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New Validated Fluorescence Quenching Based Procedure for the Determination of Cilostazol and Clopidogrel in Bulk, Tablets and Biological Fluids, With Application of Stern-volmer Equation

F. Ibrahim[1], M. Sharaf El-Din[1] and Heba Abd El-Aziz[1*]

[1]Department of Analytical Chemistry, Faculty of Pharmacy, University of Mansoura, 35516, Mansoura, Egypt.

Authors' contributions

This work was carried out in collaboration between all authors. Author FI designed the study and performed the statistical analysis. Author MSED managed the literature searches. Author HAEA managed the experimental analysis of the study. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: A very Sensitive, simple and selective Spectrofluorimetric method for the determination of Cilostazol or Clopidogrel through their reaction with fluorescein dye is developed. Determination was performed on raw material, tablet dosage form and extended for determination of cilostazol in spiked human plasma.
Study Design: Quenching reaction between fluorescein dye and tertiary amine containing drugs.
Place and Duration of the Study: This experiment was applied on analytical chemistry lab in faculty of pharmacy, analytical chemistry department, during the period (from 5 April to 10 May, 2016).
Methodology: The formed ion-pair complex between the reagent and each drug results in decrease in the fluorescence intensity of the reagent, this decrease (fluorescence quenching ΔF) is directly proportional to concentration of both drugs at the range of (0.001-0.08 μg ml^{-1}) and (0.01-

**Corresponding author: E-mail: dr_heba_abdelaziz@hotmail.com;*

0.50 µg ml^{-1}) for cilostazol (CIL) and Clopidogrel (CLP), respectively. For cilostazol (limit of detection) LOD is (0.0003µg mL-1) and (limit of quantitation) LOQ is (0.0008 µg ml^{-1}). For clopidogrel LOD is (0.0025 µg ml^{-1}) and LOQ is (0.0076 µg ml^{-1}). The affecting factors on fluorescence intensity of the product are carefully investigated and optimized. Using the optimized experimental conditions, the proposed quenching method is validated according to International Conference of Harmonization guidelines. The proposed method is perfectly applied to the analysis of commercial tablets containing each drug separately.

Results: Statistical comparisons of the results with those of the reference methods illustrate good agreement and confirm that there was no significant difference in the accuracy and precision between the proposed and reference one for both drugs, respectively. The proposed method is extended for the in vitro determination of CIL in spiked human plasma as preliminary investigation; the mean recovery (n=5) was 99.80.

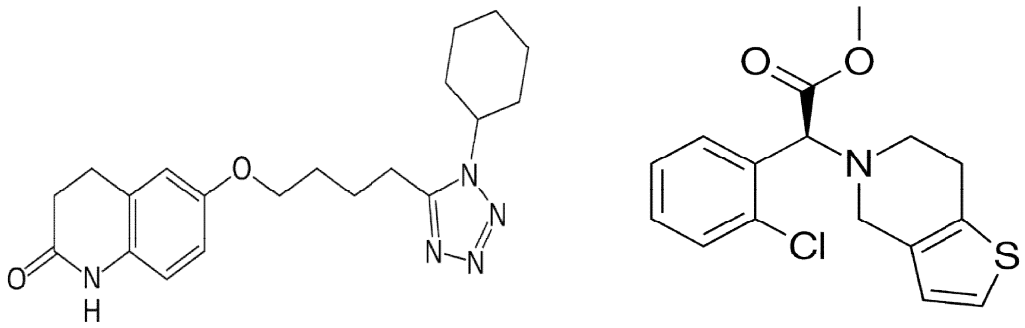
Conclusion: Owing to the simplicity, quickness and cheapness of the present study, it can be applied for the routine quality control of the studied drug in its dosage forms.

Keywords: Cilostazol (CIL); Clopidogrel (CLP); fluorescein; spectrofluorimetric; quenching; tablet dosage form.

1. INTRODUCTION

Cilostazol (CIL) (Fig. 1a); 6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1*H*)-quinolinone [1], is a quinolinone-derivative medication used in the mitigation of the symptoms of intermittent claudication in persons with peripheral vascular disease, It acts by inhibiting platelet aggregation and is a direct arterial vasodilator [2]. Clopidogrel (CLP) (Fig. 1b); ((+)-(S)-methyl 2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)acetate [1], is an oral, thienopyridine-class anticoagulant agent used to prevent blood clots in coronary artery disease, peripheral vascular disease, cerebrovascular_disease, and to limits myocardial infarction [2]. CIL is official in USP [3]. According to literature; few analytical methods for its determination were reported either alone or in combined form, including Spectrophotometry for cilostazol alone [4-7] and in combination with other drugs [8-10], HPLC Methods for cilostazol alone [11-14] and in combination with other drugs [15-16]. CLP is official in USP [3] and BP [17]. According to literature, many analytical methods were reported for its determination either alone or in combined form such as, Spectrophotometric determination of Clopidogrel alone [18-23] and in combination with other drugs [24-26], HPLC Methods for Clopidogrel alone [27-29] and in combination with other drugs [30-33] and Potentiometric determination [34-35].

