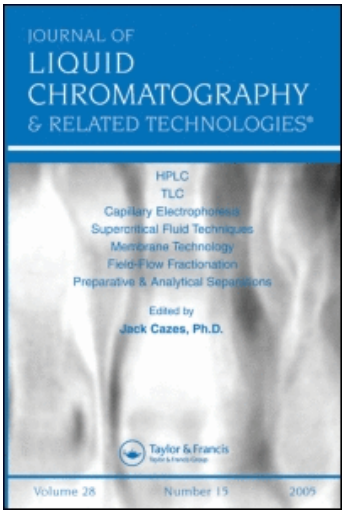

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Fathalla Belal[a]; Amina El-Brashy[a]; Manal Eid[a]; Jenny Jeehan Nasr[a]
[a] Faculty of Pharmacy, Department of Analytical Chemistry, University of Mansoura, Mansoura, Egypt

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Stability-Indicating Micellar Liquid Chromatographic Method for the Determination of Clopidogrel. Application to Tablets and Content Uniformity Testing

Fathalla Belal, Amina El-Brashy, Manal Eid, and Jenny Jeehan Nasr

Faculty of Pharmacy, Department of Analytical Chemistry,
University of Mansoura, Mansoura, Egypt

Abstract: A simple, stability-indicating, reversed-phase micellar liquid chromatographic method was developed for the analysis of the antiplatelet drug clopidogrel. Clopidogrel was determined in presence of its carboxylic acid degradation product, namely; SR26334. The analysis was carried out using a 150 mm × 4.6 mm i.d., 5 μm particle size Nucleodur MN-C18 column. The Mobile phase used was a solution containing 0.15 M sodium dodecyl sulphate and 10% n-propanol and 0.3% triethylamine in 0.02 M phosphoric acid at pH = 3.0 pumped at a flow rate of 1 mL/min with UV-detection at 235 nm. The method showed good linearity in the range of 1-20 μg/mL with limit of detection (S/N = 3) 0.06 μg/mL (1.86×10^{-7} M). The suggested method was successfully applied for the analysis of clopidogrel in bulk and in commercial tablets with average recoveries of 99.67% ± 0.94%, and 100.27 ± 0.89%, respectively. The results were favorably compared to those obtained by a reference method. The proposed method was successfully applied to the content uniformity testing of tablets. The proposed method was also applied for the determination of clopidogrel in the presence of its co-administered drug, acetyl salicylic acid, with application to synthetic mixtures and prepared tablets.

Keywords: Clopidogrel, Content uniformity testing, Degradation, Micellar liquid chromatography, Stability-indicating, Tablets

Correspondence: Manal Eid, Faculty of Pharmacy, Department of Analytical Chemistry, University of Mansoura, Mansoura 35516, Egypt. E-mail: manal_eid@yahoo.com

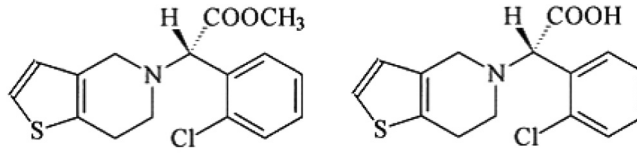
INTRODUCTION

Clopidogrel hydrogen sulphate, methyl (+)-(S)-α-(o-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(4 H)-acetate hydrogen sulfate (SR25990) is an antiplatelet agent widely used in the prevention of ischaemic stroke, myocardial infarction, and stroke.[1,2] Its carboxylic acid derivative, (+)-(S)-(o-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(4 H)-acetic acid (SR26334), which can arise by hydrolysis of the ester group both in vitro, as a result of the action of humidity and temperature in combination, and in vivo, as a result of carboxylesterase, is the main degradation product.[3] Chemical structures of clopidogrel and SR26334 are depicted in Fig. 1.

