Malaysian Journal of Chemistry, 2026, Vol. 28(1), 313-325
(Special Issue: *12th International Conference of the College of Science & 3rd Conference of Chemistry Department 2025*)

Sensitive and Accurate Determination of Clopidogrel in Pharmaceutical Preparations Using Spectrophotometric, Microfluidic and Paper-Based Colorimetric Sensor Techniques

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Clopidogrel, a widely used antiplatelet agent, requires reliable, cost-effective analytical methods for quality control. This study presents the development and validation of three new methods: spectrophotometric, microfluidic-based laser detection, and paper-based microfluidic devices (µPADs) for the analysis of clopidogrel in pharmaceutical formulations. All methods are based on the formation of a colored ion-pair complex between clopidogrel and acid alizarin black, exhibiting maximum absorbance at 512 nm. The reaction conditions, including reagent concentration, temperature, and time, were optimized, and a 1:1 drug-to-reagent stoichiometry was confirmed. The microfluidic system, fabricated from poly (methyl methacrylate) (PMMA) using laser engraving, offered high accuracy and low sample consumption. Meanwhile, the µPADs provided a portable, instrumentation-free alternative, utilizing digital image analysis (ImageJ) to quantify color intensity. Methods validation showed linearity ranges of 1-12 ppm (microfluidic), 2-16 ppm (µPADs), and 2-20 ppm (UV-Vis), with detection limits of 0.5, 1, and 1.25 ppm, respectively. Recovery rates ranged from 98.89% to 99.64%, and R^2 values exceeded 0.994 for all methods. These results demonstrate that the proposed techniques are rapid, accurate, and suitable for routine analysis of clopidogrel in resource-limited and laboratory contexts.

Keywords: Microfluidic chip, PMMA, µPADs, Clopidogrel, diode laser (532 nm)

Received: September 2025; Accepted: October 2025

Clopidogrel, approved in 1998, has the chemical formula C16H16ClNO2S and is chemically identified as methyl (+)-(S)-α-(2-chlorophenyl)-6,7-dihydrothieno [3,2-c]pyridin-5(4H)-acetate [1]. Its molecular structure is illustrated in Figure 1. Clopidogrel is recognised as a highly effective and well-tolerated agent in the management of cardiovascular conditions, particularly due to its favourable safety profile and minimal side effects [2]. Numerous analytical techniques have been reported for the determination of clopidogrel, including proton nuclear magnetic resonance (^{1}H-NMR) [3], high-performance liquid chromatography (HPLC) [4], gas chromatography-mass spectrometry (GC-MS) [5], ultraviolet-visible (UV-Vis)[6] spectroscopy, and colorimetric methods [7]. Among these, several spectrophotometric approaches have been developed, such as visual spectrophotometry, which uses clopidogrel as a reducing agent for dyes or in combination with chemical indicators for quantitative analysis [8]. These spectroscopic methods are generally characterised by their simplicity, cost-effectiveness, and ability to deliver accurate results with low detection limits. Alizarin is considered one of the most analytical reagents and indicators used in both qualitative and quantitative determination of chemical compounds [9]. It has wide applications in environmental, natural dyeing for fabric colouring, and biological fields, in addition to analytical chemistry [10]. Alizarin is considered a derivative of anthraquinone compounds. Alizarin exists in more than one derivative, the most famous of which are Alizarin Brilliant Blue R, Alizarin Cyanine Green G, Alizarin Red S, and acid alizarin black [11]. Acid alizarin black has the chemical formula C14H7NaO7, and its chemical structure is shown in Figure 1.

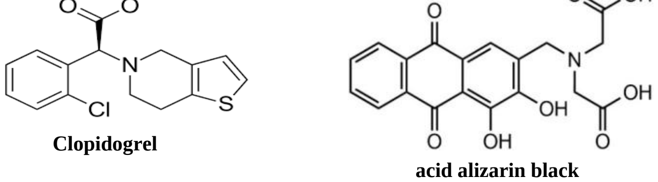


Figure 1. Chemical structure of Clopidogrel and acid alizarin black.

