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(RESEARCH ARTICLE)

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An improved and sensitive method for nitrotyrosine estimation by reverse phase-high performance liquid chromatography

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Abstract

Reactive nitrogen species such as peroxynitrite anion ($ONOO^-$), nitrogen oxide radical (NO.) may lead to nitrotyrosine formation by oxidative nitration of tyrosine. Nitrotyrosine is considered as an important marker to estimate the severity of biological oxidative damage. Therefore, in the present study, we evaluated a reverse phase-high performance liquid chromatography (RP-HPLC) method to improve the sensitivity and specificity of the nitrotyrosine estimation. The mobile phase used in the present study was methanol (pH 4.7) and 0.1% phosphoric acid (60:40%). The standard serial dilutions were injected onto the HPLC column (C-18) at a flow rate of 1 ml/min mobile phase with a time window of 6 min. The results were monitored on the Diode Array detector at wavelength 210 nm. The evaluation and quantification of output signals were made on the Chromelion software version 6.80 which controls the whole liquid chromatography system. The same standard serial dilutions were monitored spectrophotometrically at 210nm. We found the standard curves were linear over the concentration range of 0.3 to 20 ng/ml for both RP-HPLC with UV detector ($r^2 = 0.997$) and spectrophotometric ($r^2 = 0.999$) methods. In spectrophotometric method, only four standard points were detected out of seven and represented on a straight line, this shows the spectrophotometric method was unable to measure less than 2.5 ng/ml concentration. On the other hand, in RP-HPLC method, all the seven standards were lying on a straight line and HPLC machine can detect up to 1ng/ml or 1pg/µl. The present study established the highly specific, sensitive, accurate and cost-effective RP-HPLC with UV detector method for nitrotyrosine estimation.

Keywords: Nitrotyrosine; RP-HPLC; Chromelion software; Sensitive; Specific

1. Introduction

Oxidative nitration of tyrosine is mediated by reactive nitrogen species such as peroxynitrite anion ($ONOO^-$), nitrogen monoxide radical (NO.) and nitrogen dioxide, which leads to nitrotyrosine formation [1].Nitrogen monoxide is a gaseous ubiquitous free radical cellular messenger, involved in various physiological and pathological processes [2]. In the last few years, the biological activity of NO has been actively investigated. Usually in a diseased condition, oxidative stress increases the production of superoxide (O^{2-}) and NO forming peroxynitrite anion, a destructive free radical oxidant [3, 4]. Peroxynitrite anion radical production is responsible for oxidizing several lipoproteins and nitrating tyrosine residues in many proteins, thereby compromising the integrity and function of the protein [5]. Peroxynitrite undergoes reaction with carbon dioxide, protonation, isomerization and decomposition at physiological pH to give toxic products that deplete antioxidants and oxidize and nitrate, lipids, proteins and DNA [6 - 8]. This reactivity towards cellular constituents may be the basis by which overproduction of NO could exert cytotoxic effects [9]. The detailed mechanisms how peroxynitrite and other reactive nitrogen species derived from it responsible for modification of biomolecules are

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still incompletely understood. Although there are multiple targets for the reactive peroxynitrite, the presence of 3-nitrotyrosine, whether free or protein bound, has been proposed as a marker of peroxynitrite formation.

Nitrotyrosine is detected in biological fluids like plasma, lung aspirants-BALF (Broncho alveolar lining fluid) and urine. Elevated level of nitrotyrosine is detected in rheumatoid arthritis [4], septic shock [10] and coeliac disease [11]. In all these studies, nitrotyrosine was found to be undetected in healthy subjects. Nitrotyrosine is also found in various other disease-affected tissues, such as the cornea in keratoconus [12]. Peroxynitrite and/or nitrative stress could participate in the pathogenesis of diabetes [13]

