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NEW VALIDATED SPECTROFLUORIMETRIC AND SPECTROPHOTOMETRIC METHODS FOR THE DETERMINATION OF CLOPIDOGREL IN PHARMACEUTICAL PREPARATIONS USING EOSIN

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ABSTRACT

New Sensitive and accurate two spectroscopic methods were developed and validated for the determination of Clopidogrel **(CLP)** in tablet dosage forms. The spectrophotometric method (Method I) is based on the formation of red colored complex with eosin at (545 nm) in sodium acetate – acetic acid buffer of pH 3. The absorbance-concentration plot is rectilinear over the range (0.5-9 μg mL^{-1}) with LOD of (0.076 μg mL^{-1}) and LOQ of (0.23 μg mL^{-1}). The Spectrofluorimetric method (Method II) depends on the quantitative quenching effect of Clopidogrel on the native fluorescence of eosin at the same pH. The quenching effect of the formed ion-pair complex of the drug with eosin was measured at (560 nm) after excitation at (499 nm). The fluorescence-concentration plot is rectilinear over the range (0.2-6 μg mL^{-1}) with LOD of (0.0341 μg mL^{-1}) and LOQ of (0.1033 μg mL^{-1}). Under the optimized experimental conditions, the proposed methods were validated as per International Conference of Harmonization guidelines. The proposed methods were perfectly applied to the analysis of commercial tablets containing the drug. Statistical comparison of the results with those of the reference method illustrate good agreement and confirm that there were no significant difference in the accuracy and precision between the proposed and reference one respectively.

Keywords: Clopidogrel **(CLP)**, eosin, Spectrofluorimetric, Spectrophotometric, Tablet dosage form.

INTRODUCTION

Clopidogrel **(CLP)** ((+)-(S)-methyl 2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)acetate; Fig 1)(1,2,3) is an oral, thienopyridine-class antiplatelet agent used to inhibit blood clots in coronary artery disease, peripheral vascular disease, cerebrovascular disease, and to prevent myocardial infarction (heart attack) (4).The analytical methods published for its determination include use of Spectrophotometry (5-6-7-8-12),Spectrophotometry with Amlodipine (13-14),with Metoprolol (15) and with Rosuvastatin calcium (16),Derivative spectrophotometry (9-10), Spectrophotometric and Spectrodensitometric (11), RP-HPLC Method (17-18-19-20), RP-Ultra Fast Liquid Chromatography with Aspirin (21), UV-Spectroscopic and Liquid Chromatography for resolution of ternary mixture of Aspirin ,Atorvastatin and Clopidogrel (22) , RP-HPLC with Amlodipine (23,24) ,with Rivaroxaban (25), GC-MS (26) and Potentiometry (27,28).

Up till now, nothing has been published concerning the analysis of **(CLP)** depend on it's quenching effect on fluorescence of eosin hence, our target was to create and validate simple and sensitive spectrophotometric and Spectrofluorimetric methods for the determination of **(CLP)** in pharmaceutical preparations. The reagent used is available and inexpensive and its solution is stable for many weeks in refrigerator. The proposed methods depend on

measuring the absorbance value of the formed ion-pair complex between eosin and **(CLP)** at 545 nm (Method I) or measuring the quenching effect of **(CLP)** on eosin at 560 nm after excitation at 499 nm (Method II). Both methods were applied for analysis of **(CLP)** in tablets with satisfactory results.

MATERIALS AND METHODS

Experimental Apparatus

- The Spectrophotometric measurements were carried out using Shimadzu UV-Visible 1601 recording Spectrophotometer (P/N 206-67001). Recording range, (0 - 1.0); wavelength (545 nm).
- The Spectrofluorimetric measurements were done using Perkin Elmer LS 45 Luminescence Spectrometer equipped with (150) Watt Xenon arc lamp and quartz cell (1cm).
- A consort NV P901 digital pH meter (Belgium) calibrated daily with standard buffer solutions was used for measuring the pH of the buffer solutions.

