

# Simultaneous Determination of Clopidogrel and Aspirin by RP-HPLC from Bulk Material and Dosage Formulations Using Multivariate Calibration Technique

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## Abstract

A rapid, simple, and easy method for the simultaneous determination of clopidogrel and aspirin from bulk material and dosage formulations in the presence of meloxicam as internal standard has been developed. Separation was carried out on a Purospher star C<sub>18</sub> (5 μm, 250 × 4.6 mm) column at ambient temperature. The mobile phase consisted of methanol–water (80:20, v/v), the pH of the mobile phase was adjusted to 3.4 with ortho-phosphoric acid and pumped at a flow rate of 1 mL/min using isocratic pump system. Multivariate chromatographic calibration technique was subjected to high-performance liquid chromatography (HPLC) data for simultaneous quantitative analysis of binary mixtures of clopidogrel and aspirin. HPLC data based on the analyte peak areas were obtained at five wavelengths (225, 230, 235, 240, and 245 nm). The mathematical algorithm of multivariate chromatographic calibration technique is based on the use of the linear regression equations. Calibration plots for clopidogrel and aspirin were constructed at each wavelength by using the peak areas corresponding to the concentrations of each active compound. This multivariate chromatographic method was also applied to a commercial pharmaceutical dosage form containing clopidogrel and aspirin.

## Introduction

Multivariate calibration is the process of learning how to combine data from several channels in order to overcome selectivity problems, gain new insight, and allow automatic outlier detection. Multivariate calibration is the basis for the present success of high-speed near-infrared (NIR) diffuse spectroscopy of intact samples. It has shown similar advantages in, for instance, high-performance liquid chromatography (HPLC), UV–vis, and IR spectrophotometry, (transmittance, reflectance, and fluorescence), for X-ray diffraction, nuclear magnetic resonance (NMR), mass spectrometry (MS), thermal analysis, gas chromatography (GC), electrophoresis, and image analysis (tomography, microscopy), as well as other techniques (1).

Clopidogrel hydrogen sulfate (Figure 1A), or methyl (+)-(S)- $\alpha$ -(o-chlorophenyl)-6,7-dihydrothieno [3, 2-c] pyridine-5 (4H)-acetate sulphate, is a thienopyridine derivative. It is an inhibitor of ADP-induced platelet aggregation acting by direct inhibition of adenosine diphosphate (ADP) binding to its receptor and of the subsequent ADP-mediated activation of the glycoprotein GPIIb/IIIa complex (2). Clopidogrel is an analogue of ticlopidine with similar actions and uses (3–8). Clopidogrel is inactive in vitro, though its active metabolite inhibits platelet aggregation via selective binding to adenylate cyclase-coupled ADP receptors on the platelet surface (9,10). The major circulating compound is the inactive carboxylic acid derivative, which is formed by hydrolysis of the ester function by carboxylesterase (11). Clopidogrel is indicated for the reduction of atherosclerotic events in patients with atherosclerosis documented by recent stroke, recent myocardial infraction or cardiovascular disease (12).

Few methods for the determination of clopidogrel are reported in literature. A nonenzymatic and enzymatic chiral inversion of clopidogrel has been investigated in vitro using <sup>1</sup>H-NMR and a chiral HPLC spectrofluorimetric detection (13). Determination of clopidogrel in pharmaceutical dosage form by HPLC has been reported (14,15). A GC–MS method has also been reported for the analysis of the carboxylic acid metabolite of clopidogrel in plasma and serum (16).

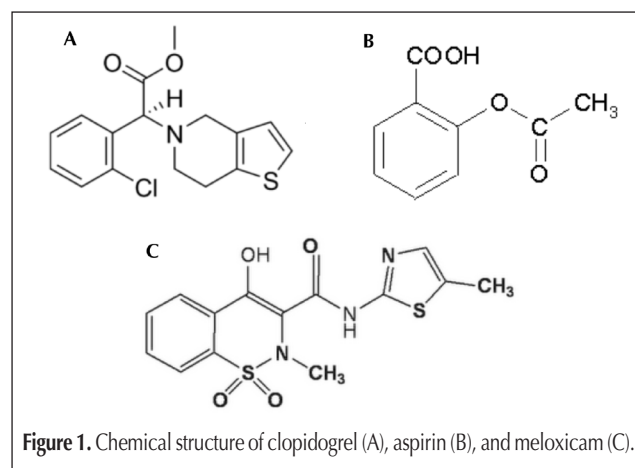


Figure 1. Chemical structure of clopidogrel (A), aspirin (B), and meloxicam (C).

