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SPECTROPHOTOMETRIC ESTIMATION OF NITRO TYROSINE BY AZO - COUPLING REACTION

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ABSTRACT

3-Nitrotyrosine has been identified using techniques like immunohistochemistry, HPLC, GCMS to demonstrate as a relevant biomarker in various disorders such as neurological, cardiovascular and others. Estimation of 3-NT by spectrophotometric method has been performed to measure 3-NT over decades. However none of the above methods have been translated to diagnose the diseases, but pathological conditions. Therefore here is an attempt to develop a method to be employed as a diagnostic tool. Current work is focussed on including preparation of 3-NT and further to derivatives using β-Napthol to yield diazotised product. All these intermediates of diazotized products have been characterized by ESI MS and the final colored dye was measured spectrophotometrically which exhibits λ max at 551 nm. The dye has been measured and compared with the direct measurement of 3-NT. The sensitivity of measurement of 3-NT increases by 10 fold, by and large we can estimate 20 µM concentration of 3-NT.

KEYWORDS: **Nitration; peroxynitrite; 3-Nitrotyrosine; azo group; β-Napthol.**

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INTRODUCTION

Nitration is a covalent modification of tyrosine residue free or protein bounded, where a nitro group is attached to one of the ortho position of the aromatic carbon with respect to hydroxyl group[1]. Peroxynitrite a potent nitrating agent brings about the process of nitration. It is generated from the rapid reaction of nitric oxide with superoxide anion radicals (O_2^-)[2]. Nitration of tyrosine residues within a protein leads[3] to the alteration of structure and function of a protein[4]. Many researchers have proposed 3-Nitrotyrosine (3-NT) as a biomarker for various diseases such as neurological disorders[5,6], CVD's[7], inflammation[8]. Peroxynitrite mediated nitration has been a topic of interest from past two decades as there is ubiquitous presence of 3-NT in many pathological conditions. Even though the number of tyrosine residues susceptible for nitration is very less (1-5 in 10,000 tyrosine residues)[9], the effect is found to be detrimental. Hence nitration in biological condition is considered a low-yield phenomenon. Therefore, the measurement of the compound is potentially important in understanding the nitration mechanism underlying many pathological conditions. 3-NT has been identified in diverse biological samples using different detection techniques. Immunohistochemical techniques like rising polyclonal and monoclonal antibodies against peroxynitrite-treated proteins have been used in numerous studies to identify 3-NT in tissue sections**.** Immunohistochemistry with these antibodies facilitates a powerful means of detection of 3-NT[10] within tissue but so far precise quantification has not been accomplished. Other techniques like HPLC[11] GCMS[12] have been employed and well exploited according to the demand. However, the lack of expertise and economical incumbrance of the tool made this approach hard to manage. In the present study, we have explicated a simple azo (-N≡N-) coupling reaction and its spectrophotometric estimation, which could be easily adopted in any clinical laboratory without compromising sensitivity. The laboratory prepared 3-NT was reduced to amino group (3-aminotyrosine) and further accorded to form an azo bond with sodium nitrite. The azo group coupled with β-Napthol forms a red colored complex. This complex is spectrophotometrically active, which could be measured at 551 nm. The quantification of the colored complex is the measure of nitro tyrosine present in the given sample which is much sensitive compare to the direct measurement of nitro tyrosine at 430 nm[13].

MATERIALS AND METHODS

Isoamyl nitrite was purchased from Sigma-Aldrich, Standard 3-NT from Alfa Aesar, β-mercaptoethanol and sodium dithionite from Merck. All the other chemicals purchased were of the highest grade available. Deionized double distilled water was used for rinsing of glassware and preparation of reagents. All spectral and absorbance measurements were done on Ocean Optics scanning Spectrophotometer (USB 4000) with 10 mm matched quartz cells.