Several methods have been reported in the literature for the determination of clopidogrel inactive metabolite SR26334 in human plasma by HPLC-UV,[4–6] HPLC-MS,[7,8] and GC-MS.[9] Only one method has been reported for the determination of clopidogrel with its impurity SR26334 in human plasma by LC-MS.[10] Only two methods have been reported for the determination of clopidogrel with its impurity SR26334 in pharmaceutical products by UV spectrophotometric methods[11] and TLC.[3,11] Clopidogrel was also determined in pharmaceuticals with its forced degradation products by HPTLC[12] and HPLC.[13,14] It was also determined with its coadministered drug, aspirin, by UV spectrophotometry,[15] chemometric spectrophotometry,[16] HPTLC,[17] and HPLC-UV.[18]

In recent years, interest in micellar liquid chromatography (MLC) has grown steadily.[19–21] Using micellar mobile phases, solutes with small and large structural differences can be separated in a single chromatogram owing to complex interactions among the solutes and the mobile and the modified stationary phases. The mobile phases are inexpensive, almost non-flammable, biodegradable, and have low toxicity and low pollution impact.[22]

In the present work, a MLC method with UV detection was used for the analysis of clopidogrel in the presence of its degradation product SR26334. This method can be applied for quality control as well as for content uniformity testing of clopidogrel tablets. Moreover, the procedure was also used for the determination of clopidogrel with its coadministered drug, acetyl salicylic acid.



Chemical structure of clopidogrel Chemical structure of SR26334

Figure 1. Chemical structures of clopidogrel and its degradation product.

EXPERIMENTAL

Apparatus

Chromatographic analyses were carried out using a Merck Hitachi Chromatograph model L-7100 equipped with a Rheodyne injector valve with a 20 μL loop, and a L-7400 UV detector. The chromatograms were recorded using a Merck Hitachi D-7500 integrator. Mobile phase was filtered using Millipore filter Sibata and degassed using Merck solvent L-7612 degasser.

Materials and Reagents

All reagents used are HPLC grade.

- Clopidogrel and its degradation product (SR26334) were kindly provided by Sanofi-Synthélabo (France).
- Diclofenac sodium was kindly provided by Sedico (Egypt) as internal standard.
- Orthophosphoric acid, 1-propanol and triethylamine (Riedel-deHäen, Sleeze, Germany).
- Sodium dodecyl sulfate (Winlab, UK).
- Acetyl salicylic acid (El-Nasr Pharmaceutical Chemicals Company (ADWIC), Egypt).
- Acetonitrile, ethanol, tetrahydofuran, and methanol (Sigma-Aldrich, Germany).
- Plavix® tablets were purchased from the Egyptian market (Sanofi-Synthélabo, France).

Preparation of Solutions

Stock solutions of 0.4 mg/mL each of clopidogrel and its degradation product were prepared in methanol. Working solutions were prepared by diluting the stock solutions with the mobile phase.

Preparation of Calibration Curves

Working solutions containing 1.0–20.0 μg/mL of clopidogrel were prepared by serial dilutions of aliquots of the stock solution together with an aliquot of internal standard solution containing 20.0 μg/mL of diclofenac sodium. 20 μL aliquots were injected (triplicate) and eluted with the mobile phase under the reported chromatographic conditions.

The average peak area ratio between the internal standard and clopidogrel versus the concentration of clopidogrel in μg/mL was plotted. Alternatively, the corresponding regression equation was derived.

Analysis of Bulk Substance

The method mentioned above was applied to the determination of the purity of clopidogrel raw material. The percentage recoveries were calculated by referring to the calibration graph previously prepared or by applying the regression equation.

Analysis of Dosage Forms

Ten tablets were accurately weighed, finely pulverized, and thoroughly mixed. An amount of pulverized tablets corresponding to 75 mg of declared active principle (calculated as clopidogrel free base) was weighed and transferred into a beaker. 80 mL of methanol were added, and the mixture was sonicated for 30 min. in an ultrasonic bath and then filtered, if necessary, into a 100-mL volumetric flask and completed to the volume with methanol. Aliquots of these solutions, together with the internal standard, were successively diluted with the mobile phase and proceeded as mentioned above. The nominal content of the tablets was obtained either from the calibration graph or from the regression equation.

