JOURNAL OF ADVANCED MEDICAL AND PHARMACEUTICAL RESEARCH

Article

Development and Validation of RP-HPLC Method for Analysis of Counterfeit Clopidogrel

Received 13th December 2023,
Accepted 25th January 2024,
Published 8th February 2024

DOI: 10.21608/JAMPR.2024.255346.1061

jampr.journals.ekb.eg

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ABSTRACT

The isocratic RP-HPLC method was developed and validated for the analysis of clopidogrel bisulfate counterfeited with acetyl salicylic acid and applied for the determination of clopidogrel bisulfate in pharmaceutical dosage forms. Clopidogrel bisulfate is commonly counterfeited with acetyl salicylic acid and it is not labeled to contain acetyl salicylic acid due to their similar pharmacological effect in inhibiting platelet aggregation. The mobile phase was composed of potassium dihydrogen phosphate buffer 0.02M (pH 2.8) and methanol 30:70 (v/v). The flow rate was 1.5mL/min. The Diode array detector was set at 220 nm. The linearity ranges were 10-70 µg/mL and 6-50 µg/mL for clopidogrel bisulfate and acetyl salicylic acid, respectively. The retention time was 2.5 min and 10.23 min for clopidogrel bisulfate and acetyl salicylic acid, respectively. The method was validated according to ICH guidelines. The developed method was successfully applied for the determination of clopidogrel bisulfate counterfeited with acetyl salicylic acid as well as the estimation of clopidogrel bisulfate in its different dosage forms.

Keywords: Isocratic HPLC; clopidogrel bisulfate; dosage forms; acetyl salicylic acid

1. INTRODUCTION

Clopidogrel bisulfate **(Figure 1a)** is a platelet inhibitor, so it is used in the treatment of cardiovascular diseases [1]. It is a pro-drug; about 85% of it is hydrolyzed by esterase to an inactive carboxylic acid metabolite, and 15% is converted by the liver cytochrome P450 enzymes to the active metabolite that irreversibly blocks the P2Y12 receptor by forming disulfide bond [2].

Clopidogrel bisulfate is adulterated with acetyl salicylic acid due to the low price of acetyl salicylic acid and its similarity in pharmacological activity in inhibiting platelet aggregation [3].

Acetyl salicylic acid (Asc) **(Figure 1b)** inhibits COX-1 and COX-2 enzymes, so it is used as an analgesic, anti-inflammatory, and antipyretic drug. Acetyl salicylic acid in small doses (75, 81, and 100 mg) is used in the prophylaxis and treatment of heart diseases such as ischemic stroke, myocardial infarction, and angina pectoris as it blocks thromboxane A2 effect on platelets, irreversibly and inhibits platelet aggregation [4].

There are several methods reported in the literature for the analysis of clopidogrel bisulfate [5], such as UV/Vis spectrophotometry, high-performance liquid chromatography (HPLC), micellar liquid chromatography

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(MLC), micellar electrokinetic chromatography (MEKC), high-performance thin layer chromatography (HPTLC), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [6]. Also, electrochemical methods were used for the analysis of clopidogrel bisulfate [7-9].

Counterfeit clopidogrel bisulfate was analyzed by different methods such as RP-HPLC, TLC, and mass spectroscopy [3,10]. Few analytical methods were reported for the simultaneous determination of clopidogrel and acetyl salicylic acid by RP-HPLC [11] and chemometric techniques [12].

The aim of this study was to develop and validate a rapid, simple, accurate, and precise RP-HPLC method for the determination of clopidogrel counterfeited with acetyl salicylic acid.

