New Photo probe for Assessment of Norepinephrine in Pharmaceutical Formulation and Serum Samples

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Abstract

A simple, sensitive and selective spectroflourimetric method for the determination of Norepinephrine (NE) is developed. The method depends on the enhancement of the fluorescence intensity of the photo probe at 351 nm in presence of different concentrations of NE in acetonitrile, at λ_{ex} = 290 nm at pH 5.01. The enhancement of the emission band of the photo probe at 351nm was found to be directly proportional to the concentration of NE with a dynamic range of $1 \times 10^{-5} - 4.4 \times 10^{-8}$ mol L^{-1} and detection limit of 2.3×10^{-8} mol L^{-1}. This method is simple, accurate and can successfully be applied to the determination of NE in pharmaceutical formulation and serum samples with remarkably satisfactory results.

Keywords: Norepinephrine; Photo probe; Enhancing; Fluorescence Intensity.

1. Introduction

Norepinephrine (NE), **Fig.(1)** is an important member of catecholamine neurotransmitters exuded by the adrenal medulla in the central nervous system of mammals , and plays crucial physiological roles in the function of the renal , hormonal , cardiovascular ,central nervous and reproductive systems **[Umasankar,Y. et al., (2009); Eisenach, J.C. et al., (1990)].**

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High level of catecholamines is interconnected with stress , a fall in blood pressure or blood volume , thyroid hormone deficiency , congestive heart failure , and arrhythmias , whereas ,low level of catecholamines is implicated towards idiopathic postural hypotension and depression **[**detection (FD) **[Lesniak, W.G. et al., (2013); Sa, M. et al., (2012)]**, electrochemical detection (ECD) **[Chen, G. et al., (2000); Chen, D.C. et al.,(2001)]**, laser-induced fluorescence detection **[Parrot, S. et al., (2004); Zhao, Y. et al., (2011)]**, mass spectrometry (MS) **[Cai, H.L. et al., (2010); Miller, A.G. et al., (2010)]** and electrochemical detection **[Lu, J.X. et al., (2000) ; Vicente-Torres, M.A. et al., (2002)] .** However, most of these methods suffer from some disadvantages including time-consuming, complicated treating process, high costs and low sensitivity. In this work, NE **Ardakania, Md. M. et al., (2010)]**. Various analytical techniques have been implemented for NE determination. These techniques include chromatographic **[Peitzsch, M. et al., (2013); Fandila, A.S. et al., (2013)]**, fluorescent **[Silva, L. et al., (2012)]**, spectrophotometric **[Hashem, E.Y. and Youssef, A.K., (2013); Wei, S. et al., (2005)]**, chemiluminescence methods **[Wang, C.Q.,(2007)]**, capillary electrophoresis **[Zhu, M. et al., (1997)]**, polarography **[Wu, Y. et al.,(1996)]**, voltammetry **[Downard, A.J. et al., (1995)]**, Liquid chromatography(HPLC) or capillary electrophoresis (CE) coupled with various detection methods, such as ultraviolet (UV) detection **[Liu, G.S. et al., (2004); Siren, H. and Karjalainen, U., (1999)]**, fluorescence concentration was determined by the ion pair between NE and photo probe the possibility of the enhancement of the photo probe fluorescence intensity sensitized by NE was established and investigated. The absorption and emission spectra of NE and photo probe were measured in acetonitrile at pH 5.01.
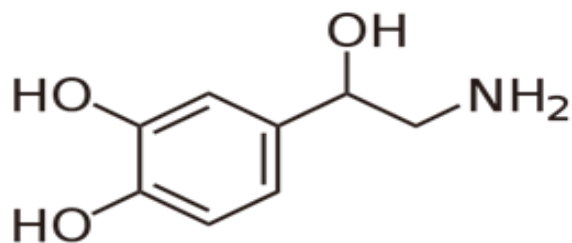