As Cilostazol and Clopidogrel have a non fluorescent structure, our aim is to develop spectrofluorimetric quenching method by reaction with highly fluorescent reagent (fluorescein). A new Spectrofluorimetric quenching method is developed with high sensitivity, simplicity and



a. Cilostazol **b. Clopidogrel**

Fig. 1. The structural formulae of the studied drugs

quickness for the determination of CIL and CLP. Fluorescein can react with CIL or CLP to form a 1:1 complex by electrostatic attraction which leads to fluorescence quenching of fluorescein dye. The fluorescence quenching values ΔF is directly proportional to concentration of either drugs in certain ranges. The reagent used is available and inexpensive and its solution is stable for many weeks in refrigerator. The proposed quenching methods depend on measuring the ΔF value of the formed ion-pair complex between fluorescein and CIL or CLP at 515.7 nm after excitation at 440 nm Fig. 2. this method was applied for analysis of CIL and CLP in their tablets separately with satisfactory results. The high sensitivity of the method enables the determination of cilostazol in human plasma with high accuracy and precision.

1.1 Experimental Apparatus

- The Spectrofluorimetric measurements were done using Perkin Elmer LS 45 Luminescence Spectrometer equipped with (150) Watt Xenon arc lamp and quartz cell (1 cm). FL WINLAB, Version 4.00.02, Copyright 2001, Perkin Elmer, Inc. UK.
- A consort NV P901 digital pH meter (Belgium, Europe) calibrated daily with standard buffer solutions was used for measuring the pH of the buffer solutions.

2. MATERIALS

All materials and reagents were of analytical grade.

1- Cilostazol (CIL) pure sample (Batch # 061500080) was kindly provided by Sedico Pharmaceutical Company, Cairo, Egypt, and used as received. Its purity was determined by official method (3) 100.18%.
2- Clopidogrel (CLP) was kindly supplied by Eva Pharma Company, Cairo, Egypt. Its purity was determined by official method (3) 99.54%.
3- Claudol tablets (100mg of cilostazol) were manufactured by Sabaa International Company for Pharmaceuticals and Chemical Industries S.A.E. (M.O.H. Reg. No: 2440/2006) and it was obtained from local pharmacy.
4- 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.

2.1 Reagents

- Fluorescein (Merck, Germany) $2x10^{-5}$M aqueous solution. (Merck, Darmstadt, Germany).
  All other reagents were obtained from El-Nasr Pharmaceutical Chemical Company (ADWIC, Cairo, Egypt).
- Borate buffer solution (0.2M) was prepared by mixing appropriate volumes of (0.2M) boric acid and (0.2M) sodium hydroxide, pH adjusted to pH 7.
- Concentrated sulfuric acid H_2SO_4 (assay: 99% purity).
- Acetic acid (assay: 96% purity).

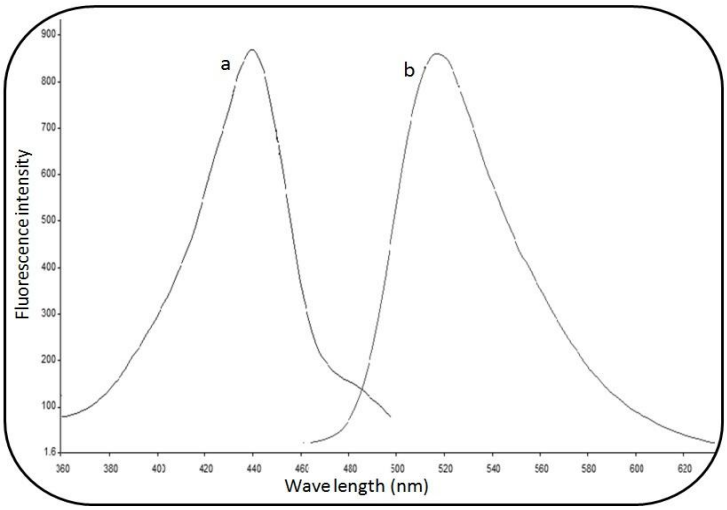


Fig. 2. Excitation and emission spectra of: (a,b) blank fluorescein ($2×10^{-5}$ M) in water

2.2 Preparation of Stock and Standard Solutions

A stock solution of (CIL) was prepared by dissolving 10 mg of Cilostazol in acetic acid: water (50:50), for (CLP) 10 mg of Clopidogrel was dissolved in 100.0 ml of acidified distilled water (add 1 ml conc. H_2SO_4 to each 100 ml distilled water) with the aid of an ultrasonic bath. Working standard solutions were prepared by suitable dilution of the stock solutions with distilled water. The standard solutions were stable for 2 months and ready for use when kept in the refrigerator.

2.3 Construction of the Calibration Curve

2.3.1 For cilostazol

To 10 ml volumetric flasks 1.2 ml of $2x10^{-5}$ M fluorescein solution was added, 1 ml of borate buffer (pH 7), then aliquots of Cilostazol covering the working concentration range (0.001-0.08 µg ml^{-1}) were added, complete to the mark with distilled water and mix well. The fluorescence intensity of the prepared solution was measured at 515.7 nm after excitation at 440 nm against an appropriate blank prepared simultaneously. The difference in the fluorescence intensity (ΔF) was plotted vs. the final concentration of the drug (µg ml^{-1}) to get the calibration graph. Alternatively, the regression equation was derived also.

2.3.2 For clopidogrel

To 10 ml volumetric flasks 1.4 ml of $2x10^{-5}$M fluorescein solution was added, 0.7 ml of borate buffer (pH 7), then aliquots of Clopidogrel covering the working concentration range (0.01-0.5 µg ml^{-1}) were added, complete to the mark with distilled water and mix well. The fluorescence intensity of the prepared solution was measured at 515.7 nm after excitation at 440 nm against an appropriate blank prepared simultaneously. The difference in the fluorescence intensity (ΔF) was plotted vs. the final concentration of the drug (µg ml^{-1}) to get the calibration graph. Alternatively, the regression equation was derived also.

