MALAYSIAN JOURNAL OF ANALYTICAL SCIENCES
Published by The Malaysian Analytical Sciences Society
ISSN 1394 - 2506

STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF ASPIRIN AND CLOPIDOGREL IN DOSAGE FORM

(Kestabilan Kaedah KCPT-Indikator bagi Penentuan Serentak Asprin dan Clopidogrel Dalam Bentuk Dos)

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Received: 13 June 2015; Accepted: 1 February 2016

Abstract

Stability-indicating High Performance Liquid Chromatographic (HPLC) method was developed for simultaneous Aspirin and Clopidogrel, A Phenomenex Gemini C-18, 5 µm column having 250mm x 4.6 mm i.d. in isocratic mode, with mobile phase containing buffer solution 0.3% orthophosphoric acid : acetonitrile (65: 35, v/v). The flow rate was 1 ml/min and effluents were monitored at 266 nm. For linearity seven points calibration curve were obtained in a concentration range from 0.030 –0.120 mg/ml for aspirin and 0.015 – 0.060 mg/ml for clopidogrel with correlation coefficient 0.9999. In the present study stability indicating HPLC method for the combination was tested by degrading the drugs together under various stress conditions like acid hydrolysis, base hydrolysis, oxidation, thermal and photolytic stress which is recommended by ICH guideline.

Keywords*:* reverse phase high performance liquid chromatography, stability indicating method, aspirin, clopidogrel

Abstrak

Kestabilan Kaedah Kromatografi Cecair Prestasi Tinggi (KCPT) – indikator telah dibangunkan bagi penentuan serentak Aspirin dan Clopidogrel. Turus Phenomenex Gemini C-18, 250mm x 4.6 mm i.d. diguna dalam mod isokratik, bersama fasa bergerak yang mengandungi larutan penimbal iaitu 0.3% asid orthofosforik : acetonitril. Kadar aliran adalah 1ml/min dan effluent dipantau pada panjang gelombang 266 nm. Tujuh titik lengkung kalibrasi dipilih bagi ujian kelinearan yang diperolehi dari julat 0.030 – 0.120 mg/ml dan 0.015 – 0.060 masing – masing bagi aspirin dan clopidogrel dengan nilai pekali korelasi adalah 0.9999. Dalam kajian ini, kestabilan kaedah KCPT- indikator bagi gabungan analit diuji di bawah pelbagai tekanan seperti hidrolisis asid, hidrolisis bes, pengoksidaan, termal dan tekanan fotolitik seperti yang dicadangkan oleh garis panduan ICH.

Kata kunci*:* kromatografi cecair prestasi tinggi fasa terbalik, kestabilan kaedah indikator, aspirin, clopidogrel

Introduction

Platelet aggregation and thrombus formation play a critical role in the initiation and development of key complications of acute coronary syndromes (ACSs). Antiplatelet therapy and antithrombotic therapy have been demonstrated to favorably modify clinical outcome, and recent trials of revascularization in ACSs have demonstrated a reduction in the frequency of major cardiac events [1 – 12]. Antiplatelet and anti-thrombin have synergistic actions that reduce the risk of spontaneous or revascularization, especially percutaneous coronary intervention (PCI)-related events. Yet, all effective antithrombotic agents also increase the risk of bleeding,

especially bleeding that results from vascular accessor associated with surgery, including coronary artery bypass grafting (CABG). The Clopidogrel in unstable angina to prevent recurrent ischemic Events (CURE) trial demonstrated that the combination of clopidogrel and aspirin was superior to aspirin alone for patients hospitalized with non-ST-elevation ACSs.

Aspirin is chemically acetylsalicylic acid (Figure 1). Its molecular formula is $C_9H_8O_4$ having molecular weight 180 g/mole [14]. It is slightly soluble in water, freely soluble in alcohol, soluble in chloroform and ether, sparingly soluble in absolute ether. Aspirin, one of the first drugs to come into common usage, is still the most widely used drug in the world, is a non-steroidal anti-inflammatory drug that exhibits anti-inflammatory, analgesic and antipyretic activities.

