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ORIGINAL ARTICLE

Stability-indicating liquid chromatography for determination of clopidogrel bisulfate in tablets: Application to content uniformity testing

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Content uniformity testing

Abstract The present study describes a simple stability-indicating reversed-phase HPLC assay for antiplatelet drug, clopidogrel bisulfate. Separation of the drug and the degradation products, under stress conditions was successfully achieved on a C-18 column utilizing 0.01 M Na_2HPO_4 (pH 4): acetonitrile in the ratio 80:20 v/v, pumped at a flow rate of 0.5 ml min^{-1} with UV detection at 235 nm. The retention time of clopidogrel was 6.84 min. The method was satisfactorily validated with respect to linearity, precision, accuracy, selectivity, sensitivity and ruggedness. The response was linear in the range of 0.2–3.5 μg ml^{-1} with detection limit 0.079 μg ml^{-1}. The suggested method was successfully applied for the analysis of clopidogrel in bulk and in commercial tablets. 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 and for determination of clopidogrel in presence of its co-administered drug, acetyl salicylic acid.

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1. Introduction

Clopidogrel hydrogen sulfate (Clp), methyl (+)-(S)-α-(o-chlorophenyl)-6,7-dihydrothieno(3,2-c)pyridine-5(4H)-acetate hydrogen sulfate (SR25990) (Fig. 1), is a new antiplatelet agent, chemically related to ticlopidine (The United States Pharmacopeia, 2007; The Merck Index, 2002). It prevents ischaemic stroke, myocardial infraction and vascular disease and demonstrated clinical efficacy superior to that of aspirin, in a large phase III trial (Moshfegh et al., 2000).

It is a carboxylic acid derivative, (+)-(S)-(o-chlorophenyl)-6,7-dihydrothieno(3,2-c)pyridine-5(4H)-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 carboxyesterase, is the

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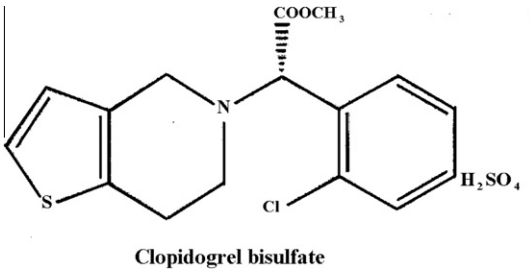

Figure 1 Chemical structure of clopidogrel bisulfate.

main degradation product (Antic et al., 2007). Few methods have been reported in literature for the determination of Clp. Recently, Clp and its impurities have been separated by normal phase HPLC using a chiral column (Rao et al., 2010). Also, the non-enzymatic and enzymatic chiral inversion of Clp has been investigated in vitro using 1H NMR and a chiral HPLC procedure (Reist et al., 2000).

Moreover, in the same article, a non-stereospecific HPLC assay method was also used to monitor the hydrolysis of Clp. The possible in vivo chiral inversion of carboxylic acid metabolite of Clp in rats was studied using (S)-α-(1-naphthyl)ethyl amine as a derivatization reagent and an HPLC method with spectrophotometric detection (Reist et al., 2000). Identification and isolation of oxidation impurity of Clp using HPLC were also reported (Mohan et al., 2008). HPLC detection of the acid metabolite of Clp (SR26334) in Wistar rat plasma (Singh et al., 2005), in dog plasma (Yang et al., 2009), and in human plasma (Bahrami et al., 2008) with application to bio-equivalence study was reported.

The analysis of carboxylic acid metabolite of Clp in plasma and serum using GC–MS (Lagorce et al., 1998), LC–MS/MS (Mitakos and Panderi, 2004; Takahashi et al., 2008) and capillary zone electrophoresis (Karazniewicz-Lada et al., 2010) methods were reported. Clp was determined in presence of its metabolite (SR26334) in human plasma by LC–MS (Patel et al., 2008; Raghundha et al., 2010; Vocilkova et al., 2009), in tablets by Micellar LC (Belal et al., 2009), HPTLC (Agrawal et al., 2003), voltammetry (Dermis and Ayadogna, 2010), UV-spectrophotometry (Zaazaa et al., 2009) and densitometry (Antic et al., 2007; Zaazaa et al., 2009; Robinson et al., 2007).

