IJPSR (2016), Vol. 7, Issue 11 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 27 May, 2016; received in revised form, 12 July, 2016; accepted, 27 July, 2016; published 01 November, 2016

DEVELOPMENT AND VALIDATION OF A RP HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ASPIRINE AND CLOPIDOGREL IN COMBINED TABLET DOSAGE FORM

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Keywords:

RP HPLC, Aspirine, Clopidogrel, ICH guideline, LOD, LOQ

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ABSTRACT: In this present study a novel, simple, economical, accurate and precise RP-HPLC method has been developed and validated for simultaneous estimation of aspirine and clopidogrel in combined tablet dosage form. Chromatographic separation was carried out on a Kromasil HD C_{18} (150 x 4.6 mm, 5µm) column with a mixture of acetonitrile and 0.1% (v/v) orthophosphoric acid aqueous solution in a ratio of 40:60 (v/v) as mobile phase at a flow rate of 1.5 ml/min. Detection was accomplished at 237 nm by employing a DAD (Diode array detector). The retention times were 2.78 and 4.99 min for aspirine and clopidogrel respectively. The method was validated according to ICH guidelines for accuracy, precision, linearity, specificity and sensitivity. Calibration plots were linear over the concentration range 10 – 70 µg/ml for both aspirine and clopidogrel with r^2>0.997. Recovery was found to be 99.2 – 99.8% and 99.2 – 99.9% for aspirine and clopidogrel respectively with RSD < 2 for both. There were no interference due to any components of pharmaceutical dosage indicating high specificity of the method. LOD and LOQ value were found to be26.2 ng/ml and 78.2 ng/ml for aspirine and 52.7 ng/ml and 152.1 ng/ml for clopidogrel reflecting high sensitivity of the method. The method was successfully employed for quantitative analysis of commercial product. Validation study revealed that method is specific, rapid, reliable and reproducible, so it can be used for routine quality control study of aspirine and clopidogrel in combined tablet dosage form.

INTRODUCTION: Aspirine (ASP) (**Fig. 1a**), chemically 2-acetoxy benzoic acid is a cyclo oxygenase inhibitor which is used as an analgesic, antipyretic, anti-inflammatory and anti thrombic agent [1]. It is one of the most commonly used anionic drugs in the world [2]. After ingestion, it rapidly hydrolyzes to salicylic acid which is primarily responsible for its pharmacological action [3]. It is included in the official monograph of both USP-NF [4] and BP [5].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.7(11).4443-48

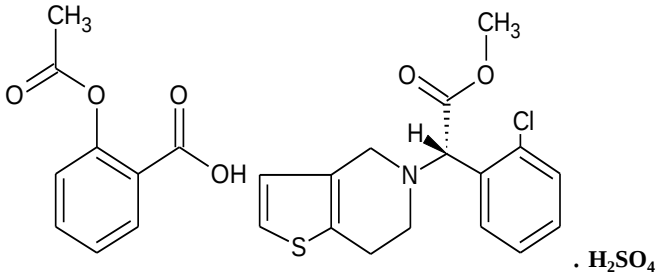
Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.7 (11).4443-48

US Food and Drug administration has approved this drug for use in secondary prevention of heart attacks and stroke due to its anti-platelet activity [4-5].

Clopidogrel bisulphate (CLO) (Fig 1b) is a USP – NF [6] enlisted drug. Its chemical name is (+)-(S)-methyl2-(2-chlorophenyl) - 2 - (6,7-dihydrothieno [3,2-*c*] pyridin-5(4*H*)-yl)acetate, sulphate which is a new thienopyridine derivative. It is an antiplatelet agent, which directly inhibits the binding of adenosine diphosphate (ADP) to its platelet receptor and blocks the subsequent ADP-mediated activation of the glycoprotein GPIIb/IIIa complex, thereby inhibiting platelet aggregation [7]. It is used to prevent ischemic stroke, myocardial infarction and vascular disease and has proved its clinical efficacy superior to that of aspirine. So, Clopidogrel is indicated for the patients with

atherosclerosis documented by recent stroke, recent myocardial infarction or cardiovascular disease [8]. The combination of ASP and CLO is used for atherosclerotic patients suffering from various heart diseases [9].