Materials

All materials and reagents were of analytical grade.
1- Clopidogrel **(CLP)** was kindly supplied by Eva Pharma Company, Cairo, Egypt.
2- Clopexagrel tablets each contain 75mg Clopidogrel **(CLP)** / tablet. Batch # CL 0149311 product of Marcyrl pharmaceutical industries El-Obour city, Egypt and it was obtained from local pharmacy**.**

Reagents

- Eosin (Riedel-De-Haen AG-D-3016 Seize 1) $4x10^{-3}$ M aqueous solution and (1×10^{-4} M) aqueous solution.
- Acetate buffer solution (0.4M) was prepared by mixing appropriate volumes of (0.4M) sodium acetate and (0.4M) acetic acid, pH adjusted to pH 3.
- Concentrated sulfuric acid H_2SO_4 (assay: 99% purity).

Preparation of stock and standard solutions

A stock solution of **(CLP)** was prepared by dissolving 20.0 mg of Clopidogrel in 100.0 mL of acidified distilled water (add 1 ml conc. H_2SO_4 to each 100ml distilled water) and was further diluted with the same solvent as appropriate. The standard solution was stable for 4 weeks and available for use when kept in the refrigerator.

Construction of the Calibration Curves

a) Spectrophotometric method (Method I)

Volumes of **(CLP)** covering the working concentration range (0.5-9 µg mL^{-1}) were transferred into a series of 10 mL volumetric flasks and 1ml of tween 80 was added. 1.5 mL of $4x10^{-3}$ M eosin solution was added to each flask, 1ml of acetate buffer (pH 3), complete to the mark with acidified distilled water and mix well. The absorbance value of each flask was measured at 545 nm against an appropriate blank prepared simultaneously. The measured absorbance values were plotted *vs.* the final concentration in µg mL^{-1} to get the calibration curve. Alternatively, the regression equation was derived.

b) Spectrofluorimetric method (Method II)

Different volumes of **(CLP)** covering the range 0.2 - 6 µg mL^{-1} were transferred into a series of 10 mL volumetric flasks and 1ml of tween 80 was added. 1.7 mL of $1x10^{-4}$ M eosin was added to each flask, 1ml of acetate buffer (pH 3), complete to the mark with acidified distilled water and mix well. The fluorescence intensity of the prepared solution was measured at 560 nm after excitation at 499 nm. The difference in the fluorescence intensity (**Δ**F) was plotted vs the final concentration of the drug (µg mL^{-1}) to get the calibration graph. Alternatively, the regression equation was derived also.

Pharmaceutical Applications

Procedure for tablets:

Twenty tablets were weighed and well pulverized to get fine and homogenous powder. Exactly weighed quantity of the powdered tablets equivalent to 20.0 mg of **(CLP)** was transferred into a small conical flask and extracted with 3 × 25 mL of acidified distilled water. The extract was filtered into a 100 mL volumetric flask. The conical flask was washed with few mLs of acidified distilled water. The washings were passed into the same volumetric flask, then completed to the mark with the same solvent. Different volumes from stock solution covering the working concentration range were transferred into a series of 10 mL volumetric flasks. The procedure mentioned under "Construction of calibration graph" was exactly done. The nominal content of the tablets was determined either from the previously plotted calibration graph or using the corresponding regression equation.

RESULTS AND DISCUSSION

Eosin has been utilized for the determination of many pharmaceutical compounds of interest through Spectrophotometric and Spectrofluorimetric Determination of Mebeverine Hydrochloride (29) and for the Determination of Dothiepin Hydrochloride (33) .Only through spectrophotometric measurement such as Spectrophotometric Determination of Amlodipine and Nicardipine ((31) , Tizanidine and Orphenadrine (32), Spectrophotometric for determination of Olanzapine and Orphenadrine by ternary complex formation with eosin and lead (30) ,

Spectrophotometric and Spectrofluorimetric determination of certain diuretics through ternary complex formation with eosin and lead (34).

The purpose of the present study was to develop simple and sensitive spectrophotometric and Spectrofluorimetric methods for the determination of **(CLP)** in its pharmaceutical formulations without prior extraction.

In this proposed study, **(CLP)** was found to form a stable ion-pair red complex with eosin at pH 3 with maximum absorbance at 545 nm **(Fig. 2)**. The formed complex is due to the electrostatic interaction between the studied drug and anionic functional group of eosin using acidic pH. The ion-pair complex which is formed due to this interaction is non fluorescent so, quenching of fluorescence of eosin increase on addition of increasing concentration of the drug. The Spectrofluorimetric quenching measurements were performed at 560 nm after excitation at 499 nm **(Fig. 3, 4)**.