Synthesis and purification of Peroxynitrite

Peroxynitrite was synthesized as per the previous procedure[14]. In brief, 50 mL of 30 % H_2O_2 (v/v) mixed with 40 mL of 5 N NaOH and 5 mL of 0.04 M Diethylene-triamine-penta-acetic acid (DTPA) was dissolved in 0.05 M NaOH by gentle mixing and made up to 100 mL further the contents were mixed with 27 mL of isoamyl nitrite and stirred continuously for 8 hrs at room temperature. Then it was washed five times with two volumes of dichloromethane to remove unreacted isoamyl nitrite and isoamyl alcohol. The resulted yellow colored aqueous phase was passed through MnO_2 column to purify peroxynitrite and remove unreacted hydrogen peroxide. Peroxynitrite was measured spectroscopically at 302 nm (ϵ = 1679 M^{-1} cm^{-1}) to determine the concentration. It was stored at -20˚ C till further use.

Preparation of 3-NT

3-NT was prepared by vortexing 3 µL of peroxynitrite (0.19 M) and 5 mL of L-tyrosine

(0.5 mM). The laboratory prepared 3-NT was compared with standard 3-NT by measuring at 430 nm [13].

HPLC detection of Laboratory prepared 3-NT

Analysis of 3-NT was achieved using an HPLC system consisted of an Agilent series 1100 equipped with a 5µ C-18 column (100mm x 2.1mm) with a guard column. An isocratic eluent of water (pH 3.1) with 10% methanol (v/v) was used at a flow rate of 1 mL/min and an Ultraviolet Detector was set at 272 nm. The injection volume was 20 µL.

Azo formation and Coupling

1 mL of 1 mM 3-NT was reacted with 100 µL of 0.25 M sodium dithionite and kept for incubation for 10 minutes in ice cold condition. 20 µL of 2 M sodium nitrite in 1 mL of 1 N HCl was slowly added to the above mixture under stirring for 15 minutes at cold condition. 50 µL of 250 mM β-napthol dissolved in 0.5 N NaOH is added slowly with constant stirring. The solution turns cherry red in color. A spectral scan was performed using UV-Vis spectrophotometer and absorption maxima were recorded.

IR studies of aminotyrosine

FT-IR spectra were measured at room temperature using a Perkin-Elmer Spectrum GX FT-IR equipped with a high-purity dried potassium bromide (KBr) beam splitter at scan range of 4000 to 400 cm^{-1}.

Mass spectral studies for daizotised product

ESI- MS was carried out using Thermo LCQ Deca XP MAX MS detector with Electro Spray Ionization (ESI). The ESI probe voltage was 4.5 kV. The nebulizing gas was Helium at 1 mL/min approx. Helium in the mass analyzer cavity was maintained at 0.1Pa (10^{-3}).

RESULTS AND DISCUSSION

Preparation of 3-NT and UV spectral analysis

Standard tyrosine was treated with peroxynitrite and result was analyzed with UV-Vis Spectrophotometer. 3-NT was measured at 430 nm [12] (Fig. 1). Comparison was made with standard 3-NT and synthesis of 3-NT was confirmed.

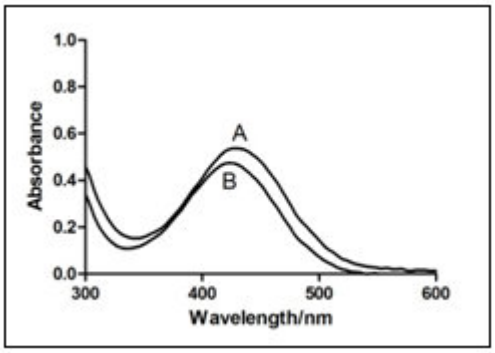


Figure 1

Absorbance spectrum of standard and laboratory prepared 3-NT: Tyrosine (0.5 mM) was nitrated with peroxynitrite (0.19 M) (A). A spectral scan was performed with UV-Vis Spectrophotometer. Comparison was made with that of commercially available 3-NT (B). Both the spectrum followed a similar trend with an absorption maxima observed at 430 nm.

HPLC analysis

The purity of standard 3-NT and laboratory prepared 3-NT was achieved by HPLC. 3-NT was eluted at 2.8 minutes for both standard and laboratory prepared 3-NT by isocratic separation method [10 % Methanol and water (v/v)] (Fig. 2).