Content Uniformity Testing

The same procedure applied for the analysis of clopidogrel in tablets was followed using one tablet as a sample. Ten tablets were analysed and the uniformity of their contents was tested by applying the official USP[1] guidelines.

Preparation of Synthetic Mixtures for Determination of Coadministered Drug

Acetyl salicylic acid (0.4 mg/mL) was prepared in methanol. Working solutions were prepared by diluting the stock solution with the mobile phase. Synthetic mixtures were prepared by adding known amounts of clopidogrel and acetyl salicylic acid and they were analyzed using the procedure outlined above. The mean percentage recoveries and relative standard deviations were calculated.

Analysis of Prepared Tablets

Prepared tablets containing 75 mg each of clopidogrel and acetyl salicylic acid were mixed with tablet excipients composed of lactose (15 mg), starch (15 mg), talc (20 mg) and magnesium stearate (10 mg) per tablet. Contents corresponding to 10 tablets were geometrically mixed then the procedure outlined above for the analysis of commercial tablets was followed.

RESULTS AND DISCUSSION

The proposed method permits the separation of clopidogrel from its degradation product (SR26334). It also permits the quantitation of clopidogrel in commercial tablets; it was possible to perform the content uniformity testing. Figure 2 shows a chromatogram indicating good resolution of SR26334 ($t_R = 6.1$ min.), clopidogrel ($t_R = 13.4$ min.), and diclofenac sodium ($t_R = 15.5$ min.). The proposed method offers high sensitivity as about 0.06 μg/mL of clopidogrel could be detected accurately.

Chromatographic Performance

A well-defined symmetrical peak was obtained upon measuring the response of eluent under the optimized conditions after thorough experimental trials that could be summarized as follows:

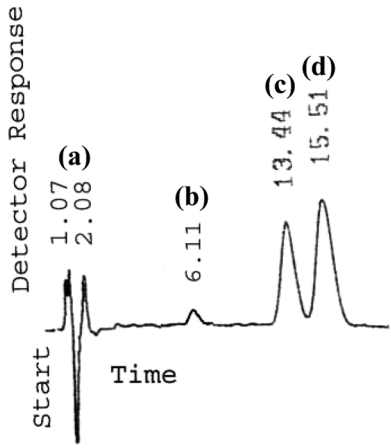


Figure 2. A chromatogram showing clopidogrel with its degradation product: (a) Solvent front; (b) 1 μg/mL SR26334; (c) 10 μg/mL clopidogrel; (d) 10 μg/mL diclofenac sodium.

Choice of Column

Two different columns were used for performance investigations, including:

- Symmetry® C18 column (250 mm × 4.6 mm i.d., 5 μm particle size), Waters, Ireland.
- Nucleodur MN-C18 column (150 mm × 4.6 mm i.d., 5 μm particle size), USA.

The experimental studies revealed that the second column was more suitable since it produced nice peaks with high resolution and a very good sensitivity.

Choice of Appropriate Wavelength

The UV detector response of clopidogrel was studied and the best wavelength was found to be 235 nm, showing the highest sensitivity.

Choice of Internal Standard

Different drugs were investigated for the choice of a suitable internal standard. These drugs include; tolterodine, lacidipine, temocapril, citalopram, amiodarone, drotaverine and diclofenac sodium. Diclofenac sodium was the best internal standard producing a well resolved peak from each of the drugs and the degradation product.

Mobile Phase Composition

Several modifications in the micellar mobile phase composition were performed in order to study the possibilities of changing the selectivity of the chromatographic system. These modifications included the change of the surfactant concentration, the concentration and type of cosurfactant, the pH, and the flow rate. The results obtained are shown in Table 1. The mobile phase was prepared using 0.3% triethylamine and 0.02 M phosphoric acid as these are the concentrations used for the preparation of this buffer solution.

pH

The effect of changing the pH of the mobile phase on the selectivity and retention times of the test solutes was investigated using mobile phases of pH ranging from 3.0–5.5 with 0.15 M SDS concentration and 10% 1-propanol. Table 1 shows that a pH of 3.0 was most appropriate, giving well-resolved peaks and the highest number of theoretical plates.