2.4 Pharmaceutical Applications

2.4.1 Procedure for tablets

Twenty tablets of each drug separately were weighed and well pulverized to get fine and homogenous powder. Accurately weighed quantities from the powdered tablets equivalent to 10.0 mg of Cilostazol and Clopidogrel were separately transferred into a small conical flask and extracted with 75 ml 50% acetic acid in case of Cilostazol and acidified distilled water in case of Clopidogrel. For each drug separately, the extract was filtered into a 100 ml volumetric flask. The conical flask was washed with few mls of the dissolving solvent. The washings were transferred through the filter paper into the same volumetric flask, and then completed to the mark with the same dissolving solvent. The procedure stated under "Construction of calibration graph" was exactly done by adding different volumes of stock solution covering the working concentration range to a series of 10 ml volumetric flasks containing the specified volume of reagent and buffer. The nominal content of the tablets were determined either from the previously plotted calibration graph or by using the corresponding regression equation.

2.5 Procedure for Cil Determination in Spiked Human Plasma

Aliquots of human plasma (1 ml) spiked with increasing concentrations of CIL were transferred into 10.0 ml screw-capped centrifugation tubes. Methanol was added to each tube so that the final volumes were the same in all tubes. After vortex mixing for 2 min., the mixtures were centrifuged at 3000 rpm for 30 min. at room temperature. The upper supernatant layers were carefully aspirated from the precipitate and the resulting solutions were filtered using syringe filters. Aliquots of 1 ml were transferred into series of 10 ml volumetric flasks containing 1.2 ml fluorescein, add 1 ml borate buffer (pH 7) and complete to mark with distilled water. A blank experiment was performed simultaneously. The difference in the fluorescence intensity (ΔF) was plotted *versus* the drug concentration in µg /ml.

3. RESULTS AND DISCUSSION

Fluorescein and eosin are dyes with similar chemical structures and both are used for imaging of cellular components. Despite there are many reports for the use of eosin for determination of many drugs through fluorescence, fluorescein was not widely used in this field. This initiates the present study for the determination of Cilostazol and Clopidogrel. Some published papers based on quenching effect of drugs on fluorescein were determination

of some cephalosporins in pharmaceutical formulations [36], bis(2-chloroethyl) sulide [37], Spectrofluorometric determination of Felodipine [38], penicillamine [39], torasemide [40] and spectrofluorimetric quenching method for determination of trandolapril [41] were presented.

The aim of the present study was to develop simple and very sensitive Spectrofluorimetric method for the determination of (CIL) and (CLP) separately in their pharmaceutical formulations.

In this proposed study, stable ion-pair complexes between fluorescein and Cilostazol or Clopidogrel were formed at pH 7. The formed complexes are due to the electrostatic interaction between the studied drugs and anionic functional group of fluorescein at neutral pH. The ion-pair complexes which are formed due to such interaction are non fluorescent so, quenching of fluorescein increases on addition of increasing concentration of each drug. The Spectrofluorimetric quenching measurements were performed at 515.7 nm after excitation at 440 nm Figs. 3, 4.

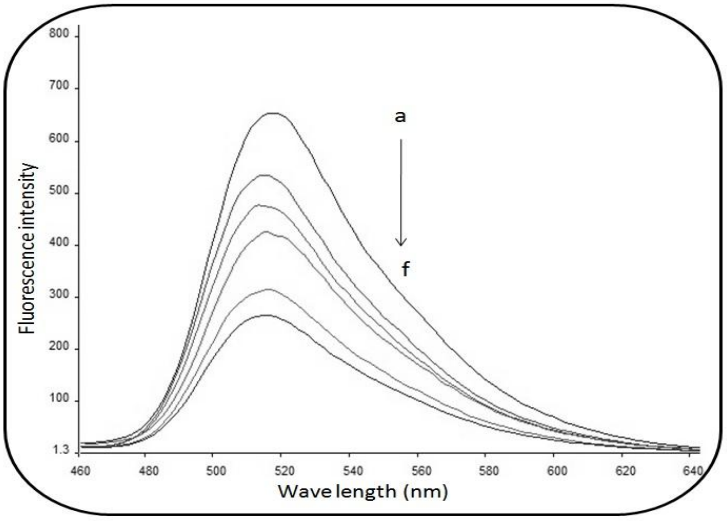


Fig. 3. Emission spectra of blank fluorescein 1.2 ml of (2×10^{-5}M) (b:f) reaction product of fluorescein (2×10^{-5}M) and (CIL) (0.001-0.08) µg mL^{-1} respectively

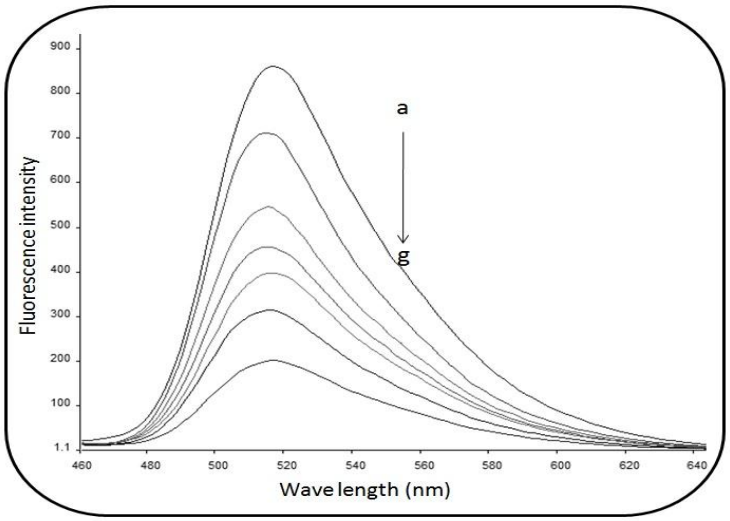


Fig. 4. (a) Emission spectra of blank fluorescein 1.4 ml of (2×10^{-5}M) (b:g) reaction product of fluorescein (2×10^{-5}M) and (CLP) (0.01-0.5) µg mL^{-1} respectively

3.1 Optimization of Experimental Conditions

The Spectrofluorimetric properties of the developed ion pair 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 fluorescein, temperature, time of reaction, different type of organized media.