Fig. (1) Structure of Norepinephrine

2. Materials and methods

2. 1. Materials

   Pure standard Norepinephrine supplied by the national organization for drug control and research (Giza, Egypt). Pharmaceutical preparation of Norepinephrine bitartrate (injection vial) produced by *Pfizer* Co. for Pharmaceuticals and chemical industries; Egypt is purchased from local market

2.2. Chemicals and Reagents

   All chemicals used are analytical-reagent of higher grade. Pure grade solvents from (Sigma- Aldrich) are used for the preparation of all solutions and during all determinations. β-(p-ethoxy)benzoylacrylic acid and thiourea were purchased from (Sigma- Aldrich). A stock solution of Norepinephrine ($1x10^{-3}$ mol L^{-1}) is freshly prepared and dissolved in ethanol and stored at 4 °C when not in use. The working standard solutions of ($3x10^{-5}$ – $1x\ 10^{-9}$ mol L^{-1}) are freshly prepared by appropriate dilution with acetonitrile. Azothizole (photo probe) stock solution (1×10^{-5} mol L^{-1}) is prepared by dissolving Azothizole with a small amount of ethanol in 25 ml measuring flask, then diluting to the mark with ethanol. The fluorescence intensity was measured at λ_{ex}/ λ_{em} = 290/351 nm. Stock and working solutions were stored at 0 – 4 °C when not in use. In all experiments, clean and sterilized volumetric flasks (10 mL) were used.

2.3. Apparatus

   All fluorescence measurements are carried out on Prekin Elmer LS 45 spectrofluorophotometer in the range (290 – 750 nm with attenuator 30%). The absorption spectra are recorded with Thremo UV-Visible double-beam spectrophotometer. All pH measurements are made with Crison instruments S.A.E.08328 ALELLA-Barcelona (EU)**.**

2.4. General procedure

2.4.1. Preparation of Norepinephrine solutions.

   To 10 ml clean and sterilized measuring flasks, the standard solutions of Norepinephrine were prepared by different additions of (1×10^{-3} mol L^{-1}) Norepinephrine solution to give different concentrations of Norepinephrine. The solutions were diluted to the mark with

acetonitrile at room temperature. The above method was used for the subsequent measurements of absorption, emission spectra, effect of pH and effect of solvents. The fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em}$ = 290/351 nm.

2.4.2. Preparation of 5-(p-ethoxy) benzoyl methyl-2-diazo- α –naphthol-4-hydroxy thiazol.

The reaction of β-(p-ethoxy)benzoylacrylic acid with thiourea gives 2-amino-4-hydroxy-5-(p-ethoxy) benzoyl methyl thiazole. The diazotization of the 2-amino-4-hydroxy-5-(p-ethoxy) benzoyl methyl thiazole with α- sodium naphthoate in the presence of nitrous acid and HCl give the azo compound 5-(p-ethoxy) benzoyl methyl-2-diazo- α -naphthol-4-hydroxy thiazol (photo probe) **Fig.(2)**.
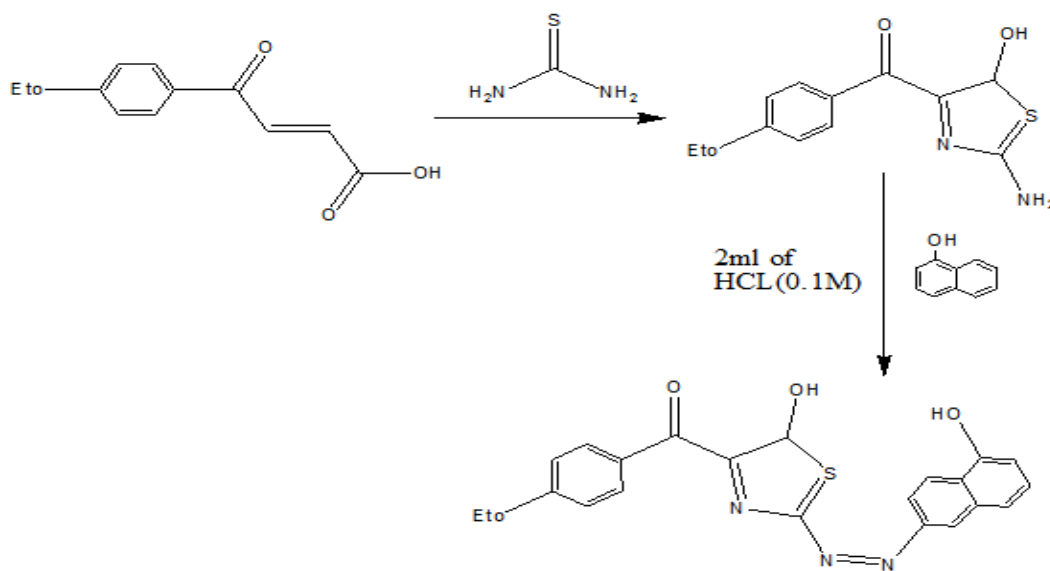