Table 1. Effect of experimental parameters on the chromatographic performance of clopidogrel

	Mass distribution ratio (D_m)		Relative retention (α)	Number of theoretical plates (N)		Resolution (R)
Parameter	Cp*	Dg**	Cp/Dg	Cp	Dg	Cp/Dg
pH						
3.0	13.48	6.59	2.04	8600	9470	12.48
3.5	12.78	3.87	3.30	5840	3690	17.18
4.0	11.13	2.17	5.13	6580	2800	20.37
5.0	16.06	1.90	8.45	7110	7420	26.43
5.5	17.52	1.96	8.94	6700	7220	28.13
Concentration of SDS (M)						
0.075	22.82	8.53	2.67	9270	8550	18.99
0.10	18.88	9.65	1.96	6580	7560	12.80
0.12	14.98	7.76	1.93	6360	4680	12.18
0.15	12.48	6.35	1.96	7340	8730	11.03
0.18	10.96	5.42	2.02	6670	3420	11.18
Concentration of 1-propanol (%)						
6	14.81	8.41	1.76	6340	6240	9.13
8	13.77	7.04	1.95	5430	4470	11.42
10	12.23	5.79	2.11	7070	7440	12.65
12	12.25	5.06	2.45	5700	6050	14.24
15	9.35	3.02	3.10	5250	3180	21.51
Nature of cosurfactant						
Methanol	18.81	12.88	1.46	3470	4360	5.44
Ethanol	17.56	11.70	1.50	5510	5820	6.28
n-Propanol	12.27	5.98	2.05	6850	7580	11.12
Acetonitrile	19.55	12.53	1.56	6760	6590	7.54
Flow rate (mL/min)						
0.7	13.19	5.89	2.24	5980	5650	13.52
1.0	11.78	5.13	2.30	6590	6070	13.05
1.2	11.57	4.99	2.32	6320	4390	13.03
1.5	11.51	4.90	2.35	4760	5770	10.84
1.7	11.52	4.90	2.35	5880	2930	12.92

*Cp is Clopidogrel.
**Dg is the degradation product.

pHs higher than 5.5 resulted in very late peaks for clopidogrel. This may refer to the fact that clopidogrel is converted to a more polar compound in the higher pH range as the carboxylic group is ionised. As it

becomes more polar, it exerts higher affinity towards the stationary phase, thus, it is eluted at increased retention time, giving broad peaks.

Concentration of Surfactant

The effect of changing the concentration of surfactant on the selectivity and retention times of the test solutes was investigated using mobile phases containing concentrations of 0.075–0.18 M of sodium dodecyl sulfate and containing 10% n-propanol and buffered at pH 3. Table 1 shows that 0.15 M SDS was the best, giving well-resolved peaks and highest number of theoretical plates. Retention times increased when concentration of surfactant decreased.

Concentration of Cosurfactant

The effect of changing the concentration of cosurfactant on the selectivity and retention times of the test solutes was investigated using mobile phases containing concentrations of 6–15% of 1-propanol and containing 0.15 M SDS and buffered at pH 3. Table 1 shows that 10% of 1-propanol was the best, giving well-resolved peaks and the highest number of theoretical plates. Hence, a small amount of 1-propanol is added to accelerate and control the elution of the drugs.

Type of Cosurfactant

The effect of changing the type of cosurfactant on the selectivity and retention times of the test solutes was investigated using mobile phases containing 10% of either methanol, ethanol, 1-propanol, acetonitrile, or tetrahydrofuran. Table 1 shows that 10% of 1-propanol was the best, giving well-resolved peaks and the highest number of theoretical plates. Tetrahydrofuran gave no defined peaks.