Optimization of Experimental Conditions

The Spectrofluorimetric and Spectrophotometric properties of the developed complex as well as the different experimental parameters affecting its development and stability were carefully studied and optimized. Such factors were changed individually while the others were kept constant. These factors include; the pH, type of buffer, volume of buffer, volume of eosin and time of reaction, different type of organized media.

Addition of non ionic surfactants was found to be important to prevent complex precipitation and also enhance reproducibility of this method. Accordingly, the complex stability was achieved and precipitate formation was avoided with good precision.

A. Effect of pH

Complex formation between the studied drug and eosin mainly depend on pH, as it affects the ionization of eosin. The effect of pH of acetate buffer on the absorbance intensity was studied over the pH range 2.3–5. This study shows that absorbance intensity remain constant around pH 3. Above pH 3.2 the absorbance intensity starts to decrease gradually, so pH 3 is the optimum pH of choice **Fig. (5)**.

B. Effect of volume of acetate buffer

The volume of acetate buffer was studied from 0.5:2.5 ml. the absorbance intensity remain constant and show negligible change until 1.5 ml after which gradual slight decrease in absorbance occur. 1ml of acetate buffer was chosen as the optimum volume of buffer **Fig. (6)**.

C. Effect of volume of eosin

The optimum volume of the reagent was obtained by study different volumes from 0.2 to 3 ml of eosin solution. For Spectrofluorimetric method; it was found that 1.7 mL of eosin (1×10^{-4} M) was suitable to develop the maximum ΔF (fluorescence intensity of eosin-fluorescence intensity of the formed complex). For the Spectrophotometric method 1.5 mL of eosin (4×10^{-3} M) was enough to provide the maximum absorbance **Fig (7), Fig (8)**.

D. Effect of different organized media.

Different organized media are tested for the one which give the highest absorbance, 0.1%tween80 was found to be the most suitable surfactant **Fig (9).**

E. Effect of time of reaction

The effect of time on formation and stability of the formed complex was studied and it was found that in Spectrophotometric method (I): the complex is formed instantaneously and was found to be stable for 48 h without any turbidity, but in Spectrofluorimetric method (II): the quenching effect occur immediately and remain stable and constant for at least 15 min.

Analytical Performance

The absorbance-concentration plot was found to be linear over the range of 0.5-9.0 µg mL^{-1}. While the difference in the fluorescence (ΔF) - concentration plot was linear over the range of 0.1-6.0 µg mL^{-1}. Linear regression analysis of the data is found in Table 1. Linear regression analysis of the data provides the following equations:

$A = 0.039 + 0.094C$ (r=0.9999)

$\Delta F = 72.82 + 135.0C$ (r=0.9999)

Where A is the absorbance in 1-cm cell, C is the concentration of the drug (µg mL^{-1}), ΔF = the native fluorescence of eosin solution (F^o) - fluorescence of the resultant complex (F), and r is the correlation coefficient.

The limit of quantitation (LOQ) was calculated according to ICH Q2 Recommendation (35) by finding the lowest concentration that can be measured, below which the calibration curve is non-linear and was found to be 0.232 and 0.103 µg $mL^{-1}$for methods I and II respectively. LOQ was calculated from the following equation (2007): LOQ = 10 S_a / slope

The limit of detection (LOD) was also calculated according to ICH Q2 Recommendation (35) and was detected to be 0.0766 and 0.034 µg mL^{-1} for methods I and II respectively. LOD was calculated from the mentioned equation (2007): LOD = 3.3 S_a / slope

The proposed methods were assessed by computing the accuracy as percent relative error and precision as percent standard deviation (RSD %) (Table1).

Validation of the method

The proposed methods were validated for linearity, specificity, accuracy, precision & robustness.

Linearity:

Under the mentioned experimental conditions, the calibration graphs for the two methods were designed by plotting the absorbance value in method I or difference in the fluorescence intensity (ΔF) in method II vs concentration in µg mL^{-1}. The regression plots showed a linear correlation between ΔF or absorbance values and drug concentrations over the range present in Table 1. Regression equations, intercepts, slopes and correlation coefficients for the calibration data are presented in Table 1.