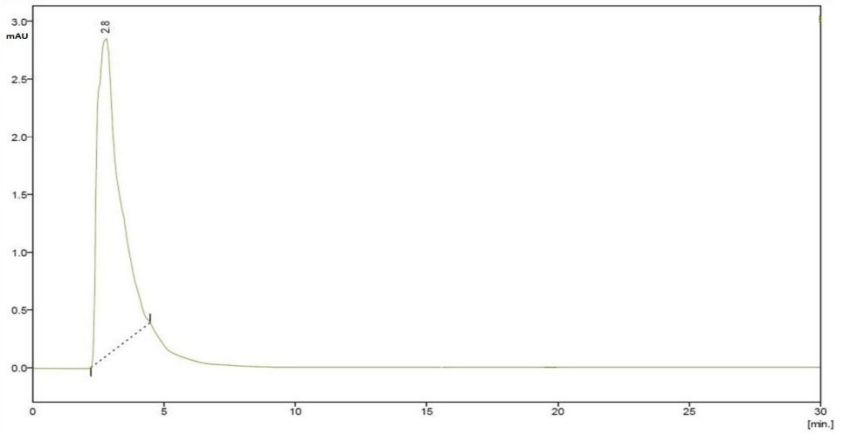


Figure 2a

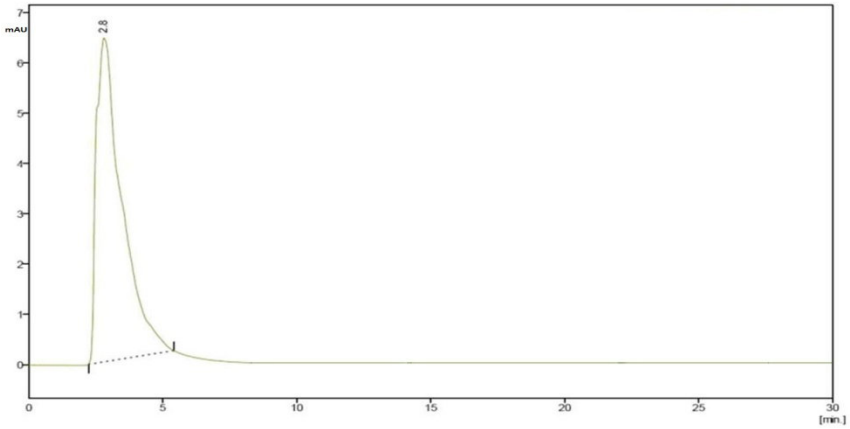


Figure 2b

Figure 2
HPLC Chromatograms of 3-NT. Standard 3-NT (2a) and laboratory prepared 3-NT (2b) in methanol (10%):water system at pH 3.1

ESI- MS

The ESI-MS spectra of Laboratory prepared 3- NT was found to be $[M]^+$ 226.9 (Fig. 3) which was well matched with the calculated mass $[M]^+$ 226.1.

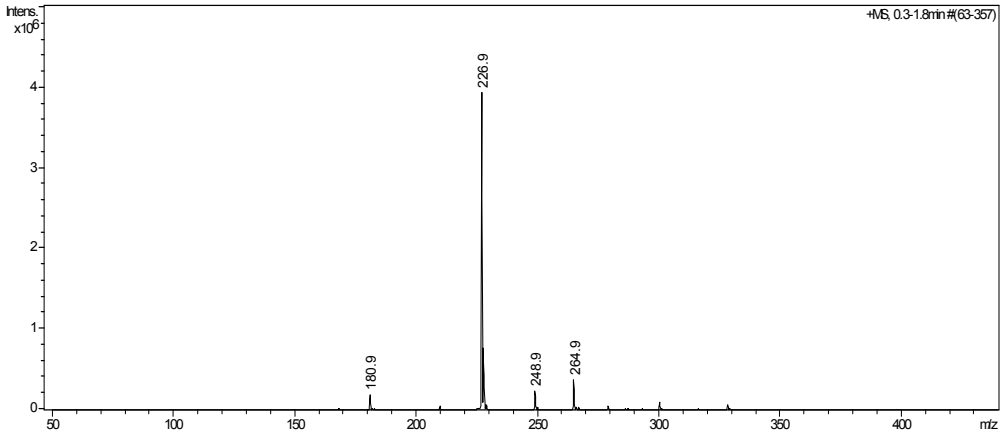


Figure 3
ESI MS of laboratory prepared 3-NT. $[M]^+$ of 3-NT was found to be 226.9, $[M+23]^+$ 248.9 and $[M+39]^+$ 264.9