3.1.1 Effect of volume of fluorescein

By studying different volumes from 0.2 to 2 ml of (2×10^{-5} M) fluorescein solution, the optimum volume of the reagent was obtained. It was found that 1.2 ml and 1.4 ml of fluorescein were suitable for formation of the complex and development of quenching value (ΔF) of fluorescein upon its reaction with Cilostazol and Clopidogrel, respectively Fig. 5a, 5b.

3.1.2 Effect of PH

Acetate buffer over the pH range 3.5-5.5 was studied. It was found that no quenching effect of the fluorescence of fluorescein was observed moreover; such acidic pH values make a sharp decrease in the fluorescence of the reagent itself. Borate buffer over the pH range 6-10 was studied. It was found that increasing pH values in the studied range result in gradual increase in the quenching effect on fluorescein (ΔF) up to pH 7, after which a slight decrease in the quenching effect was observed. At pHs more than 8.5, there was no quenching effect as the complex between drug and reagent was decomposed giving the reagent in non combined form which give very high fluorescence intensity the same as blank, so pH 7 is the optimum pH of choice Fig. 6.

3.1.3 Effect of volume of borate buffer

The volume of borate buffer was studied from 0.2:2.0 ml. It was observed that the quenching effect of both drugs on fluorescence intensity of fluorescein increased by increasing the volume of buffer to 1.2 ml. At higher values, a slight decrease in ΔF was observed. 1 ml is the optimum volume of buffer for Cilostazol and 0.7 ml for Clopidogrel Fig. 7.

3.1.4 Effect of different surfactants

Different surfactants were used; the results were satisfactory without using surfactant. For example, using SDS (0.1M) makes a sharp quenching for the reagent and shift emission wave length to 523 nm. Using tween80 (0.1%) makes a slight quenching of the reagent and intensifies the quenching effect of both drugs on the reagent. Generally, surfactants tend to disrupt ground state complex formation which lead to a minimum concentration range linearity and the results become non quantitative.

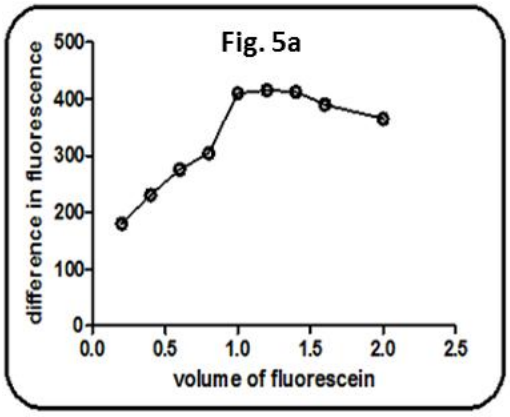


Fig. 5a. Effect of volume of fluorescein on fluorescence quenching intensity using (0.005 ug/ml) of cilostazol

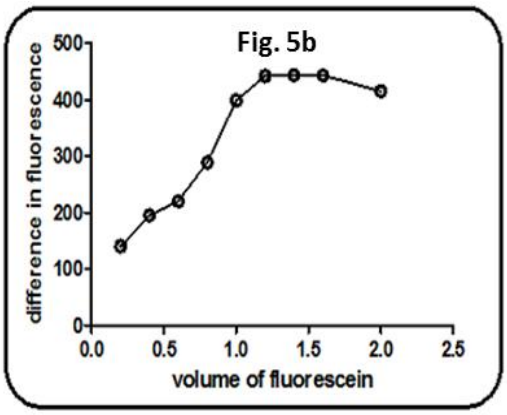


Fig. 5b. Effect of volume of fluorescein on fluorescence quenching intensity using (0.1 ug/ml) of clopidogrel

3.1.5 Effect of time on reaction

Quenching effects of Cilostazol and Clopidogrel on fluorescein occur immediately and remain stable and constant for at least 20 min for Clopidogrel then ΔF gradually decrease, but in case of Cilostazol the complex remain stable for 35 min or more.

3.1.6 Effect of diluting solvent

Maximum fluorescence quenching was obtained upon diluting in water which adds a great advantage to the proposed method.