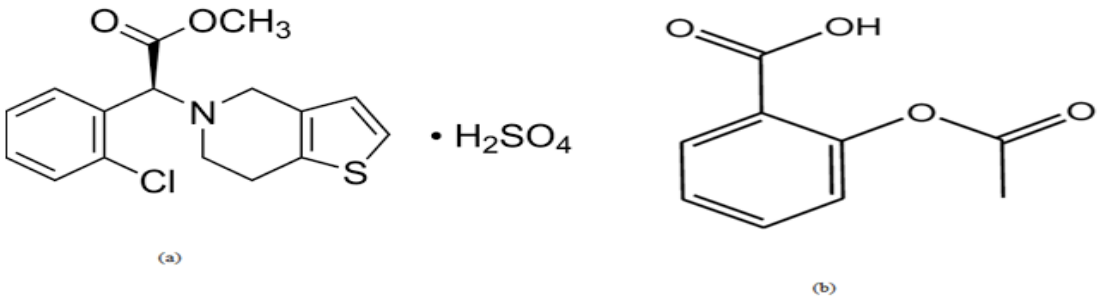

Figure 1. Chemical structure of (a) clopidogrel bisulfate and (b) acetyl salicylic acid.

2. EXPERIMENTAL

2.1. Apparatus and software

Dionex Ultimate 3000 RS Thermo Scientific HPLC system (Sunnyvale, CA, USA) with diode array detector (DAD), Quaternary RS pump, RS auto-sampler injector, and Thermo stated RS column was used. The software Dionex Chromeleon version 7.1.2.1478 was used for the recording and integration of chromatograms. HANA instruments pH 211 microprocessor pH Meter with double junction glass electrode was used. The sonicator used was 3510 Branson.

2.2. Materials and reagents

Clopidogrel bisulfate (99.90 %) and acetyl salicylic acid (99.75%) were obtained from Sigma Company for pharmaceutical industries, Quesna, Menuofia, Egypt. Methanol of HPLC grade was obtained from Cornell lab, Cairo, Egypt. Dihydrogen phosphate was obtained from El-Gomhouria Co. For Trading Drugs, Chemicals & Medical Supplies (23 El Sawah Street, Cairo, Egypt).

Preparation of phosphate buffer

Phosphate buffer (0.02M) was prepared by dissolving 1.36 gm of potassium dihydrogen phosphate in distilled water in a 500 mL volumetric flask, then sonicated for 15 minutes. Then the volume was completed to 500 mL with distilled water. pH was adjusted to 2.8 with phosphoric acid. The buffer solution was filtered using a 0.45µm filter membrane and then sonicated for 30 minutes for degassing.

Pharmaceutical Dosage Forms

Borgavix® tablets (Borg Pharmaceutical Industries, Alexandria, Egypt) labeled to contain 98 mg of clopidogrel bisulfate per tablet (equivalent to 75mg of clopidogrel).

Myogrel plus® tablets (Adwia Co. S.A.E. 10th of Ramadan city, Egypt) were used. Each tablet is labeled to contain 75 mg of acetyl salicylic acid and 98 mg of clopidogrel bisulfate (equivalent to 75 mg of clopidogrel).

2.3. Preparation of standard solutions

2.3.1. Standard stock solution

A standard stock solution of 200µg/mL was prepared for each of clopidogrel bisulfate and acetyl salicylic acid in methanol by weighing 20 mg of each drug and dissolving in methanol in two separate 100 mL volumetric flasks. Finally, the volume was completed to the mark with methanol.

2.3.2. Standard working solutions

Different volumes (0.5, 1.5, 2, 2.5, and 3.5) mL of clopidogrel bisulfate and (0.3, 0.5, 1.5, 2, and 2.5) mL of acetyl salicylic acid were taken from the standard stock solution of each drug into 10 mL volumetric flasks and the volume was completed to the mark with methanol to obtain different concentrations (10, 30, 40, 50 and 70) µg/mL and (6, 10, 30, 40 and 50) µg/mL for clopidogrel bisulfate and acetyl salicylic acid, respectively to construct the calibration curves.

2.4. Optimum chromatographic conditions

The column was Thermo scientific ODS HYPERSIL (250 x 4.6 mm and particle size of 5µm). The mobile phase was composed of methanol and phosphate buffer 0.02M (pH 2.8) with a ratio 70:30 (v/v). The flow rate was 1.5mL/min. Diode array detector (DAD) was used at 220 nm. The injection volume was 10 µL at ambient temperature.