Diazotisation

3-NT was reduced to 3-aminotyrosine with sodium dithionite [15]. The disappearance of peak from 430 nm indicates complete reduction of 3-NT (Fig. 4). Addition of sodium nitrite in acidic condition forms the diazonium ion (-N≡N). The diazonium salt produced is coupled with a coupling agent β-Napthol [16]. Coupling reaction was carried out to develop a prominent colour, after it reacts with nitrosonium ion to give colored dye which is measured at a wavelength of 551 nm (fig. 5). Though the absorbtion maxima are at different wavelength the absorbance of this compound is almost ten times increased than that of the absorbance of 3-NT. Thus as the sensitivity for 3-NT is increased upon derivatization, the method could be employed in determining the concentration of 3-NT even at lower concentration.

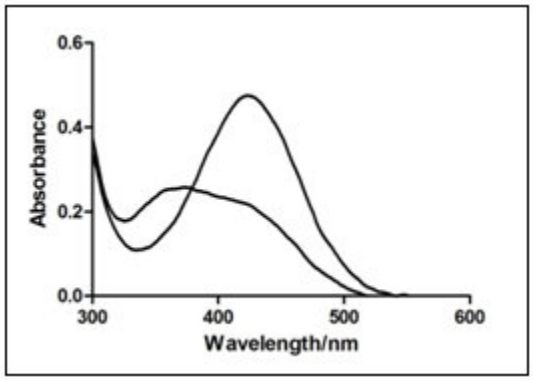


Figure 4
Overlay absorbance spectrum of 3-NT and 3-aminotyrosine: The λ max of 3-NT was observed at 430 nm. After the reduction to 3-aminotyrosine λ max at 430 nm was unseen.

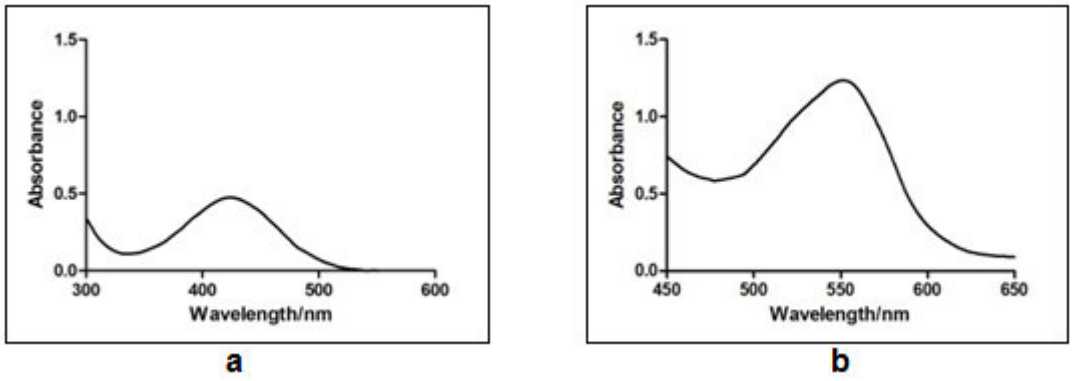


Figure 5
Absorbance spectra of a: 3-NT and b: diazotised product of 3-NT. The λ max of 3-NT is 430 nm and that of diazotised product 551 nm.

FT-IR studies for aryl amino tyrosine

Salts of primary amines show strong, broad absorption between 3400-3330 and 3320-3250 cm^{-1} arising from asymmetrical and symmetrical stretching in the NH_3^+ group, where as aryl amines exhibits slightly higher stretching frequencies than alkylamine, hence the 3452.7 cm^{-1} (Fig. 6) [17].