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Various procedures for quantitative determination of aspirin have been investigated by different workers. Aspirin has been simultaneously determined with paracetamol, salicylic acid, phenobarbitol, caffeine, and other substances in pharmaceutical dosage forms including effervescent tablets by HPLC (17,18). Techniques of spectrophotometry and electrophoresis have also been utilized to quantitate aspirin in plasma, urine and different dosage forms (19). In a delayed release tablet, aspirin has been simultaneously determined with salicylic acid using second derivative UV spectrophotometry (20). An assay of acetylsalicylic acid and three of its metabolites has been performed in human plasma and urine using non-aqueous capillary electrophoresis with reversed electroosmotic flow (21). But no method has been reported for the simultaneous determination of aspirin and clopidogrel. In this paper simultaneous quantitation of aspirin and clopidogrel from bulk materials and dosage formulation is reported.

### A single chromatographic regression equation

The basis of the model can be explained starting from simple linear regression function. A linear regression function between two variables, concentration and peak area, for the chromatographic determination of the  $X$  analyte at  $\lambda_i$  wavelength can be defined by the equation:

$$A_{X_i} = b_{X_i} C_X + a_{X_i}$$

Where,  $A_{X_i}$  is the peak area of the  $x$  analyte at  $\lambda_i$  wavelength,  $C_X$  is the concentration of the  $x$  analyte (the concentration units are  $\mu\text{g/mL}$  in the newly developed method),  $b_{X_i}$  is the slope and  $a_{X_i}$  is the intercept of the regression equation. This intercept value indicates the difference between the ideal and calculated system.

### Multivariate chromatographic calibration technique

Multivariate chromatographic calibration technique is a powerful mathematical tool for optimizing chromatographic multivariate calibration and elimination of fluctuations coming from instrumental and experimental conditions. This multivariate chromatographic calibration contains reduction of multivariate linear regression functions to univariate data set. Under optimized conditions, the applied numerical method provides considerable resolving power and sensitivity for the quantitative analysis, quality control, and routine analysis of subject compounds. The mathematical algorithm of this approach is explained as follows:

If the absorbance of an analyte ( $X$ ) is measured at five wavelength set as in our case ( $\lambda = 225, 230, 235, 240, \text{ and } 245 \text{ nm}$ ), following chromatographic linear regression equations can be written for each analyte in the mixture.

$$\begin{aligned} A_{\lambda 225} &= a \times C_X + k_1 \\ A_{\lambda 230} &= b \times C_X + k_2 \\ A_{\lambda 235} &= c \times C_X + k_3 \\ A_{\lambda 240} &= d \times C_X + k_4 \\ A_{\lambda 245} &= e \times C_X + k_5 \end{aligned} \quad \text{Eq. 1}$$

Where  $A_x$  represent the peak area of the analyte,  $a, b, c, d,$  and  $e$  are the slopes of linear regression functions of the analyte,  $k_1, k_2, k_3, k_4,$  and  $k_5$  are the intercept of linear regression functions at five selected wavelengths and  $C_X$  represents the concentration of analyte. The five-equation system (1) can be summarized as:

$$A_T = a \times C_X + b \times C_X + c \times C_X + d \times C_X + e \times C_X + K_T \quad \text{Eq. 2}$$

It can be simplified to

$$A_T = C_X (a + b + c + d + e) + K_T \quad \text{Eq. 3}$$

Where  $A_T$  and  $K_T$  represents the sum of absorbance obtained and sum of intercepts of regression equations at five-wavelength set respectively. The concentration of the  $x$  analyte in a mixture can be calculated by using the following equation.

$$C_X = A_T - K_T / (a + b + c + d + e) \quad \text{Eq. 4}$$

This procedure is the mathematical basis of the multivariate chromatographic approach for a single component, two-component, and multi-component analysis. In this case, the multivariate chromatographic calibration model contains the use of linear regression function based on the relationship between peak area ratio and concentration of analyte. The algorithm of this calibration model was repeated for the prediction of unknown concentration of each analyte in a mixture containing two or more active compounds.