The validity of the method was evaluated by statistical assessment of the regression lines by finding standard deviation of the residual ($S_{y/x}$), standard deviation of the intercept (S_a) and standard deviation of the slope (S_b). The small values of the figures refer to the low scattering of the points around the calibration graphs and indicate that the method is highly precise. (Table 1)

Accuracy:

Statistical analysis of the data, obtained by the proposed and the reference methods for **(CLP)** using Student's t-test and variance ratio F-test, proves no significant difference between the performance of the two methods regarding the accuracy and precision respectively (Table 1).

Precision:

a. Repeatability

The repeatability was calculated from three replicate analysis of Clopidogrel under the complete analytical procedure. Intra-day precision of assays were done using (0.5, 3.0, 7.0 µg mL-1) for spectrophotometric method and using (0.1, 3.0, 6.0 µg mL-1) for quenching fluorimetric method, and the results are shown in (Table 2).

b. Intermediate precision:

It was done for the same concentrations mentioned under repeatability through repeated analysis of the drug in pure form for a period of three successive days (Table 2).

Robustness of the method

The robustness of the proposed methods were demonstrated by the resistance of the proposed methods to deliberate minor change in experimental parameters such as pH 3 ± 0.2 and change in the volume of eosin, (4×10^{-3} M), using 1.5 ± 0.2 mL. These minor changes that may happen during the experimental operation didn't greatly affect the accuracy and precision of the methods. The proposed methods were applied to the determination of the studied drug in its dosage forms including tablets, so the proposed methods were tested for specificity and accuracy for tablets.

Specificity

The specificity of the methods was examined by observing the effect of any interference that may occur from the common tablet exciepients, such as talc, lactose, starch and magnesium stearate. From the obtained results, these exciepients didn't interfere with the proposed method so the proposed methods were specific.

Accuracy

The results of the proposed methods were compared with those obtained using the reference method (6). Statistical analysis (36) of the results obtained using Student's t-test and variance ratio F-test showed no significant difference between the performance of the two methods regarding the accuracy and precision, respectively (Table 2).

Mechanism of the reaction

Using the limiting logarithmic method (1964), the stoichiometry of the reaction between Clopidogrel and eosin was studied. The absorbance intensity of the reaction product was alternatively measured in the presence of either eosin or **(CLP)**. Plots of log [eosin] **vs.** log A and log [**CLP**] **vs.** log A gave two straight lines, the slopes of the two plots were 1.09:1.24 for eosin: **(CLP)** respectively (Fig. 10). From this we can conclude that, the molar reactivity of the reaction is 1:1 for eosin: **(CLP)**. Based on the resulted molar ratio, a schematic proposal for the reaction pathway between **(CLP)** and eosin is seen in the following scheme:

CONCLUSION

The present two proposed methods are sensitive and selective for the determination of Clopidogrel without interference from common tablet exceipients. The present study is simple, rapid and inexpensive, so provide an economic method that can be applied for the routine quality control of the studied drug in its dosage forms. The use of water as diluting solvent is environmentally friendly; also the ion-pair formed is measured directly without need for prior extraction with organic solvent.

Table1: Performance data of the proposed methods.

Parameter	Spectrophotometric Method (I)	Spectrofluorimetric Method (II)	Ref. method (6)
- Concentration range (µg mL^{-1}).	0.5-9	0.1-6	40-70
- LOD (µg mL^{-1}).	0.0766	0.0341	
- LOQ (µg mL^{-1}).	0.2320	0.1033	
- Correlation coefficient (r).	0.9999	0.9999	
- Slope	0.0940	135.00	
- Intercept	0.0386	72.80	
- $S_{y/x}$	2.5×10^{-3}	1.95	
- S_a	2.2×10^{-3}	1.39	
- S_b	4×10^{-4}	0.415	
- % Error	0.298	0.482	
- % RSD	0.668	0.96	
- Mean found (%)	99.76	99.47	99.54
- ± SD.	0.67	0.96	0.27
- Student's t-value.	0.078 (2.57)	0.125 (2.57)	
- Variance ratio F-test.	4.93 (19.16)	12.99 (19.16)	
- Applications.	Tablets	Tablets	