(a) **(b)**

FIG 1: CHEMICAL STRUCTURES OF (a) ASPIRINE (b) CLOPIDOGREL BISULPHATE

Literature survey revealed several assay methods for ASP and CLO individually or in combination with other drugs by UV spectrophotometry [10-12], High performance liquid chromatography (HPLC) [13-26], RP HPLC-UV [27], Reverse phase -Ultra fast liquid chromatography (RP-UFLC) [28-29], Gas Chromatography-Mass Spectrometry (GC-MS) [30] and LC-MS/MS [31]. However every method has its own limitation. Thereby, an attempt has been taken to develop and validate a simple, economic, reliable, reproducible, accurate and precise RP-HPLC based method for simultaneous estimation of ASP and CLO in combined tablet dosage form where validation of the analytical method has been performed in accordance with ICH guideline [32].

MATERIALS AND METHOD:

Apparatus and chromatographic condition:

The chromatographic condition consisted of a PerkinElmer Flexar HPLC integrated with DAD (Diode Array detector). Chromatographic separation was carried out on a Kromacil-100 HD,C18 column (15cm x 4.6mm i.e., 5 µm) with mobile phase composed of acetonitrile and 0.1% (V/V) orthophosphoric acid aqueous solutionin a ratio of 40:60 (v/v) at flow rate of 1.5 ml/min. The mobile phase was prepared freshly, filtered, sonicated before use and the detection wavelength was set at 237 nm. The injection volume was 20 µl.

Chemicals and Reagents:

The pharmaceutical grade pure samples of aspirine (99.20%) was supplied by Zhejiang Tianu Pharma co. Ltd. China, clopidogrel bisulfate (99.12%) from INFA, Italy. HPLC grade acetonitrile (Scharlau Chemie S. A., Spain) and analytical grade orthophosphoric (Scharlau Chemie S. A., Spain) were used. Commercial samples for analysis were purchased from pharmacy outlets in Dhaka, Bangladesh, labels claiming 75 mg of aspirine and 75 mg of Clopidogrel. Proper storage condition and shelf life were confirmed.

Preparation of Stock Solutions of Standard ASP and CLO:

Stock solutions of 0.5 mg/ml ASP and 0.5 mg/ml CLO were prepared in mobile phase. Serial dilution of the stock solutions were carried out using mobile phase at concentration range of 10 – 70µg/ml for both ASP and CLO. This solution was subjected to liquid chromatographic analysis.

Analysis of Tablet Formulation:

Twenty tablets were weighed accurately and grounded to power. Powder equivalent to 50 mg of ASP and 50 mg of CLO were weighed and transferred to a 100 ml volumetric flask. The powder sample was dissolved in mobile phase by intermittent shaking and sonication. A filtered portion was diluted to mobile phase to get a final concentration of 50µg/ml for both ASP and CLO. All the solutions were filtered through 0.2 micron disk filter prior to inject through HPLC.

RESULT AND DISCUSSION:

Optimization of HPLC method:

During optimization of HPLC method two different columns (Gracesmart ODS, 250 mm x 4.6 mm, 5µ and Kromasil HD C18, 150 mm x 4.6 mm, 5µ) were tried, both of them gave sharp peaks but to decrease the retention time smaller one was selected for final analysis. Different mobile phase compositions were tested for better sharpness and acceptable resolution like acetonitrile and phosphate buffer (pH 3, 4), acetonitrile with acetate buffer (pH 3, 4), acetonitrile and aqueous solution of orthophosphoric acid mixture, but the later one was selected for analysis due to better separation of peaks and most importantly this combination is easy to prepare, less time consuming and column friendly because no salt was used. Flow rate of mobile phase ranging from 1.0 ml/min – 2.0 ml/min were tried but 1.5 ml/min was optimized

because at 1.0 ml/min retention time is high and at 2.0 ml/min pump pressure is a risky factor. Analysis at several wavelength were performed but 237 nm was selected due to optimum intensity of both of the peaks.