Microfluidic chips were initially fabricated using glass or silicon substrates with standard photolithography techniques [12]. However, their use is limited by high cost, complex and hazardous fabrication processes, and restricted channel design flexibility [13]. In contrast, polymers are cheaper, easier to process, and suitable for large-scale fabrication methods such as injection molding and hot stamping [14]. Their advantages—low cost, ease of fabrication, mechanical strength, chemical stability, flexibility, and biocompatibility—make them ideal materials for microfluidic devices [15]. Among these, polymethyl methacrylate (PMMA) is widely used due to its low cost, excellent optical clarity, and favorable electrical and mechanical properties. Moreover, PMMA is recyclable, as it can thermally depolymerize into its monomer, methyl methacrylate, and its relatively low hydrophobicity supports stable electroosmotic flow under an electric field [16]. Paper-based microfluidic colorimetric technology is widely used in routine analyses due to its simplicity, visual readout, and suitability for remote applications [17]. Over the past two decades, colorimetric methods have advanced with miniaturized platforms such as paper-based microfluidic analytical devices (µPADs), which transport microliter to nanoliter volumes through capillary action [18]. Compared to conventional silicon, polymer, or glass micro devices, µPADs offer notable advantages, including easy fabrication without cleanroom facilities [19], low-cost materials, no need for pumps, environmental friendliness, and in-situ analysis [20,21]. The integration of paper substrates with colorimetric detection provides low cost, portability, simple fabrication, and minimal reagent use, making µPADs ideal for point-of-need pharmaceutical quality control. This study aims to develop and evaluate a reliable method for the determination of the antiplatelet drug clopidogrel through its reaction with acid alizarin black reagent, employing three analytical techniques: conventional spectrophotometry, microfluidic chip-based analysis, and paper-based microfluidic sensing.

EXPERIMENTAL

Apparatus

All absorbance spectral measurements were made using a UV-Visible double beam spectrophotometer device (Shimadzu, type 1800, Japan) with a 1 ml cell, wavelength range 190–1100 nm, and spectral bandwidth 2 nm. Constructed laser microfluidic sensor, provided by a diode laser (532nm, 20 mW, lens focal length 1.5 mm), double syringe pump, detector made by UNO, wax printer (Xerox Color Qube 8900S) and wax inks (ColorQube), oven, Gentec, China PH100& PH20 series A digital pH meter (HANNA Bench type) with a combined saturated calomel glass electrode was used for pH measurements.

Reagents and Materials

All reagents and chemicals used were of analytical or pharmaceutical grade.

Clopidogrel standard solution, pharmaceutical grade, was provided from SDI, Samaraa, Iraq, which was reported to be 99.5 % purity. A stock standard solution containing 100 ppm of clopidogrel was prepared by dissolving the weighed amount of 0.1 g in distilled water.

Acid alizarin black was prepared by dissolving 0.1g of dye (99% purity) in distilled water and diluting to 100 ml to prepare 1000 ppm as a stock solution.

Design and Fabrication of the Microfluidic Laser System

A two-channel microfluidic device was designed and fabricated using poly (methyl methacrylate) (PMMA) as the substrate material. The layout of the microfluidic chip is illustrated in Figure 2a. The device comprises two inlets and a single outlet to facilitate controlled fluid manipulation. Fabrication was carried out using CO_2 laser engraving, which allowed for precise patterning of the PMMA layers. Two PMMA sheets, each measuring 2 mm in thickness and cut to dimensions of 2 cm × 5 cm, were prepared. The upper layer contains the inlet and outlet ports, while the lower layer was patterned to form the microfluidic channels using laser ablation in a wafer-like design. The assembled structure of the final microfluidic chip is presented in Figure 2b.

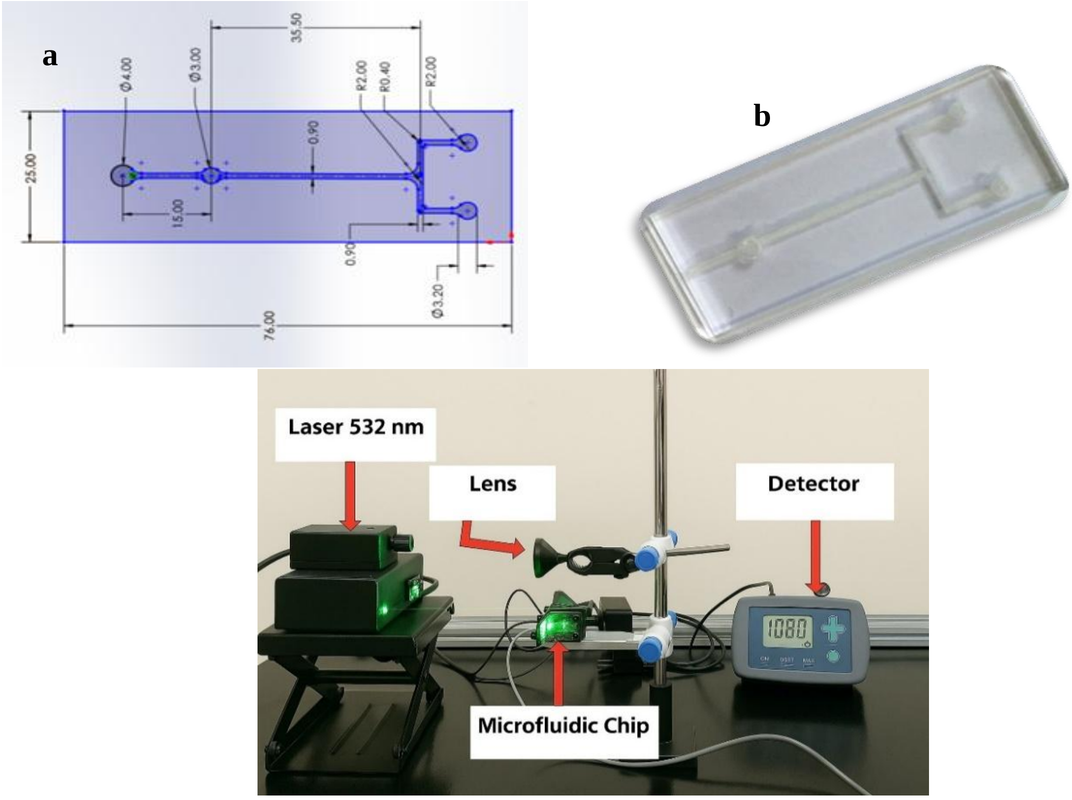
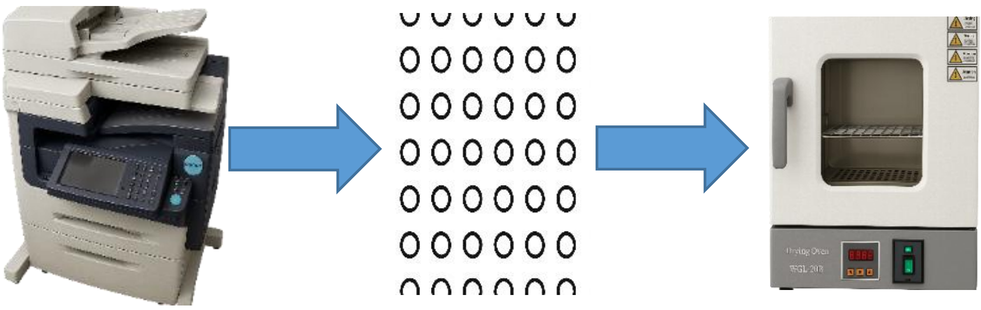