Table 2: Validation of the proposed methods for the determination of (CLP) in pure form

Sample concentration	% recovery (repeatability)	% recovery Intermediate precision
Spectrophotometric method	99.60	102.00
0.5 µg mL^{-1}	100.00	99.60
	102.00	99.00
X`	100.53	100.20
± SD	1.29	1.59
%RSD	1.29	1.59
% Error	0.74	0.92
3 µg mL^{-1}	99.63	99.00
	99.66	98.60
	101.00	100.66
X`	100.10	99.42
± SD	0.78	1.09
%RSD	0.78	1.09
% Error	0.45	0.63
7 µg mL^{-1}	100.40	100.14
	100.57	99.71
	99.85	100.28
X`	100.27	100.04
± SD	0.38	0.30
%RSD	0.38	0.30
% Error	0.22	0.17

Sample concentration	% recovery (repeatability)	% recovery Intermediate precision
Spectrofluorimetric method	98.00	98.50
0.1 µg mL^{-1}	101.00	99.60
	99.00	100.00
X`	99.33	99.37
± SD	1.53	0.78
% RSD	1.53	0.78
% Error	0.89	0.45
3 µg mL^{-1}	100.33	101.00
	98.66	99.00
	99.63	99.66
X`	99.54	99.89
± SD	0.84	1.02
%RSD	0.84	1.02
% Error	0.49	0.59
6 µg mL^{-1}	100.16	99.80
	99.83	100.50
	100.33	99.66
X`	100.11	99.99
± SD	0.25	0.45
%RSD	0.25	0.45
% Error	0.15	0.26

Table 3 (a): determination of (CLP) in commercial tablets by Spectrophotometric method

Preparation	Spectrophotometric method		Ref. Method (6)	
	Amount taken (µg mL^{-1})	% Found	Amount taken (µg mL^{-1})	%Found
Clopexagrel tablets (75.0 mg (CLP) /Tablet)	3.00	99.41	40.00	99.84
	6.00	105.52	50.00	100.24
	9.00	99.78	60.00	99.89
X^{-} ± SD		99.90 ± 0.57	99.99 ± 0.22	
Student's t test		0.25		
Variance ratio F test		6.72		

Table 3 (b): Application of the proposed Spectrofluorimetric method for the determination of (CLP) in commercial tablets.

Preparation	Spectrofluorimetric method		Ref. Method (6)	
	Amount taken (µg mL^{-1})	% Found	Amount found (µg mL^{-1})	% Found
Clopexagrel tablets (75.0 mg (CLP) / Tablet)	0.20 1.00 3.00	98.80 100.34 99.97	40.00 50.00 60.00	99.84 100.24 99.89
X^{-} ± SD		99.70 ± 0.80	99.99 ± 0.22	
Student's t test		0.596		
Variance ratio F test		13.6		

The tabulated values of t and F are (2.78) and (19.00) respectively, at p = 0.05 (Miller, J. N.; Miller, J. C. 2005) (36).