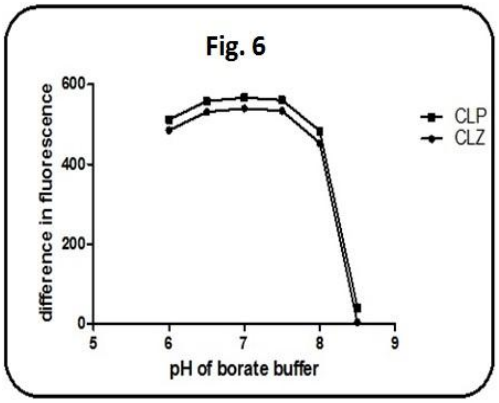


Fig. 6. Effect of pH of borate buffer on fluorescence quenching intensity of both cilostazol (0.005 ug/ml) and clopidogrel (0.1 ug/ml)

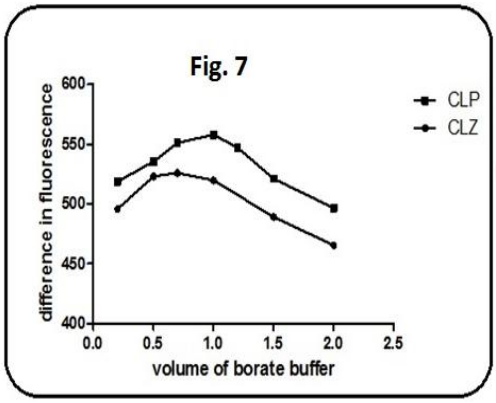


Fig. 7. Effect of volume of borate buffer on fluorescence quenching intensity of both cilostazol (0.005 ug/ml) and clopidogrel (0.1 ug/ml)

3.2 Analytical Performance

A plot of the difference in the fluorescence (∆F) and concentration was found to be linear over the range of 0.001-0.08 µg ml^{-1} and 0.01-0.5 µg ml^{-1} for Cilostazol and Clopidogrel, simultaneously. Linear regression analysis of the data is found in Table 1. Linear regression analysis of the data provides the following equations:

∆F = 52.01+ 5340.04C (r=0.9999) → For Cilostazol

∆F = 83.05+ 602.87C (r=0.9999) → For Clopidogrel

Where ∆F (difference in fluorescence) = the native fluorescence of fluorescein solution (F^o) - fluorescence of the formed ion pair complex (F).

C is the concentration of Cilostazol or Clopidogrel and r is the correlation coefficient.

The limit of quantitation (LOQ) was calculated according to ICH Q2 Recommendation [42] by finding the lowest concentration that can be measured, below which the calibration curve is non-linear and was found to be 0.0008 and 0.0076 µg $ml^{-1}$for Cilostazol and Clopidogrel, 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 [42] and was detected to be 0.0003 and 0.0025 µg ml^{-1} for Cilostazol and Clopidogrel, respectively. LOD was calculated from the stated equation(2007):

$$LOD = 3.3\ S_a / slope$$ (where S_a is the slope of intercept).

Evaluation of the proposed quenching method was done by calculating the accuracy as percent relative error and precision as percent relative standard deviation (RSD %) (Table1).

3.3 Validation of the Method

The proposed method was validated for linearity, specificity, accuracy, precision & robustness.

3.3.1 Linearity

Under the mentioned experimental conditions, the calibration graphs for the two drugs were designed by plotting difference in the fluorescence intensity (∆F) vs. concentration in µg ml^{-1}. The regression plots showed a linear correlation between ∆F and drugs concentration over the range present in Table 1. Regression equations, intercepts, slopes and correlation coefficients for the calibration data are illustrated in (Table 1).

Table 1. Performance data of the proposed quenching methods showing high accuracy in relation to reference methods

Parameter	Proposed method		Reference method (3)	Reference method (19)
	Cilostazol	Clopidogrel	Cilostazol	Clopidogrel
- Concentration range (μg mL^{-1}).	0.001-0.08	0.01-0.5	0.1-4	40-70
- LOD (μg mL^{-1}).	0.0003	0.0025		
- LOQ (μg mL^{-1}).	0.0008	0.0076		
- Correlation coefficient (r).	0.9999	0.9999		
- Slope	5340.03	602.87		
- Intercept	52.01	83.05		
- $S_{y/x}$	0.731	0.785		
- S_a	0.44	0.46		
- S_b	10.50	1.97		
- % Error	0.55	0.587		
- % RSD	0.95	1.31		
- Mean found (%)	99.66	100.34	100.18	99.54
± SD.	± 0.95	± 1.31	± 0.94	± 0.27
- Student's t-value.	0.76 (2.45)	0.9 (2.77)		
- Variance ratio F-test.	1.00 (19.25)	13 (19.00)		
- Applications.	Tablets	Tablets		

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 prove that the method is highly precise. (Table 1)

3.3.2 Accuracy

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

3.3.3 Precision

3.3.3.1 Repeatability

The repeatability was calculated from three replicate analyses of Cilostazol and Clopidogrel under the complete analytical procedure. Intra-day precision of assays were done using (0.005, 0.008, 0.05 μg ml^{-1}) for Cilostazol and using (0.01, 0.05, 0.1 μg ml^{-1}) for Clopidogrel, and the results are shown in (Table 2).

3.3.3.2 Intermediate precision

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

3.4 Robustness of the Method

The robustness of the proposed method was demonstrated by the resistance of the proposed method to neglicable minor change in experimental parameters such as pH 7 ± 0.2 and change in the volume of fluorescein, (2×10^{-5} M), using 1.2±0.2 ml for Cilostazol and 1.4±0.2 ml for Clopidogrel. These minor changes that may occur during the experimental operation didn't greatly affect the accuracy and precision of the method.

3.5 Validation for Tablets

The proposed method was applied for the determination of the two studied drugs in their dosage forms, separately, so the proposed method was tested for specificity and accuracy for tablets.

3.5.1 Specificity

The specificity of the method was determined by observing the influence of any interference that may occur from the common tablet excepients, such as talc, lactose, starch and magnesium stearate. The obtained results show that excepients didn't interfere with the proposed method so the proposed method was specific.