2.5. Construction of calibration curve

Mixtures of clopidogrel bisulfate and acetyl salicylic acid were prepared in the concentration range 10-70 and 6-50 µg/mL for clopidogrel bisulfate and acetyl salicylic acid, respectively by taking different volumes (0.5-3.5 µL) and (0.3-2.5 µL) from standard stock solution of clopidogrel bisulfate and acetyl salicylic acid, respectively in 10 mL volumetric flasks. Then the volumes were diluted to the mark by methanol, 10 µL of each prepared mixture of clopidogrel bisulfate and acetyl salicylic acid were injected triple at the optimum chromatographic conditions, and the area under the curve was calculated. The calibration curves were constructed by plotting the area under the curve versus the corresponding concentrations for each drug. The regression equations were calculated.

2.6. *Preparation of dosage forms*

Borgavix® tablets (each tablet is labeled to contain 98 mg of clopidogrel bisulfate) and Myogrel plus® tablets (each tablet is labeled to contain75 mg of acetyl salicylic acid and 98 mg of clopidogrel bisulfate) were used.

For each type of these commercial tablets, ten tablets were weighed and finely powdered. An accurate amount of the powder equivalent to one tablet was weighed, dissolved in methanol, and transferred quantitatively into a 100 mL volumetric flask, and the volume was completed to the mark with methanol and filtered. 0.5 mL of the filtrate was transferred into a 10 mL volumetric flask, and the volume was completed to the mark using methanol. A 0.45 µm membrane filter was used for filtration before injection in HPLC. 10 µL of each prepared concentration was injected triple at the optimum chromatographic conditions. The assay solution of borgavix® contained 49 µg/mL of clopidogrel bisulfate while that of Myogrel plus® contained 49 µg/mL of clopidogrel bisulfate and 37.5µg/mL of acetyl salicylic acid.

3. RESULTS AND DISCUSSION

3.1. Method development

Several trials were made for the development of the isocratic RP- HPLC method for the determination of clopidogrel bisulfate counterfeited with acetyl salicylic acid. Several ratios of organic solvent (methanol) were investigated as 55%, 65%, 70%, and 75%. It was found that 70% methanol was the optimum. Potassium dihydrogen phosphate buffer (0.02M) was tried at different pH (3, 3.5, and 2.8). pH 2.8 was the optimum. Several flow rates were tried as 1, 1.2, and 1.5 mL/min. The flow rate of 1.5mL/min was the optimum.

Clopidogrel bisulfate was adulterated with acetyl salicylic acid, so the isocratic RP- HPLC method was developed and validated for its determination in counterfeited tablets. Thermo scientific ODS HYPERSIL column (250 x 4.6 mm and particle size of 5µm) was used. The mobile phase was composed of potassium dihydrogen phosphate buffer 0.02M (pH 2.8) and methanol 30:70 (v/v). The flow rate was 1.5mL/min. The Diode array detector was set at 220 nm. The injection volume was 10 µL at ambient temperature.

The retention time was 2.5 min and 10.23 min for clopidogrel bisulfate and acetyl salicylic acid, respectively **(Figure 2)**. Three mixtures were prepared for clopidogrel bisulfate that was adulterated with acetyl salicylic acid by ratios 30, 40, and 50%. The method was applied to the analysis of dosage form.

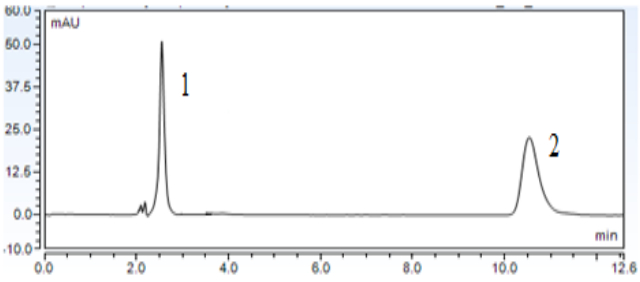

Figure 2. Chromatogram obtained by isocratic RP-HPLC for mixture of clopidogrel bisulfate 70µg/mL (peak2) and its adulterant acetyl salicylic acid 30µg/mL (peak1) using mobile phase 0.02M phosphate buffer (pH 2.8): methanol (30: 70, v/v), flow rate 1.5mL/min and DAD at 220 nm.