Linearity:

The linearity for HPLC method was determined at five concentration level ranging from 10-70 µg/mlfor both ASP and CLO. The calibration curve was constructed by plotting peak area against respective concentration of ASP and CLO. The plots of peak area Vs respective concentration of ASP and CLO were found to be linear in the range of 10-70.0 µg/ml with coefficient of correlation (r^2) 0.9998 and 0.9999 for ASP and CLO respectively (**Table 1**)

TABLE 1: LINEARITY RANGE STUDY RESULTS FOR ASP AND CLO

Parameters	ASP	CLO
Linearity range	10 – 70 µg/ml	10 – 70 µg/ml
Correlation coefficient (r^2)	0.9998	0.9999
Slope	30883	14417
Intercept	290.43	-2456.24

Recovery:

Recovery studies were performed at three different levels 80, 100 and 120%. Placebo/matrix were spiked with known amount of ASP and CLO. At each level of the amount three determinations were performed and the results obtained were compared with expected results. Recovery for pharmaceutical formulations should be within the range of 100±5%. The percent R.S.D. of individual measurements was also determined. High recovery and satisfactory RSD value at each level (**Table 2**) indicates that this method can be used for routine analysis of ASP and CLO in combination drugs.

TABLE 2: RECOVERY STUDY RESULTS FOR ASP AND CLO (n=3)

Label Claim (mg/tablet)	Level of Addition (%)	Amount Added (mg)	Recovery (%) (Mean ± SD)	%RSD (Average)
ASP (75)	80	60.0	99.3±0.12	0.22
	100	75.0	99.2±0.24	
	120	90.0	99.8±0.29	
CLO (75)	80	60.0	99.9±0.26	0.32
	100	75.0	99.2±0.37	
	120	90.0	99.2±0.34	

Precision:

Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day) for 2 consecutive days. Six replicates from a homogenous sample of ASP and CLO were analyzed in the same day (intra-day precision) and 2 consecutive days (inter-day precision). %RSD of six determinations should be less than 2% at each day and day to day assay variation should be less than 1%. The specifications were absolutely conformed according to data presented on **Table3** indicating high precision of the method for simultaneous estimation of ASP and CLO from combined tablet dosage form.

TABLE 3: INTRA - DAY AND INTER – DAY PRECISION RESULT FOR ASP AND CLO. (n=6)

Compound	Intra-day precision (Repeatability)		Inter day precision		
	Assay (%) ± SD	%RSD	Assay (%) ± SD	% RSD	Difference in assay (%) between day1 and day2
ASP	99.3±0.27	0.27	99.1±0.34	0.34	0.20
CLO	99.1±0.29	0.29	98.9±0.35	0.35	0.20

Sensitivity:

Limit of detection (LOD) and limit of quantitation (LOQ) were determined from the signal-to-noise ratio. LOD and LOQ were calculated using 3.3σ/s and 10σ/s formulae, respectively, where, σ is the standard deviation of the peak areas and *s* is the slope of the corresponding calibration curve. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 26.2 ng/ml and 78.2 ng/ml for ASP and 52.7 ng/ml and 152.1 ng/ml for CLO. Lower LOD and LOQ value indicates high sensitivity of the method.

Ruggedness and Robustness: Ruggedness study was accomplished between two analyst, column and instrument. Robustness of the assay was carried out by deliberate change in few parameters like flow rate of mobile phase and mobile phase ratio. SD in retention time of ASP and CLO was found within the acceptable limit and tailing factors

of the peaks corresponding to ASP and CLO was found to be < 1.5.

