A highly selective and sensitive spectrofluorimetric method for the assessment of 3-nitrotyrosine in serum using (Eu(TTA)₃Phen) photo probe

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A simple, accurate and fast method was developed for the assessment of 3-nitrotyrosine as a biomarker for the early diagnosis of liver cirrhosis with minimal hepatic encephalopathy (MHE) using a (Eu(TTA)₃Phen) photo probe. 3-Nitrotyrosine can remarkably quench the luminescence intensity of the (Eu(TTA)₃Phen) complex in DMSO at pH = 9 and λₘₐₓ = 617 nm. The quenching of the luminescence intensity of (Eu(TTA)₃Phen) complex particularly the electrical emission band at λₘₐₓ = 617 nm is used for the assessment of 3-nitrotyrosine in different serum samples of patients with liver cirrhosis.

1. Introduction

3-Nitrotyrosine has an IUPAC name ((2S)-2-amino-3-(4-hydroxy-nitrophenyl)propanoic acid), Fig. 1a. Hepatic encephalopathy is a brain dysfunction caused by acute or chronic liver disease. It is of two types: overt and covert type. The overt type is characterized by bedside characteristic clinical features and does not need sophisticated investigations for diagnosis.¹ Covert or minimal hepatic encephalopathy (MHE) is characterized by an examination of normal mental and neurological status. It can be diagnosed by sophisticated psychometric tests, e.g., paper-and-pencil psychometric tests, inhibitory control test, critical flicker frequency and the stroop smartphone application.²,³ Up to 80% of patients with cirrhosis have MHE. Its presence is associated with poor quality of life, inability to drive, traffic violation and accidents. Within 3 years, ~50% may develop overt hepatic encephalopathy.¹,⁴ MHE is commonly found with advanced liver disease, history of overt hepatic encephalopathy, esophageal varices and alcohol abuse as etiology of liver cirrhosis.⁵

It is probable that MHE is a marker of advanced liver failure because it is associated with a shorter survival time, particularly among patients with high concentrations of ammonia after oral glutamine load.⁶,⁷ For this reason, MHE has been proposed as an indication for liver transplantation. Currently, the “gold standard” for the diagnosis of MHE is the psychometric hepatic encephalopathy score (PHES).⁸ However, “gold standard” method is time consuming and needs adjusting for time. Therefore, MHE is not routinely diagnosed in most clinical settings because of lack of simple procedures, and most patients with MHE remain undiagnosed and untreated. Hence,
there is a need for a simple diagnostic test that can be performed routinely in the laboratory to detect MHE in patients with liver cirrhosis. It would be useful to have some peripheral biomarkers that could be measured in blood samples and reflect the presence of MHE in cirrhotic patients in clinical practice. In the last decade, 3-nitrotyrosine has been used as a marker for MHE. There are many different methods for the determination of 3-nitrotyrosine such as, gas chromatography-mass spectrometry (GC-MS) or LC-MS analysis, either LC-MS or affinity approaches (immunoassays). For a non-derivative analysis, either LC-MS or affinity approaches can be used. However, both techniques have their limitations as well. Most LC-MS methods have an additional step of purifying obtained extracts using a solid-phase extraction (SPE) technique, hence increasing costs and time required for sample preparation. Regarding immunoassays, the biggest concern is their insufficient selectivity and specificity due to cross-reactivity with 3-nitrotyrosine and other structurally similar metabolites, but the immunoassay sensitivity is not optimal either. These shortcomings make it difficult to compare the immunoassay results obtained in different studies. In this study, 3-nitrotyrosine was determined by the quenching of the luminescence intensity of the Eu(TTA)₃Phen photo probe at λ_em = 617 nm in the presence of different concentrations of 3-nitrotyrosine in the blood serum samples of patients with MHE.