Nitrotyrosine has been known in biological samples using numerous detection techniques. Polyclonal and monoclonal antibodies raised against peroxynitrite-treated proteins have been used in various studies to spot nitrotyrosine in tissue sections. Beckman et al have reported an immunohistochemical technique, using specific antibodies to 3-nitrotyrosine, to detect and localize nitrated proteins in several diseases and pathological conditions, however, very few methods have been reported for quantifying trace amounts of 3-nitrotyrosine in physiological fluids or protein hydrolysates [14 -17]. Shigenaga et al. used an indirect procedure in which 3-nitrotyrosine is converted to N-acetyl-3-aminotyrosine which is then assayed using HPLC with electrochemical detection [18]. Several methods are commonly employed for the determination of protein in biological samples [16–19]. Unfortunately, many compounds that are constituents of biological buffers interfere with these methods, limiting their application.

Therefore, in the present study, we have introduced a new analytical method in which reverse phase-high performance liquid chromatography (RP-HPLC) with UV detector was used to boost the sensitivity and specificity of the nitrotyrosine estimation. The separation of a complex of other interfering compounds by RP- HPLC technique has led to a reduction in observed nitrotyrosine levels in various biological fluids. The present study was designed to make nitrotyrosine estimation easy to use, sensitive, rapid and economical at laboratory scale.

2. Material and methods

2.1. Reagents

Chemicals and solvents used throughout this study such as methanol and phosphoric acid were of high quality and HPLC grade, purchased from Sigma Aldrich (St. Louis, MO, USA). We obtained nitrotyrosine standard from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Standard preparation

A stock solution of 5mg/ml nitrotyrosine standard was prepared in 0.1N HCL. Working standard solutions (0.3 – 20 ng/ml) were prepared by diluting the stock solution in water.

2.3. HPLC analysis

The HPLC system used throughout this study consisted of a quaternary pump (Ultimate 3000, Thermo Fischer Scientific, USA), a manual sample injector with a 20µl sample loop, a degasser and photodiode array detector (Thermo Fischer Scientific, USA). The output signals were evaluated and quantified by Chromelion software version 6.80 which controls the whole liquid chromatography system. The column used was a reversed phase, Acclaim™ 300, C18 column (150 x 4.6 mm i.d., 3µm particle size, 300 Å diameter), procured from Sigma Aldrich (St. Louis, MO, USA).. The mobile phase, which was used in the present study was methanol (pH 4.7) and 0.1% phosphoric acid (60:40%). The samples were passed at a flow rate of 1 ml/min mobile phase with a time window of 6 min. The results were monitored on UV-Vis detector at 210 nm wavelength.

2.4. Spectrophotometric estimation

All the serial dilutions of nitrotyrosine standards were measured by multimode reader, Spectramax,M2e (Molecular Devices, Sunnyvale, CA, USA) at absorbance 210 nm.

2.5. Nitrotyrosine extraction/Recovery

To 50µl matrix spiked with varying standard concentrations (0.625, 1.25 and 2.5 ng/ml), add 250µl 4N HCL and then incubate at 70 °C for 10 min for protein precipitation. After then place at room temperature for 10 min and centrifuge at 2500 rpm for 5 min. remove the supernatant and add 300µl distilled water, followed by filtration through 0.2µ syringe filter and then used for nitrotyrosine estimation and recovery by HPLC system.

2.6. Linearity

A stock solution of 5 mg/ml nitrotyrosine standard was prepared and then solutions of different concentrations (0.3, 0.625, 1.25, 2.5, 5, 10, 20 ng/ml) for construction of calibration plots were prepared from this stock solution. The mobile phase was filtered through a 0.45µ membrane filter and passed through column at 1 ml/min for column equilibration; the baseline was monitored continuously during this process. Detection was carried out at HPLC-UV detector at 210 nm. The prepared dilutions were injected in sequence, peak area was calculated for all the dilution, and concentration was plotted against peak area. The same procedure was also carried out spectrophotometrically at 210nm, to find out the difference in sensitivity between both the methods. In the later spectrophotometric method, absorbance was calculated for each dilution, and calibration curve was plotted between concentration and absorbance.

