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A Validated Stability Indicating HPTLC Method for Determination of Aspirin and Clopidogrel Bisulphate in Combined Dosage Form

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Abstract

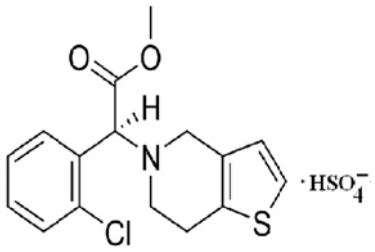
A sensitive, selective, precise and stability indicating (in accordance with ICH guidelines) High-Performance Thin Layer Chromatographic method of analysis for Aspirin and Clopidogrel bisulphate was developed, to resolve drugs response from that of their degradation products. The method employed TLC aluminum plates precoated with silica gel 60 F_{254} as the stationary phase. The solvent system consisted of carbon tetrachloride-acetone (6: 2.4 *v/v*). This system was found to give compact spots for both Aspirin and Clopidogrel bisulphate (R_f value 0.13±0.02, 0.78±0.02 respectively). Both the drugs were subjected to stress test conditions like acid/ alkali/ neutral hydrolysis, oxidation, dry heat treatment and photo degradation. The spots for product of degradation were well resolved from the spot of respective drugs. Densitometric analysis of drugs was carried out in the absorbance mode at 220 nm. The linear regression data for the calibration plots showed good linear relationship with r^2 0.9991 and 0.9866 in the concentration range of 200-600 ng/spot and 300-600 ng/spot for Aspirin and Clopidogrel bisulphate, respectively. The results indicate that the drugs are susceptible to degradation, to different extent in different conditions.

Keywords:

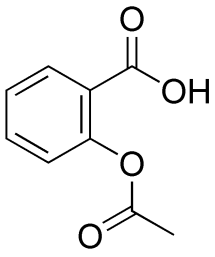
Aspirin; Clopidogrel bisulphate; HPTLC; Stability indicating

1. Introduction

Aspirin (2-acetyloxy benzoic acid) is cyclo oxygenase inhibitor and Clopidogrel bisulphate (methyl (s)-2-chlorophenyl (4,5,6,7- tetrhydothioeno-[3,2-C]pyridine-5-yl) acetate bisulphate) an ADP antagonist are two major antithrombogenic agents [1-3], that are widely used for the treatment and prevention of cerebro and cardiovascular conditions such as stroke. Combined use produces enhanced therapeutic effect. Aspirin and Clopidogrel both are esters and hydrolysis leads to inactivation or decreased therapeutic activity [4].



Clopidogrel bisulphate



Aspirin

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A survey of literature revealed spectrometric determination of Aspirin in biological fluids [5]. RP-HPLC methods were reported for the simultaneous estimation of Aspirin, Paracetamol, Caffeine [6] and Aspirin with Atorvastatin [7]. Spectrofluromertric method is also reported for the estimation of Aspirin and Dipyridamole [8]. Colorimetry [9], HPLC [10], HPTLC [11] and Gas chromatographic [12] methods were also described in the literature for the estimation of Clopidogrel bisulphate. RP-HPLC [13] method for analysis of Aspirin and Clopidogrel in combination was reported. However till now in our knowledge, no HPTLC method for the simultaneous estimation and for stability study of Aspirin and Clopidogrel bisulphate in combination has so far been reported. The present work describes the development of a simple, precise and accurate method for the simultaneous estimation of Aspirin, Clopidogrel bisulphate and their degradation products in bulk drugs and marketed formulation. The Validation and forced degradation studies were carried out as per ICH guidelines [14, 15]

2. Experimental

2.1. Materials

Working standards of Aspirin (Purity 100.99%) and Clopidogrel (Purity 99.81%) were provided as a gift sample by Sidmak Laboratories Pvt Ltd, Valsad, India and used without further purification. The drugs were received along with certificate of analysis. All the other reagents used were of analytical grade. Chloroform (AR grade), Toluene (AR grade), Methanol (AR grade), Acetone (AR grade), Carbon tetrachloride (AR grade), Acetic acid (AR grade), Dimethyl Sulfoxide (AR grade), Dichloromethane (AR grade), Benzene (AR grade), Tri ethanolamine (AR grade) were purchased from Thomas Baker (chemicals) Pvt Limited, India.