## Experimental

### Chemicals and reagents

Clopidogrel bisulphate was kindly donated by Getz Pharma (Pvt.) Ltd., (Karachi, Pakistan). Aspirin reference standard was obtained from Reckitt Benckiser Pakistan Ltd., (Karachi, Pakistan). Pidogrel-AP 75 tablets labeled to contain 75 mg clopidogrel and 75 mg aspirin and Pidogrel- AP 150 tablets whose declared contents are 75 mg clopidogrel and 150 mg aspirin were obtained from a local market in Karachi, Pakistan. All analytical grade reagents used were obtained from Merck (Pvt.) Ltd., (Karachi, Pakistan). Methanol gradient grade (Tedia, **City, State**) and deionized filtered water was used to prepare mobile phase.

### Apparatus and chromatographic conditions

The chromatographic system consisted of Shimadzu model LC-10AT VP pump with a SPD-10A, UV-vis detector. Chromatographic system was integrated via Shimadzu model CBM-102 Communication Bus Module to a Pentium IV. The separation was performed on a Purospher start,  $C_{18}$  ( $5 \mu\text{m}, 250 \times 4.6 \text{ mm}$ ) column at ambient temperature. The samples were introduced through a rheodyne injector valve with a  $20\text{-}\mu\text{L}$  sample loop. The mobile phase consisted of  $\text{CH}_3\text{OH-H}_2\text{O}$  (80:20%, v/v) and pumped at a flow rate of 1 mL/min using isocratic pump system. Mobile phase was filtered through a  $0.45\text{-}\mu\text{m}$  Millipore filter and degassed ultrasonically before use. The eluents were monitored at 225, 230, 235, 240, and 245 nm.

### Preparation of stock and working solutions

Stock solutions of 100 µg/mL of clopidogrel and aspirin were prepared in methanol. The working standard solutions were prepared by dilution of the stock solutions with the same solvent to reach a concentration range of 0.25–50 µg/mL. These solutions were used in the preparation of calibration curves. All the solutions were prepared each day and protected from light. The stock solutions were stored at 4°C.

### Sample preparation

Ten Pidogrel-AP 75 tablets (each containing 75 mg clopidogrel and 75 mg aspirin) manufactured by Highnoon Laboratories, Ltd., (Lahore, Pakistan) were accurately weighed and finely powdered in a mortar. An amount of the tablet mass equivalent to one tablet was transferred to a 100 mL volumetric flask, dissolved in methanol; the volume was then completed to the mark with the same solvent. A portion of this solution was then filtered through a Whatman filter paper no. 41 and diluted to 50, 25.0, 12.5, 5.0, 1.0, and 0.25 µg/mL.

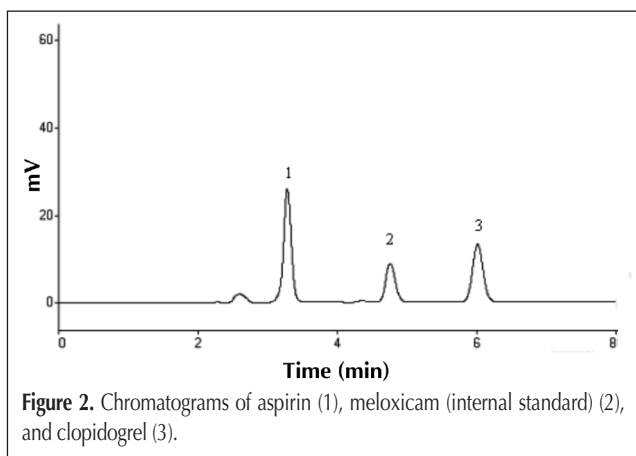


Figure 2. Chromatograms of aspirin (1), meloxicam (internal standard) (2), and clopidogrel (3).