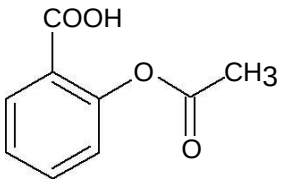

Figure 1. Chemical structure of aspirin

Clopidogrel bisulfate, chemicall y it is [S - (a)(2 - chlorophenyl) - 6,7 - dihydrothieno (3,2-C) pyridine-5 (4H) acetic acid methyl ester sulphate] (Figure 2). The empirical formula of clopidogrel bisulfate is $C_{16}H_{16}ClNO_2S•H_2SO_4$ and its molecular weight is 419.9 g/mole [14]. It is a white to off-white powder. It is practically insoluble in water at neutral pH but freely soluble at pH 1. It also dissolves freely in methanol, dissolves sparingly in methylene chloride and is practically insoluble in ethyl ether. It has a specific optical rotation of about +56°. The structural formula is as follows:

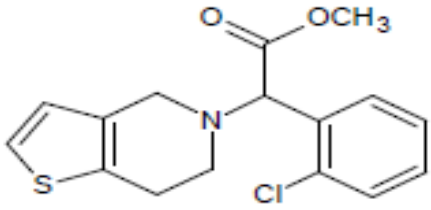

Figure 2. Chemical Structure of clopidogrel

The combination of clopidogrel with aspirin provides enhanced prevention of atherothrombotic events by blocking the platelet aggregation by both ways. (ADP pathway and collagen induced pathway) and it shows a synergistic antiplatelet action in controlling the ischemic events.

Literature reveals that various methods simultaneous determination of aspirin and clopidogrel in pharmaceutical formulation HPTLC [5 – 6]. Various publications are available regarding determination method of aspirin and clopidogrel but most of the methods are applicable to alone aspirin or clopidogrel in pharmaceutical dosage form or in bilogical fluids. Only three methods are reported for the simulteneous determination of apirin and clopidogrel. One is semi-micro column HPLC-UV method for simultaneous determination of clopidogrel metabolite, aspirin and salicylic acid in rat plasma. Second is a spectrophotometric method, which is able to determine aspirin and clopidogrel in combine dosage form and third is simple high performance liquid chromatography, which applicable to routine quality control sample analysis. The separation is performed by high

performance liquid chromatography for reasons of robustness and familiarity of analysts with this technique. To our knowledge, no stability-indicating analytical method for the determination of aspirin and clopidogrel in combine dosage forms has been published.

In the present study stability indicating HPLC method for the combination was tested by degrading the drugs together under various stress conditions like acid hydrolysis, base hydrolysis, and oxidation, thermal and photolytic stress which is recommended by ICH guideline.

Materials and Methods

Pharmacopoeial grade standards of aspirin and clopidogrel bisulphate were provided by Blue Cross laboratory. A tablet containing 150 mg aspirin and 75 mg clopidogrel was commercially available. HPLC grade acetonitrile, methanol and water were obtained from Spectrochem Pvt. Ltd., Mumbai (India). Analytical grade hydrochloric acid, sodium hydroxide pellets, orthophosphoric acid and hydrogen peroxide solution (30 % v/v) were obtained from Ranbaxy Fine Chemical, New Delhi (India).

HPLC instrumentation and conditions

The chromatographic system used to perform development and validation of this assay method was comprised of (SHIMADZU, HPLC) with LC- 20AT (VP series) pump, with software Spinchrom and UV-Visible detector SPD-20A (VP series). Chromatographic analysis was performed on a Phenomenex Luna C8 (250 mm 4.6 mm i.d., 5 µm particle size) column. The mobile phase was consisted of 0.3 % orthophosphoric acid (v/v) - acetonitrile (65:35, v/v). The flow rate of the mobile phase was adjusted to 1.0 ml/min and the injection volume was 20 µl. Detection was performed at 226 nm.

In the presence work, an analytical method based on LC using UV detection was developed and validated for assay determination of aspirin and clopidogrel in tablet formulation. The analytical conditions were selected, keeping in mind the different chemical nature of aspirin and clopidogrel. The development trials were taken by using the degraded sample of each component was done, by keeping them in various extreme conditions.

The column selection has been done on the basis of backpressure, resolution, peak shape, theoretical plates and day-to-day reproducibility of the retention time and resolution between aspirin and clopidogrel peak. After evaluating all these factors, C8 (2) (250 mm 4.6 mm i.d., 5 µm particle size) column was found to be giving satisfactory results. The selection of buffer based on chemical structure of both the drugs.