3.2. Method validation

The developed isocratic RP-HPLC method was validated as per ICH guidelines [13].

3.2.1. System suitability

System suitability parameters for the developed isocratic RP-HPLC method for the determination of clopidogrel bisulfate counterfeited with acetyl salicylic acid were calculated, and the results were acceptable regarding the reference values, as shown in **(Table 1)**.

Table 1. System suitability parameters for clopidogrel bisulfate counterfeited with acetyl salicylic acid by the proposed isocratic RP-HPLC method.

Parameter	Clopidogrel Bisulfate	Acetyl salicylic acid	Reported values [14]
t_r (min)	10.23	2.5	-
NTP	3499	2987	> 2000
Asym	1.60	1.12	< 2
k`	9.23	1.50	2-10

t_r: retention time, NTP: number of theoretical plates, Asym: asymmetry factor of the peak, and k`: capacity factor.

3.2.2. Linearity

Clopidogrel bisulfate and acetyl salicylic acid showed linearity at concentration ranges of 10-70 µg/mL and 6-50 µg/mL, respectively. The regression equations for these drugs had a good correlation coefficient, as shown in **(Table 2)**.

Table 2. Regression parameters for determination of clopidogrel bisulfate counterfeited with acetyl salicylic acid by the proposed isocratic RP-HPLC method.

Regression parameters	Clopidogrel bisulfate	Acetyl salicylic acid
Linearity range (µg/mL)	10-70	6-50
r^2	0.9997	0.9998
a	0.108	0.195
b	0.146	0.227
S_a	0.074	0.050
S_b	0.002	0.002
$S_{(y/x)}$	0.016	0.016

r^2: coefficient of determination, a: intercept, b slope, S_a: standard deviation of intercept, S_b: standard deviation of slope, $S_{(y/x)}$: residual standard deviation.

3.2.3. Limit of detection (LOD) & Limit of quantitation (LOQ)

The values of LOQ, LOD for clopidogrel bisulfate and acetyl salicylic acid were calculated from the next equations:

$$LOQ = (10x\ \sigma) / b$$

$$LOD = (3.3x\ \sigma) / b$$

where σ is the standard deviation of blank that was determined by measuring the absorbance of solvent (methanol) triple at the wavelength of each drug, and b is the slope of the calibration curve.

LOD values were found to be 0.044 and 0.034 µg/mL, while LOQ values were found to be 0.132 and 0.103 µg/mL for clopidogrel bisulfate and acetyl salicylic acid, respectively.

3.2.4. Accuracy and precision

Three mixtures of clopidogrel bisulfate adulterated with acetyl salicylic acid by percentages 30%, 40%, and 50% were prepared within the linearity range and determined triple on the same day and for three days. The average of % recovery ± SD indicated the accuracy of the developed method **(Table 3)**. %RSD was determined for intraday and inter-day precision as presented in **(Tables 4 and 5)**.

Table 3. Evaluation of accuracy for determination of clopidogrel bisulfate counterfeited with acetyl salicylic acid by the proposed isocratic RP-HPLC method.

Drug	Conc. taken (µg/mL)	*Mean Found conc.(µg/mL)	% Recovery	Mean% Recovery ± SD
Clopidogrel bisulfate	50	49.56	99.13	99.14 ± 0.18
	60	59.38	98.96	
	70	69.53	99.32	
Acetyl salicylic acid	50	49.62	99.25	99.26± 0.33
	40	39.57	98.94	
	30	29.88	99.59	

*Mean for three determinations, SD: standard deviation

3.2.5. Robustness

Robustness was evaluated for the developed isocratic RP-HPLC method by changing pH by ±0.2 and the organic solvent ratio (methanol) by ±2%. % RSD for three concentrations was less than two, so the developed method proved to be robust, as shown in **(Table 6)**.

3.2.6. Selectivity

The selectivity of the developed method was studied by the determination of clopidogrel bisulfate in the presence of excipients. The selectivity was confirmed by the good mean % recovery ± SD **(Table 7)** as well as by comparing the chromatograms of the tablet and that of a standard solution of clopidogrel bisulfate drug regarding peak position and shape **(Figure 3)**; it was found that they were identical. This indicates that there is no interference from excipients, and the method was selective for the determination of clopidogrel bisulfate in dosage form.