2.2. Instrumentation

Chromatographic separation of drugs were performed on Merck TLC plates precoated with silica gel 60 F_{254} (10 cm ×10 cm with 250 µm layer thickness) from E. Merck, Germany. The samples were applied onto the plates as a band with 4 mm width using Camag 100 µl sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland). Linear ascending development was carried out in a twin trough glass chamber (for 10 x 10 cm). Densitometric scanning was performed using Camag TLC scanner 3 in the range of 200-400 nm and operated by winCATS software (V 1.4.3, Camag).

2.3. Selection of Detection Wavelength

After chromatographic development bands were scanned over the range of 200-400 nm and the spectra were overlain. It was observed that both the drug showed considerable absorbance at 220 nm. So, 220 nm was selected as the wavelength for detection (Fig.1).

2.4. Method development

Method development, for resolution of Aspirin and Clopidogrel in combination, was started with development of densitogram with neat solvents and combinations of Chloroform, Toluene, Methanol, Acetone, Carbon tetrachloride, Dimethyl Sulfoxide, Dichloromethane and Benzene. Finally Methanol and toluene used in the ratio of 1 : 1 *v/v* as mobile phase, resulted in Aspirin and Clopidogrel bisulphate getting separated with R_f 0.46 and 0.82 respectively, but the major problem arose when NaOH was added to the sample for stress degradation study, NaOH peaks are very large and coincided with that of the Clopidogrel bisulphate. Various other combinations of same mobile phase were used like 7 : 3, 3 : 7 *v/v*. Neat

chloroform was tried (Aspirin R_f 0.18, Clopidogrel R_f 0.82) but these did not help. Trials were also taken to neutralize NaOH after stress degradation by adding equivalent amount of HCl directly and by using indicators (bromothymol blue, methyl red etc.); NaOH was replaced with KOH but no significant difference was obtained. Other combinations were also used like toluene, methanol and chloroform in the ratio of 5: 3: 2, 5: 2: 3, 5: 4: 1 and 4: 4: 2 *v/v/v*, in all cases solvent front peak was very prominent. A literature survey revealed that Clopidogrel elutes with R_f 0.33 when mobile phase, carbontetracloride: chloroform: acetone in the ratio of 6: 4: 0.15 *v/v/v* was used. Aspirin R_f in this mobile phase was found to be 0.09. We changed the mobile phase ratio to 4 : 2 : 4; 6 : 2 : 2; 4 : 2 : 6 *v/v/v;* R_f values of Aspirin and Clopidogrel bisulphate were found to be 0.16 , 0.77; 0.13, 0.82 ; 0.10, 1 respectively, but still solvent front response was big and was interfering with the peak of Clopidogral bisulphate. Hence carbon tetrachloride and acetone was tried as mobile phase in the ratio of 6: 2.4, here peaks were good and no solvent front was obtained. In this mobile phase Aspirin and Clopidogrel bisulphate had R_f value 0.13 and 0.78 respectively.