2. Experimental

2.1. Apparatus

The absorption spectra were recorded on a double beam PerkinElmer Lambda 25 UV-visible spectrophotometer fitted with a tungsten halogen lamp for operation in the visible range and a deuterium lamp for operation in the UV range. All luminescence measurements were recorded on a Meslo-PN (222-263000) Thermo Scientific Lumina fluorescence spectrometer in the range of 190–900 nm. The pH was measured using a pHs-JAN-WAY 3040 research pH meter. The separation of serum samples was carried out by centrifuging the sample for 15 min at 4000 rpm.

2.2. Materials

Pure standards, 3-nitrotyrosine, phenylalanine, valine, tyrosine, tryptophan, citrulline, albumin, glucose, urea and methionine were purchased from Sigma Aldrich. Human serum samples were collected from two hospitals, namely Al-Kasr-EL-Aini new teaching hospital (Cairo, Egypt) and Ain Shams Specialized Hospital (Cairo, Egypt). Sample collection was performed in accordance with WHO (World Health Organization) approved protocol for human specimen collection. All experiments were performed in accordance with the Guidelines “Ministry of Health and Population, Egypt” and approved by the ethics committee at “Ain Shams” university. Informed consents were obtained from the human participants of this study.

2.3. Preparation of standard solutions

A stock solution of 3-nitrotyrosine (1.0 × 10⁻³ mol L⁻¹) was freshly prepared by dissolving 5.60 mg in 25 mL pure DMSO. A more diluted solution (1.0 × 10⁻⁵, 1.0 × 10⁻⁷ mol L⁻¹) was prepared by appropriate dilution with DMSO. Stock and working solutions are stored at 4°C when not in use. An Eu³⁺ ion stock solution (1.0 × 10⁻⁴ mol L⁻¹) was prepared by dissolving 2.47 mg Eu(TTA)₃Phen in a small amount of deionized water and the volume was completed to the mark in a 25.0 mL measuring flask. The pH was adjusted to 8.24 using a phosphate buffer solution. The above-mentioned working solutions were used for the subsequent measurements of absorption spectra, emission spectra, effect of pH and solvents. The fluorescence intensity was measured at λ_em/λ_exc = 375/617 nm.

2.4. Calibration curve

After the preparation of the solution of 3-nitrotyrosine in DMSO as described above, 1.0 mL of the photo probe Eu(TTA)₃Phen was put in the cell and different concentrations of 3-nitrotyrosine were added to the cell of the spectrofluorimetric device, followed by recording the luminescence spectra at the selected excitation wavelength λ_exc = 375 nm.

2.5. Proposed method

The blood was allowed to clot by leaving it 20 min undisturbed at room temperature. The clot was removed by centrifugation at 4000 rpm for 15 min and then the supernatant was decanted, which was designated as serum. The serum was transferred into a clean tube and then kept at -20°C. Furthermore, 100 µL of the serum was added to 1.00 mL of Eu³⁺ stock solution (1.0 × 10⁻⁴ mol L⁻¹) in DMSO and the pH was adjusted to 8.24 in the cell of the spectrofluorimetric device. The luminescence intensity of the Eu³⁺ photo probe was measured before and after the addition of the serum solution. The change in the luminescence intensity was used for the determination of 3-nitrotyrosine in the serum sample.

2.6. Standard method

2.6.1 Assay principle. A 3-nitrotyrosine quantitation kit used was a competitive ELISA. The unknown protein nitrotyrosine sample or nitrated BSA standards were first added to a nitrated BSA preabsorbed EIA plate. After a brief incubation, an anti-nitrotyrosine antibody was added, followed by an HRP conjugated secondary antibody. The protein nitrotyrosine content in an unknown sample was determined by comparing with a standard curve that was prepared from predetermined nitrated BSA standards.