TABLE 4: ROBUSTNESS STUDY RESULTS FOR ASP AND CLO (n=6)

Parameters	ASP		CLO	
	RT	TF	RT	TF
		A. Flow rate		
1.4	2.82	1.12	5.05	1.37
1.5	2.78	1.11	4.99	1.35
1.6	2.72	1.08	4.84	1.31
Mean ± SD	2.77 ± 0.05	1.1±0.016	4.96 ± 0.11	1.34±0.025
		B. Mobile phase ratio (Aqueous: organic)		
39:61	2.74	1.12	5.02	1.38
40:60	2.75	1.11	4.98	1.38
41:59	2.72	1.08	4.93	1.36
Mean ± SD	2.74 ± 0.013	1.10 ± 0.017	4.98 ± 0.037	1.37 ± 0.014

Specificity:

For testing the interference of placebo/matrix in the peak region of ASP and CLO, drug product (Placebo+ ASP + CLO) and only Placebo (mixture of all the ingredients of drug product except ASP and CLO)were run through the column, but no trace of peaks were found within the peak region of ASP and CLO due to Placebo. So this method can be used selectively used for ASP and CLO combination product. (**Fig.2** and **3**).

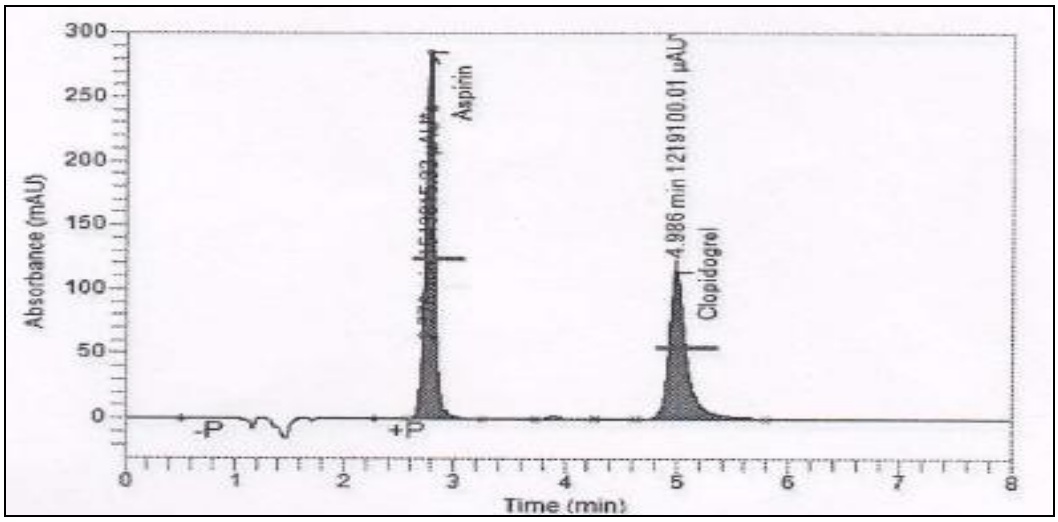


FIG. 2: CHROMATOGRAM OF DRUG PRODUCT CONTAINING ASP, CLO AND EXCIPIENTS

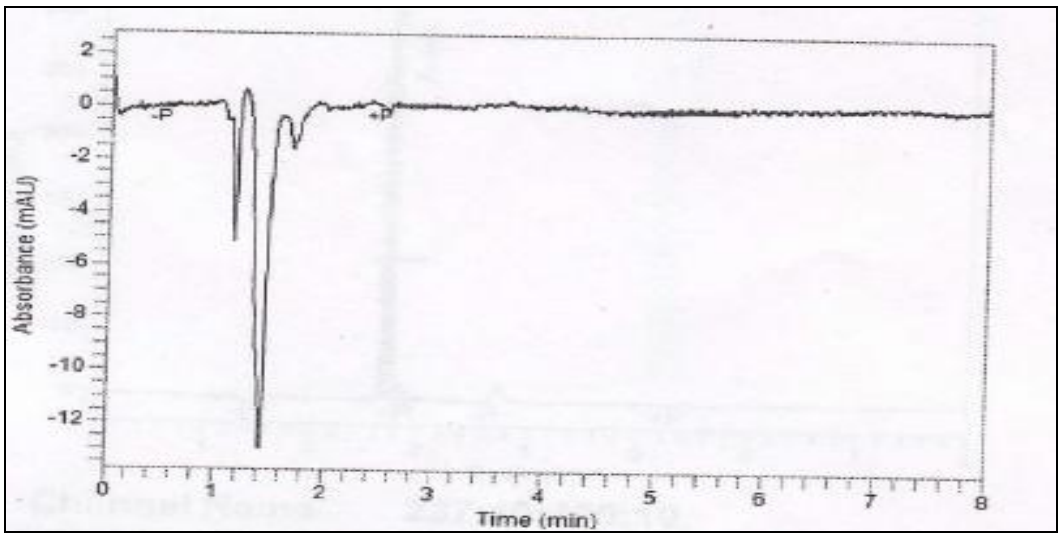


FIG. 3: CHROMATOGRAM OF PLACEBO CONTAINING ALL EXCIPIENTS EXCEPT ASP AND CLO

System Suitability:

The system suitability was evaluated by six replicate analyses of ASP and CLO mixture at a concentration of 50µg/ml for both ASP and CLO. The acceptance criteria were a R.S.D. of peak areas and retention times less than 2%, Theoretical plate numbers (N) at least 2500 for each peak and tailing factors (T) less than 1.5 for ASP and CLO. From table 5 all the system suitability parameters are within the acceptable range indicating the suitability of the method for estimation of ASP and CLO from combined product.

TABLE 5: SYSTEM SUITABILITY STUDY RESULT (n = 6)

Compound	Peak Area (%RSD)	Retention time (%RSD)	Tailing Factor (Average)	Theoretical plate (Average)
ASP	0.058	0.12	1.11	6997
CLO	0.063	0.15	1.37	6155

Stability of Solution:

Stability of solution was evaluated by injecting the same solution containing ASP and CLO at 24 hours interval. The solution was stored at room temperature. Day to day variation in parameters like peak area, retention time, tailing factor, theoretical plate counts were within the acceptable range indicating that the prepared solution can be used for two days. (**Table 6)**