Flow Rate

The effect of flow rate on the formation and separation of peaks of the studied compounds was studied and a flow rate of 1 mL/min was optimal for good separation in a reasonable time (Table 1).

Validation of the Method

Optimal Chromatographic Separation Conditions

- Column: Nucleodur MN-C18 column (150 mm × 4.6 mm i.d., 5 μm particle size), Machery-Nagel, Germany.

- Mobile Phase: A solution containing 0.15 M sodium dodecyl sulfate and 10% 1-propanol and 0.3% triethylamine in 0.02 M phosphoric acid of pH = 3.0.
- Flow rate: 1 mL/min
- Detector wavelength: 235 nm
- Internal standard: Diclofenac sodium (a 0.04 mg/mL stock solution was prepared in methanol).

Concentration Ranges and Calibration Graphs

Under the above-described experimental conditions, a linear relationship was established by plotting clopidogrel concentrations against peak area ratio for clopidogrel to the internal standard. The concentration range was found to be 1–20 μg/mL. Linear regression analysis of the data gave the following equation:

$$P = -0.028 + 0.071\ C \quad (r = 0.9999)$$

where C is the concentration of clopidogrel in μg/mL and P is the peak area ratio.

The high value of the correlation coefficient (*r*-values >0.999) with small intercept indicate good linearity of the calibration graph. Statistical analysis of the data gave small values of the standard deviation of the residuals, ($S_{y/x}$) 2.29×10^{-3}, of slope, (S_b) 1.19×10^{-4}, and of intercept, (S_a) 1.43×10^{-3} and the % relative error, (% Er) 0.31%.[23]

Limit of Quantitation (LOQ) and Limit of Detection (LOD)

The limit of quantitation (LOQ) was determined by establishing the lowest concentration that can be measured according to ICH Q2B recommendations[24] below which the calibration graph is non linear and was found to be 1.0 μg/mL.

The limit of detection (LOD) was determined by establishing the minimum level at which the analyte can be reliably detected (S/N = 3); it was found to be 0.06 μg/mL (1.86×10^{-7} M).

Accuracy and Precision

The proposed method was evaluated by studying the accuracy as percent relative error (% Er) and precision as percent relative standard deviation (% RSD) using three preparations with suitable concentrations, as shown in Table 2. The intra-day (n = 3) and inter-day (n = 3) accuracy, calculated as % Er, was found to be within 0.11–0.32% and 0.17–0.41% for clopidogrel, respectively. The repeatability of the assay was found to be within

Table 2. Accuracy and precision data for clopidogrel using the proposed method

Parameter	Clopidogrel concentration, μg/mL		
	5	10	15
Intra-day*	99.62	100.39	100.09
% Recovery	99.06	100.67	99.81
	100.18	99.83	99.71
Mean ($\overline{X}$)	99.62	100.30	99.87
±S.D.	0.56	0.43	0.19
% R.S.D.	0.56	0.43	0.19
% Er.	0.32	0.25	0.11
Inter-day*	99.62	100.39	100.09
%Recovery	100.46	100.95	99.71
	101.04	99.69	99.53
Mean ($\overline{X}$)	100.37	100.34	99.78
±S.D.	0.71	0.63	0.29
% R.S.D.	0.71	0.63	0.29
% Er.	0.41	0.36	0.17

N.B. Each result is the average of three separate determinations.
*Intra-day: within the day.
Inter-day: consecutive days.

0.19–0.56 (n = 3) at 5, 10, and 15 μg/mL. The reproducibility of the assay at the same concentration levels was found to be within 0.29–0.71 (n = 3).