Table 2. Precision of the proposed quenching methods for the determination of (CIL) and (CLP) in pure form

Parameter	% recovery (repeatability)	% recovery intermediate precision
Cilostazol	100.20	100.50
0.005 µg mL^{-1}	99.50	100.15
	98.78	99.50
X`± SD	99.49	100.05
	0.71	0.51
%RSD	0.71	0.51
% Error	0.41	0.29
0.008 µg mL^{-1}	99.60	99.50
	99.99	99.70
	100.30	100.00
X`	99.96	99.73
± SD	0.35	0.25
%RSD	0.35	0.25
% Error	0.20	0.15
0.05 µg mL^{-1}	99.50	100.10
	100.30	99.54
	99.20	98.99
X`	99.67	99.54
± SD	0.57	0.56
%RSD	0.57	0.56
% Error	0.33	0.32
Clopidogrel	100.40	99.80
0.01 µg mL^{-1}	99.58	100.1
	99.72	99.30
X`	99.90	99.73
± SD	0.44	0.40
% RSD	0.44	0.41
% Error	0.25	0.23
0.05 µg mL^{-1}	99.45	99.70
	99.99	100.50
	100.13	100.10
X`	99.86	100.10
± SD	0.36	0.40
%RSD	0.36	0.40
% Error	0.21	0.23
0.1 µg mL^{-1}	100.12	99.20
	99.60	99.64
	99.70	100.30
X`	99.81	99.71
± SD	0.28	0.55
%RSD	0.28	0.55
% Error	0.16	0.32

3.5.2 Accuracy

The results of the proposed method were compared with those obtained using the reference methods [3,19]. Statistical analysis [43] 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 (Tables 3, 4).

3.6 Validation of CIL in Spiked Human Plasma

The proposed method was successfully applied for determination of CIL in spiked human plasma. That proved that the proposed method was of high sensitivity Fig. 8, the results of assay were summarized in (Table 5). Under the above mentioned experimental conditions, a linear relationship was obtained by plotting difference in the fluorescence intensity (∆F) vs. concentration in µg ml^{-1}. Linear regression analysis of the data yielded the following equation:

$$\Delta F = 334.59 + 12674.22C (r=0.9999) \quad (CIL)$$

Where: ∆F represents the difference in the fluorescence, C is the concentration of the drug in µg/mL and r refers to the correlation coefficient.

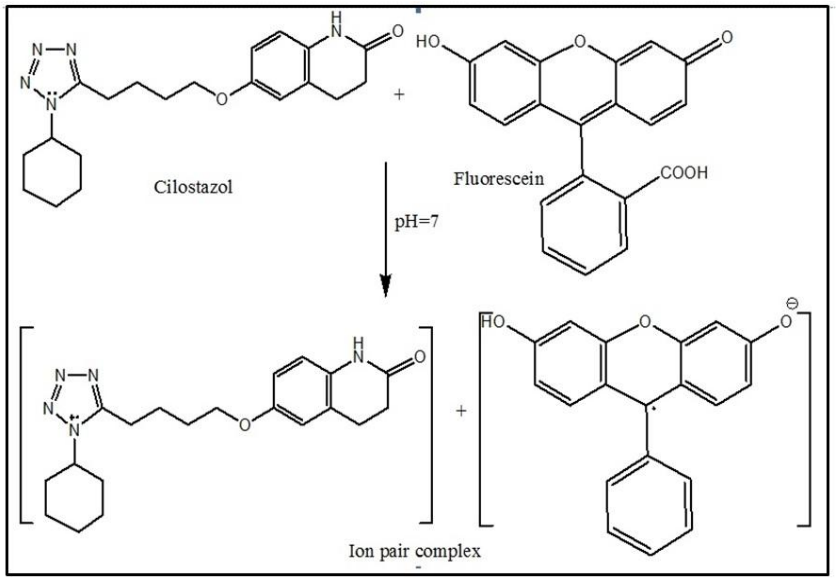
The high value of the correlation coefficient (r) indicates the good linearity of the calibration curve performed in human plasma.

3.7 Reaction Mechanism

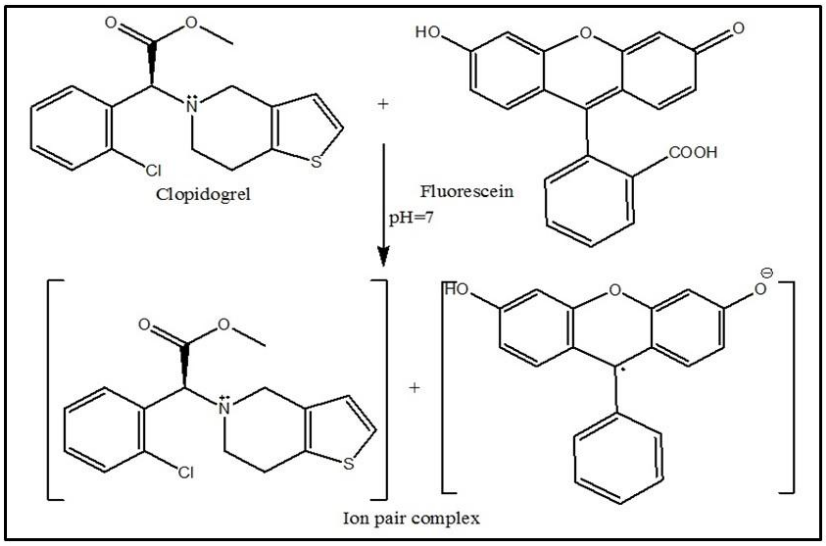
Using the limiting logarithmic method (1964), the stoichiometry of the reaction between Fluorescein and Cilostazol or Clopidogrel was studied. The ∆F (difference in the fluorescence) between fluorescein and the formed complex was alternatively measured in the presence of either fluorescein or (CIL) and fluorescein or (CLP), respectively. Plots of log [fluorescein] vs. log ∆F and log [CIL] or log [CLP] vs. log ∆F produce two straight lines, the slopes of the two plots were 0.45:0.56 for fluorescein: (CIL) respectively and 0.55: 0.41 for fluorescein: (CLP) respectively Figs. 9, 10. From this we can conclude that, the molar reactivity of the reaction is 1:1 for fluorescein and Cilostazol and for fluorescein and Clopidogrel Scheme (1 and 2).

