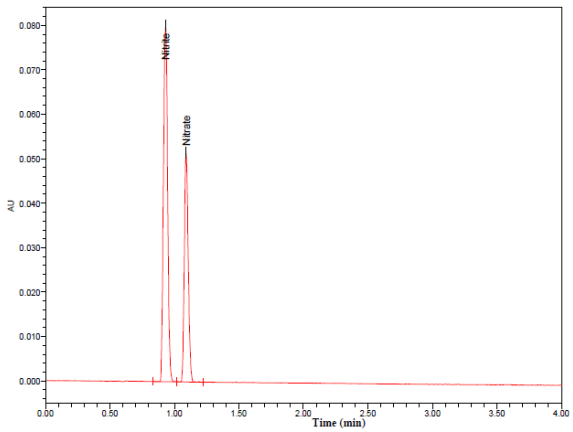

Fig. 2: Chromatogram of nitrite and nitrate in Milli-Q® water

This method was an improved method compared to the previously reported methods by Romitelli *et al.*, Jedlickova *et al.* and Griess assay**.** The linearity of this method was assessed using aqueous standards. Calibration curves for both nitrite (4-500 µM) and nitrate (6-400 µM) were found linear over the range. The linearity was expressed by the following equation: (a) nitrite y = 5648.5x-62972 (r^2 = 0.9998), (b) nitrate y = 7278.8x-87835 (r^2 = 0.9988) (y = peak area, x = concentration of corresponding anion). The mean values of retention time±SD for nitrite and nitrate determined in standard mixtures were: (a) nitrite 0.933±0.01 min (RSD = 0.1072%) (b) nitrate 1.091±0.01 min (RSD = 0.0916%). The recovery of nitrite and nitrate was 97.57±4.55 and 94.41±6.75 while the lower limit of quantification (LLOQ) was 4 µM and 6 µM respectively. The criteria for LLOQ was based on the analytical response at LOQ must be five times the baseline noise.

The described method used a different technique for determination of nitrite and nitrate ions; the differences between the present method, Griess assay [7], Romitelli *et al.* [5] and Jedlickova *et al.* [6] methods are: in Griess assay, direct estimation of nitrate is not possible until and unless it is reduced to nitrite. An additional pilot study for dilution finding to fit the sample in the selected linearity range of 20-100 µM is required [7]. Hence, large sample volume of more than 160 µl ultra-filtered plasma is indispensable. HPLC method reported by Romitelli *et al.* used a run time of around or more than 30 min with an additional 20 min column washing repeated after each sample analysis, and an injection volume of 200 µl which is quite high and for that large sample volume is required which is difficult to harvest and is time-consuming especially in small laboratory animals viz., rat and mice. Lastly Jedlickova *et al.* described the method using two different detectors PDA UV-Vis and electrochemical. This required two different runs where time and sample requirement is very high.

Hence to surmount the limitations of the above methods, an improved method was established with the minimum injection volume of 1.5 µl using Acquity™ UPLC with single PDA detector for simultaneous quantification of nitrite and nitrate in rat plasma.

The UPLC-MS/MS bioanalytical method for simultaneous determination of nitrite and nitrate ion was developed in rat plasma. The method was rapid, accurate, linear and sensitive enough to detect low concentration of 4 µM (nitrite) and 6 µM (nitrate) with simultaneous quantification in rat plasma sample. It overcomes the limitations of the reported methods of Jedlickova *et al.*, Romitelli *et al.* and Griess assay. The merits of the present method are.

(a) Short analysis time (4 min)

(b) Low sample size i. e. injection volume of 1.5 µl

(c) Low cost as flow rate is 0.1 ml/min

(d) Simultaneous estimation of nitrite and nitrate

(e) Wide linearity range, hence no dilution finding study is required

(f) No sample pre-treatment unlike derivatization or pre-column treatment or enzymatic reaction

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CONFLICTS OF INTERESTS

The authors declare no conflict of interest.

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