Figure 2. Two-Channel Microfluidic Design. (a) Schematic created using SolidWorks, with a thickness of 2 mm. (b) Final fabricated microfluidic chip. (c) microfluidic system.

Fabrication of Microfluidic Paper-Based Analytical Devices (µPADs)

The microfluidic paper-based analytical devices (µPADs) were fabricated following the protocol established by Carrilho et al. [22], utilizing a wax printing technique. A Xerox ColorQube 8900S printer with ColorQube wax-based inks was used to print the device patterns onto Whatman Grade 1 filter paper. Based on modifications proposed by Peters et al. [23], the printed paper was coated with wax on the front side. To create hydrophobic barriers, the wax-printed sheets were placed in an oven at 65 °C for 15 minutes, allowing the wax to penetrate through the thickness of the porous filter paper and form well-defined channel boundaries. The final design consisted of six circular detection zones in each line, optimized for multiple sample analyses as shown in Figure 3.



(a) (b) (c)

Figure 3. Fabrication of Microfluidic Paper-Based Analytical Devices (µPADs).

Fabrication process of the paper-based microfluidic device: (a) wax printer used for pattern printing, (b) filter paper immediately after wax printing, (c) filter paper placed in oven for heat treatment to allow wax penetration and formation of hydrophobic barriers.

Optimization of Complex Formation

Spectroscopic method /to optimize the conditions for complex formation, various volumes of the reagent were tested against a fixed concentration of the drug to determine the optimal reagent amount. The influence of pH on the absorbance of the resulting complex was evaluated by adding drops of 0.1 N hydrochloric acid and 0.1 N sodium hydroxide, covering a pH range from 2 to 14. Additionally, the impact of reaction time on complex stability and absorbance was assessed over a period ranging from 0 to 180 min. Temperature effects were also investigated within the range of 25-65°C to determine thermal influence on complex formation. The stoichiometric relationship between the drug and reagent was established using Job's method of continuous variation, where equimolar solutions (50 mM) were mixed in varying proportions. The absorbance was recorded for each mixture, and the corresponding maximum absorption wavelength (λmax) was determined for each molar ratio.

PMMA Microfluidic/The effect of the amount of reagent used was studied by adding increasing concentration of reagent with fixed concentration of (25 µg. ml^{-1}), then the intensity was measured for each complex using a diode laser (532nm) at 3.5 mw and a detector. The effect of time was studied in order to signify the stability of the complex colored product at the studied optimum conditions. The effect of time was studied for 25 µg mL^{-1} drug and 25 µg mL^{-1} reagent with a range 0-180 min at room temperature.

Paper-based analytical devices(µPADs)/ The effect of the amount of reagent used was studied by adding different concentrations of reagent with (2.5 µl) of drug at a concentration of 25 µg. ml^{-1}, then the intensity was measured for all solutions using the ImageJ program. The effect of time was studied in order to signify the stability of the complex colored product at the studied optimum conditions. The effect of time was studied for 25 µg mL^{-1} drug and 25 µg mL^{-1} reagent with a range of (0-180) min at room temperature.