2.7. Precision

Precision was determined as both repeatability and intermediate precision, in accordance with the International Council for Harmonisation (ICH) recommendations.

2.8. Limit of Quantification (LOQ) and Limit of Detection (LOD)

LOQ and LOD were calculated by the standard deviation (σ) method. LOQ and LOD were determined from the slope, S, of the calibration plot, Sy/x, by use of the formulae

$$LOQ = 10 \times \sigma/S. \text{ and}$$

$$LOD = 3 \times \sigma/S$$

σ = Standard deviation, S = Slope

2.9. Efficiency

Efficiency was calculated by using the following formula;

$$N = 16 (tR/Wb)2$$

(N = Efficiency, tR = Retention time, Wb= Peak Width)

3. Results and discussion

Peroxynitrite formation is being increasingly proposed as a contributor to reactive nitrogen species generation in various human diseases [10-14]. The evidence presented for peroxynitrite participation usually includes the demonstration of increased levels of nitrotyrosine under those conditions. Indeed, this has been often the only evidence presented the assumption that nitrotyrosine estimation is one of the good prognostic biomarker of peroxynitrite formation [20-22]. Nitrotyrosine level may be considered as an important marker for evaluation of nitric oxide under in vivo conditions. It also reflects the level of free radicals present in the clinical sample. It is one of the stable product because of its longer half life, also we have confirmed by measuring nitrotyrosine level after about a week by both the methods viz: RP-HPLC and spectrophotometric method, and we found no significant difference in the levels. In the present study, we have established an RP-HPLC-UV method with greater precision, accuracy and validation according to ICH guidelines. This method is simple, specific, reliable and cost-effective. One of the best and important outcome of the present study, we have not used any kind of buffer in the mobile phase as a sometimes buffer (salts) creates overestimation of the particular analyte, which will create an error in estimation.

The standards having concentrations 0.3 to 20 ng/ml nitrotyrosine were prepared. Each of these standard solutions was injected thrice onto the HPLC column and the peak area was calculated using Chromelion software. Chromatograms of all the seven standards of nitrotyrosine were shown in Figure 1. Also absorbance of all the seven standards was taken by multimode reader. Calibration curve was prepared by plotting peak area (y) versus nitrotyrosine standard concentration (x) for RP-HPLC with UV detector method and OD_{210nm} (y) versus nitrotyrosine standard concentration (x) for spectrophotometric method (Figure 2). We found an excellent relationship between peak area and nitrotyrosine standard concentration ($r^2 = 0.997$) and between OD_{210nm} versus nitrotyrosine standard concentration ($r^2 = 0.999$). Both the methods were accurate while the limit of detection and limit of quantitation was excellent for RP-HPLC method. In spectrophotometric method, only four standard points were detected out of seven and represented on a straight line, this shows spectrophotometric method limitations, unable to measure less than 2.5 ng/ml concentration. In case of RP-

HPLC method, all the seven standards were lying on a straight line and HPLC machine can detect upto 1ng/ml or 1pg/µl. As we know nitrotyrosine is very less produced in the living system, its quantification by spectrophotometric method is challenging. Hence, in the present study, we establish simple and sensitive RP-HPLC method for nitrotyrosine quantification. We have also checked the precision and repeatability of the RP-HPLC method of intra and inter day variations, by injecting nitrotyrosine standards in triplicates and found no significant difference. We have calculated the efficiency of the method as mentioned in the methodology section, which is approximate 9000 (N), this value was confirmed by HPLC Chromelion software, which generates number of theoretical plates, confirms the efficiency of the method. One more interesting thing we have extrapolated in the present study, we confirm whether the peak at retention time 3.5 was of nitrotyrosine only or a mixture of tyrosine and nitrotyrosine. We have prepared the mixture of nitrotyrosine and tyrosine standards, and injected onto the column, we observed two sharp peaks at different retention times. This reaffirms that peak came at retention time 3.5 was of pure nitrotyrosine (Figure 3). We have also standardized nitrotyrosine estimation by spiking standard on to the matrix and observed more than 90% recovery (Figure 4).