2.6.2 Assay protocol. All reagents were prepared and mixed thoroughly before use. Each protein sample including nitrated BSA and blank were assayed in duplicate. 50.0 µL of the unknown protein sample or nitrated BSA standard were added to the wells of the EIA plate. The samples were incubated at room temperature for 10 min on an orbital shaker. 50 µL of the diluted anti-nitrotyrosine antibody was added to each well and incubated at room temperature for 1.0 hours on an orbital shaker. Micro well strips were washed 3 times with 250 µL Wash Buffer per well with thorough aspiration between each wash. After the last wash, the wells were emptied and tapped micro well strips on an absorbent pad or paper towel to remove excess...
Wash Buffer. 100 μL of the diluted secondary antibody–enzyme conjugate was added to all the wells. The wells were incubated at room temperature for 1 h on an orbital shaker. Micro well strips were washed 3 times according to step 4 above, and we proceeded immediately to the next step. The substrate solution should be warmed to 30 ºC. 100 μL of the substrate solution was added to each well, including the blank wells. The wells were incubated at room temperature on an orbital shaker. Actual incubation time varied from 2.0–30 min. The enzyme reaction was stopped by adding 100 μL of the Stop Solution into each well, including the blank wells. Results were read immediately (color will fade over time). The absorbance of each micro well was read on a spectrophotometer using 450 nm as the primary wavelength.

3. Results and discussion

3.1. Absorption and emission spectra

The absorption spectra of Eu(TTA)₃Phen complex, 3-nitrotyrosine and 3-nitrotyrosine in the presence of the photo probe are shown in Fig. 2. The absorption spectrum of (1.0 × 10⁻⁵ mol L⁻¹) [Eu(TTA)₃Phen] shows (π → π*) transition band at 345 nm. Upon the addition of (1.0 × 10⁻⁵ mol L⁻¹) of 3-nitrotyrosine, no shift was observed in the band. This indicates that no complexation between the photo probe and 3-nitrotyrosine in the ground state occurred. The characteristic peaks of Eu³⁺ ion appear at (⁴D₀ → ⁵F₂ = 580 nm, ⁴D₀ → ⁷F₁ = 590 nm, ⁴D₀ → ⁷F₂ = 617 nm, ⁵D₀ → ⁷F₁ = 650 nm, ⁵D₀ → ⁷F₂ = 690 nm and ⁵D₀ → ⁷F₃ = 710), as shown in Fig. 3. The effect of the solvent on the luminescence intensity of the photo probe [Eu(TTA)₃Phen] was studied under the conditions optimized above. The high luminescence intensity of the photo probe was observed in aprotic solvents such as DMSO and DMF than in protic solvents such as ethanol. This could be attributed to the efficient quenching of the excited state of the photo probe by interactions with high-energy vibration oscillators such as O–H groups, as shown in Fig. 3. The pH of the medium has a great effect on the luminescence intensity of the [Eu(TTA)₃Phen] complex. The pH meter has been adjusted using the phosphate buffer solution (Na₂HPO₄). The pH has been adjusted using NH₄OH and HCl solutions. The optimum pH value where the peak at λ_em = 617 nm has the highest intensity was obtained at pH = 8.24, as presented in Fig. 4. The validity and selectivity photo probe Eu(TTA)₃Phen to 3-nitrotyrosine was tested by studying the influence of a series of interfering species such as, NaCl, KCl (2.0 × 10⁻³ mol L⁻¹), uric acid (0.08 g L⁻¹), urea (0.06 g L⁻¹), total protein (0.01 g L⁻¹), (0.06 g L⁻¹) triglyceride, glucose (0.08 g L⁻¹) and (1.0 × 10⁻⁴ mol L⁻¹) of each amino acids such as, tyrosine, tryptophan, phenylalanine, valine, citrulline and methionine on the luminescence spectrum of Eu(TTA)₃Phen in the presence of (1.0 × 10⁻⁵ mol L⁻¹) 3-nitrotyrosine. The tolerable limit was defined as the concentration of the individually added species causing a deviation less than 5.0% of the luminescence intensity of the Eu(TTA)₃Phen photo probe at the optimum conditions. The results indicated no significant change in the luminescence intensity of the Eu(TTA)₃Phen photo probe, as shown in Fig. 5. The luminescence emission spectrum of the photo probe exhibits a strong luminescence intensity band at 617 nm with a broad and structureless shape. The emission spectra of [Eu(TTA)₃Phen] complex in different concentrations of 3-nitrotyrosine is shown in Fig. 6. After the addition of different concentrations of 3-nitrotyrosine to [Eu(TTA)₃Phen] in DMSO, the intensity of the characteristic peak at λ_em = 617 nm of Eu³⁺ was quenched, which indicates that 3-nitrotyrosine is a good...
quencher of the luminescent Eu$^{3+}$ complex. A correlation between the emission intensity of the photo probe [Eu(TTA)$_3$-Phen] complex at $\lambda_{em}$ 617 nm and 3-nitrotyrosin was obtained by applying the Stern–Volmer plot in with ($\frac{F_0}{F_0 - C_0}$) versus $[3$-nitrotyrosine$]$; where, $k_{sv}$ is the Stern–Volmer constant, which is determined from the slope of the fitted data and equals to 0.0148 L mol$^{-1}$C$_0$1. One over $k_{sv}$ equals to $C_1/2$ (half quenching concentration), $C_1/2 = \frac{1}{k_{sv}} = 67.56$ mol L$^{-1}$. $R_0$ is the critical transfer distance, which is the average distance between the donor and the acceptor at which the probability of fluorescence quenching is just equal to 0.5 excited state, $R_0 = 7.35/(C_1/2)^{1/3} = 1.81$ Å, indicating the electron transfer mechanism of quenching (Fig. 7). A linear correlation was found between ($\frac{F_0}{F_0 - C_0}$) at $\lambda_{em} = 617$ nm, and the concentration of 3-nitrotyrosine in the ranges given in Table 1 and the regression parameters are summarized in Table 1. The limit of detection (LOD) and quantitation (LOQ) were also calculated according to ICH guidelines$^{38-42}$] and presented in Table 1.