Fig.2: Scheme for synthesis of the photo probe

2.4.2.1. Characterization of of 5-(p-ethoxy) benzoyl methyl-2-diazo- α -naphthol-4-hydroxy thiazol

IR, υ (cm^{-1}) υ_{OH} (3422), υ_{CH2}(2853), $\upsilon_{C=O}$(1652), $\upsilon_{C=N}$(1619), $\upsilon_{N=N}$(1477**)**

^{1}H-NMR (DMSO-d$_6$, ppm) : 9 (s, 1H ,OH), 8.8 (s,1H,OH), 8-8.2 (s,2H,NH$_2$)

3.6 (s, 2H, CH$_2$) ,4.2 (q, 2H, CH$_2$O, J=11.2), 1.3 (t,3H, CH$_3$, J=13.6).

EI-M m/z(%) 313 ($C_{15}H_{11}N_3O_3S$, 100%), 285 ($C_{14}H_{11}N_3O_2S$,37%) ,263 ($C_{13}H_{12}NO_3S$, 7%), 157 ($C_{10}H_7NO$,14%),149 ($C_9H_9O_2$, 32%), 121 (C_8H_9O,28%) ,95 (C_6H_7O, 28%) ,66 (C5H6,31%)

Elemental analysis CHNS, Anal. Calc. for $C_{23}H_{19}N_3O_4S$ (433): C, 63.7; H, 4.38; N, 9.7; S, 7,0. Found   C,63.3 ;   H,4.2 ; N,10,0; S,7.2

 2.5. 1. Determination of Norepinephrine in pharmaceutical formulation

   An appropriate volume was taken from Norepinephrine bitartrate (injection vial) ($1×10^{-3}$ mol L^{-1}) to obtain ($3×10^{-4}$ mol L^{-1}) in 50 mL acetonitrile then further dilution to the linear range concentration. Solutions were standing for about 10–15 min. The concentration of the drug was determined by using three concentrations for each sample from the corresponding calibration graph.

2.5. 2. Determination of Norepinephrine in serum sample

   To a 0.5 ml of serum in a glass stoppered tube, 0.5 ml of distilled water was added followed by addition of 1 mL of trichloroacetic acid for protein separation. The tube was stoppard and shaken slowly for 20 min to extract the protein. The two phases were allowed to settle and supernatant, then the aqueous layer was pipette out and discarded **[Mattingly, D., (1964)]**. 1 mL of the extracted serum was transferred into a calibrated 10 mL measuring flask containing 1 mL of ($1x10^{-5}$ mol/L) photo probe then diluted to the mark with acetonitrile at pH 5.01. The fluorescence intensity of the test solution is measured before and after addition of 1 mL of the extracted serum at 351 nm. The change in the fluorescence intensity is used for determination of Norepinephrine in serum sample.