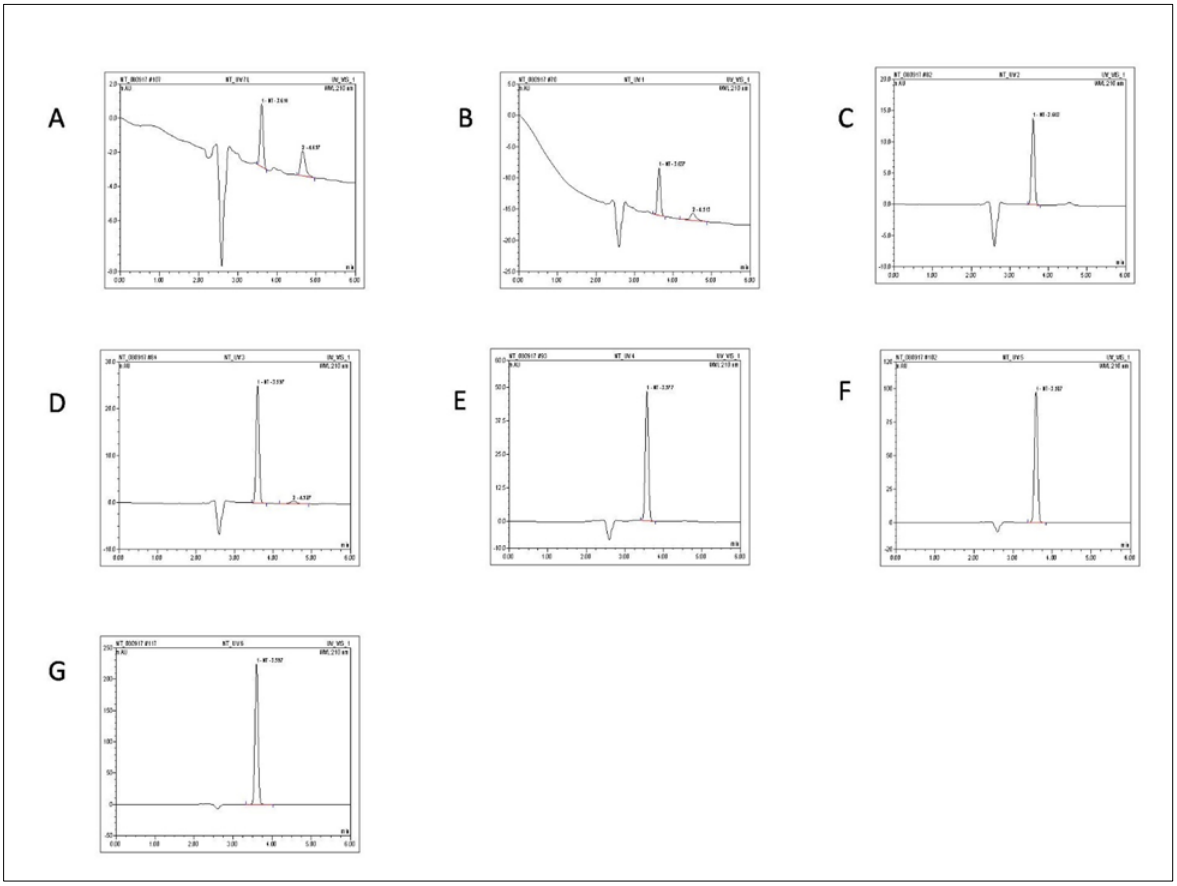

Figure 1 Chromatograms of all the seven standards of nitrotyrosine concentration (0.3 – 20ng/ml)