Calibration Curves

Spectroscopic method / a precisely measured volume of clopidogrel standard solution was transferred into a series of 1 mL volumetric flasks. To each flask, 60 ppm of acid alizarin black reagent was added, followed by dilution to the mark with distilled water. The solutions were mixed thoroughly to ensure complete reaction. The absorbance of each mixture was recorded at 650 nm against a reagent blank prepared under identical conditions but without the drug.

PMMA Microfluidic/ The concentration of slandered clopidogrel was then determined using either the established calibration curve or the corresponding regression equation. Accurately measured different concentrations of drug solution were injected into the microfluidic chip's channel with 25 µg mL^{-1} of reagent.

Paper-based analytical devices (µPADs)/ accurately measured fixed volume (2.5µl) from different concentrations of drug solution, which was injected into a microfluidic paper with 2.5 µl of 25 µg mL^{-1} of reagent. The amount of drug present in each standard pharmaceutical preparation was computed from the corresponding calibration graph or regression equation.

Analysis of Commercial Medications

Spectroscopic method /Commercial clopidogrel tablets, each containing 75 mg of active ingredient, were utilized for the pharmaceutical formulation assay. Twenty tablets were accurately weighed, finely powdered, and the average weight was calculated. A portion of the powder equivalent to 12.5 mg of clopidogrel was dissolved in ethanol and transferred into a 100 mL volumetric flask. The solution was diluted to the mark with ethanol and filtered using Whatman No. 42 filter paper. The absorbance of the resulting solution was measured at the maximum wavelength of the formed complex.

PMMA Microfluidic/drug solutions were prepared by taking a fixed volume (0.1ml) from (100) µg. ml^{-1} from a pharmaceutical commercial sample with a series of concentrations of the standard drug in a 1ml volumetric flask, then inject this mixture into the microfluidic chip's channel with 25 µg mL^{-1} of reagent in another channel.

Paper-based analytical devices (µPADs)/drug solutions were prepared by taking a fixed volume (2.5µl) from (100) µg. mL^{-1} from a pharmaceutical commercial sample with a series of concentrations of standard drug in a 1 ml volumetric flask, then inject this mixture into microfluidic paper with (2.5µl) of 25 µg mL^{-1} of reagent. Each commercial drug's (%) recovery was determined using the formula.

Validation

Statistical parameters of the proposed analytical methods were calculated using OriginPro 2021 and IBM SPSS Statistics 21. Several value statistical parameters were calculated, including the intercept, correlation coefficient (r), standard deviation (SD), regression equation, limit of detection (LOD), and limit of quantitation (LOQ) for both standard and pharmaceutical formulations.

RESULTS AND DISCUSSION

Determination of Clopidogrel Concentration Using the Spectroscopic Method. Spectroscopic analysis was conducted by scanning the absorbance across the wavelength range of 190-900 nm. The results revealed characteristic absorption peaks at specific wavelengths: clopidogrel exhibited a maximum absorbance (λmax) at 250 nm, acid alizarin black showed a peak at 650 nm, and the clopidogrel-alizarin complex demonstrated a distinct λmax at 512 nm. These findings confirm the successful formation of the colored complex and the feasibility of using 512nm for the quantitative determination of clopidogrel via complexation with acid alizarin black.

Mechanism of Complex Formation

The formation of an ion-association complex occurs through the interaction between an electron-donating species and an electron-accepting species. This interaction is primarily driven by electrostatic attraction, which enhances the stability of the formed complex and leads to an observable increase in color intensity [24, 25]. Ion-association complexes are widely employed in the detection and analysis of pharmaceutical and organic compounds using various chromogenic reagents [26].

In the context of this study, the amide functional group in clopidogrel becomes protonated upon hydration, acquiring a partial positive charge. Concurrently, the carboxyl group in acid alizarin black donates a proton, becoming negatively charged. This facilitates an electrostatic interaction between the positively charged site of the drug and the negatively charged site of the reagent, resulting in the formation of a stable ion-pair complex [27]. The formation of this complex is responsible for the observed chromogenic shift, which is exploited for analytical detection.