3. Results and Discussion

3.1 Spectral characteristics

   The absorption spectrum of (1 x 10^{-5} mol/L) photo probe in acetonitrile is shown in **Fig. (3).** The spectrum (1) in **Fig. (3).** shows an intense broad band at (272 nm) due to the $\pi{\rightarrow}\pi^*$ transition in the photo probe. Upon addition of different concentrations of the Norepinephrine to the photo probe in acetonitrile (spectra 2-4) the equilibrium maintained (the intensity of the band 272 nm is increased . The result reveals that the charge transfer

ion pair was formed between Norepinephrine and the photo probe in the ground state and this is observed in **Fig. (3).**

The emission spectra **Fig.(4)** of the photo probe in the acetonitrile at pH 5.01 showed the characteristic emission band at 351 nm **Fig.(4)**. The fluorescence intensity of the photo probe (acetonitrile and pH 5.01, λ_{ex}=290)   at 351 nm increased upon adding different concentration of   Norepinephrine and these confirming the charge transfer from Norepinephrine to the photo probe in acetonitrile at λ_{ex}=290
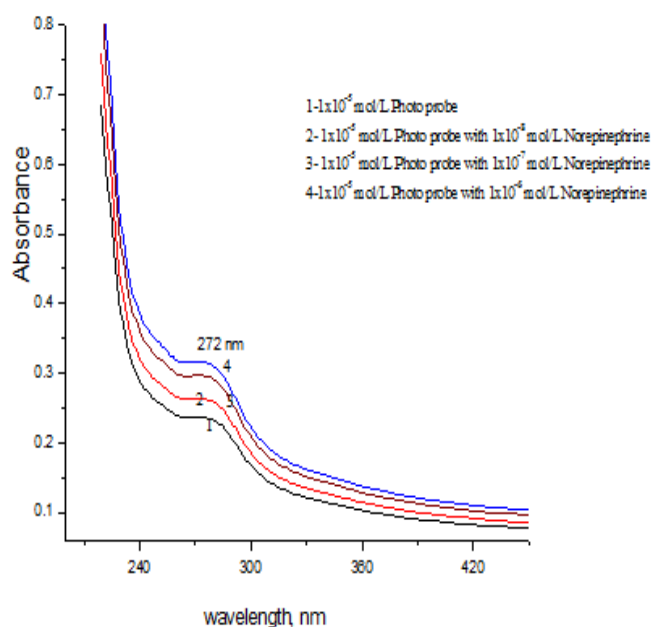

Fig. (3) Absorption spectra of photo probe in the presence of different concentrations of Norepinephrine.
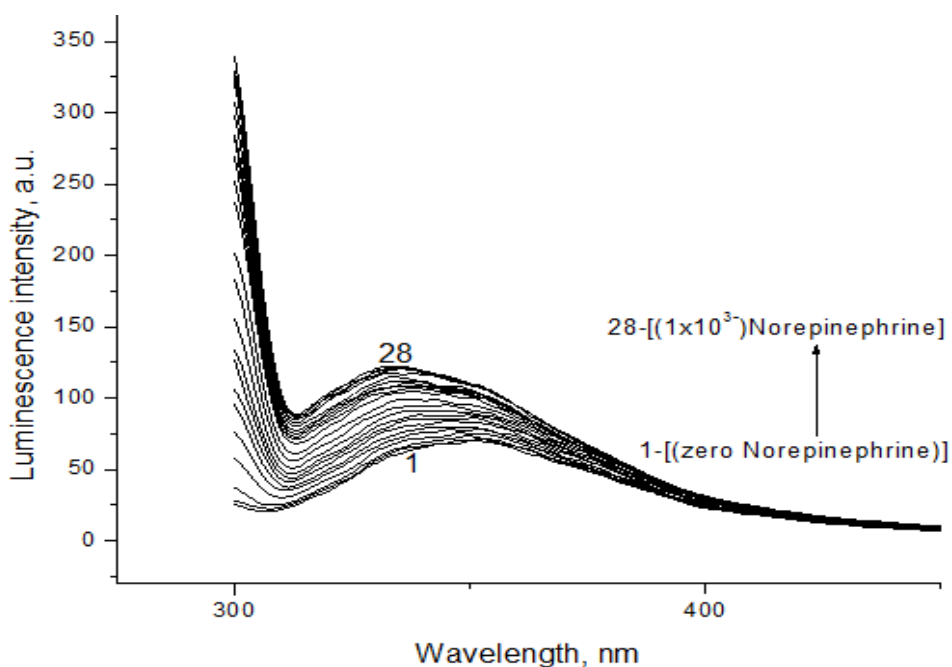
Fig. (4) Fluorescence spectra of photo probe in the presence of different concentrations of Norepinephrine in acetonitrile at λ_{ex} =290.