Preparation of stock solution for stress studies

The degradation samples were prepared by transferring powdered tablets, equivalent to 150 mg aspirin and 75 mg clopidogrel into a 250 ml round bottom flask. Then prepared samples were employed for acidic, alkaline and oxidant media and also for thermal and photolytic stress conditions. After the degradation treatments were completed, the stress content solutions were allowed to equilibrate to room temperature and diluted with mobile phase to attain 0.075 mg/ml of aspirin and 0.0375 mg/ml of clopidogrel concentration.

Results and Discussion

The acidic pH range was found suitable for solubility, resolution, stability, theoretical plates and peak shape of both components. Best results were obtained with 0.3 % orthophosphoric acid solution improved the peak shape of aspirin and clopidogrel. Finally, by fixing 0.3 % orthophosphoric acid (v/v) and mobile phase composition consisting of a mixture of 0.3% orthophosphoric acid (v/v) into ratio 65:35, v/v. Optimized mobile phase proportion was providing good resolution between aspirin and clopidogrel and also for degradation product which is generated during force degradation study. For the selection of organic constituent of mobile phase, acetonitrile was chosen to reduce the longer retention time and to attain good peak shape. Figure 1 and Figure 2 represent the Chromatograms of standard and test preparation respectively.

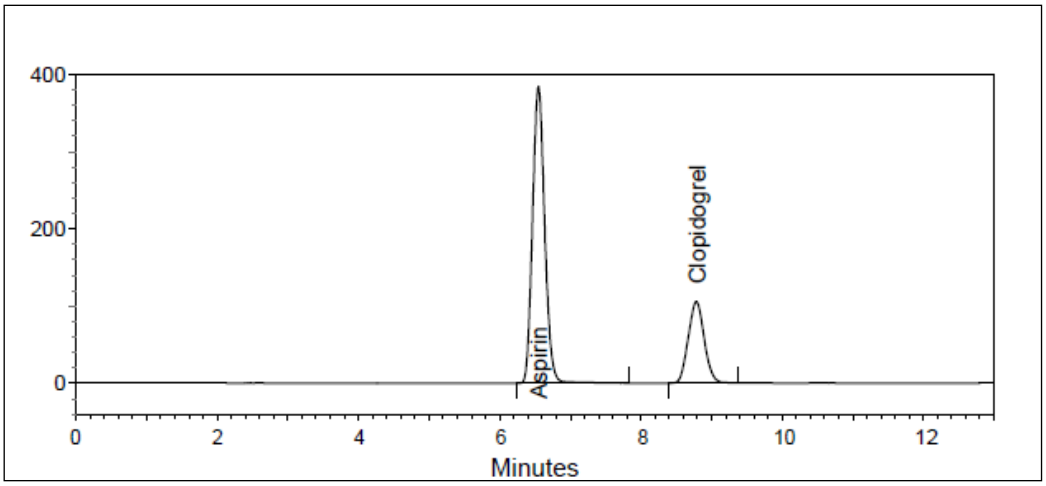

Figure 1. Chromatogram of standard preparation (0.08mg/ml)

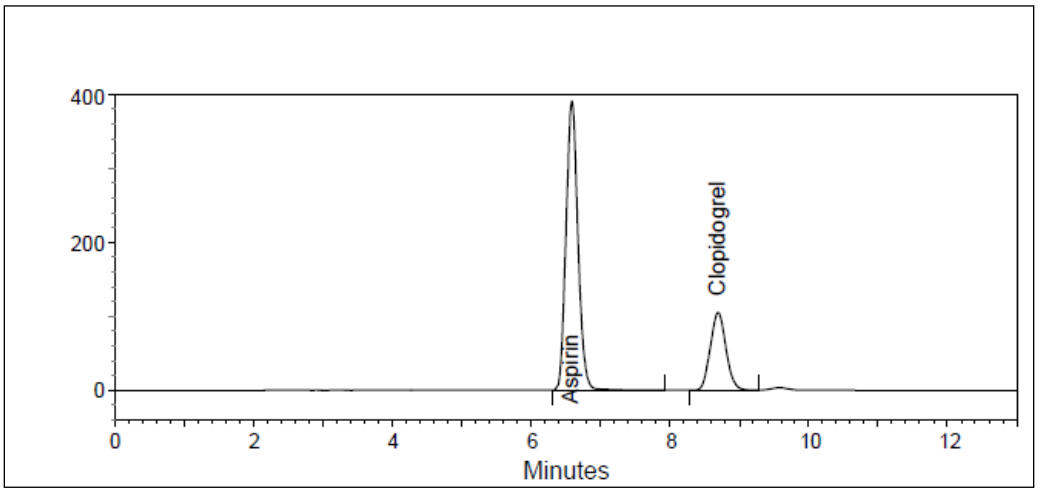

Figure 2. Chromatogram of test preparation (0.08mg/ml)