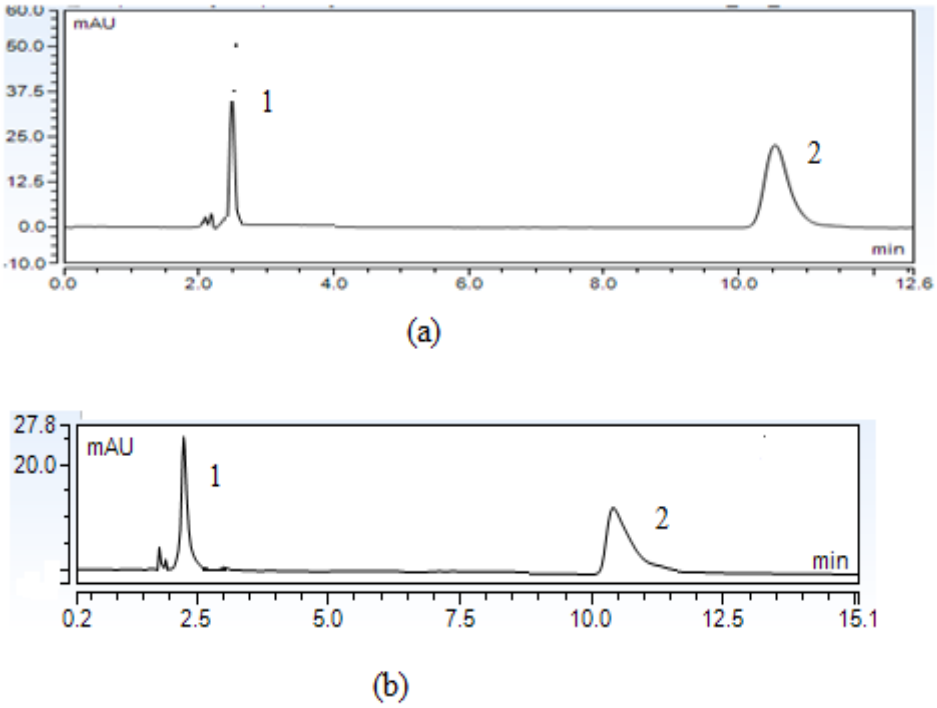

Figure 3. HPLC chromatogram obtained by the developed method for: (a) Mixture of acetyl salicylic acid 30µg/mL (peak 1) and clopidogrel bisulfate 70µg/mL (peak 2), (b) Myogrel plus® tablet solution containing 37.5µg/mL acetyl salicylic acid (peak 1) and 49µg/mL clopidogrel bisulfate (peak 2).

Table 4. Results of intraday precision for determination of clopidogrel bisulfate counterfeited with acetyl salicylic acid by the proposed isocratic RP-HPLC method.

Clopidogrel bisulfate					**Acetyl salicylic acid**				
Conc. taken (µg/mL)	Conc. found (µg/mL)			%RSD	Conc. taken (µg/mL)	Conc. found (µg/mL)			%RSD
50	50.03	49.41	49.26	0.82	50	49.98	49.67	49.23	0.76
60	59.70	59.01	59.42	0.58	40	39.81	39.28	39.63	0.68
70	69.95	68.15	70.48	1.76	30	30.00	29.69	29.95	0.56

%RSD: relative standard deviation.

Table 5. Results of inter-day precision for the determination of clopidogrel bisulfate counterfeited with acetyl salicylic acid by the proposed isocratic RP-HPLC method.

	Clopidogrel bisulfate			Acetyl Salicylic acid		
Conc. Taken (µg/mL)	50	60	70	50	40	30
Conc. found (µg/mL) day1	49.56	59.38	69.53	49.62	39.57	29.88
Conc. found (µg/mL) day2	50.24	59.88	68.70	50.50	40.21	30.16
Conc. found (µg/mL) day3	50.15	60.07	69.05	50.19	39.45	29.91
Mean found Conc. (µg/mL)	49.99	59.78	69.09	50.11	39.74	29.98
%RSD	0.74	0.60	0.60	0.89	1.02	0.50

SD: standard deviation, %RSD: relative standard deviation

Table 6. Results of the robustness of the proposed isocratic RP-HPLC method for determination of clopidogrel bisulfate counterfeited with acetyl salicylic acid.