3.2 Effect of Solvent

The effect of the solvent on the fluorescence intensities of $(1 \times 10^{-5}$ mol L^{-1}) photo probe and $(1 \times 10^{-5}$ mol L^{-1}) of Norepinephrine was studied under the conditions optimized above **Fig.(5)**. The high fluorescence intensity of the photo probe was observed in aprotic solvent like acetonitrile this may be attributed to the good charge transfer from Norepinephrine to photo probe. The higher intensity of the photo probe was obtained in case of acetonitrile. Also, the fluorescence intensities for the photo probe in aprotic solvent like DMSO, acetonitrile and DMF are stronger than in protic solvent like water and ethanol. This could be attributed to the protic solvents prevent the charge transfer from the Norepinephrine to photo probe in the excited state. The protic solvents shield the Norepinephrine from the photo probe and the quenching of the fluorescence intensity at 351 nm is occurred.
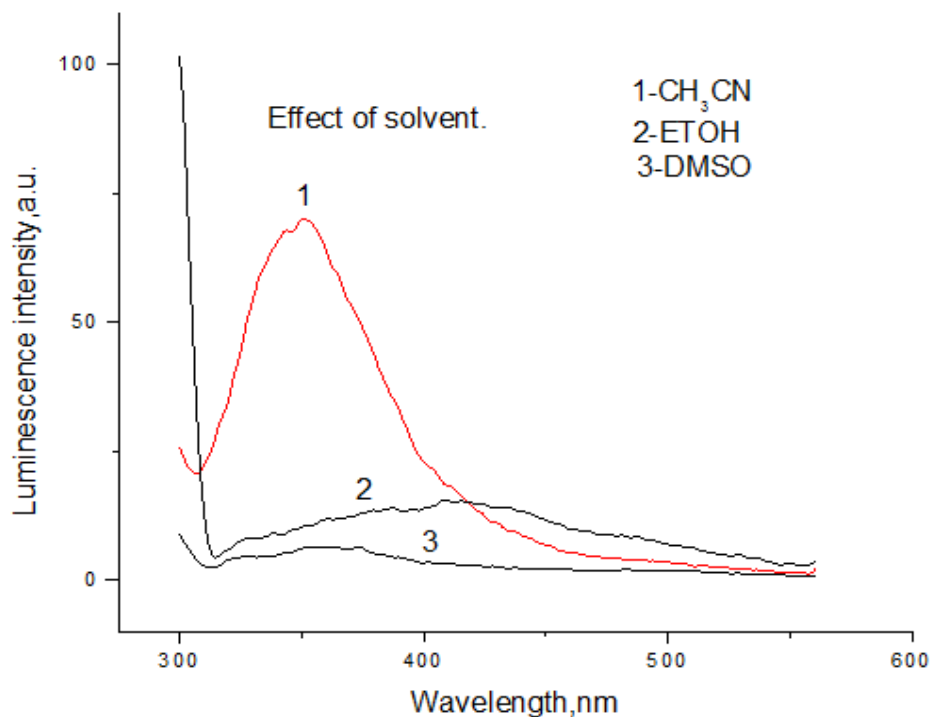
Fig. (5) Fluorescence spectra of photo probe in different solvent at λ_{ex} =290.