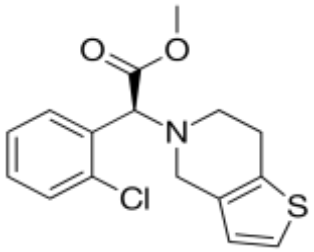


Figure 1: Structure of Clopidogrel

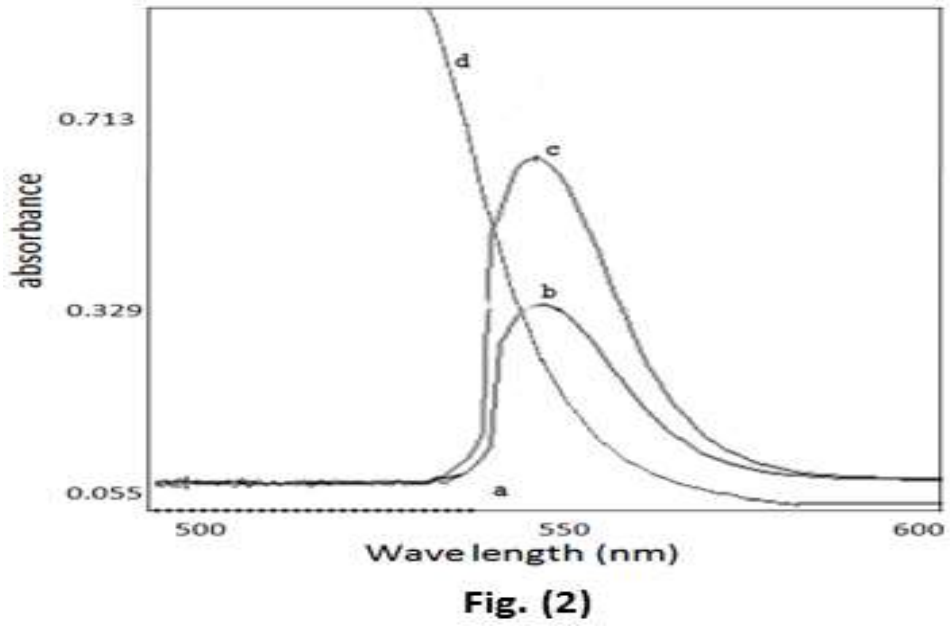


Figure 2: Absorption spectra of (a) Clopidogrel only (3 µg mL-1)
(b) Reaction product of Clopidogrel (3 µg mL-1) with (4×10-3M) Eosin at pH 3.
(c) Reaction product of Clopidogrel (6 µg mL-1) with (4×10-3M) Eosin at pH 3.
(d) Blank eosin (4 × 10-3M) at pH 3.

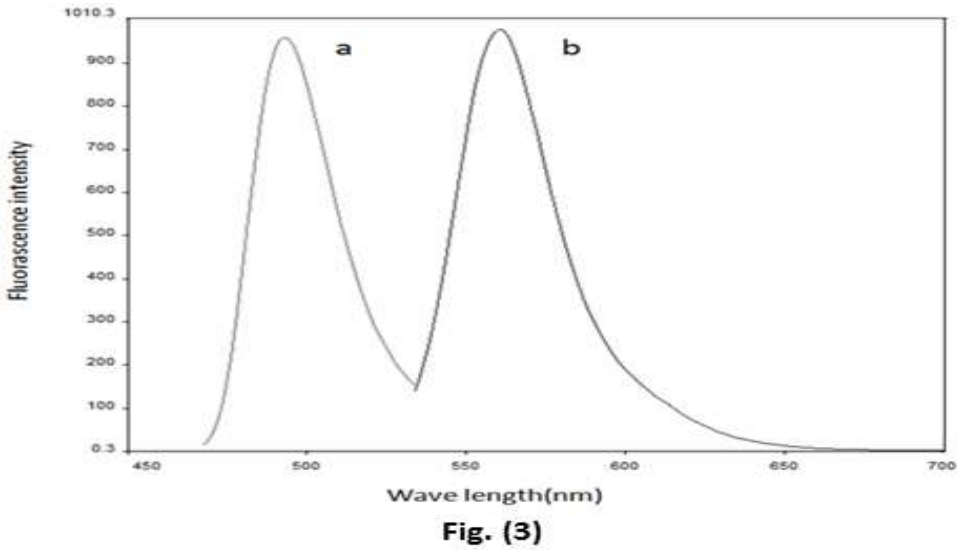


Fig. (3)

Figure 3: Excitation and emission spectra of: (a,b) Blank eosin (1×10-4 M) at pH 3