TABLE 6: SOLUTION STABILITY RESULT (n = 6)

Parameters (Mean ± SD)	ASP (50 µg/ml)		CLO (50 µg/ml)	
	Day1	Day2	Day1	Day2
Peak area	1543815±852	1543201±889	1219100±763	1218963±782
Retention time	2.77±0.0034	2.77±0.0039	4.99±0.0075	4.99±0.0078
Tailing factor	1.11±0.0012	1.11±0.0015	1.35±0.0016	1.36±0.0018
Theoretical plate	6981±18	6992±23	6149±18	6132±21

Market product analysis:

Two brands of ASP and CLO combination from commercial market were tested according to this method and satisfactory result was found. **Table7**.

TABLE 7: COMMERCIAL PRODUCT ASSAY RESULTFOR ASP AND CLO (n=3)

Compound	Label claim	Brand 1		Brand 2	
		Average drug content (mg)	% of drug content	Average drug content (mg)	% of drug content
ASP	75.0 mg	74.84	99.79±0.11	74.65	99.53±0.16
CLO	75.0 mg	74.82	99.76±0.21	74.95	99.93±0.075

CONCLUSION: The developed HPLC method is simple, economic, specific, accurate and precise for the simultaneous estimation of ASP and CLO in combined tablet dosage form. The developed method offers good resolution between ASP and CLO. It was successfully validated in terms of system suitability, linearity, range, precision, accuracy, specificity, LOD, LOQ and robustness according to ICH guidelines. So, the described method is suitable for routine analysis and quality control of pharmaceutical preparations comprising these drugs either as individual or in combination.

ACKNOWLEDGEMENT: We are thankful to Veritas Pharmaceuticals Ltd. Bangladesh for providing working standard of both Aspirin and Clopidogrel Bisulfate.

CONFLICT OF INTEREST: None

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How to cite this article:
Al Masud A and Begum I: Development and validation of a RP HPLC method for simultaneous estimation of aspirine and clopidogrel in combined tablet dosage form. Int J Pharm Sci Res 2016; 7(11): 4443-48.doi: 10.13040/IJPSR.0975-8232.7(11).4443-48.