3.3 Effect of pH

Norepinephrine contains several functional groups that ionize at different values of pH. The good result was obtained at pH 5.01 due to suitable configuration of Norepinephrine at this pH for the good charge transfer between Norepinephrine and the photo probe which enhances the fluorescence intensity of the photo probe at 351 nm.

3.4 Interference in the detection of Norepinephrine

We examined the effects of interfering species commonly observed in biological samples. The concentration of Norepinephrine was maintained at ($1x\ 10^{-5}$ mol/L) and the pH was adjusted to 5.01. Potential interference was studied by new photo probe with the selected amines and drugs at a concentration of ($1x10^{-5}$ mol/L). the results reveal that there is no detectable interference in case of the amines like (dopamine, epinephrine and drugs like (ethosuximide, primidone, phenobarbital, phenytoin, carbamazepine, theophylline, procainamide, propranolol, quinidmne, diazepam, chlordiazepoxide, acetaminophen, and

acetylsalicylic acid). This attributed to the high selectivity of the photo probe for the Norepinephrine at pH 5.01.

3.5 Calibration curve and detection limit

The effect of the concentrations of the norepinephrine on the fluorescence intensity of photo probe is shown in **Fig.(4)**. As can be clearly seen from the figure and under the optimal conditions, the fluorescence intensity is increased linearly with the concentrations of norepinephrine over the range (1.0×10^{-5} - 4.4×10^{-8} mol L^{-1}) with a correlation coefficient of 0.989 **Fig. (6)**.
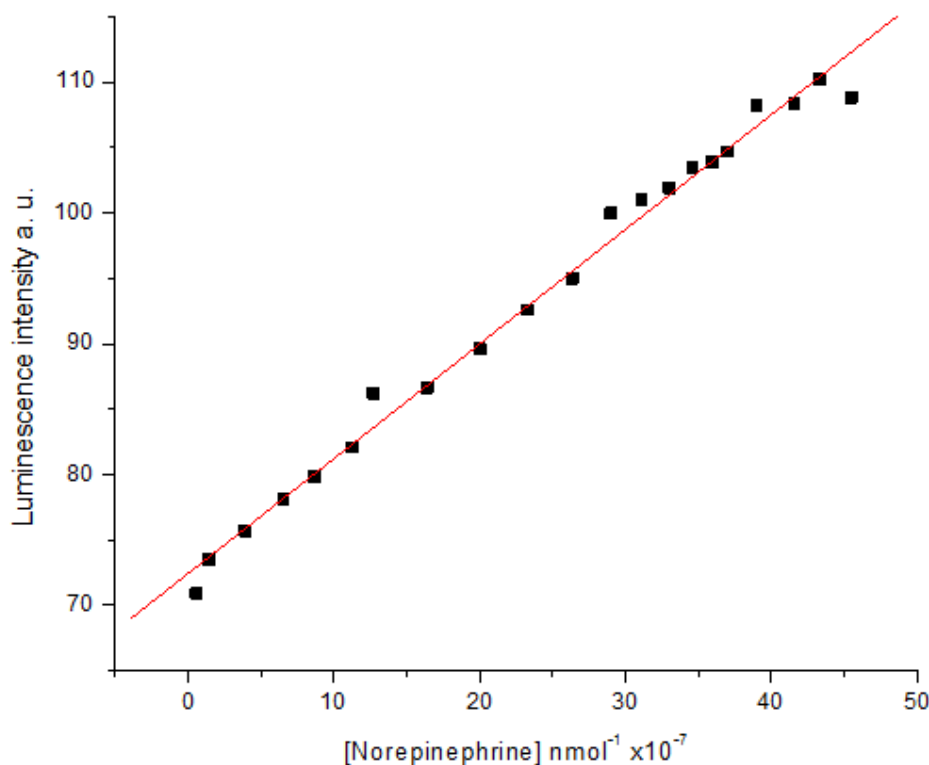
Fig. (6). Linear relationship between fluorescence intensity of photo probe and the concentrations of Norepinephrine.