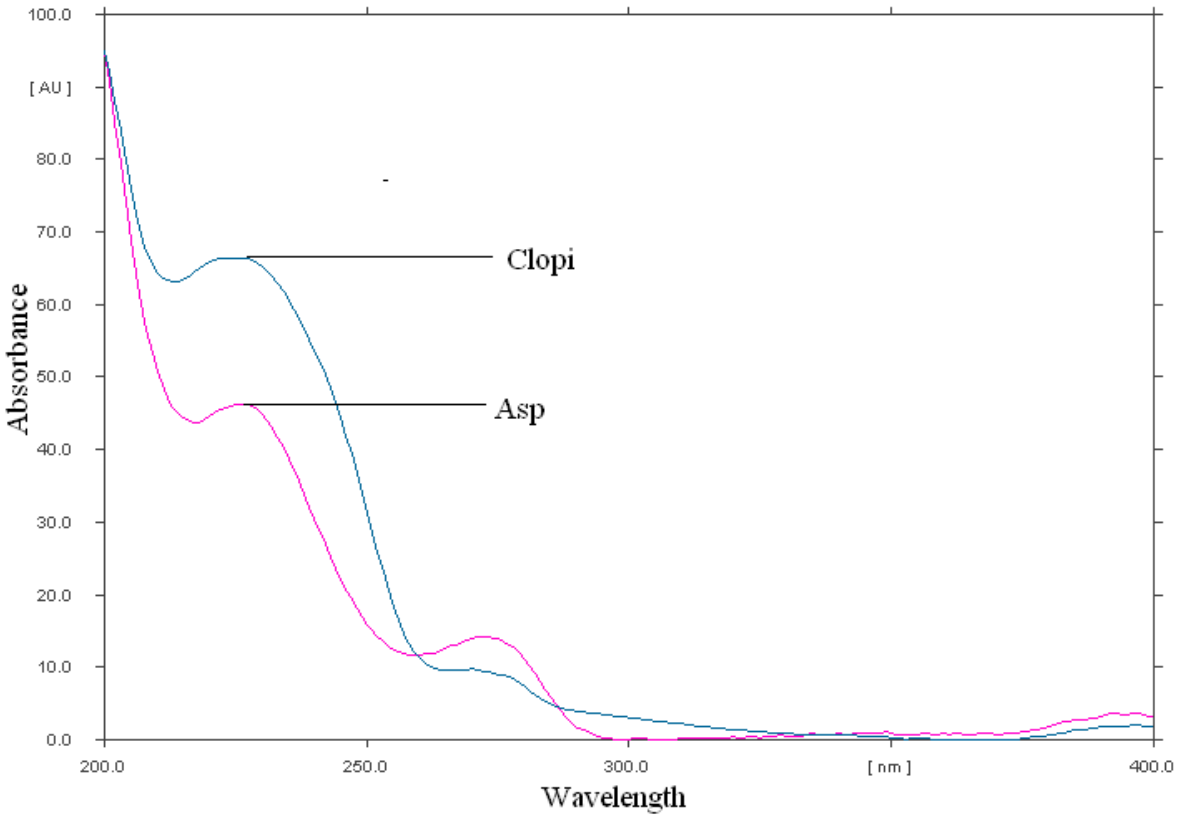

Fig 1. UV absorbance Spectra of Aspirin (Asp) and Cloidogrel bisulphate (Clopi).

2.5. Calibration curve

Individual stock solutions, of Clopidogrel bisulphate and Aspirin (1 mg mL^{-1}), were prepared in methanol. Different volumes of stock solution were spotted on the TLC plate to obtain concentrations 300-600 ng $spot^{-1}$ and 200- 600 ng $spot^{-1}$ of clopidogrel bisulphate and Aspirin, respectively. The data of peak area versus drug concentration was treated by linear least square regression analysis.

2.6. Assay

Tablet (labeled to contain 75 mg of clopidogrel bisulphate and aspirin each) sample was prepared by dispersing the equivalent amount of triturated powder in methanol. After

appropriate dilution it was filtered through whatmann filter paper no. 41 and spotted on the TLC plate with standard solution of same concentration on the adjoining track. Average of three readings showed % assay was 97.80 and 99.18 for Aspirin and Clopidogrel respectively.

2.7. Stress degradation of Aspirin and Clopidogrel bisulphate

2.7.1. Degradation under base catalyzed hydrolytic condition

i. At ambient temperature:

25 mg of each drug was weighed separately and dissolved in a volumetric flask of 25 mL with, methanol. To 1mL of this solution, 0.5mL of 0.05 N NaOH was added and final volume was made up to 10mL with methanol. 300 ng of sample was spotted at time 0, 30, 60 min with a reagent blank spotted in adjoining track.

ii. With reflux:

Accurately weighed 25 mg of drug was separately dissolved in 25 mL of methanol, then to 5 mL of this solution, 5 mL of 0.05 N NaOH was added and final volume was made up to 50 mL with methanol, final solution was refluxed for 1 hr and 3 hr. Each sample was spotted against the appropriately diluted standard solution.