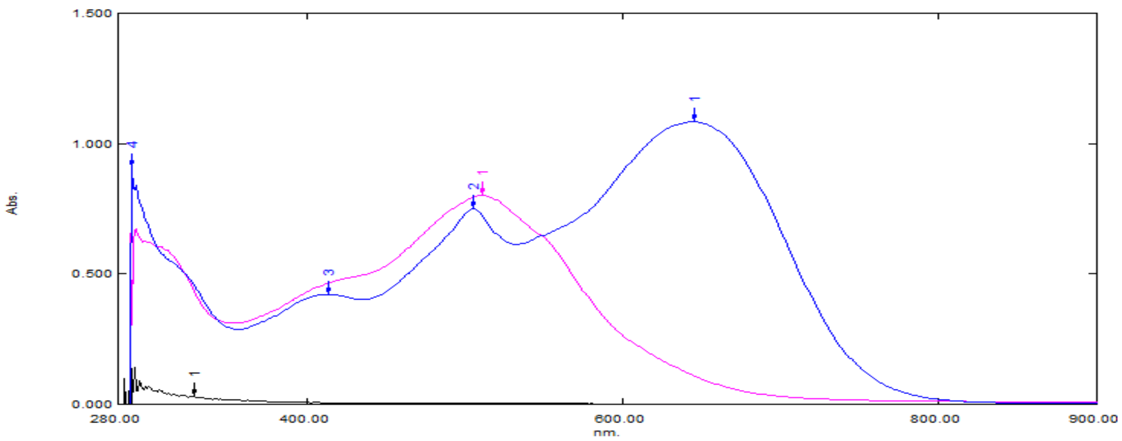


Figure 4. UV-Vis spectrum for acid alizarin black and clopidogrel complex (A) reagent, (B) complex, and (C) drug.

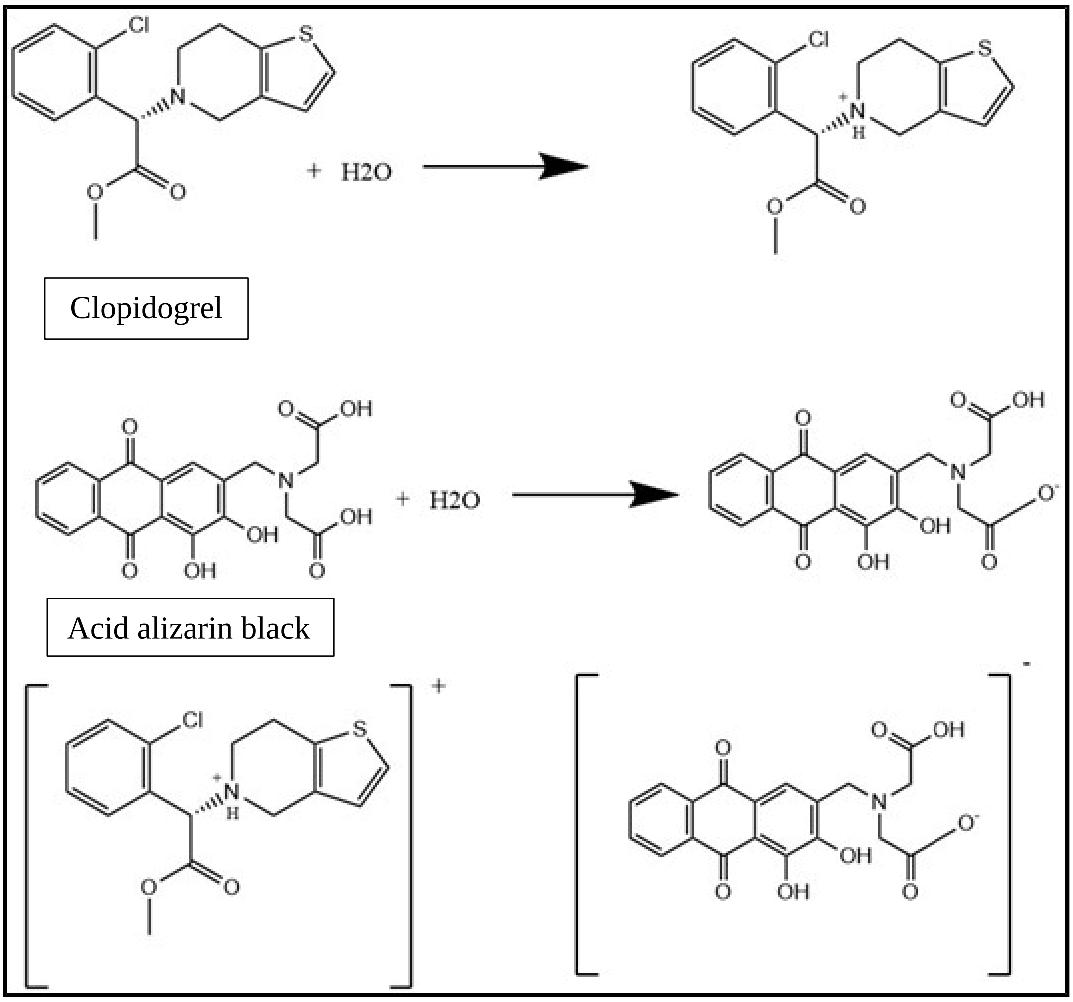


Figure 5. Suggested ion-association mechanism for the clopidogrel complex with acid alizarin black [28,29,30].