The detection limit (LOD) and quantification detection limits were calculated according to ICH guidelines **[International Conference on Hormonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, (2005)]** using the formulae: LOD = 3.3 S/b and LOQ = 10 S/b, (where S is the standard deviation of blank luminescence intensity values,

and b is the slope of the calibration plot) are also presented in **Table 1**. The comparison of the proposed photo probe for the determination of Norepinephrine with other published methods indicate that the developed method has good stability, lower limit of detection (2.3×10^{-8} mol L^{-1}) and wide linear range of application (1.0×10^{-5} - 4.4×10^{-8} mol L^{-1}) as shown in **Table 2**.

Table 1. Sensitivity and regression parameters for photo probe.

Parameter	values
λ_{em}, nm	351
Linear range, mol L^{-1}	$1 \times 10^{-5} - 4.4 \times 10^{-8}$
Limit of detection (LOD), mol L^{-1}	2.3×10^{-8}
Limit of quantification (LOQ), mol L^{-1}	7.8×10^{-8}
Regression equation, Y*	
Intercept (a)	68
Slope (b)	4.0×10^{8}
Standard deviation	2.66
Variance (Sa^2)	7.01
Regression coefficient (r)	0.989

*Y=a+bX, Where Y is luminescence intensity, X is concentration in n mol L^{-1} , a is intercept, b is slope.

Table 2: Comparison of different determination methods for the (Norepinephrine) with the proposed method

Method	Linear range (mol l^{-1})	Detection limit (mol l^{-1})	References
Competitive enzyme-linked immunosorbent assay	$3.2 \times 10^{-8} - 3.2 \times 10^{-3}$	1.0×10^{-9}	[Kim, J. et al., (2008)]
A novel composite of molecularly imprinted polymer-coated PdNPs	$5.0 \times 10^{-7} - 8.0 \times 10^{-5}$	1.0×10^{-7}	[Chen, J. et al., (2015)]
Electrochemical sensor was prepared by modification of carbon paste electrode with a nanostructured mesoporous material	$7.0 \times 10^{-8} - 2.0 \times 10^{-3}$	4.0×10^{-8}	[Mazloum-Ardakani, M. et al., (2012)]
A disposable screen-printed electrode modified with MWNTs-ZnO/ chitosan	$5.0 \times 10^{-7} - 3.0 \times 10^{-5}$	2.0×10^{-7}	[Wang, Y. et al., (2015)]
Spectrofluorimetric method	$4.4 \times 10^{-8} - 1.0 \times 10^{-5}$	2.3×10^{-8}	**Present work**

3.6 Accuracy and precision of the method

To compute the accuracy and precision, the assays described under "general procedures" were repeated three times within the day to determine the repeatability (intra-day precision) and three times on different days to determine the intermediate precision (inter-day precision) of the method. These assays were performed for pharmaceutical formulation and serum samples. The results of this study are summarized in **Table 3**. The percentage relative standard deviation (%RSD) values were in range of (1.71-2.33%) (intra-day), (2.67- 3.22%) (inter-day), for pharmaceutical formulation and serum samples indicating high precision of the method. Accuracy was evaluated as percentage relative error (RE) between the measured mean concentrations and the taken concentrations of drug. Bias {bias % = [(Concentration found - known concentration) x 100 / known concentration]} was calculated at each concentration and these results are also presented in **Table 3**. Percent relative error (%RE) values were in range of (1.3-2.1 %) (intra-day) and (2.7- 3.0%)(inter-day) for pharmaceutical formulation and serum samples demonstrates the high accuracy of the proposed method.