The results of the proposed method were favorably compared with those obtained using the reference method.[18] Statistical analysis of the results obtained by the proposed and reference methods showed no significant difference in the performance of the two methods using student's t-test and variance ratio F-test (Table 3). The proposed procedure offers additional advantages over the reference procedure in that the proposed is more sensitive with good accuracy and precision. The reference method depends on the RP-HPLC determination of clopidogrel using a C_{18} column and a mobile phase composed of acetonitrile:methanol:20 mM phosphate buffer at pH 3 (50:7:43 v/v) at a flow rate of 1 mL/min. Detection was carried out at 240 nm.[18]

Applications

Dosage Form Analysis

The proposed method was successfully applied to the assay of clopidogrel in commercial tablets (Plavix®) and also in its prepared tablets with

Table 3. Statistical analysis of the results obtained by the proposed and reference methods for pure samples of clopidogrel

	Proposed method n = 9		Reference method[18] n = 5	
Parameters	Conc. (μg/mL)	R (%)	Conc. (μg/mL)	R (%)
	1	97.40	10	97.10
	2	99.15	20	100.40
	5	99.62	30	100.94
	8	100.45	40	100.87
	10	100.39	50	99.15
	12	100.24		
	15	100.09		
	18	99.83		
	20	99.86		
Mean ($\overline{X}$)		99.67		99.69
±S.D.		0.94		1.62
Variance		0.88		2.62
Students *t*-value	0.029			
Variance ratio *F* value	2.977			

Tabulated *t*- and *F*-values at p=0.05 are: 1.782 and 3.838, respectively [25].

aspirin. The average percent recoveries of different concentrations were based on the average of three replicate determinations. The results shown in Table 4 are in good agreement with those obtained with the reference method.[18] Figure 3 shows a chromatogram indicating well resolved peaks of clopidogrel. Degradation of clopidogrel is easily detectable

Table 4. Assay of Clopidogrel in formulations using the proposed and reference methods

	Plavix 75 mg/tab.		Prepared tablets 75 mg/tab.	
Parameters	Proposed	Ref.[18]	Proposed	Ref.[18]
Recovery (%)	101.24	97.12	101.96	97.54
	99.34	99.19	99.80	99.90
	100.79	100.30	101.38	99.63
	99.73	100.60	100.53	100.77
Mean ($\overline{X}$)	100.27	99.30	100.92	99.46
±S.D.	0.89	1.58	0.95	1.37
Variance	0.79	2.50	0.90	1.88
Students *t*-value	1.070		1.751	
Variance ratio *F*-value	3.164		2.089	

Tabulated *t*- and *F*-values at p=0.05 are 1.943 and 9.277, respectively.

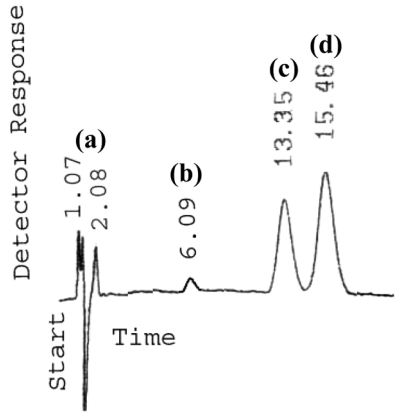


Figure 3. A chromatogram showing clopidogrel in Plavix tablets: (a) Solvent front; (b) 1 μg/mL SR26334; (c) 10 μg/mL clopidogrel; (d) 10 μg/mL diclofenac sodium.

and can be determined quantitatively as shown by the addition of standard known concentration of the degradation product to the tablet (Fig. 3). Therefore, the proposed method can be used for the quality control of the tablets.

Table 5. Results of content uniformity testing of clopidogrel tablets using the proposed method

Parameter	Percentage of the label claim
Data	99.00
	99.50
	99.10
	98.68
	100.89
	98.58
	99.40
	99.10
	100.40
	98.58
Mean ($\overline{X}$)	99.32
±S.D.	0.77
% RSD	0.77
% Error	0.24
Acceptance value (AV) [1]	1.848
Max. allowed AV (L1) [1]	15

Content Uniformity Testing

Due to the high precision of the proposed method and its ability to rapidly estimate the concentration of the drug in a single tablet extract with sufficient accuracy, the method is ideally suited for content uniformity testing which is a time-consuming process when using conventional assay techniques. The steps of the test were adopted according to the USP[1] procedure. The acceptance value (AV) was calculated for each of the commercially available tablets and it was found to be smaller than the maximum allowed acceptance value (L1). The results demonstrated excellent drug uniformity as shown in Table 5.