Determination of Clopidogrel Concentration using the Fabricated Laser-Based

Microfluidic Sensor

The concentration of clopidogrel was evaluated using the fabricated microfluidic chip constructed from polymethyl methacrylate (PMMA) and irradiated with a 532 nm diode laser. The incident laser beam passed through the solution within the microchannel, and the transmitted intensity was measured using a UNO laser power meter (mW scale).

A clear inverse relationship was observed between laser intensity and clopidogrel concentration; higher concentrations resulted in lower transmitted light intensity due to increased absorption and scattering within the channel [31]. This principle underpins the chip's ability to perform sensitive, real-time monitoring of clopidogrel levels based on optical detection.

Determination of Clopidogrel Concentration using µPADs

The detection of clopidogrel via microfluidic paper-based analytical devices (µPADs) involves an immediate colorimetric reaction upon applying the drug and reagent to the surface of wax-patterned filter paper. Once the reaction develops, an image of the detection zone is captured using a smartphone camera. The image is then processed using the ImageJ software, which separates the image into the three primary color channels: red, green, and blue. The channel with the highest color intensity is identified, typically associated with the most prominent spectral response of the formed complex. The mean color intensity of the selected channel is then calculated. Since the color intensity decreases with increasing clopidogrel concentration, an inverse relationship is established between the drug concentration and measured intensity, allowing for quantitative analysis based on a calibration curve.

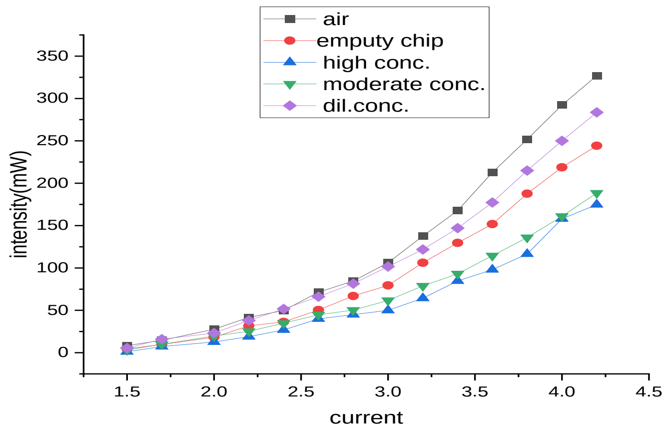


Figure 6. Relationship between Laser Intensity and Increasing Clopidogrel Concentration.

Optimization

According to the data in the three methods, the optimal reagent concentration is 25 ppm, as complexes formed at this concentration exhibit the highest absorbance. Beyond this concentration, the absorbance stabilizes, and the solution adopts the color of the reagent. Due to the basic nature of the reagent and the presence of ionizable active groups, the complex's color in acidic media directly reflects the reagent's color, while absorbance slightly decreases under reduced acidity.

The effect of time on complex stability indicates that absorbance gradually decreases over extended periods, as prolonged exposure leads to partial decomposition of the complexes. This behavior is attributed to the relatively weak ion-association interactions between the drug and the reagent compared to covalent bonds. Similarly, temperature influences complex stability, with absorbance decreasing at higher temperatures due to thermal decomposition of the complexes, again reflecting the weak nature of the ion-association interactions. Mole ratio analysis revealed that the complexes form in a 1:1 stoichiometric ratio (drug: reagent), as evidenced by the highest absorbance observed at this ratio.

Calibration Curve

The linearity of clopidogrel detection was assessed using three analytical approaches. In the UV-Vis spectrophotometric method, a calibration curve was established over the concentration range of 2-20 ppm, based on absorbance measurements at the maximum wavelength (λmax) of 512 nm. For the laser-based microfluidic system, employing a 532 nm diode laser, a linear response was observed within the range of 1-12 ppm. In contrast, the paper-based microfluidic analytical device (μPAD) demonstrated a linear detection range of 2-16 ppm, where the complex exhibited its strongest signal in the blue channel during RGB image analysis using Image J software. These differences in linear ranges reflect the physical and technical characteristics of each analytical platform. Microfluidic devices offer enhanced sensitivity at low concentrations, while conventional UV–visible spectrophotometry is more suitable for higher concentrations. Paper-based microfluidic devices provide a balance between sensitivity and practicality. These results confirm the suitability of each method for reliable and quantitative estimation of clopidogrel within the specified concentration ranges.

Table 1. Summary of Optimization Parameters.

λ_{max} (nm)	**512**
Conc. of reagent	25 ppm
Complex color	pink
Temp (ºC)	35ºC
Reaction time	immediately
Mole ratio	1:1
pH	5

Analysis of Commercial Clopidogrel Tablets

The analysis of the marketed clopidogrel formulation was performed using the standard addition method to assess the accuracy and applicability of the proposed techniques. A known volume (1 mL) of a 100 ppm clopidogrel commercial medication was added to each volumetric flask containing the clopidogrel standard solution and the acid alizarin black reagent. The absorbance was then measured using three techniques: UV-Visible spectrophotometry at 460 nm, the microfluidic laser-based sensor at 532 nm, and the paper-based microfluidic device analyzed via blue-channel intensity in the RGB image analysis software.