Table 3: Evaluation of intra-day and inter-day accuracy and precision

Sample	Norepi nephrin e taken*	Intra-day accuracy and precision (n=3)			Inter-day accuracy and precision (n=3)		
		Norepinep hrine Average Found* ±CL	%RE	%RS D	Norepinephrine average found*±CL	%R E	%RSD
Norepinephri ne bitartrate	1.0	1.013 ±0.07	1.3	1.71	1.027±0.22	2.7	2.67
Serum sample	1.0	1.021±0.20	2.1	2.33	1.030±0.34	3.0	3.22

The values are mulitiplied by 10^{-7} mol L^{-1} for method. RE, Percent relative error, %RSD, relative standard deviation and CL, Confidence limits were calculated from: CL = ± tS/√n. (The tabulated value of t is 4.303, at the 95% confidence level; S = standard deviation and n = number of measurements

3.7 Analytical application. Determination of Norepinephrine in different samples

The analytical utility of the proposed spectroflourimetric method was tested by measuring the concentration of Norepinephrine in pharmaceutical formulation (Norepinephrine bitartrate) and serum samples. The results obtained are summarized in **Table 4**. Good agreement between the average values obtained by the developed procedure (94-108%, RSD 1.98-2.34%) and the standard method (99.33-99.78, RSD 1.19-1.23%) **[British Pharmacopoeia, (1999)]** and no significant differences between the two methods. A comparison between the values of mean for the samples using the standard method with that obtained by the developed method revealed no significant differences between the two methods.

Table 4: Determination of (Norepinephrine) in pharmaceutical preparation and serum samples using photo probe

Drug	Taken (x 10⁻⁷ M)	Found (x 10⁻⁷ M)	Average *	Average recovery ± R.S.D. (%)	B.P. (LC)
Norepinephrine bitartrate *Pfizer* Co	1.0	1.12, 1.13, 0.99	1.08	108 ± 1.98	99.33± 1.19
Serum sample	1.0	0.87, 1.05, 0.89	0.94	94 ± 2.34	99.78± 1.23

*Average of three measurements.

4. Conclusion

The developed method provides an excellent selectivity for Norepinephrine at pH 5.01. The method is sensitive and provides a wide linear dynamic range of Norepinephrine concentrations. By measuring the fluorescence intensity of the photo probe under the optimal conditions. A detection limit of (2.3×10^{-8} mol L^{-1}) was achieved. The interference caused by other amines and drugs analogue for Norepinephrine is minimized in the developed method compared to the reported methods

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الملخص العربي

تعيين تركيز عقار النورابينفرن فى الصورة الدوائية لة وفى عينات الدم المختلفة

باستخدام متراكب الازوسيزول

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1. قسم الكيمياء- كلية البنات- جامعة عين شمس
2. قسم الكيمياء ـ كلية العلوم- جامعة عين شمس

يتنـاول اسـتحداث طريقـة ضـوئية جديـدة باسـتخدام متراكـب الازوسـيزول الـذائب فـي مـذيب الايثـانول وعنـد الاس الهيدروجينى 5.01 لتعين عقار النورابينفرن وتم دراسة طيف الانبعاث الخاص بهذا المتراكب مـع تركيزات مختلفة من النورابينفرن ورسم علاقة بينهم ووجد انها علاقة طردية فى المـدي التركيزى ($4.4x10^{-8}$ - $1x10^{-5}$) مولارى وأقل حد للاستجابة ($2.3x10^{-8}$) مولارى كما تم دراسة ايضـا تـاثير الاس الهيدروجينى ــ نـوع المـذيب المستخدم- نسبة تداخل هذا العقار مع الامينات والعقاقير الاخرى— والنسبة المؤية لاسترجاع والثبـات وعمل تطبيقات لهذا العقار في الصورة الدوائية لة و بعض عينات الدم ومقارنة هذة الطريقة بالطريقة القياسية .