2.7.2. Degradation under acid catalyzed hydrolytic condition

i. At ambient temperature

25 mg of each drug was weighed separately and dissolved in, a volumetric flask of 25 mL with, methanol then to 1 mL of this solution 0.5 mL of 0.05 N HCl was added and final volume was made up to 10 mL with methanol. 300 ng of each sample was spotted at time 0, 80 and 120 min with a reagent blank spotted simultaneously.

ii. With reflux:

Accurately weighed 25 mg of drug was separately dissolved in 25 mL of methanol, then to 5 mL of this solution, 5 mL of 0.05 N HCl was added and final volume was made up to 50 mL with methanol, final solution was refluxed for 1 hr and 2 hr. 300 ng of each sample was spotted against the standard solution after appropriate dilution.

2.7.3. Degradation under neutral hydrolytic condition

Accurately weighed 30 mg of drug was separately dissolved in 10 mL of methanol, then to 5 mL of this solution, 0.5/ 2.5 mL water was added and final volume is made up to 50mL with methanol Final solution was refluxed for 1 hr/ 2 hr respectively. 300 ng each sample was spotted. The standard solution in methanol was also spotted after appropriate dilution.

2.7.4. Oxidative Degradation:

Accurately weighed 25 mg of drug was separately dissolved in 25 mL of methanol. Then to 5 mL of this solution, 1 mL of 30% H_2O_2 was added and final volume was made up to 50mL with methanol. Final solution was refluxed for 1hr. 300 ng each sample was spotted. The standard solution in methanol was also spotted after appropriate dilution.

2.7.5. Degradation by dry Heat

Accurately weighed 25 mg of drug was separately transferred to the 25 mL of volumetric flask, and was kept in oven at 100 ^{0}C for 1 hr, after that sample was appropriately diluted to spot 300ng of each drug.

2.7.6. Photo-degradation

Working Standards of both the drugs are exposed to UV light up to illumination of 200 watt hr m^{-2} followed by visible light up to illumination of 1200 lux-hr. The drugs were then dissolved & diluted appropriately to spot 300 ng of each drug.

2.8. Stress degradation of Formulation

Sample was exposed to stress condition as mentioned under study for bulk drugs. Then the sample was filtered & appropriate volume was spotted on to TLC plate.

3. Results and Discussion

The TLC procedure was optimized with a view to develop a stability indicating method for pure drugs. The mobile phase consisting of carbon tetrachloride- acetone (6: 2.4, *v/v*) gave good resolution, sharp and symmetrical peaks with R_f value of 0.13 and 0.78 for Aspirin and Clopidogrel bisulphate respectively (Fig.2). Also the spots were compact and not diffused. It was observed that prewashing of TLC plates with methanol followed by drying and activation, and pre-saturation of TLC chamber with mobile phase for 20 min ensured good reproducibility for peak shapes and areas of drugs.