The percentage recovery of clopidogrel was calculated using the following equation [32]:

% Recovery= (A-B) / C × 100

Where A is the expected total amount of drug, B is the amount of drug found based on pre-analysis, and C is the amount of bulk drug added.

The recovery results, summarized in Table 2, indicated high accuracy and minimal matrix interference across all methods, affirming the reliability of the developed protocols for determining clopidogrel in pharmaceutical preparations.

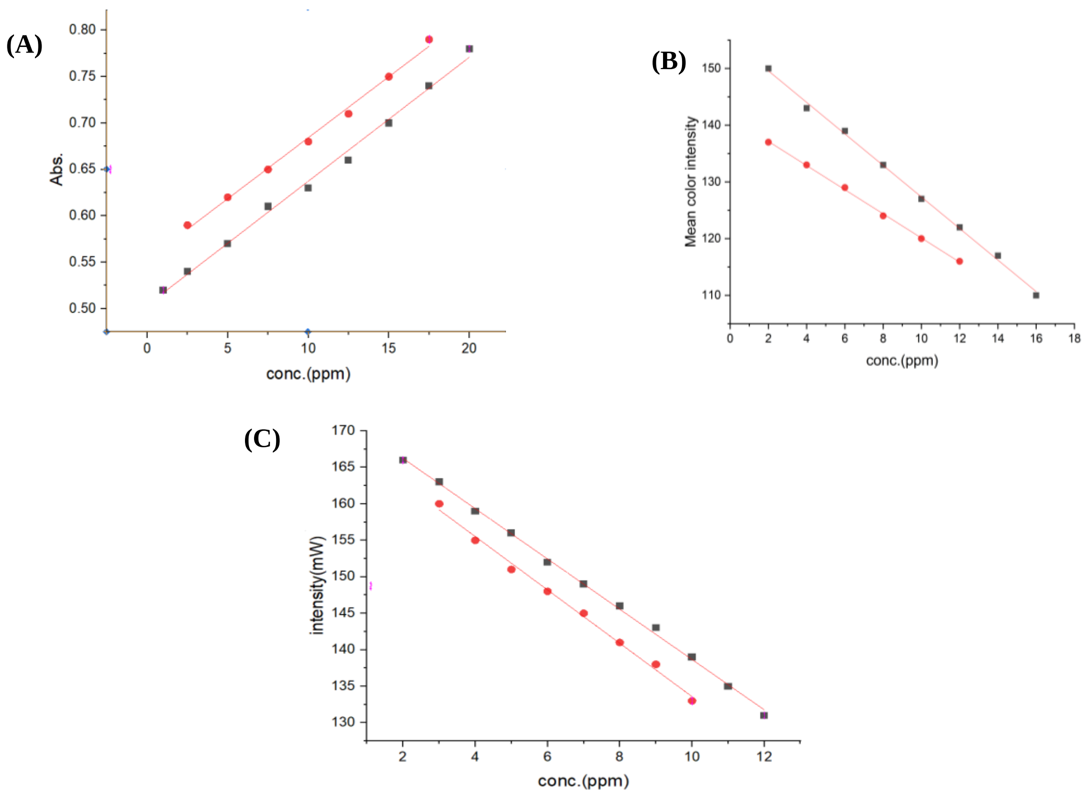


Figure 7. Standard calibration curve and standard addition plot for clopidogrel determination using (A) UV-Visible spectrophotometric method, (B) paper-based microfluidic analytical device (μPADs) method, (C) microfluidic sensing-based laser technique.

Table 2. Analysis of tablet Clopidogrel in tablets (PLAVINEER 75mg).

Methods	Actual content found	%Recovery
Spectrophotometric	74.72	99.64
Microfluidic	74.49	99.32
μpads	74.17	98.89

Validation of the Developed Methods

The validation of the proposed analytical methods was performed based on linear regression analysis using the least squares method. All Statistical parameters were calculated using Microsoft Excel 2016, Origin Pro 2021, and IBM SPSS Statistics 21 [33, 34].

The validation parameters, including regression equations, correlation coefficients, LOD, LOQ, and linearity ranges for clopidogrel in all three methods (spectroscopic, microfluidic, and paper-based microfluidic), are summarized in Table 3. These results confirm the methods' reliability, sensitivity, and suitability for pharmaceutical analysis. The limit of detection (LOD) and limit of quantification (LOQ) were determined using an empirical method by serial dilution of the lowest concentration within the linear range and measuring its absorbance [35]. Since this was done using the approximate method, the resulting LOD and LOQ values were higher than the minimum concentration within the linear range.