In order to determine whether the analytical method or assay were stability- indicating, aspirin and clopidogrel combine tablets were stressed under various conditions to conduct forced degradation studies. Regulatory guidance in ICH Q2A, Q2B, Q3B and FDA 21 CFR section 2 11 all require the development and validation of stability-indicating potency assays. Unfortunately, the current guidance documents do not indicate detailed degradation conditions in stress testing. However, the used forced degradation conditions, stress agent concentration and time of stress, were found to effect degradation and not complete degradation of active materials. The discovery of such conditions was based on development trial.

Acidic condition

Acidic degradation study was performed by heating the drug content in 1 N HCl (50 ml) at 80 °C for 1 hour and mixture was neutralized with 1 N NaOH solution. The drug content was found to be degrading up to 16.93 % in acidic condition. Clopidogrel was more susceptible to acid hydrolysis under experimental conditions. In all degradation conditions the drug degrades as observed by by the decreased area in the peak of the drug when

compared with peak area of the same concentration of the undegraded drug, with giving one additional degradation peak at 9.02 min.

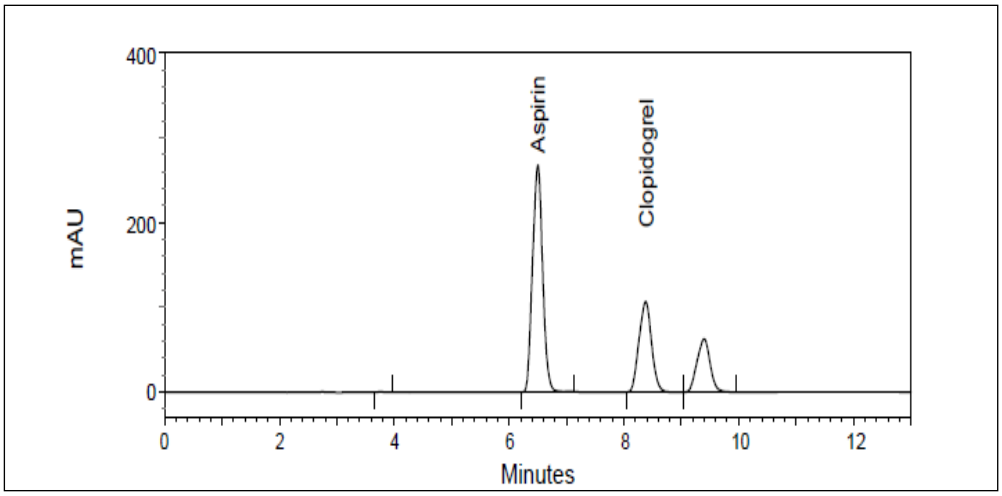

Figure 3. Chromatogram of acidic forced degradation study

Alkaline condition

Alkaline degradation study was performed by heating the drug content in 1 N NaOH (50 ml) at 80 °C for 1 hour and mixture was neutralized with 1 N HCl solutions. In alkali degradation, it was found that around approximately 22 % of the drug was degraded (Figure 4). Aspirin was more susceptible to alkaline hydrolysis under experimental conditions. In all degradation conditions the drug degrades as observed by the decreased area in the peak of the drug when compared with peak area of the same concentration of the undegraded drug, with giving one additional degradation peak at 4.72 min.