## Results

### Multivariate chromatographic calibration technique

This method is based on the simultaneous use of the linear regression functions obtained by plotting the concentration versus ratio of peak area (analyte area/internal standard area) in the working concentration range. The five linear regression functions at wavelengths of 225, 230, 235, 240, and 245 nm for individual active compounds were calculated by using relationships between the multivariate HPLC data and concentration set (standard series of drug solutions). For each active compound, the formulated numerical HPLC approach explained previously was constructed from the total slope and intercept values were obtained from the individual calibration functions. The unknown concentration of clopidogrel and aspirin in tablets was determined by the equation (4) using the sum of peak areas obtained at five wavelengths for samples.

### Classical HPLC

A typical HPLC chromatogram of clopidogrel and aspirin along with internal standard meloxicam is shown in Figure 2. Aspirin appeared at retention time of 3.2, clopidogrel at 6.0, and internal standard at 4.7. Five different wavelengths (225, 230, 235, 240, and 245 nm) were selected to record these chromatograms under optimized chromatographic conditions (Figure 3). The results of this multivariate calibration technique applied to reference drugs and dosage formulations are shown in Table I.

In the wavelength points, linear regression functions for each active compound were obtained from the HPLC data. The calculated linear regression functions and their statistical parameters are presented in Table II. The correlation coefficients of regression equations were found to be satisfactory in the application of the developed algorithm.

Table I. Multivariate Precision and Accuracy of Clopidogrel and Aspirin

Added conc. (µg/mL)	Found conc. (µg/mL)					Multivariate calibration	% Accuracy					Multivariate calibration	
	225	230	235	240	245		225	230	235	240	245		
<i>Clopidogrel</i>													
0.25	0.242	0.246	0.249	0.249	0.247	0	96.98	98.44	99.69	99.67	99.14	0	
1.0	0.985	0.985	0.999	1.00	0.994	0	98.55	98.58	99.94	100.10	99.43	0	
5.0	4.95	5.01	5.00	4.96	4.99	6.57	99.11	100.32	100.01	99.32	99.97	131.43	
12.5	12.12	12.82	12.64	12.90	12.63	12.57	97.01	102.60	101.15	103.27	101.11	100.56	
25.0	25.10	25.00	25.35	24.40	25.17	26.26	100.40	100.02	101.40	97.61	100.70	105.04	
50.0	50.05	50.03	50.02	49.96	50.35	49.23	100.11	100.07	100.04	99.92	100.71	98.46	
						Mean	98.69	100.00	100.37	99.98	100.17	108.87	
						RSD	1.47	1.50	0.71	1.84	0.78	3.50	
						RE	1.30	-0.005	-0.37	0.01	-0.17	-8.87	
<i>Aspirin</i>													
0.25	0.250	0.252	0.252	0.257	0.259	0.196	100.09	101.00	100.85	103.02	103.95	78.46	
1.0	1.00	1.01	0.994	1.00	1.03	0.023	100.07	101.07	99.42	100.02	103.30	1.21	
5.0	4.99	4.99	5.00	4.98	5.01	3.482	99.80	99.96	99.70	99.70	100.03	69.64	
12.5	12.06	12.34	13.06	12.86	12.97	13.28	96.48	98.73	104.49	102.88	103.78	106.29	
25.0	25.10	24.97	25.33	24.81	25.08	20.24	100.41	99.91	101.32	99.24	100.34	80.99	
50.0	49.76	49.77	49.77	50.01	48.47	50.46	99.92	99.92	100.96	100.03	96.95	100.92	
						Mean	99.46	100.09	100.96	100.81	100.39	72.91	
						RSD	1.48	0.86	1.86	1.66	2.73	51.84	
						RE	0.53	-0.09	-0.81	-1.39	-1.39	27.08	

### Method validation

Under optimum experimental conditions, linear correlation between the peak areas and applied concentration (plotted at five wavelengths) was found in the concentration range 0.25–50 ( $\mu\text{g/mL}$ ), as confirmed by the correlation coefficients (Table II). The peak area ( $y$ ) is proportional to the concentration of clopidogrel and aspirin ( $x$ ) following regression equation. The experimentally determined LOD and LOQ for clopidogrel and aspirin were ranged 0.499–4.103  $\mu\text{g/mL}$  and 0.013–2.60  $\mu\text{g/mL}$  respectively (Table II).