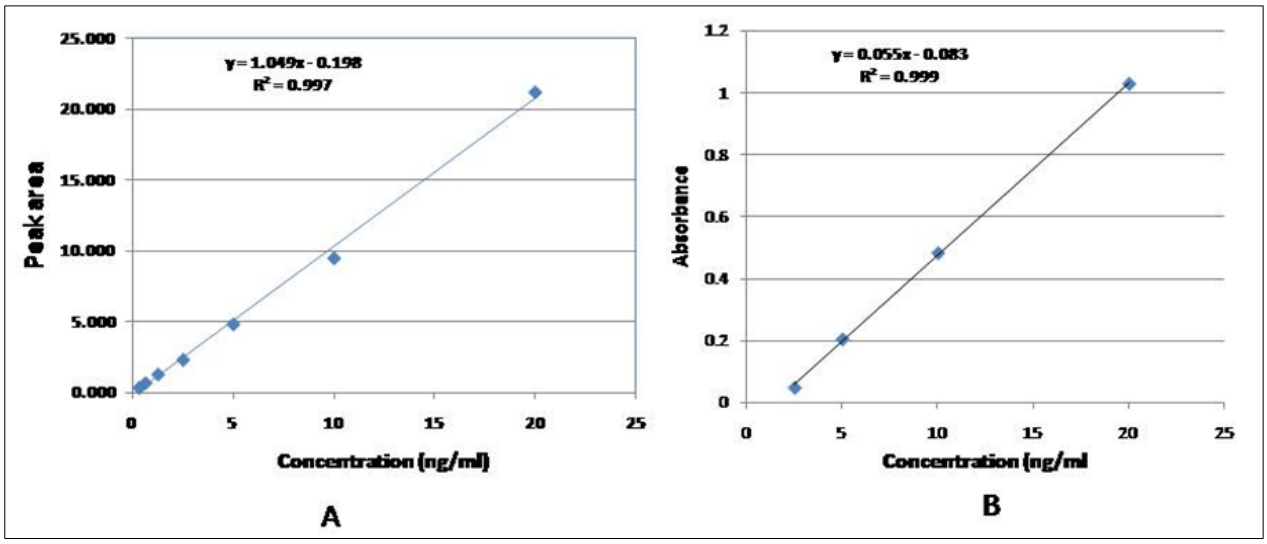

Figure 2(A) Calibration curve for RP-HPLC method by plotting peak area (y) versus nitrotyrosine standard concentration (x) (B) Calibration curve for Spectrophotometric method by plotting absorbance OD_{210nm} (y) versus nitrotyrosine standard concentration (x)