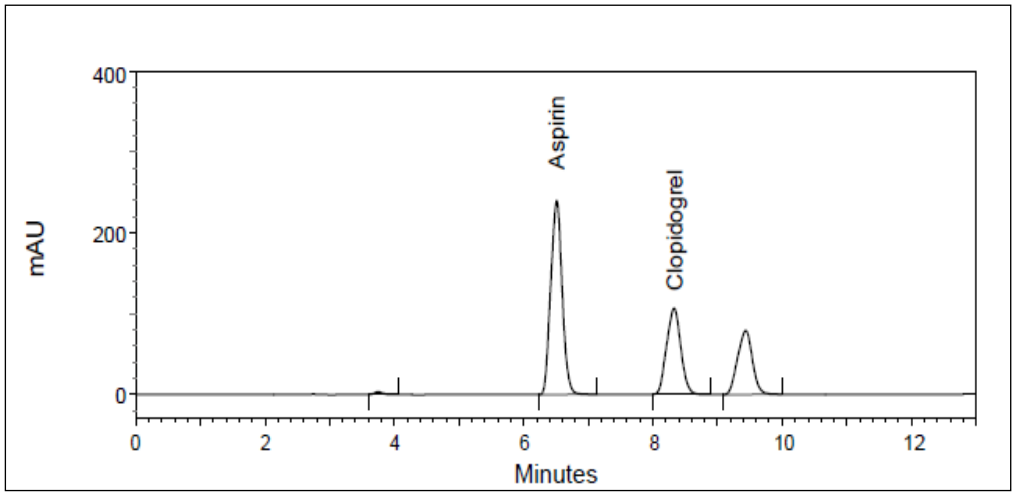

Figure 4. Chromatogram of alkali forced degradation study

Oxidative condition

Oxidation degradation study was performed by heating the drug content it's in initial concentration carried out in 1% v/v hydrogen peroxide no significant changes then will increase into 3% v/v hydrogen peroxide at 80 °C for 30 minutes. In oxidative degradation, it was found that approximately 15.84 % of drug was degraded (Figure 5). .

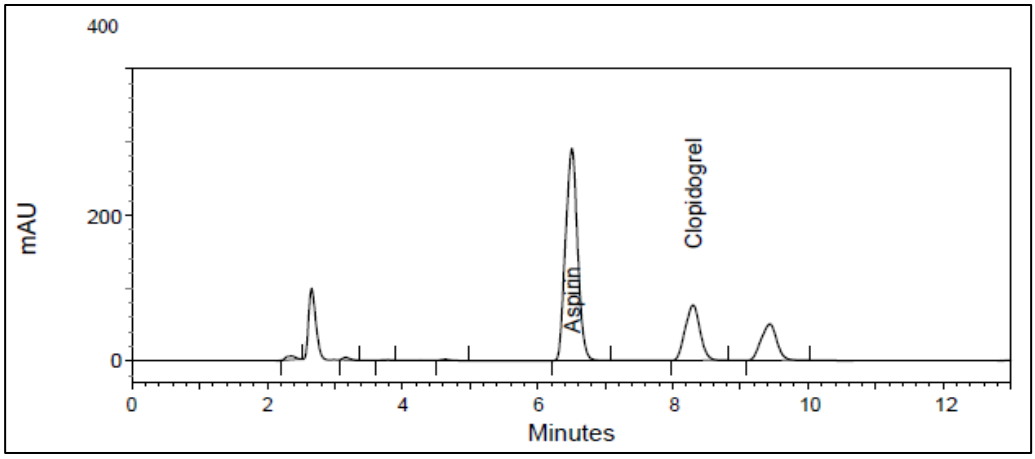

Figure 5. Chromatogram of oxidative forced degradation study

Thermal condition

Thermal degraded samples wherever degradation possible from about 1% to 30%.Preferably, the following stress conditions are was performed by exposing solid drug at 80 °C for 72 hours. Resultant chromatogram of thermal degradation study (Figure 6) indicates that aspirin is found to be slightly stable under thermal degradation condition. Only 7.0 % of drug content was degraded.