3.8 Quenching Mechanism

The fluorescence quenching mechanisms usually contain either dynamic or static quenching, which are caused by diffusion and ground-state complex formation. In order to further clarify the fluorescence quenching mechanism induced by each drug, the Stern-Volmer equation [44-45] gets used to evaluate the results.



Scheme 1. The proposed mechanism of reaction between cilostazol and fluorescein



Scheme 2. The proposed mechanism of reaction between clopidogrel and fluorescein

Table 3. Determination of (CIL) in commercial tablets by proposed quenching method

Preparation	Cilostazol		Ref. method (3)	
	Amount taken (µg mL^{-1})	% Found	Amount taken (µg mL^{-1})	%Found
Claudol tablets	0.005	100.00	0.20	101.65
(100.0 mg	0.01	99.00	0.50	98.94
(CIL) /Tablet)	0.04	100.01	1.00	100.20
X̄± SD	99.67 ± 0.58		100.26 ± 1.36	
Student's t test	0.70			
Variance ratio F test	5.52			

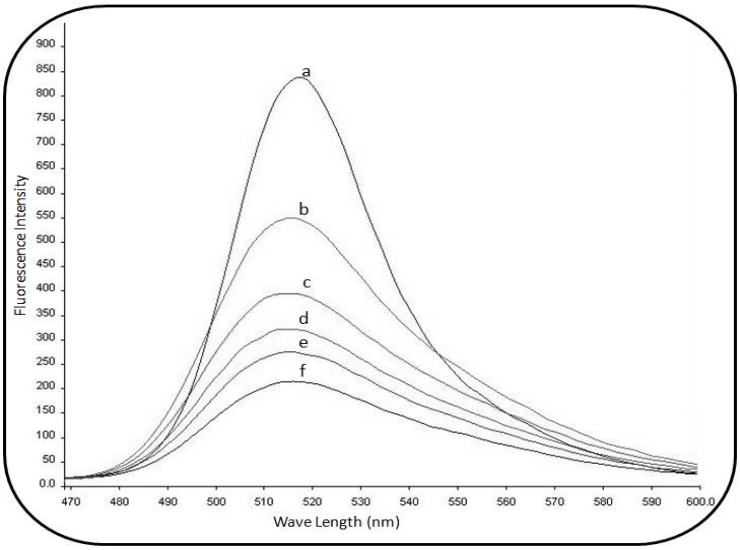


Fig. 8. Assay of CIL in spiked human plasma, where: (a) Emission spectra of blank fluorescein (2×10^{-5}M) (b:f) reaction product of fluorescein (2×10^{-5}M) and (CIL) (0.002-0.008-0.015-0.02-0.03) µg mL^{-1} in spiked human plasma

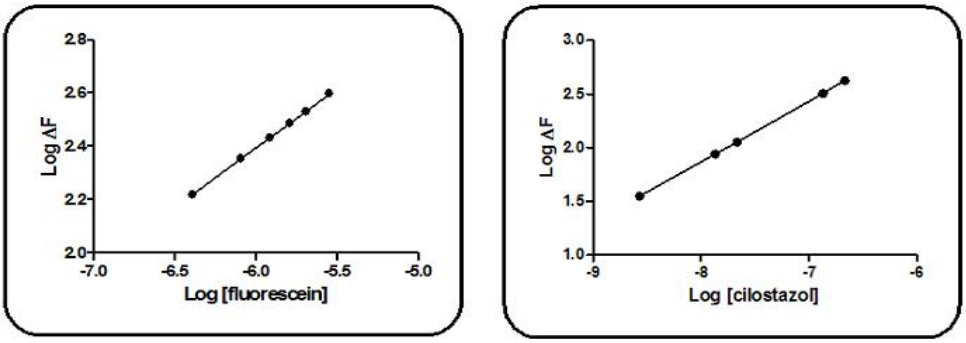


Fig. 9. Stoichiometry of the reaction between CIL and fluorescein (2×10^{-5}M) using limiting logarithmic method