The United States Pharmacopoeia (USP) (The United States Pharmacopeia, 2007) recommended a reversed phase HPLC method with UV detection at 220 nm for determination of Clp. Clp was also determined by LC–MS/MS (Shin and Yoo, 2007) and in its tablets by HPLC with UV detection using C-8 column (Mitakos and Panderi, 2002) and C-18 column (Sippel et al., 2008). Clp was also determined with its co-administered drug, aspirin by UV spectrophotometry (Anandakumar et al., 2007), chemometric spectrophotometry (Rajput et al., 2008), HPTLC (Patel et al., 2008) and HPLC-UV detection (Anandakumar et al., 2002). Two HPLC methods, with UV detection (Mitakos and Panderi, 2002; Sippel et al., 2008) were reported for determination of Clp in presence of its forced degradation products. One method (Mitakos and Panderi, 2002) used a semi-micro C-8 BDS column that is not common in routine analysis. The second one (Sippel et al., 2008) used C-18 column which is most commonly used in daily routine analysis but was sensitive to NaOH and light degraded products only. In our research a sensitive C-18 column was used for determination of Clp in presence of all induced degradation products, using NaOH, HCl, H_2O_2 and light, moreover, small amounts (0.2–3.5 μg ml^{-1}) were determined with good results and short time (t_R of Clp = 6.84 min) compared to other HPLC methods (Belal et al., 2009; Sippel et al., 2008). The method was rapid, accurate, and simple and it can be used for quality control as well as for content uniformity testing of clopidogrel tablets. The suggested method was also used for the determination of Clp with its co-administered drug, acetyl salicylic acid.

2. Experimental

2.1. Apparatus

Chromatographic analyses were carried out using LC system (Perkin–Elmer, USA) consisted of series 200 Vacuum Degasser, series 200 LC pump, series 200 variable-wavelength UV detector and series 200 autosampler fitted with 200 μl sample loop. The LC separation was performed on a Waters μ Bondapak C-18, 300 × 3.9 mm ID, 10 μm column. The mobile phase consisted of a mixture of 0.01 M Na_2HPO_4, adjusted to pH 4 and acetonitrile in the ratio: 80:20 v/v. The mobile phase was filtered by passing through 0.45 μm pore size membrane filter (Millipore, Millex-HV, USA) and degassed prior to use. The samples were also filtered using 0.45 mm disposable filter. The flow rate was 0.5 ml min^{-1}. All determinations were performed at ambient temperature with a detection wavelength at 235 nm.

2.2. Materials

2.2.1. Pure standards

(a) *Clopidogrel bisulfate: used* as received (Sanofi-Synthelabo, France).
(b) *Acetyl salicylic acid: used* as received (El-Nasr Pharm. Chem. Co., ADWIC, Egypt).

2.2.2. Pharmaceutical dosage forms

The following commercially available pharmaceutical dosage forms were analyzed:

(1) Myogrel® tablets are labeled to contain clopidogrel bisulfate equivalent to 75 mg of clopidogrel, manufactured by ADWIA Co., SAE, 10th of Ramadan City, Egypt, Batch No. 080243.
(2) Stroka® tablets are labeled to contain clopidogrel bisulfate equivalent to 75 mg of clopidogrel, manufactured by Multi-APEX Pharma Co., SAE, Badr City, Cairo, Egypt, Batch No. 2300809.

2.3. Chemicals and reagents

All chemicals used throughout this work were of analytical grade and the solvents were of HPLC grade:

(a) Acetonitrile and methanol (Merck, Germany).
(b) Hydrochloric acid (BDH, Germany).
(c) Sodium hydroxide (BDH, Germany).
(d) Hydrogen peroxide (Winlab, UK).
(e) Dibasic sodium phosphate (Winlab, UK).
(f) Ortho phosphoric acid (BDH, Germany).

2.4. Preparation of standard stock solutions

Stock standard solutions of clopidogrel bisulfate and acetyl salicylic acid were prepared in methanol of concentration 0.5 mg ml^{-1} for each separately. Working solutions were prepared by serial dilution of the stock solution with the mobile phase.

2.5. General assay procedure

2.5.1. Preparation of the calibration graph
Working solutions containing 0.2–3.5 μg ml^{-1} of Clp were prepared by serial dilution of aliquots of the stock solution and triplicate 10 μl injections were made of each solution and the peak areas of Clp were plotted against the corresponding concentration in μg ml^{-1} to obtain the calibration graph. Alternatively, the corresponding regression equation was derived.