### 4. Accuracy and precision

To compute the accuracy and precision, the assays described under general procedures were repeated three times within a day to determine the repeatability (intraday precision) and three times on different days to determine the intermediate precision (inter day precision) of the method. These assays were performed for three levels of the analyte. The results of this study are summarized in Table 2. The percentage relative standard deviation (% RSD) values were $\leq 0.056-0.1\%$ (intraday) and $\leq 0.054-0.097\%$ (inter day) for patients. The inter day values indicated high precision of the method. Accuracy was evaluated as percentage relative error (RE) between the measured mean concentrations and the taken concentrations of 3-nitrotyrosine. Bias (bias%) was calculated at each concentration, as presented in Table 2. Percent relative error (% RE) values of $\leq 0.29-4.60$ (intraday) and 0.18–2.80% (inter day) for the patient states demonstrate the high accuracy of the proposed method. The obtained results are summarized in Table 2. There was good agreement between the average values obtained by the developed procedure (8.534 ± 0.0089) and the standard spectrophotometric method (8.57 ± 0.004) without recording any significant differences between

| Parameter | Values |
|-----------|--------|
| $\lambda_{em}$ nm | 617 |
| Linear range, mol L$^{-1}$ | 2.3 × 10$^{-4}$ to 1.0 × 10$^{-9}$ |
| Limit of detection (LOD), mol L$^{-1}$ | 2.6 × 10$^{-10}$ |
| Limit of quantification (LOQ), mol L$^{-1}$ | 7.8 × 10$^{-10}$ |
| Intercept ($a$) | 0.626 ± 0.071 |
| Slope ($b$) | 0.0152 ± 1.84742 × 10$^{-4}$ |
| Standard deviation | 0.431 |
| Variance (sa$^2$) | 0.186 |
| Regression coefficient ($r^2$) | 0.998 |

Fig. 5 Effect of foreign species on the selectivity of the photo probe Eu[TTA)$_3$Phen to 3-nitrotyrosine.