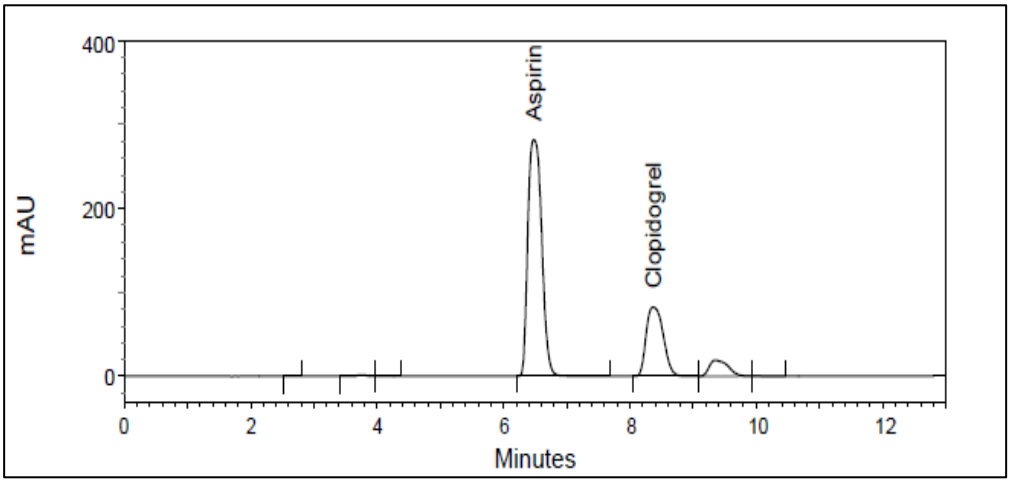

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Figure 6. Chromatogram of thermal degradation study

Photolytic condition

Exposure of drug molecules may produce photolytic degraded products. The rate of photo degradation depends upon the

intensity of incident light and quantity of light absorbed by the drug molecule. Photolytic degradation is carried out by exposing the drug substance (in solid as well as in the solution form) or drug product to a combination of visible and UV light. Photolytic degradation study was performed by exposing the drug above specific photolytic condition. Drug content was found to be more stable than other stress condition stable in UV-light. Aspirin is stable under UV light compare to clopidogrel. (Figure 7).

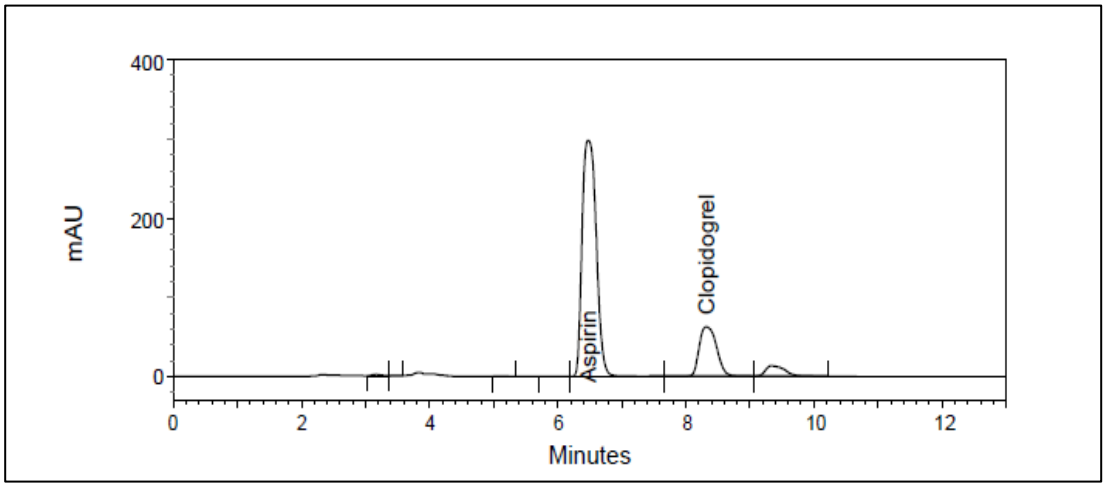

Figure 7. Chromatogram of UV-light degradation study

Linearity

For linearity seven points calibration curve were obtained in a concentration range from 0.030 – 0.120 mg/ml for aspirin and 0.015 – 0.060 mg/ml for clopidogrel, respectively. The response of the drug was found to be linear in the investigation concentration range and the linear regression equation for aspirin was y = 60026378.57x + 51410.11 with correlation coefficient 0.9999 (Figure 8) and for clopidogrel was y = 44544414.03x - 1890.29 with correlation coefficient 0.9999 (Figure 9). Where x is the concentration in mg/ml and y is the peak area in absorbance unit.