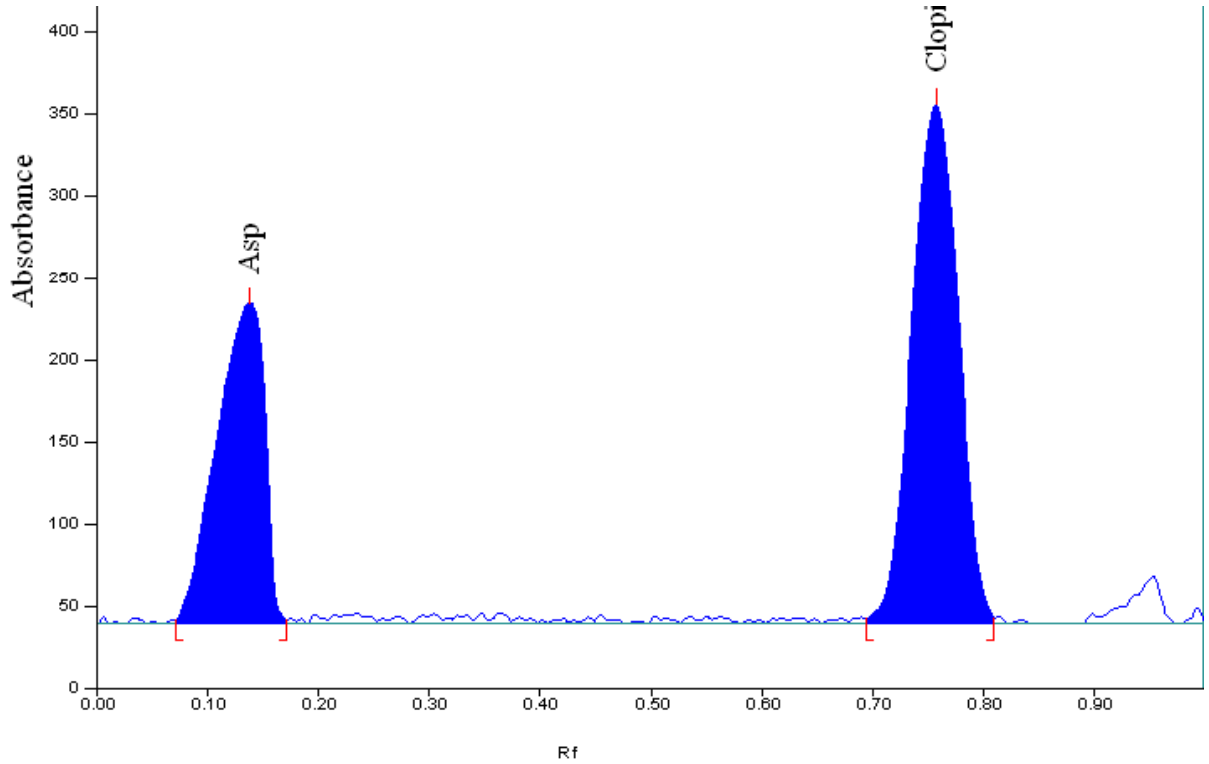

Fig.2. Representative densitogram of Aspirin (Asp) and Clopidogrel bisulphate (Clopi) at R_f 0.13 and 0.78 respectively.

3.1. Validation parameters

Method was validated as per ICH guideline with respect to linearity, range, accuracy precision, limit of detection, limit of quantification and results are shown in Table 1.

3.2. Stress degradation

3.2.1. Base induced-degradation

The densitogram of the base degraded samples for Aspirin showed additional peak at R_f value of 0.05. The peak area of the drug was found to be reducing as compared to the initial area but there was no significant change for Clopidogrel bisulphate under mild basic condition. On increasing strength of base together with heat there was also significant change for Clopidogrel bisulphate peak area; indicating that Aspirin degrades in basic condition much faster than Clopidogrel bisulphate.

Table 1. Validation parameters.

Parameters	Aspirin	Clopidogrel bisulphate
Beer's law range (ng/spot)	200-600	300-600
Regression equation $y^* = mx + C$	y = 24.32x + 1400	y = 24807x – 1025
r^{2*}	0.9991	0.9866
Accuracy* (% Recovery)	97.5 - 102%	98.5 - 101.2%
Precision* (%RSD)	Less than 1.5%	Less than 1.5%
LOD	8.23 ng $spot^{-1}$	8.79 ng $spot^{-1}$
LOQ	27.14 ng $spot^{-1}$	29.01 ng $spot^{-1}$

Note: * mark indicates average of 6 readings

3.2.2. Acid induced-degradation

The densitogram of the acid degraded samples for Aspirin and Clopidogrel bisulphate showed additional peaks at R_f value of 0.05 and 0.09, respectively. The peak area of the drug was found to be reducing as compared to the initial area indicating that Aspirin and Clopidogrel bisulphate both undergo degradation in acidic conditions, further study showed Clopidogrel bisulphate under going faster degradation than Aspirin.

3.2.3. Neutral and Oxidative-degradation

Chromatogram of the drugs exposed to water and H_2O_2 with and without reflux revealed that only Aspirin peak area decreased significantly with time i.e. only Aspirin is susceptible under these conditions.

3.2.4. Dry heat degradation

The densitogram of the drug exposed to dry heat (Aspirin and Clopidogrel bisulphate) showed no additional peak, also there was no significant change in peak area of the drugs was observed. It indicated that Aspirin and Clopidogrel bisulphate both did not undergo degradation in dry heat condition.

3.2.5. Photo-degradation

Upon exposure to UV light, color and texture of both drugs changed Aspirin became sticky and deep in color where as Clopidogrel bisulphate color was completely changed to deep brown. From the densitogram of the drug exposed to UV and Visible light only Clopidogrel bisulphate showed significant change in peak area.