Co-Administered Drug

The proposed method allows determination of clopidogrel in presence of its co-administered drug, acetyl salicylic acid, as shown in Fig. 4. In order to assess the validity and applicability of the developed method, recovery

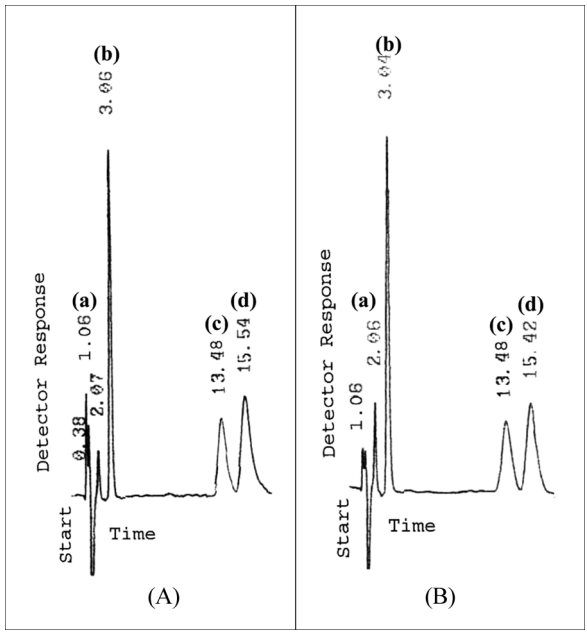


Figure 4. A chromatogram showing clopidogrel with its coadministered drug acetyl salicylic acid. (A) Synthetic mixture: (a) Solvent front; (b) 10 μg/mL acetyl salicylic acid; (c) 10 μg/mL clopidogrel; (d) 10 μg/mL diclofenac sodium. (B) Prepared tablet: (a) Solvent front; (b) 10 μg/mL acetyl salicylic acid; (c) 10 μg/mL clopidogrel; (d) 10 μg/mL diclofenac sodium.

Table 6. Recovery data obtained for different synthetic mixtures of clopidogrel and acetyl salicylic acid

	Clopidogrel			Acetyl salicylic acid		
Mixture no.	Added (μg/mL)	Found (μg/mL)	% R	Added (μg/mL)	Found (μg/mL)	% R
1	12.00	11.97	99.75	8.00	8.09	101.12
2	10.00	10.23	102.30	10.00	9.89	98.90
3	10.00	10.01	100.10	6.00	5.95	99.17
4	10.00	10.05	100.50	8.00	8.07	100.87
5	8.00	8.09	101.12	10.00	9.99	99.90
6	8.00	7.98	99.75	12.00	12.21	101.75
7	6.00	6.07	101.17	10.00	9.90	99.00
Mean ($\overline{X}$)			100.67			100.10
±S.D.			0.93			1.15
% RSD			0.92			1.15
% Error			0.35			0.43

studies were performed by analyzing synthetic mixtures of each drug in different ratios. The mean percentage recoveries (± RSD) indicating good accuracy and precision were found to be 100.67 ± 0.92 for clopidogrel and 100.10 ± 1.15 for acetyl salicylic acid, as shown in Table 6.

CONCLUSION

The proposed method for the determination of clopidogrel based on the use of micellar liquid chromatography with spectrophotometric detection was shown to be reliable, simple, accurate, sensitive, and precise. Moreover, the method is fast and feasible. The proposed method was found to have a limit of detection of 0.06 μg/mL and a limit of quantitation of 1.00 μg/mL.

The proposed method is stability-indicating as it is suitable for the determination of clopidogrel in presence of its degradation product, SR26334. In addition, it is suitable for the determination of clopidogrel in commercial tablets. Thus, it can be used for the quality control of clopidogrel tablets with excellent application of content uniformity test. It also offers the possibility to determine clopidogrel in the presence of the co-administered drug, acetyl salicylic acid.

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