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

2.5.3. Procedure for preparation of degradation products
Degradation products were prepared by dissolving 25 mg of Clp in 10 ml methanol and adding 40 ml of 1 M HCl, of 1 M NaOH and of 5% (v/v) H_2O_2, each separately in a 100 ml volumetric flask. The solution mixtures were heated in a water bath at 80 °C for 1 h, neutralized with 2 M NaOH and 2 M HCl for the acidic and alkaline degradation, respectively. The volumes were completed with methanol and working solutions were diluted with the mobile phase and the steps were followed as described under "general assay procedure".

2.5.4. Analysis of dosage forms
Ten tablets were accurately weighed, finely pulverized and thoroughly mixed. An amount corresponding to 50 mg of Clp 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 into a 100-ml volumetric flask and completed to volume with methanol. Aliquots of this solution were successively diluted with the mobile phase and proceeded as mentioned above. The nominal content of the tablets was calculated either from the calibration graph or from the regression equation.

2.5.5. Content uniformity testing
The same procedure applied for the analysis of Clp in tablets was followed using one tablet, as a sample. Ten tablets of each dosage were analyzed and the uniformity of their contents was tested by applying the official USP (The United States Pharmacopeia, 2007) guidelines.

2.5.6. Preparation of synthetic mixtures for determination of co-administered drug
Acetyl salicylic acid (0.5 mg ml^{-1}) 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 Clp and acetyl salicylic acid and they were analyzed using the procedure outlined above. The mean percentage recoveries and relative standard deviations were calculated.

3. Results and discussion

The proposed method permits the separation of Clp from its degradation products using HCl, NaOH, H_2O_2 and light. It also permits the quantitation of Clp in commercial tablets; it was possible to perform the content uniformity testing. Figs. 2–4 show chromatograms indicating good resolution of Clp and its degradation products. The proposed method offers high sensitivity, as about 0.079 μg ml^{-1} of Clp could be detected accurately.

3.1. Chromatographic performance

A well-defined symmetrical peak was obtained upon measuring the response of eluent and the optimized conditions after

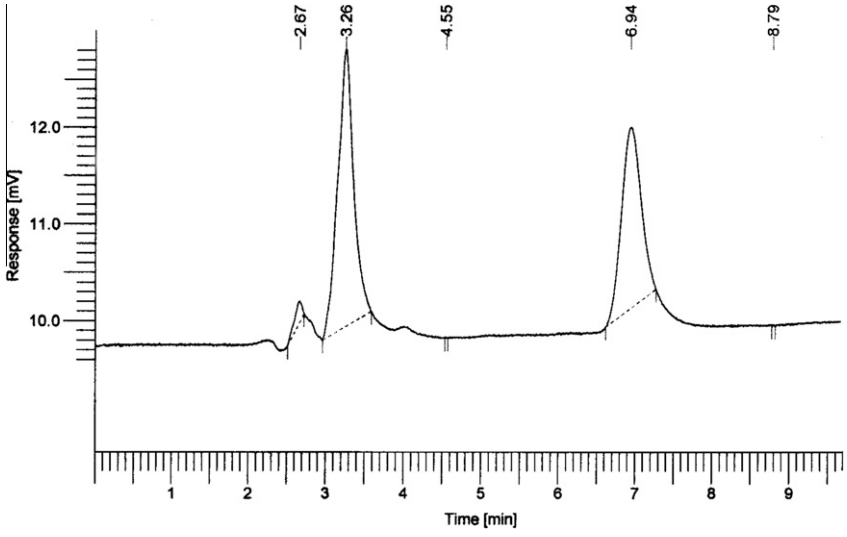

Figure 2 Clopidogrel and NaOH degradation product chromatogram.