4. Conclusion

From the above study we can conclude that the Aspirin and Clopidogrel bisulphate undergo degradation to different extent under different, above mentioned, stress conditions (Table 2). Also the spectrum of product of degradation was completely different form the spectrum of original drug as shown in Fig.3. and non interference by the degraded product can be confirmed by peak purity values for Aspirin peak after degradation having correlation coefficient r (s, m) 0.9924 and r (m, e) 0.9982 Similarly for the Clopidogrel bisulphate r(s, m) 0.9981 and r(m, e) 0.9973. It confirms that degradation product of drug can be separated from that of drug by this method. Since a formulation of same combination is available in market, this method can be used for the stability study of marketed formulations also. The study on formulation revealed that the formulation is more stable than the bulk drug (Table 3).

Table 2. Result of stress degradation study for bulk drugs.

Stress degradation conditions			Percentage degradation (%)	
			Aspirin	Clopidogrel bisulphate
With Base	Without reflux (0.05 N NaOH)	30 min	74.20	0.0
		60 min	80.37	0.0
	With reflux (1N NaOH)	180 min	100	88.62
With Acid	Without reflux (0.05 N HCl)	80 min	0.0	6.65
		120 min	11.07	24.06
	With reflux (0.05 N HCl)	60 min	16.75	11.62
		120 min	32.51	99.0
With water	Reflux (0.5mL in 50 mL)	60 min	33.36	0.0
		180 min	33.91	0.0
	Reflux (2.5mL in 50 mL)	60 min	100	0.0
With Oxidising Agent (H_2O_2, 30%)	Reflux	60 min	12.17	0.0
Dry Heating (100^0 C)		60 min	0.0	0.0
Photo stability		UV 200 watt-hr m^{-2}	19.8	12.38
		VIS 1200 lux-hr	4	5.68

Table 3. Result of stress degradation study for Tablet.

Stress degradation conditions			Percentage degradation (%)	
			Aspirin	Clopidogrel bisulphate
With Base	Without reflux (0.05 N NaOH)	30 (min)	11.03	0.0
	With reflux (1N NaOH)	180 (min)	100	34.21
With Acid	With reflux (0.05N HCl)	60 (min)	5.3	8.52
With Water	Reflux (0.5mL in 50 mL)	180 (min)	11.76	0.0
With Oxidizing Agent (H_2O_2, 30%)	Reflux (1 mL in 50 mL)	60 (min)	6.22	0.0
Dry Heating (100^0 C)		60 (min)	0.0	0.0
Photo stability		UV-200 (watt-hr/m^2)	21.0	10.6
		VIS 1200 (Lux-hr)	4.1	3.8