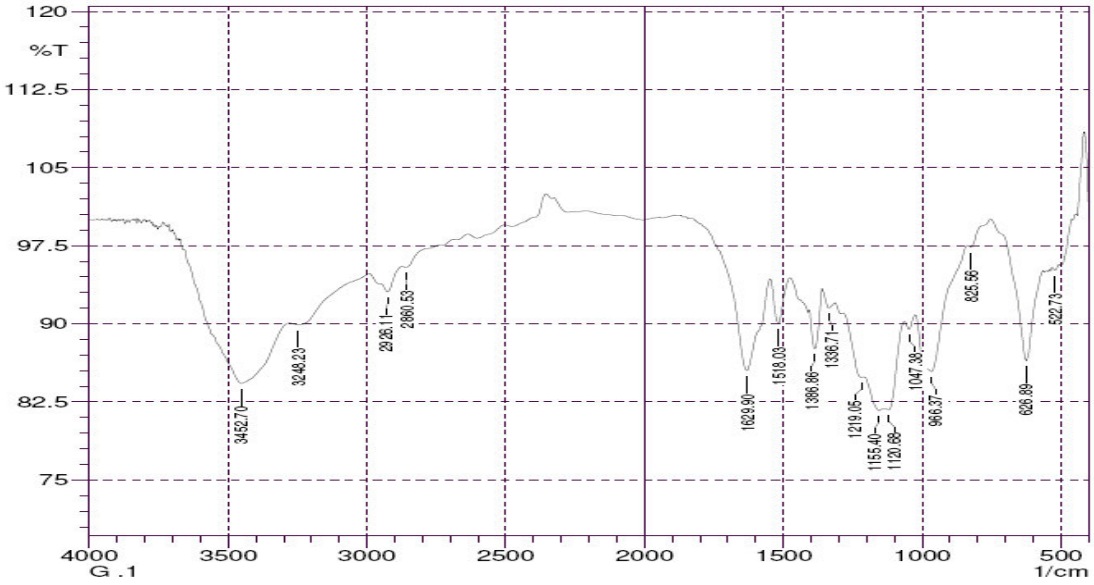


Figure 6
FTIR spectra of 3-aminotyrosine

ESI MS for diazotized product

The ESI-MS spectrum obtained for diazotized product is $[M+H]^+$ is 352.4 (Fig. 7) as the calculated mass $[M]^+$ 351.3. It provides information on the products of diazotization reaction as well as aminotyrosine $[M+23]^+$ 219.4, 3-NT $[M+23]^+$ 249.3, tyrosine $[M+23]^+$ 204.4 etc. Based on the existing ESI MS data and also previous knowledge of diazotization reaction [16] with primary arylamines, plausible reaction pathway has been constructed (Scheme I).