Fig. 6 Luminescence emission spectra of the [Eu(TTA)$_3$Phen] complex in the presence of different concentrations of 3-nitrotyrosine in DMSO at $\lambda_{ex} = 375$ nm and pH = 9.0.

Fig. 7 Linear relationship between ($\frac{F_0}{F_0 - C_0}$) and the concentration of 3-nitrotyrosine $\lambda_{em} = 617$ nm.
Table 2  Evaluation of intra-day and inter-day accuracy and precision

| Sample   | Standard method average nM | Intra-day accuracy and precision (n = 3) | Inter-day accuracy and precision (n = 3) |
|----------|----------------------------|----------------------------------------|----------------------------------------|
|          |                           | Average found nM ± CL | % RE | % RSD | Average found nM ± CL | % RE | % RSD |
| Health (1) | 8.3                        | 8.39 ± 0.89           | 1.08 | 0.072 | 8.3 ± 0.90            | 1.33 | 0.073 |
| Health (2) | 7.9                        | 7.84 ± 0.92           | 0.76 | 0.077 | 7.9 ± 0.92            | 0.25 | 0.076 |
| Health (3) | 7.5                        | 7.54 ± 0.94           | 0.53 | 0.08  | 7.5 ± 0.96            | 2.80 | 0.082 |
| Health (4) | 9.1                        | 9.24 ± 0.85           | 1.54 | 0.063 | 9.1 ± 0.86            | 1.21 | 0.067 |
| Health (5) | 6.8                        | 6.82 ± 0.99           | 0.29 | 0.088 | 6.8 ± 0.98            | 2.06 | 0.086 |
| Patient (1) | 177.8                      | 177.66 ± 0.93         | -0.07 | 1.78 | 178.8 ± 0.94          | 0.56 | 1.79  |
| Patient (2) | 109.9                      | 109.74 ± 0.79         | -0.14 | 1.56 | 110.9 ± 0.78          | 0.90 | 1.55  |
| Patient (3) | 206.3                      | 206.01 ± 0.105        | -0.14 | 1.1  | 207.3 ± 0.104         | 0.48 | 1.97  |
| Patient (4) | 111.1                      | 111.8 ± 0.79          | 0.63 | 1.56 | 112.0 ± 0.77          | 0.81 | 1.54  |
| Patient (5) | 170.1                      | 170.9 ± 0.80          | 0.47 | 1.05 | 171.1 ± 0.80          | 0.58 | 1.58  |

$^a$ CL, confidence limits: $CL = \pm tS/(n)^{1/2}$, the tabulated value of $t$ is 4.303, at the 95% confidence level. $S$, standard deviation. $N$, number of measurements. % RE, percent relative error. % RE = [(concentration proposed − concentration known)/concentration known] × 100. % RSD, relative standard deviation. % RSD = /[average measurements] × 100.

Table 3  Comparison of the spectrofluorimetric technique with some existing methods for the determination of 3-nitrotyrosine

| Method                                      | Detection limit | Reference |
|---------------------------------------------|-----------------|-----------|
| (GC/MS) Isotope dilution liquid, chromatography-electro spray, ionization tandem, mass spectrometry | (1.73) nM | 25        |
| HPLC-UV method, combined with pre-column, cloud point extraction (CPE) | (1.83) nM | 26        |
| GC tandem MS | (5–15) nM | 27        |
| GC-MS HPLC-fluorescence detector, 470 nm | 2.677 ± 1.540 v nM | 28        |
| ELISA based on the sandwich | 4.46 ± 4.49 nM | 29        |
| Photo probe method, 3-nitrotyrosine-Eu(TTA)_3Phen | 573.54 ± 142.86 nM | 31        |
|                                        | 1.6 ± 0.19 nM | The present work |

Conflicts of interest
There are no conflicts to declare.

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