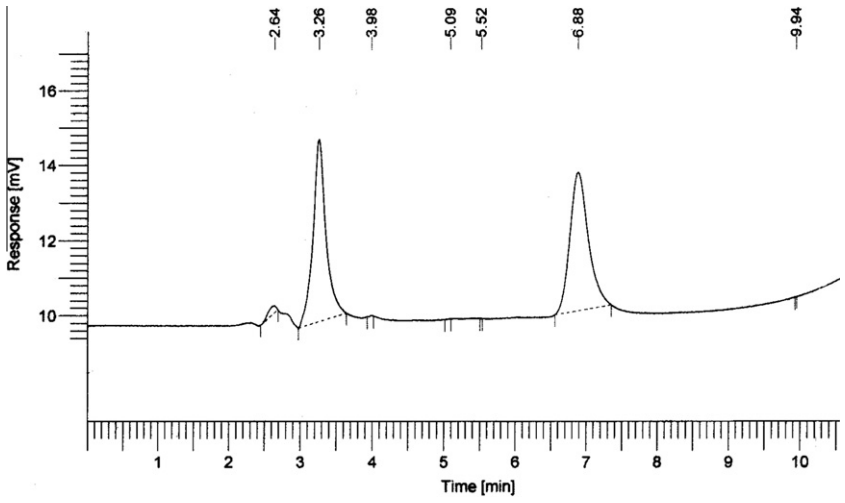

Figure 3 Clopidogrel and HCl degradation product chromatogram.

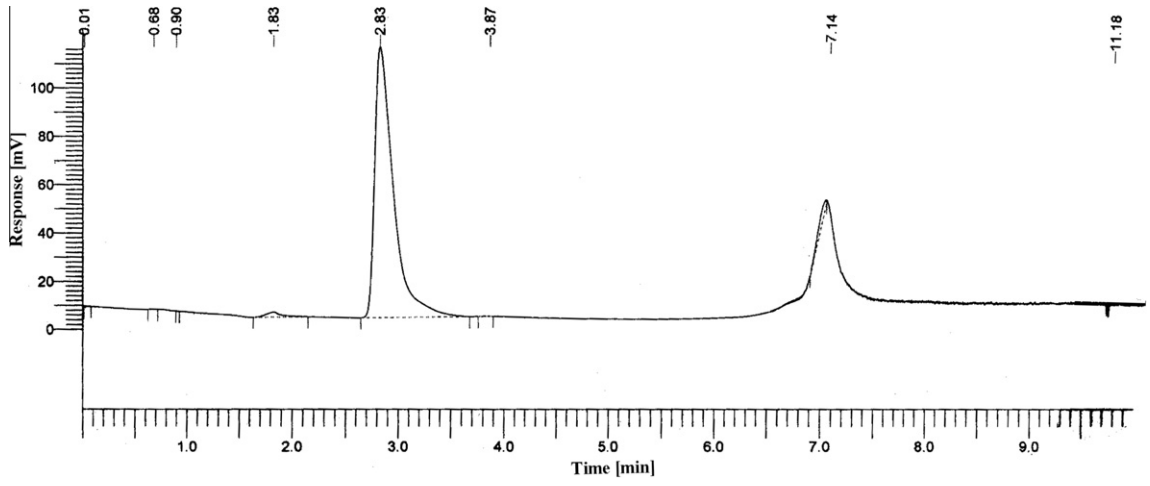

Figure 4 Degradation of clopidogrel in H_2O_2.

through experimental trials that could be summarized as in the following sections.

3.1.1. Choice of column

Columns from different sources were evaluated. Three different columns were used for performance investigation:

(a) Waters μ Bondapak, C-18, 300 × 3.9 mm, 10 μm (USA).
(b) Nova-pak, C-18, 300 × 3.9 mm, 4 μm (USA).
(c) Spherical, C-18, 150 × 3.9 mm, 5 μm (USA).

The experimental studies revealed that the first column was more suitable, since it produced the best chromatographic performance and acceptable peak characteristic including tailing factor, number of theoretical plates, capacity factor, high resolution and a very good sensitivity.

3.1.2. Choice of appropriate wavelength

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

3.1.3. Chromatographic characteristics

Table 1 shows the different compositions of the mobile phase, pH, and the flow rate. As indicated from the table, the composition of mobile phase; acetonitrile/phosphate buffer (pH 4) with ratio 20:80 v/v and a flow rate of 0.5 ml min^{-1} were the appropriate conditions that were giving well-resolved peaks and highest number of theoretical plates.

3.2. Validation of the method

The optimal chromatographic separation conditions are:

Column: Waters μ Bondapak C-18, 300 × 3.9 mm, 10 μm.
Mobile phase: a solution containing 0.01 M Na_2HPO_4 (pH 4) and acetonitrile in the ratio 80:20 v/v.
Flow rate: 0.5 ml min^{-1}.
Detector wavelength: 235 nm.