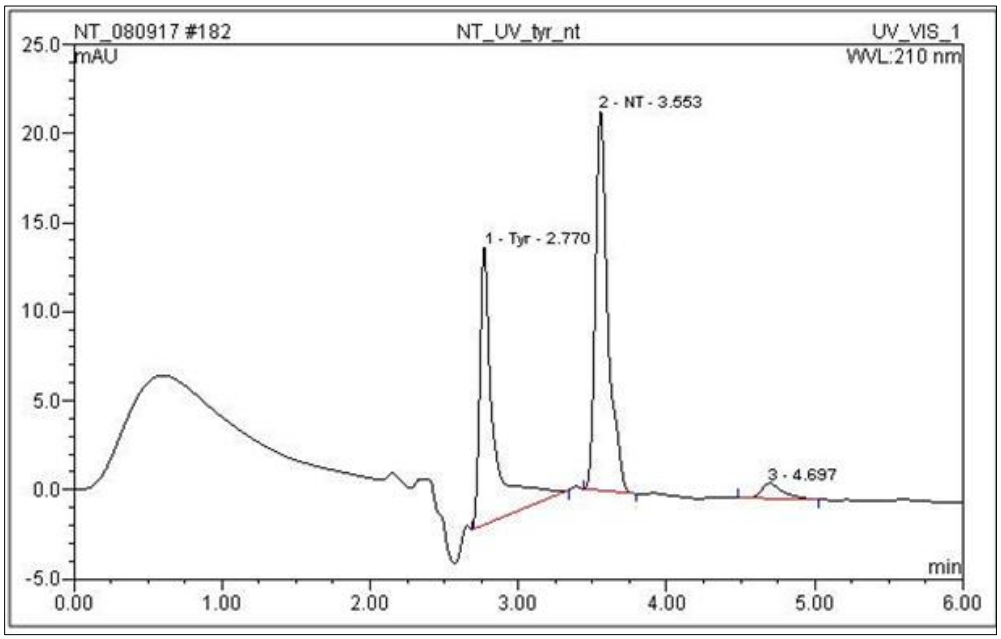

Figure 3 Chromatogram of mixture of tyrosine and nitrotyrosine standard

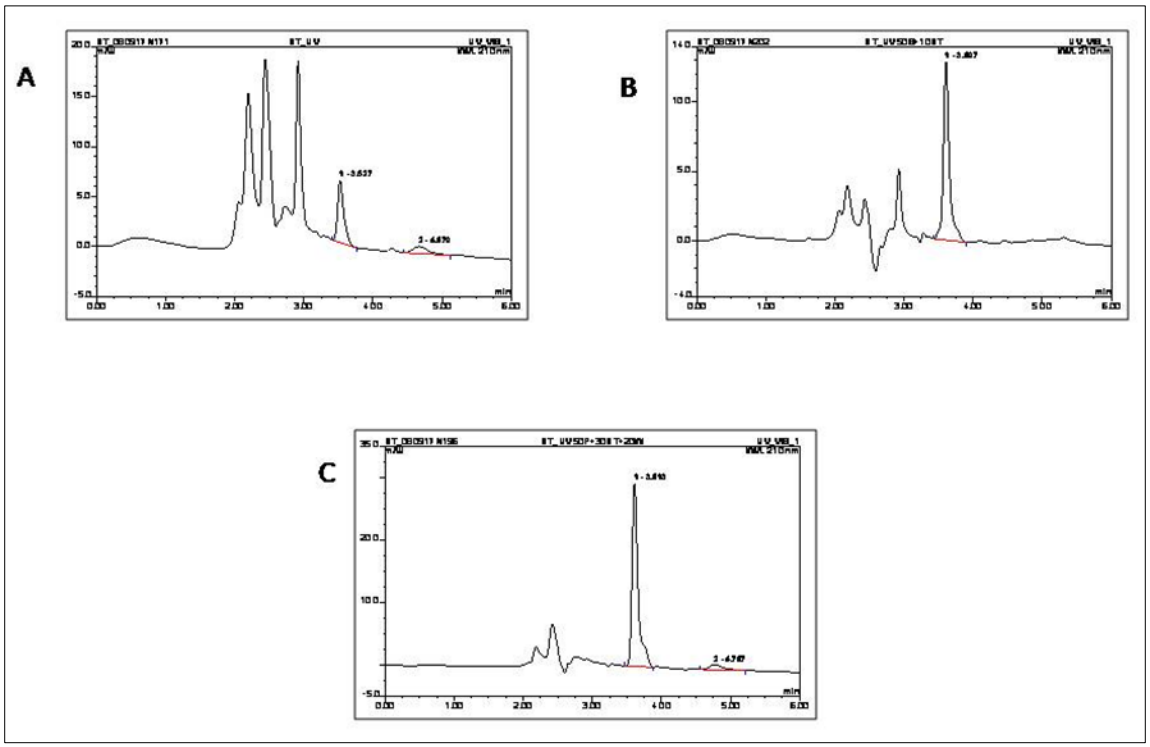

Figure 4 Chromatograms of nitrotyrosine estimation from matrix by spiking (a) 0.625 ng/ml(b) 1.25 ng/ml(c) 2.5 ng/ml

4. Conclusion

The present study established the highly specific, sensitive, accurate and cost-effective RP-HPLC with UV detector method for nitrotyrosine estimation. This method makes it possible to estimate the severity of biological oxidative damage by measuring the level of nitrotyrosine.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

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