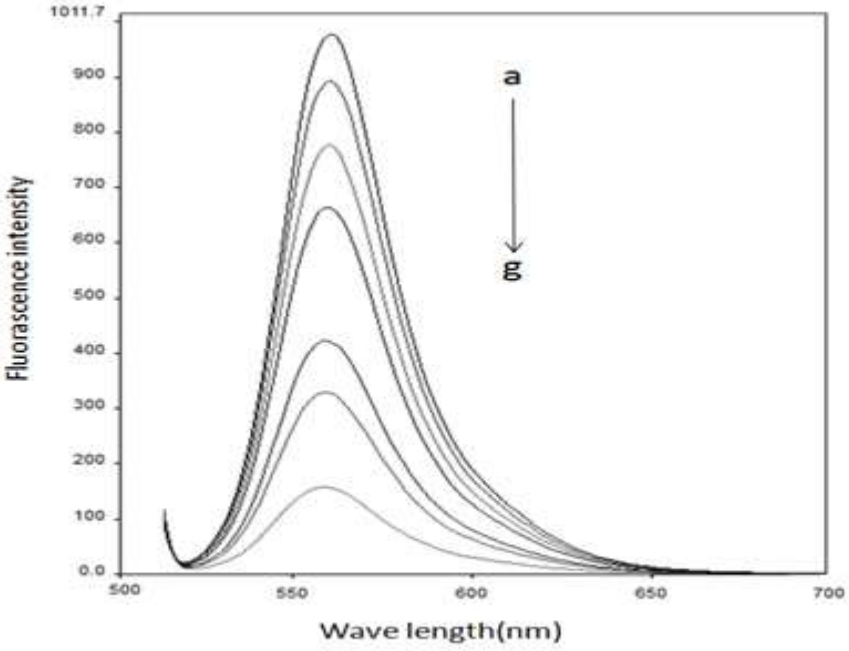


Fig. (4)

Figure 4:

(a) Emission spectra of blank eosin (1×10^{-4}M)

(b) (b:g) Reaction product of eosin (1×10^{-4}M) and **(CLP)** (0.2 – 0.5 – 1.0 - 2.0 - 3.0 - 5.0) μg mL^{-1} respectively.

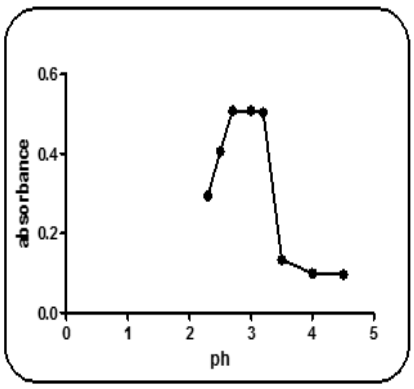


Fig (5)

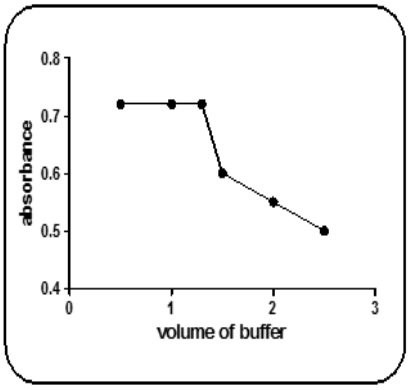


Fig (6)

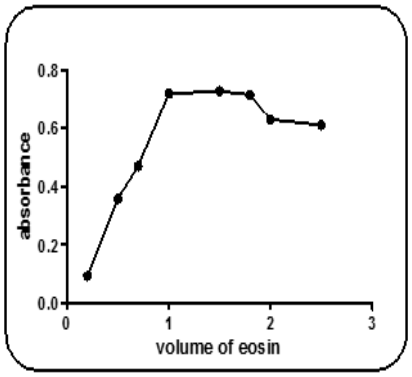


Fig (7)

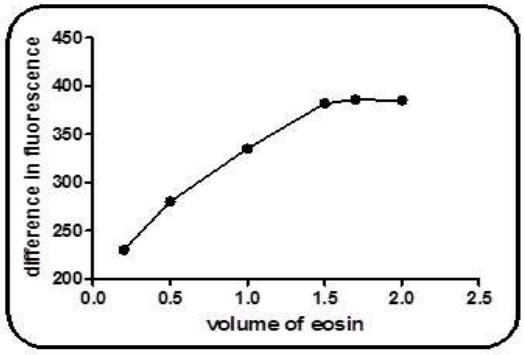


Fig. (8)