3.2.1. Degradation studies

In order to assure the selectivity and provide an indication of the stability-indicating properties of the proposed method,

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

Parameters	Retention time (min) ± SD[a]	Number of theoretical plates (N)
pH		
3	3.27 ± 0.010	2970
3.5	6.09 ± 0.018	5133
4	6.05 ± 0.023	6507
4.5	6.01 ± 0.021	1627
5	5.44 ± 0.019	2959
Acetonitrile ratio (%)		
10	3.25 ± 0.015	1878
15	5.23 ± 0.025	6102
20	6.05 ± 0.023	6507
25	5.68 ± 0.025	6238
30	4.26 ± 0.024	5999
40	3.12 ± 0.031	3218
Flow rate (ml min^{-1})		
0.5	6.84 ± 0.024	8416
0.8	6.12 ± 0.018	5828
1.0	5.64 ± 0.032	4936
1.2	4.42 ± 0.027	3845
1.5	3.45 ± 0.028	2113

[a] Average of three values.

Table 2 Recovery of clopidogrel bisulfate after stress degradation.

Condition	Time (h)	Clp (recovery %)	t_R (min) ± SD[a]
1 M HCl	1	78	3.27 ± 0.020
1 M NaOH	1	42	3.29 ± 0.052
5% (v/v) H_2O_2	1	80	2.85 ± 0.019
Light	7 (days)	98.72	–

[a] t_R of the degraded products, average of three values.

Table 3 Relative retention times of the degraded products and acetyl salicylic acid.

Compound	t_R ± SD (% RSD)	Relative t_R[a]
Clopidogrel	6.83 ± 0.027 (0.395)	–
Deg. with HCl	3.27 ± 0.020 (0.612)	0.4788
Deg. with NaOH	3.29 ± 0.052 (1.581)	0.4817
Deg. with H_2O_2	2.85 ± 0.019 (0.667)	0.4173
Acetyl salicylic acid	3.44 ± 0.054 (1.570)	0.5029

[a] With respect to clopidogrel.

forced degradation studies were performed under various stress conditions, with 1 M HCl, 1 M NaOH, and 5% (v/v) H_2O_2 at 80 °C for 1 h. Moreover, a solution of Clp in methanol was exposed to diffused day light for a period of 7 days, Figs. 2–4 show the retention times of the degraded products. The acid stressed samples showed approximately (22%) degradation (t_R = 3.27 min), the base stressed samples showed approximately (58%) degradation (t_R = 3.29 min) and Clp in 5% (v/v) H_2O_2 showed 20% degradation and t_R = 2.85 min. No significant degradation was observed in samples of Clp that were left in day light for 7 days.

Table 2 shows the concentration of Clp after stress degradation and the relative retention times of the degraded products to Clp are shown in Table 3.

As indicated from Table 3, the relative retention times are less than 0.5; this means that well-separated peaks of Clp and its degraded products were obtained under the chromatographic conditions discussed.

3.2.2. Concentration ranges and calibration graphs

Under the above-described experimental conditions, a linear relationship was established by plotting Clp concentrations against the corresponding peak areas. The concentration range was found to be 0.2–3.5 μg ml^{-1}. Linear regression analysis of the data gave the following equation:

$$P = 11808.98C - 52.371 \quad (r = 0.9996)$$

where C is the concentration of Clp in μg ml^{-1} and P is the peak area. Statistical analysis (Miller and Miller, 1993) of the data gave: S_b = 134.1648, S_a = 282.4196, where S_b and S_a are the standard deviations of slope and intercept, respectively.

3.2.3. Limit of quantitation (LOQ) and limit of detection (LOD)

LOQ was determined by establishing the lowest concentration that can be measured according to ICH Q 2 recommendations (ICH Q 2, 2005) below which the calibration graph is non linear and was found to be 0.2 μg ml^{-1}. LOD was determined by establishing the minimum level at which the analysis can be reliably detected (S/N = 3); it was found to be 0.079 μg ml^{-1}.

3.2.4. 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 4. The intra-day

Table 4 Accuracy and precision data for clopidogrel using the proposed method.