Accuracy and Precision for Proposed Methods

The accuracy of the developed analytical methods was evaluated by applying the standard addition method. Known amounts of clopidogrel standard were spiked into pre-analysed pharmaceutical samples at different concentration levels. The percent recovery of the added drug served as a measure of accuracy, ensuring the reliability of the proposed methods in detecting clopidogrel content [36, 37].

Precision was assessed through both intra-day and inter-day variations. Intra-day precision was determined by analysing six replicates of each sample within a single day, while inter-day precision involved repeating the same procedure over six consecutive days. The results were expressed as mean ± standard deviation (SD) and percentage relative standard deviation (%RSD), in accordance with accepted analytical validation guidelines [38, 39].

Table 3. Validation parameters for the developed analytical methods for clopidogrel analysis.

Validates	Spectroscopic	Microfluidic	μPADs
Intercept (a)	0.5037	173.12	155.14
Slope (b)	0.01336	-3.4455	-2.779
Standard deviation of the intercept (S_a)	0.019	6.135	5.498
Standard deviation of slope (S_b)	0.00082	0.122	0.098
Standard deviation(sd)	0.084	6.211	12.75
Linear range (ppm)	2-20	1-12	2-16
R^2	0.9948	0.9983	0.9979
Regression equation	Y=0.01336x +0.5037	y = -3.4455x + 173.12	y = -0.556x + 155.14
Limit of detection (LOD)	1.25	0.5	1
Limit of quantitation (LOQ)	3.78	1.51	3.33

Table 4. Accuracy data of the determination of clopidogrel in developed methods.

Methods	Amount of commercial drug (µg/ml)	Amount of standard drug (µg/ml)	Recovery	Percent Recovery (%)
Spectroscopic	2	2	9.78	97.8
	2	6	9.95	99.5
	2	10	9.89	98.9
Microfluidic	2	2	9.95	99.5
	2	6	9.98	99.8
	2	10	9.87	98.7
µPADs	2	2	9.93	99.3
	2	6	9.89	98.9
	2	10	9.88	98.8

Table 5. Intra-Day and Inter-Day Precision Data for the Determination of Clopidogrel Using the Proposed Analytical Methods.

No. of scans	Spectroscopic		Microfluidic		µPADs	
	Standard drug	Commercial drug	Standard drug	Commercial drug	Standard drug	Commercial drug
Scan -1	0.840	0.841	150	143	140.6	135.1
Scan-2	0.840	0.844	151	144	143	135.6
Scan-3	0.842	0.838	151	141	142.8	133
Scan-4	0.843	0.848	152	142	141	133.5
Scan-5	0.841	0.842	149	143	143	134
Scan-6	0.840	0.837	153	144	140	134
Average	0.841	0.842	151	142.833	141.733	134.20
Sd	0.001	0.004	1.291	1.067	1.1447	1.079
%RSD	0.089	0.312	0.85	0.747	0.830	0.800

Comparison between Analytical Methods

To evaluate and compare the precision and sensitivity of the developed analytical techniques, statistical analyses were conducted. A t-test was performed to assess any significant differences between the proposed methods and the standard spectrophotometric technique, as summarized in Table 6. Additionally, an F-test was applied to compare the variances and determine the reproducibility of the methods.

Furthermore, Table 7 presents a comparative analysis of the developed spectroscopic, microfluidic, and paper-based microfluidic methods with other previously reported spectrophotometric techniques for the determination of clopidogrel. The comparison highlights differences in detection limits, linear range, and operational advantages, offering insights into the relative performance and applicability of each method in pharmaceutical analysis.

Table 6. Statistical comparison using the t-test for the proposed methods and the standard spectrophotometric method.

t-test Spectroscopic	t-test Microfluidic	t-test µpads	t-test* Tablet value
1.82	**1.24**	**1.017**	**4.303**

***D.F=2 and 95% confidence limit**

Table 7. Statistical comparison using the F-test to evaluate variance between the proposed methods and the standard spectrophotometric method.

f-test between spectroscopic and microfluidic	f-test between spectroscopic and µPADs	f-test tablet value
2.73	**3.577**	**19**

***D.F=2,2 and 95% confidence limit**

CONCLUSION

The developed methods for the determination of clopidogrel are characterized by their simplicity, cost-effectiveness, sensitivity, and operational ease. These techniques do not require the use of auxiliary reagents, and the employed reagent is both readily available and efficient. The resulting complex demonstrates notable stability at ambient temperature without the need for heating, supporting the practical utility of these methods. Analytical results revealed high accuracy and precision, with excellent recovery values for pharmaceutical formulations. Furthermore, the approaches, particularly the paper-based microfluidic and polymer-based microfluidic systems, are aligned with green analytical chemistry principles, offering environmentally friendly, sensitive, and reliable alternatives suitable for routine pharmaceutical analysis.

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