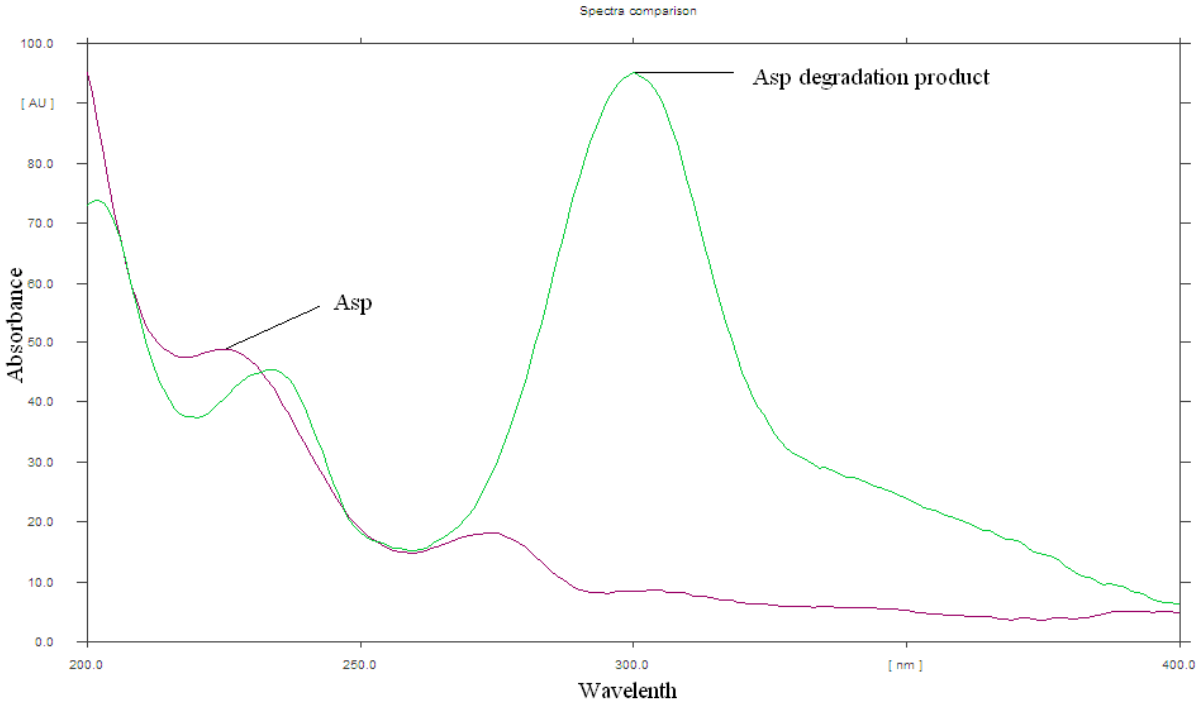

Fig.3. Overlain Spectra of Aspirin (ASP, R_f 0.13) and degradation product of aspirin after base catalysed hydrolysis (R_f 0.05).

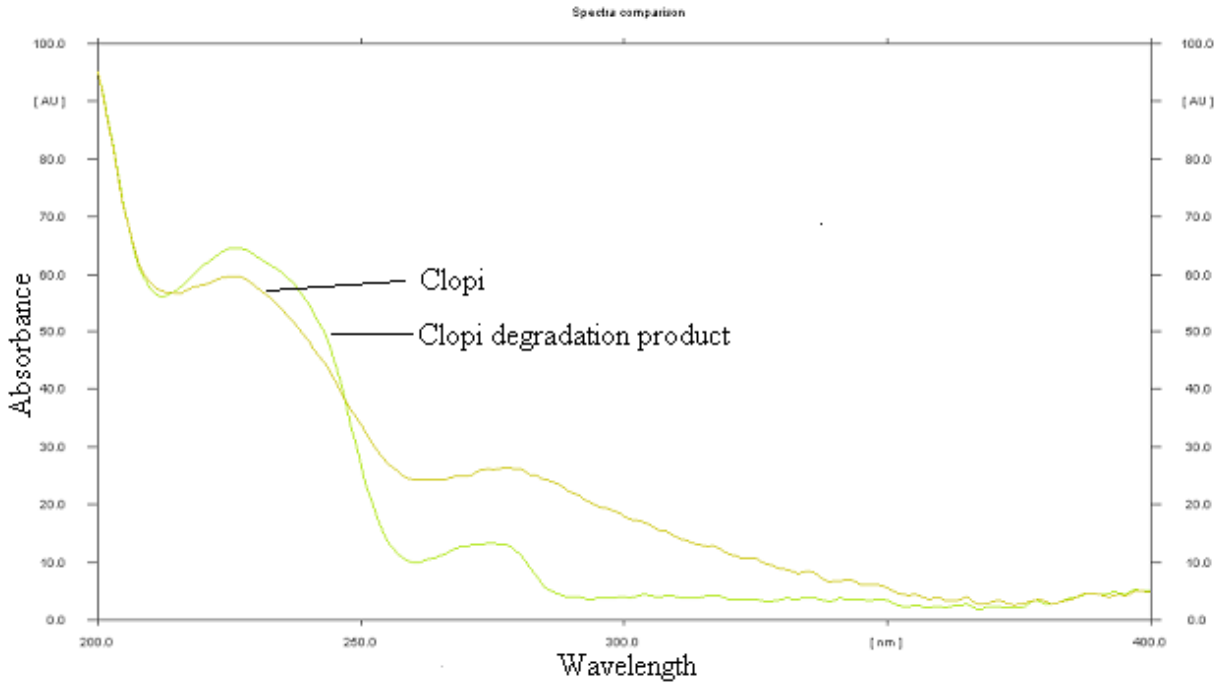

Fig.4. Overlain spectra of clopidogrel bisulphate (clopi, R_f 0.78) and degradation product of clopidogrel bisulphate (R_f 0.09) after base hydrolysis.

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