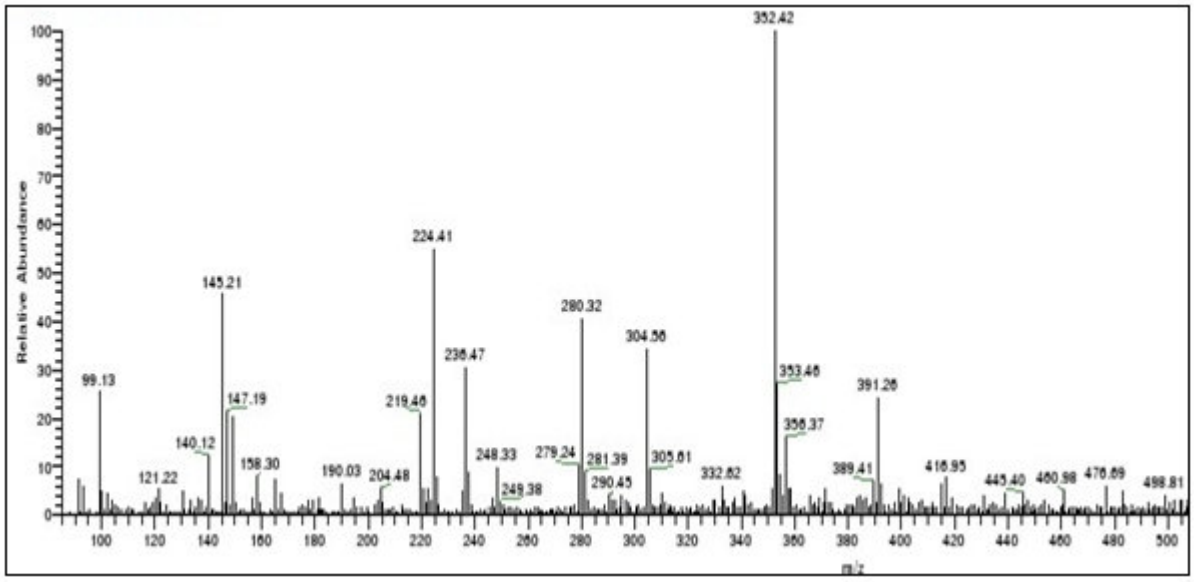
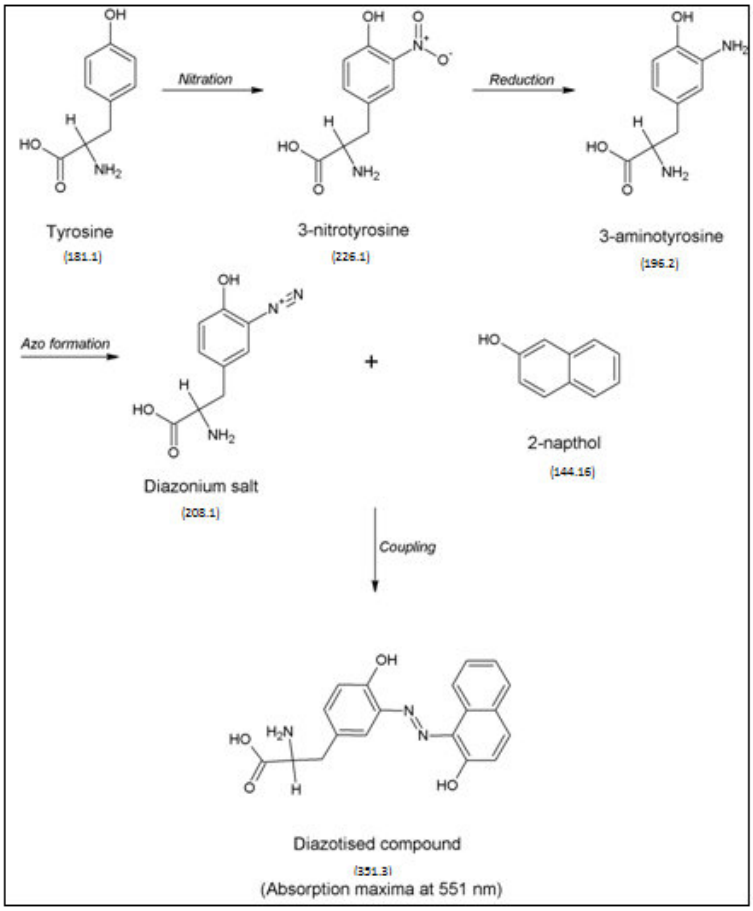


Figure 7

ESI MS of diazotized product. m/z value for diazotised product obtained was $[M+H]^+$ 352.4, aminotyrosine $[M+23]^+$ 219.2 , 3-NT $[M+23]^+$ 249.1, and tyrosine $[M+23]^+$ 204.1



Scheme I

Schematic diagram of the mechanism of reaction

CONCLUSION

Nitration is considered as a potential biomarker for many pathological conditions. Quantification of 3-NT by simple spectrophotometric method is challenging as the 3-NT is very less produced in living system [9]. Hence quite sensitive method is always a requirement, therefore the current research focused on developing a sensitive method for 3-NT. It is successful in quantifying ~20 µM concentration of 3-NT through diazotisation procedure. The exact quantification may be a questionable as 3-NT is not directly measured. However the limit of detection of 3-NT is achieved through this procedure as 3-NT is reduced and further diazotised. Hence the probability of quantification can be even lower than the measured concentration of 3-NT (5 µM). The sensitivity of detecting 3-NT is ~10 fold higher when compared to measurement of 3-NT directly.

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