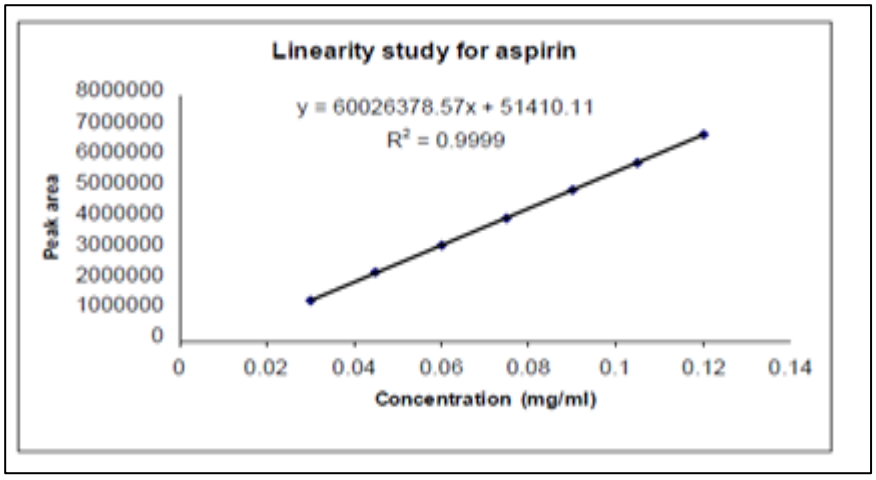

Figure 8. Linearity curve for aspirin

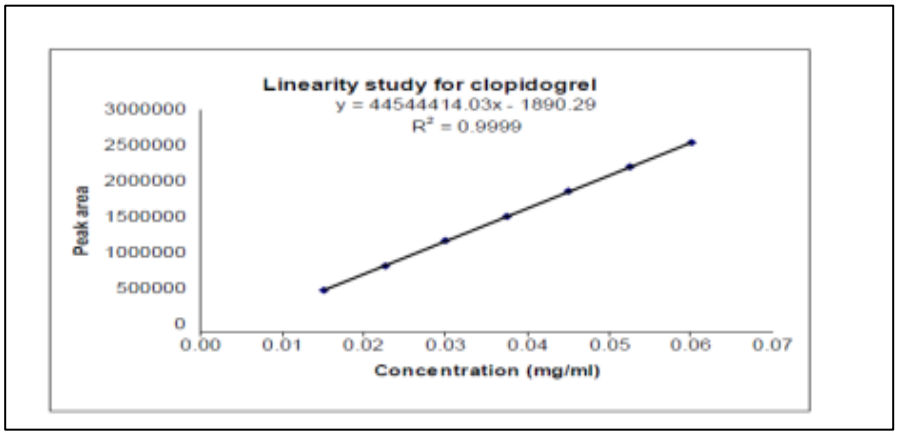

Figure 9. Linearity curve for clopidogrel

Detection and Quantification Limit (LOD and LOQ)

The limit of detection and limit of quantification were evaluated by serial dilutions of aspirin and clopidogrel stock solution in order to obtain signal to noise ratio of 3:1 for LOD and 10:1 for LOQ. The LOD value for aspirin and clopidogrel were found to be 0.05 ppm and 0.15 ppm, respectively and the LOQ value 0.2 ppm and 0.3 ppm, respectively. Chromatogram of LOD study for aspirin and was illustrated in Figure 10.

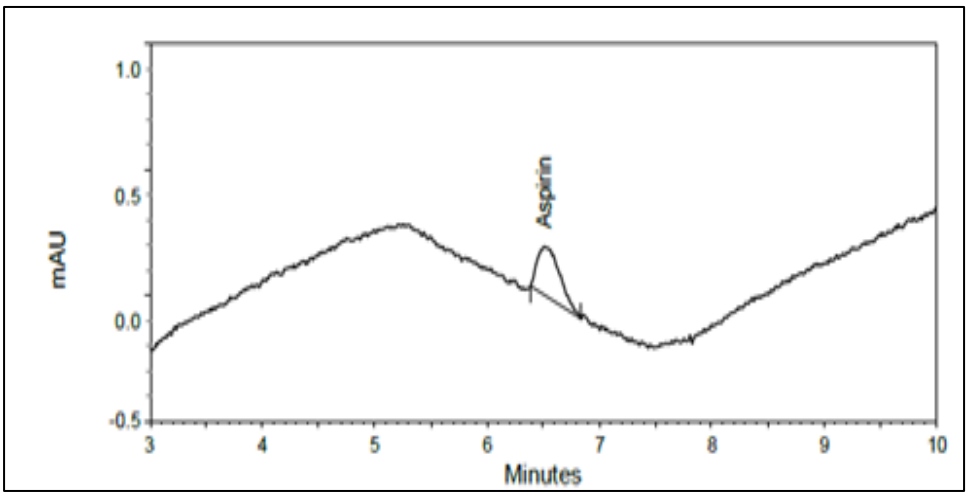

Figure 10. Chromatogram of LOD study of aspirin

Precision

Data obtain from precision experiments are given in Table 1 for intraday and interday precision study for both aspirin and clopidogrel. The RSD values for intraday precision study and interday precision study was < 2.0 % for aspirin and clopidogrel. It was confirmed that the developed method was precise for analysis. Results of precision in this study was summarized in the Table 1 below