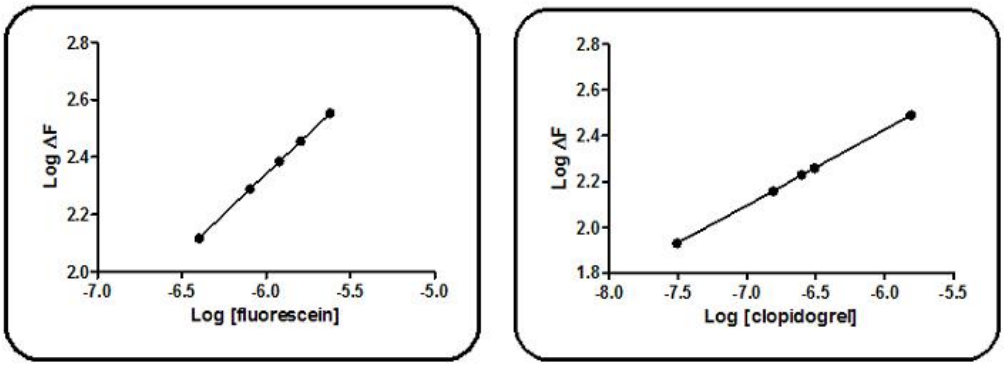


Fig. 10. Stoichiometry of the reaction between CLP and fluorescein (2×10^{-5}M) using limiting logarithmic method

Table 4. Determination of (CLP) in commercial tablets by proposed quenching method

Preparation	Spectrofluorimetric method		Ref. Method (19)	
	Amount taken (µg mL^{-1})	% found	Amount taken (µg mL^{-1})	% found
Clopexagrel tablets (75.0 mg (CLP) / Tablet)	0.05	98.60	40.00	99.84
	0.1	100.90	50.00	100.24
	0.3	99.93	60.00	99.89
$\bar{X}$ ± SD	99.81 ± 1.16		99.99 ± 0.22	
Student's t test	0.25			
Variance ratio F test	8.99			

The tabulated values of t and F are (2.78) and (19.00) respectively, at p = 0.05 (Miller, J. N.; Miller, J. C.) [43]

Table 5. Assay of (CIL) in spiked human plasma by proposed quenching method

Parameter	Amount taken (µg/mL)	Amount found (µg/mL)	% Found
	0.002	0.0020	100.00
	0.008	0.0078	97.50
	0.015	0.0150	101.33
	0.02	0.0200	100.50
	0.03	0.0290	99.67
$\bar{x}$		99.80	
± SD		1.43	
% RSD		1.43	
% Error		0.640	

Table 6. Stern-volmer quenching constants for the CIL-fluorescein and CLP-fluorescein systems

System	Temperature T/K	Stern-volmer equation (Q µmol L^{-1})	Correlation coefficient (r)	K_{sv} (L mol^{-1})	K_q (L mol^{-1} S^{-1})
CIL-Fluorescein	303	$F/F_0=0.99+6.03\times10^6[Q]$	0.9990	6.03×10^6	1.47×10^{15}
	323	$F/F_0=0.95+4.0\times10^6[Q]$	0.9995	4.0×10^6	0.97×10^{15}
CLP-Fluorescein	303	$F/F_0=1.05+5.2\times10^5[Q]$	0.9979	5.2×10^5	1.20×10^{14}
	323	$F/F_0=1.02+3.4\times10^5[Q]$	0.9995	3.4×10^5	0.83×10^{14}

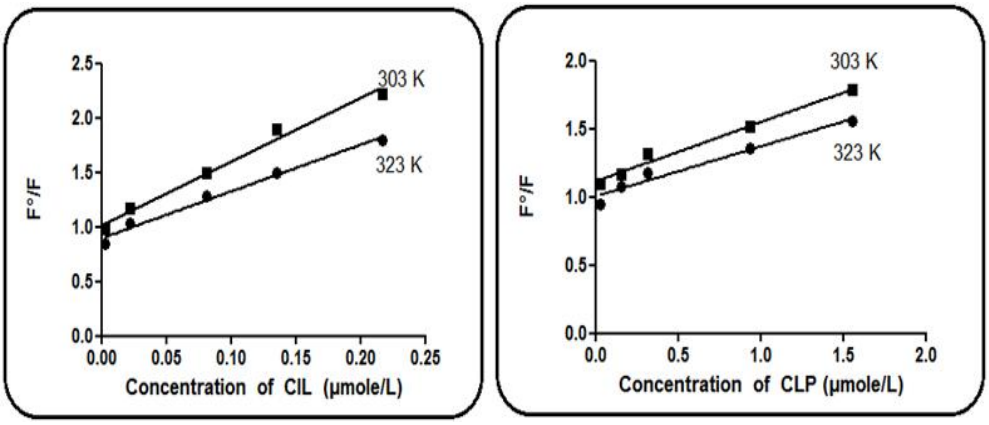


Fig. 11. The Stern-Volmer plots of CIL-Fluorescein and CLP-Fluorescein systems at different temperatures

$$\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + K_q \tau_0 [Q]$$

Where F_0 and F represent the steady-state fluorescence intensities in the absence and presence of the quencher, [Q] is the concentration of quencher, K_{sv} is stern volmer constant, K_q is the quenching rate constant and τ_0 is the average lifetime of the molecule without any quencher which in case of fluorescein equal 4.1 ns [46]. Fig. 11 Shows stern-volmer plot at two different temperatures with intercept equal 1. Kq is equal to Ksv divided by $\tau 0$. (Table 6) shows that the minimum value for quenching rate constant was (0.84×10^{14} L mol^{-1} S^{-1}) which is much greater than the maximum quenching rate constant (2×10^{10} L mol^{-1} S^{-1}). The results indicate that the reaction between both of our drugs and fluorescein is static quenching mechanism.

4. CONCLUSION

The present proposed procedure is sensitive and selective for the determination of both studied drugs without interference from common tablet excipients. Owing to the simplicity, quickness and cheapness of the present study, it 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 adds a great advantage to the proposed method; also the ion-pair formed is measured directly without need for prior tedious extraction processes with organic solvent.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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