Precision data on the intra- and inter-day variations for different concentrations were less than 2% (RSD), indicating sufficient precision. Mean recoveries of aspirin and clopidogrel from pharmaceutical dosage were ranged 99.46–100.96% and 98.69–100.37% with RSD below 2% and relative error also < 2%

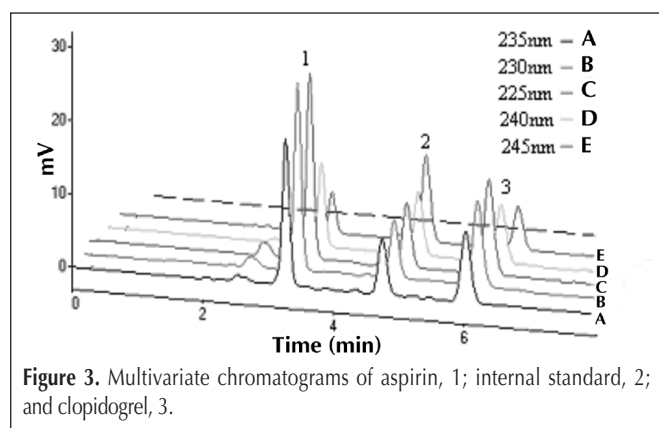
**Table II. Calibration Curves and LOD and LOQ of Clopidogrel and Aspirin at Different Wavelengths\***

Conc. range ( $\mu\text{g/mL}$ )	$\lambda$ (nm)	Regression equation	$r^2$	LOD ( $\mu\text{g/mL}$ )	LOQ ( $\mu\text{g/mL}$ )
<i>Analyte: Clopidogrel</i>					
0.25–50	225	$Y = 2648.37x + 10466.88$	0.9964	1.747	4.103
	230	$Y = 2518.86x + 5991.46$	0.9981	0.499	1.514
	235	$Y = 1968.83x + 4086.67$	0.9980	0.749	2.270
	240	$Y = 1667.26x + 6389.11$	0.9924	0.574	1.742
	245	$Y = 1180.36x + 4996.62$	0.9935	0.706	2.140
<i>Analyte: Aspirin</i>					
0.25–50	225	$Y = 3958.42x + (-3977.21)$	0.9922	0.234	0.712
	230	$Y = 2864.83x + 3602.92$	0.9931	0.013	0.039
	235	$Y = 3025.77x + (-5015.24)$	0.9625	0.141	0.427
	240	$Y = 1853.49x + (-893.73)$	0.9989	0.858	2.60
	245	$Y = 893.73x + (-75.41)$	0.9979	0.385	1.168

\* Correlation of coefficient =  $r^2$ , Limit of detection = LOD, Limit of quantitation = LOQ.

**Table III. Assay of Clopidogrel and Aspirin in Different Tablets**

Brand name	Aspirin			Clopidogrel		
	Claimed	Found	% Contents	Claimed	Found	% Content
Pidogrel-AP 75	75.0 mg	74.80 mg	99.73	75.0 mg	75.055 mg	100.06
Pidogrel-AP 150	150.0 mg	150.55 mg	100.36	75.0 mg	75.35 mg	100.46



**Figure 3.** Multivariate chromatograms of aspirin, 1; internal standard, 2; and clopidogrel, 3.

for all analyzed concentrations (Table I), confirming the accuracy of the method.

### Tablet analysis

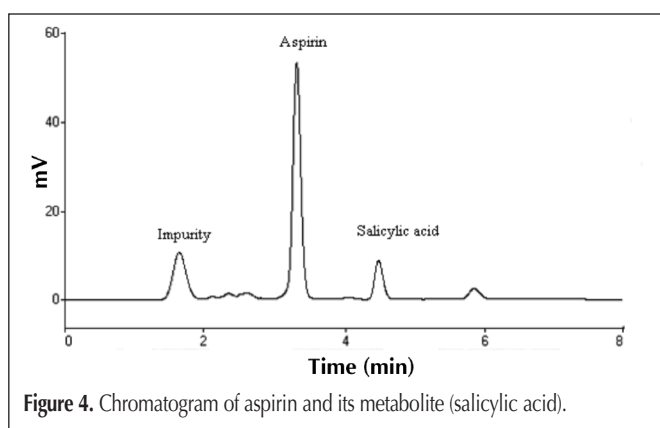
Validity of the proposed method was tested for pharmaceutical preparation by assaying aspirin and clopidogrel tablets. One formulation was labeled to contain 75 mg clopidogrel and 75 mg aspirin and the other labeled to contain 75 mg clopidogrel and 150 mg aspirin. Other substances present in the matrix did not alter the shape of the peaks. The results (Table III) produced are complying with the label claim of commercial pharmaceutical dosage form.