Parameters	Clopidogrel bisulfate		Acetyl salicylic acid	
	**Conc. found (µg/mL)	%RSD	**Conc. found (µg/mL)	%RSD
MeoH 68%	70.83	0.58	30.32	0.60
* MeoH 70%	69.05	1.07	29.91	1.03
MeoH 72%	69.02	0.49	30.02	0.22
pH 2.9	70	0.59	30.2	0.97
* pH 2.8	68.70	0.36	30.16	0.75
pH 2.6	68.97	1.34	30.14	0.80

Concentration taken is 70 µg/mL clopidogrel bisulfate and 30 µg/mL acetyl salicylic acid. RSD: relative standard deviation, *: optimum condition, **: mean concentration found.

Table 7: Assay Results for determination of clopidogrel bisulfate in Borgavix® tablet by the developed isocratic HPLC method

Parameters	Developed HPLC method			Reported UV spectrophotometric method [15]		
Conc. taken (µg/mL)	49.00			40.00		
Conc. found (µg/mL)	48.7	48.9	48.02	40	39.49	39.49
Mean % recovery ± SD	99.08±0.95			99.25±0.42		
F-test	5.18			(19)*		
t-test	0.284			(2.77)*		

*Tabulated values for t-test and F-test at 95% confidence interval.
Tablet labeled to contain 98mg clopidogrel bisulfate (75mg clopidogrel).

3.3. Application of the developed method to the dosage form

3.3.1. Assay of tablet for clopidogrel bisulfate from the market

Borgavix® tablet containing clopidogrel bisulfate showed good recovery within the compendial range (98-102%) and tolerable standard deviation of not more than 2 for assay sample measured triple. t-test was accepted as the calculated t-value did not exceed the tabulated value (2.77); also F-test was accepted as the calculated F value was less than the tabulated value (19) by comparing the results of the developed RP-HPLC method to a reported method [15] as shown in **(Table 7)**.

3.3.2. Assay of tablet for clopidogrel bisulfate and acetyl salicylic acid from the market

Myogrel plus® tablet containing clopidogrel bisulfate and acetyl salicylic acid showed good recovery and tolerable standard deviation of not more than 2 for the assay sample measured triple. There was no significant difference between the developed method and the reported method 1 regarding t-test and F-test **(Table 8)**.

Table 8. Assay Results for determination of clopidogrel bisulfate and acetyl salicylic acid in Myogrel plus® by the developed isocratic HPLC.

Parameters	**Developed HPLC method**						**Reported HPLC method [1]**					
	Clopidogrel bisulfate			Acetyl salicylic acid			Clopidogrel bisulfate			Acetyl salicylic acid		
Conc. taken (µg/mL)	49			37.5			49			37.5		
Conc. found (µg/mL)	48.79	48.48	49.24	37.57	37.43	37.14	48.69	49.12	49.54	37.37	37.03	37.37
Mean % recovery ± SD	100.25±0.58			98.79±0.53			100.24±0.86			99.67±0.33		
F-test	1.24			1.24			(19)*					
t-test	0.84			0.73			(2.77)*					

* Tabulated values for t-test and F-test at 95% confidence interval.
Each (Myogrel plus®) tablet labeled to contain 98 mg Clopidogrel bisulfate (75 mg clopidogrel) and 75 mg acetyl salicylic acid.

4. CONCLUSION

A simple isocratic RP-HPLC method was developed for the determination of clopidogrel bisulfate counterfeiting with acetyl salicylic acid. It was successfully applied for the determination of clopidogrel bisulfate in its dosage form and the simultaneous determination of clopidogrel bisulfate and acetyl salicylic acid in its dosage form with acceptable accuracy and precision.

FUNDING

The authors declare that no fund was received for this study.

CONFLICT OF INTEREST

The authors declare no conflict of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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