Table 1. Results of precision study

Set	Aspirin (%Assay)		Clopidogrel (%Assay)	
	Intraday (n = 6)	**Interday (n = 6)**	**Intraday (n = 6)**	**Intraday (n = 6)**
1	99.1	100.2	99.3	99.6
2	100.0	99.9	98.7	99.6
3	99.6	100.5	98.6	100.1
4	99.5	100.3	99.0	100.1
5	100.3	101.0	100.0	100.6
6	99.1	100.8	99.5	100.7
Mean	99.6	100.5	99.2	100.1
Standard deviation	0.48	0.40	0.53	0.47
% RSD	0.48	0.40	0.53	0.47

Accuracy

Recovery of aspirin and clopidogrel were determined at three different concentration levels. The mean recovery for aspirin was 99.12 – 99.83% and 98.20 – 100.35 % for clopidogrel (Table 2). The result indicating that the method was accurate.

Table 2. Results of accuracy study

Level (%)	Amount Added Concentration (mg/ml)	Amount Found Concentration (mg/ml)	Recovery[a] (%)	RSD[a] (%)
Aspirin				
50	0.03751	0.03721	99.22	0.07
100	0.07497	0.07432	98.12	0.23
150	0.11250	0.11232	99.83	0.05
Clopidogrel				
50	0.01874	0.01840	98.20	0.19
100	0.03748	0.03695	98.59	0.14
150	0.05627	0.05647	100.35	0.24

[a] *Each value corresponds to the mean of three determination*

Robustness

The result of robustness study of the developed assay method was established in Table 3 and Table 4, respectively. The result had shown that during all variance conditions, assay value of the test preparation solution was not affected and it was in accordance with that of actual. System suitability parameters were also found satisfactory hence the analytical method would be concluded as robust method.

Table 3. Evaluation data of robustness study of aspirin

	% Assay	System Suitability Parameters		
		Theoretical	Asymmetry	% RSD
Flow 0.9 ml/min	100.5	6460	1.05	0.22
Flow 1.1 ml/min	100.3	5661	1.05	0.09
0.28 % H_3PO_4-ACN (65:35, v/v)	100.0	6117	1.00	0.40
0.32 % H_3PO_4-ACN (65:35, v/v)	99.7	5588	1.02	0.30
0.3% H_3PO_4-ACN (63:37,v/v)	100.2	5475	1.12	0.19
0.3% H_3PO_4-ACN (67:33,v/v)	100.1	5838	1.04	0.20
Column change (different lot)	100.4	5425	1.05	0.34

Table 4. Evaluation data of robustness study for clopidogrel

Robust Conditions	% Assay	System Suitability Parameters			
		Theoretical	Asymmetry	% RSD	Resolution
Flow 0.9 ml/min	98.5	6975	1.07	0.67	5.75
Flow 1.1 ml/min	100.0	5992	1.06	0.39	5.14
0.28 % H_3PO_4-ACN(65:35,v/v)	98.8	6899	1.03	1.03	6.35
0.32 % H_3PO_4-ACN(65:35,v/v)	99.2	6113	1.03	0.69	5.6
0.3% H_3PO_4-ACN(63:37,v/v)	99.5	5850	1.11	0.70	4.31
0.3% H_3PO_4-ACN (67:33,v/v)	99.0	6185	1.04	0.30	5.71
Column change (different lot)	100.0	5996	1.04	0.25	4.97

Conclusion

The method was validated for all validation parameters as per ICH guidelines. The linearity range for aspirin and clopidogrel was 0.030 – 0.120 mg/ml for aspirin and 0.015 – 0.060 mg/ml for clopidogrel with correlation coefficient 0.9999. The % RSD for intra-day precision was < 2%. The developed and validated stability indication HPLC method is found to be linear, accurate, precise, specific and robust, confirming the stability indicating method for the simultaneous estimation of aspirin and clopidogrel.

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