Parameters	Clp concentration (μg ml^{-1})		
	0.5	1	2
Intra-day	98.44	101.39	100.81
	100.34	101.01	99.08
	101.56	100.73	101.12
Mean ($\bar{x}$)	100.11	101.04	100.34
SD	1.57	0.33	1.10
% RSD	1.57	0.33	1.10
% Er	0.91	0.19	0.64
Inter-day	99.84	101.34	102.11
	100.65	100.58	101.82
	102.04	101.48	100.46
Mean ($\bar{x}$)	100.84	101.13	101.46
SD	0.24	0.48	0.88
% RSD	1.23	0.48	0.87
% Er	0.71	0.28	0.50

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

Table 5 Statistical analysis of the results obtained by the proposed and reference methods for pure samples of clopidogrel bisulfate.

Parameters	Proposed method		Reference method[a]	
	Concentration (μg ml^{-1})	Recovery[b] (%)	Concentration (μg ml^{-1})	Recovery[b] (%)
	0.2	97.34	4	98.84
	0.5	98.82	8	101.73
	1.0	101.42	12	99.78
	1.5	100.35	16	102.52
	1.8	101.22	20	101.85
	2.0	99.56		
	2.2	98.78		
	2.5	100.46		
	3.0	99.88		
	3.5	101.65		
N		10		5
Mean ($\bar{x}$)		99.95		100.94
SD		1.36		1.56
Variance		1.86		2.43
Student's *t*-value		1.27 (2.16)[c]		
Variance ratio *F*-value		1.31 (3.63)[c]		

[a] Belal et al. (2009).
[b] Each result is the average of three separate determinations.
[c] Tabulated *t* and *F* values at $p = 0.05$ (Miller and Miller, 1993).

Table 6 Assay of clopidogrel bisulfate in tablets using the proposed and reference methods.

Parameters	Myogrel tablets®		Stroka tablets®	
	Proposed	Reference[a]	Proposed	Reference[a]
Recovery[b] (%)	101.31	101.42	100.28	98.87
	101.82	102.01	101.35	101.23
	100.88	99.14	99.01	101.88
	102.52	101.48	98.98	100.84
Mean ($\bar{x}$)	101.63	101.01	99.91	100.71
SD	0.71	1.28	1.14	1.30
Variance	0.50	1.63	1.30	1.68
Student's *t*-value	0.85		0.93	
Variance ratio *F*-value	3.26		1.29	

Tabulated *t* and *F* values at $p = 0.05$ are 2.45 and 9.28, respectively (Miller and Miller, 1993).
[a] Belal et al. (2009).
[b] Each result is the average of three separate determinations.

($n = 3$) and inter-day ($n = 3$) accuracy, calculated as % Er, was found to be within 0.19–0.91% and 0.28–0.71% for Clp, respectively. The repeatability of the assay was found to be within 0.33–1.57 ($n = 3$) at 0.5, 1, and 2 μg ml^{-1}. The reproducibility of the assay at the concentration levels was found to be within 0.48–1.23 μg ml^{-1} ($n = 3$).

The results of the proposed method were favorably compared with those obtained using the reference method (Belal et al., 2009). Statistical analysis (Miller and Miller, 1993) 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 5). The proposed procedure offers additional advantages over the reference procedure; in that it is simple and more sensitive with good accuracy and precision and carried out in short time (t_R of Clp = 6.84 min) with low consumption of mobile phase (flow rate 0.5 ml min^{-1}) compared to the reference one (1 ml min^{-1}). Moreover, it does not need the use of an internal standard which consumes time for preparation. The reference method (Belal et al., 2009) depends on formation of Micellar LC (t_R = 13.4 min).

3.3. Applications

3.3.1. Dosage form analysis

The proposed method was successfully applied to the assay of Clp in commercial tablets (Myogrel® and Stroka®). The average percent recoveries of different concentrations were based on the average of three replicate determinations. The results shown in Table 6 are in good agreement with those obtained by the reference one (Belal et al., 2009).

3.3.2. 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

Table 7 Results of content uniformity testing of clopidogrel tablets using the proposed method.

Parameters	Label claim[a] (%)	
	Myogrel tablets®	Stroka tablets®
	100.84	101.32
	98.78	100.53
	97.64	101.64
	99.53	98.48
	101.34	101.42
	98.68	98.65
	97.56	101.48
	100.68	97.84
	99.35	98.52
	101.66	101.61
	99.61	100.15
	1.47	1.57
Mean ($\bar{x}$)	1.48	1.57
% RSD	0.47	0.50
Acceptance value (AV)[b]	3.53	3.77
Max. allowed AV (LI)[b]	15	

[a] Each result is the average of three separate determinations.
[b] USP (2007).