### Discussion

Multivariate calibration technique based on regression analysis is established for the quantitative multiresolution of a mixture containing clopidogrel and acetylsalicylic acid. The mathematical algorithms of multivariate calibrations as namely multi-linear regression calibration are based on the use of the linear regression equations at five-wavelength set in the range of 225–245 nm. In this technique, we are increasing the amount of data by using more wavelength points for calibration, which gives results that are closer to actual values

The developed multivariate model was then applied to HPLC linear regression functions for the multi mixture analysis. The quantitative determination of clopidogrel and aspirin in samples was done by the constructed multivariate chromatographic calibration method.

To optimize the operating conditions for HPLC-UV detection of clopidogrel and aspirin, a number of parameters such as column type, mobile phase compositions, pH, effectors (phosphoric or acetic acid) and flow rate were varied. Conditions that had a shorter retention time,  $C_{18}$  reversed-phase analytical column, mobile phase methanol–water 80:20 (v/v) pH 3.4 (adjusted with phosphoric acid) and flow rate = 1 mL/min were chosen as optimal. Meloxicam as an internal standard is suitable in our case as shown in Figure 2, distinct and appropriate with retention time neither too short nor too long. System suitability data obtained at different wavelengths is shown in Table IV.



**Figure 4.** Chromatogram of aspirin and its metabolite (salicylic acid).



**Table IV. System-Suitability Data for Separation of Clopidogrel and Aspirin under the Optimum Separation Conditions\***

Wavelength (nm)	k	AS	N	RS
Drug: Clopidogrel				
225	1.65	1.07	7296	0.58
230	1.33	1.06	7100	0.55
235	1.65	1.07	7341	0.50
240	1.33	1.07	7327	4.73
245	2.47	1.07	7702	2.60
Drug: Aspirin				
235	0.44	0.96	4236	0.77
230	0.27	0.96	4214	2.67
225	0.44	0.96	4269	2.83
240	0.27	0.96	4237	2.69
245	1.0	0.95	4309	0.20

\* Mobile phase = MeOH-H<sub>2</sub>O (80:20), pH 3.40 flow rate = 1 mL/min; K = capacity factor; AS = peak asymmetry; N = number of theoretical plates; RS = resolution.

In order to evaluate the selectivity of method, excipients were added to prepare synthetic binary mixture. These include avicel PH (microcrystalline cellulose), magnesium stearate, hydroxypropylmethyl cellulose, lactose, and cornstarch. There was no interference of these excipients in analysis

Aspirin rapidly hydrolyzes in various aqueous, organic, and biological media with the resultant degradation products being salicylic acid and acetic acid. During the study, the decomposition of aspirin also took place when its solution was kept at room temperature for 4–6 h. A separate peak of degradative product (salicylic acid) along with aspirin was obtained (Figure 4).

All the subjected wavelength points were included into the calculations. The results showed some variations from each other for the classical HPLC and unsatisfied results at lower concentrations for multivariate HPLC data (Table I).

## Conclusions

In this study, a multivariate approach has been applied to high-performance liquid chromatographic analysis. This technique was subjected to HPLC data for simultaneous quantitative analysis of binary mixtures and a commercial tablet formulation containing clopidogrel and aspirin. Successful chromatographic separation of clopidogrel, aspirin, and meloxicam (internal standard) was accomplished. Validity of the proposed method was also proved by assaying pharmaceutical preparation containing aspirin and clopidogrel. In our case, classical HPLC provided better results as compared to multivariate HPLC. Still at higher concentrations of analyte, multivariate technique provided acceptable results. The proposed method has been proved to be convenient and effective for the quality control of clopidogrel and aspirin in pharmaceutical dosage forms.

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