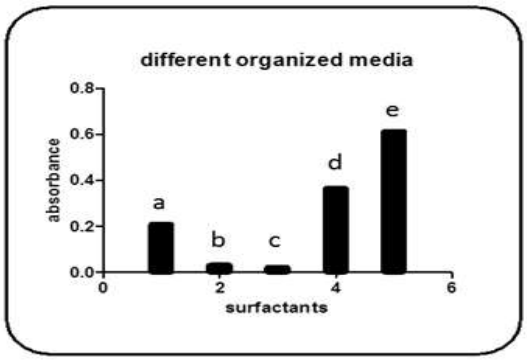
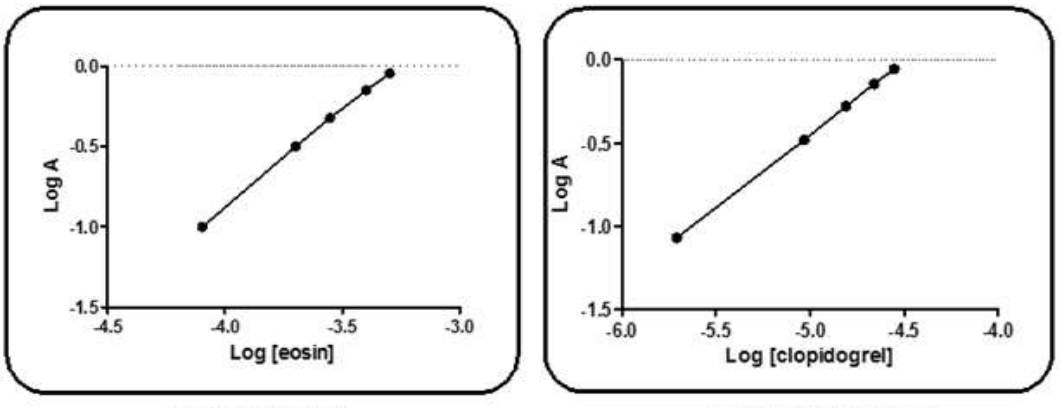


Fig. (9)

Fig (5): Effect of different pH of acetate buffer on formation of complex with **(CLP)** 6.0 µg mL^{-1}.
Fig (6): Effect of volume of acetate buffer pH 3 on formation of complex with **(CLP)** 6.0 µg mL^{-1}.
Fig (7): Effect of volume of eosin (4×10^{-3} M) on formation of complex with **(CLP)** 6.0 µg mL^{-1}**.**
Fig (8): Effect of volume of eosin (1×10^{-4} M) on formation of complex with **(CLP)** 1.0 µg mL^{-1}**.**
Fig (9): Effect of different organized media on formation of complex with **(CLP)** 6.0 µg mL^{-1}**.**where :a)0.3%tween80,b)0.3%sodium dodecyl sulphate,c)0.3%cetrimide,d)0.3%methyl cellulose,e)0.1%tween80 .

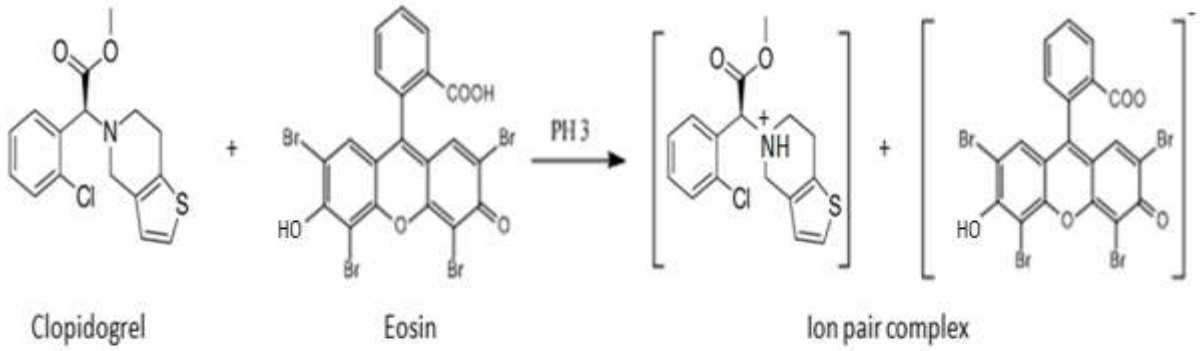


Log [eosin] vs log A.

Log [Clopidogrel] vs log A.

Fig. (10)

Fig. (10): Stoichiometry of the reaction between clop and eosin (4×10^{-3} M) using limiting logarithmic method.



Scheme 1: The proposal of the mechanism for the reaction between **(CLP)** and eosin.

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