Table 8 Recovery data obtained from different synthetic mixtures of clopidogrel bisulfate and acetyl salicylic acid.

Mix. No.	Clopidogrel bisulfate		Acetyl salicylic acid	
	Added ($\mu g\ ml^{-1}$)	Recovery[a] (%)	Added ($\mu g\ ml^{-1}$)	Recovery[a] (%)
1	3	97.05	2	101.36
2	1	97.45	1	100.48
3	2	100.67	3	98.82
4	1	99.84	3	97.63
5	2	98.78	4	101.45
6	3	101.68	5	99.77
Mean ($\bar{x}$)		99.23		99.92
SD		1.82		1.50
% RSD		1.84		1.50
% Er		0.75		0.61

[a] Each result is the average of three separate determinations.

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 procedure. The acceptance value (AV) was calculated for each of the commercially available tablets and was found to be smaller than the maximum allowed acceptance value (LI). The results are shown in Table 7.

3.3.3. Co-administered drug

The proposed method allowed determination of Clp in presence of its co-administered drug, acetyl salicylic acid. In order to assess the validity and applicability of the developed method, recovery 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 99.23 ± 1.84 for clopidogrel and 99.92 ± 1.50 for acetyl salicylic acid with retention time 3.44 min for the latter compound (Table 3 and Fig. 5) (see Table 8).

4. Conclusion

The purpose of this study is to develop and validate a LC method that allows simple, specific, accurate and reproducible determination of clopidogrel in tablets (Myogrel® and Stroka®). The proposed method is fast, feasible and has a limit of detection of 0.079 $\mu g\ ml^{-1}$ and a limit of quantitation of 0.2 $\mu g\ ml^{-1}$.

The proposed method is stability-indicating as it is suitable for the determination of clopidogrel in presence of its degradation products under all stress conditions using HCl, NaOH, H_2O_2 and light. In addition it is suitable for the determination of clopidogrel in commercial tablets. Thus, it can be used for quality control of clopidogrel tablets with excellent application to content uniformity test. It also offers the possibility to determine clopidogrel in the presence of the co-administered drug, acetyl salicylic acid in short time.

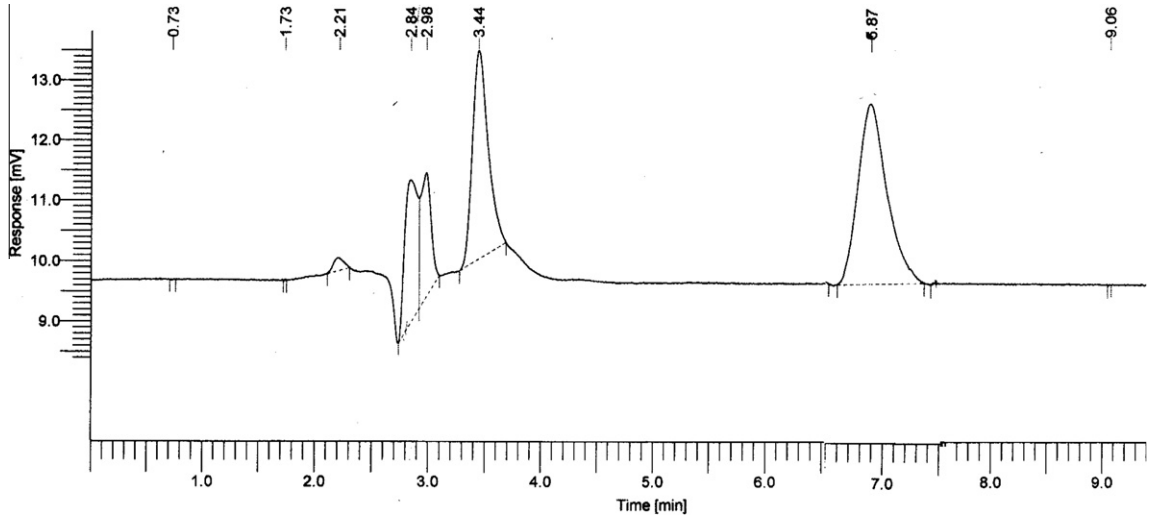

Figure 5 Clopidogrel and acetyls chromatogram.

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