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A validated LC method for the determination of clopidogrel in pharmaceutical preparations

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Abstract

A stability indicating, reversed-phase high-performance liquid chromatographic method was developed and validated for the determination of clopidogrel in pharmaceutical dosage forms. The determination was performed on a semi-micro column, BDS C8 (250 × 2.1 mm i.d., 5 μm particle size); the mobile phase consisted of a mixture of 0.010 M sodium dihydrogen phosphate (pH 3.0) and acetonitrile (35:65, v/v), pumped at a flow rate 0.30 ml min^{-1}. The UV detector was operated at 235 nm. The retention times for clopidogrel and naproxen, which was used as internal standard, were 3.08 and 6.28 min, respectively. Calibration graphs are linear (r better than 0.9991, $n = 6$), in concentration range 1.00–3.00 μg ml^{-1} for clopidogrel. The intra- and inter-day RSD values were less than 1.96%, while the relative percentage error E_r was less than 2.0% ($n = 5$). Detection and quantitation limits were 0.12 and 0.39 μg ml^{-1}, respectively. The method was applied in the quality control of commercial tablets and content uniformity test and proved to be suitable for rapid and reliable quality control.

Keywords: Clopidogrel; Liquid chromatography; Pharmaceutical dosage forms; Stability indicating

1. Introduction

Clopidogrel hydrogen sulfate, methyl (+)-(*S*)-α-(*o*-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridin-5(4H)-acetate hydrogensulfate (SR25990, Fig. 1) is a new thienopyridine derivative chemically related to ticlodipine. It has been shown to prevent ischaemic stroke, myocardial infraction and vascular disease and demonstrated clinical efficacy superior to that of aspirin, in a large phase III trial [1]. Thus, clopidogrel is indicated for the reduction of atherosclerotic events in patients with atherosclerosis documented by recent stroke, recent myocardial infraction or cardiovascular disease [2].

Clopidogrel is an entantiopure carboxylic ester of *S*-configuration. The *R*-enantiomer is devoid of antithrombotic activity and can evoke convulsions at high doses of animals [3]. It is rapidly absorbed and undergoes extensive metabolism and metabolic activation, as evidenced by the absence of detectable amounts of unchanged drug in plasma [4,5]. The parent compound is inactive

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in vitro, while its active metabolite inhibits platelet aggregation via selective binding to adenylate cyclase-coupled ADP receptors on the platelet surface [6,7]. The major circulating compound is the inactive carboxylic acid derivative, which is formed by hydrolysis of the ester function by carboxylesterase [8].

Few methods for the determination of clopidogrel have been reported in literature. Recently, the nonenzymatic and enzymatic chiral inversion of clopidogrel has been investigated in vitro using ^{1}H-NMR and a chiral HPLC procedure [9]. Moreover, in the same article, a nonstereospecific HPLC assay method was also used to monitor the hydrolysis of clopidogrel. The possible in vivo chiral inversion of carboxylic acid metabolite of clopidogrel in rats was also studied using (*S*)-α-(1-naphthyl)ethylamine as a derivatization reagent and an HPLC method with spectrofluorimetric detection [9]. For the analysis of the carboxylic acid metabolite of clopidogrel in plasma and serum a GC-MS method has also been reported [10]. To our knowledge, no article related to the determination of clopidogrel in pharmaceutical dosage forms has ever been mentioned in literature or in Pharmacopoeias.

The focus of the present study was to develop and validate a rapid reversed-phase high-performance liquid chromatographic method for the quality control of clopidogrel in pharmaceutical preparations. A semi-micro (2.1 mm i.d.) BDS C8 analytical column was used to perform the experiments. One of the most important advantages of the use of semi-micro columns is the reduction of solvent usage as they operate at lower flow rates (0.10–0.50 ml min^{-1}) [11–15]. The reduction in column diameter from a standard 4.6-mm i.d. column to a 2.1-mm i.d. column increases sensitivity at a constant injection volume [16,17]. The proposed method is applicable as well for routine analysis and content uniformity test of clopidogrel in tablets and complies well with the validation requirements in the pharmaceutical industry.

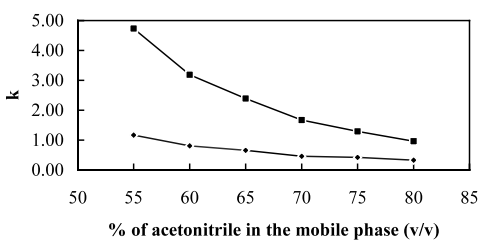

Fig. 1. Plots of the capacity factor, *k*, vs. acetonitrile concentration in the mobile phase of naproxen (◆) and clopidogrel (■).

2. Experimental

2.1. Equipment

A high-performance liquid chromatographic system consisted of a GBC model LC1126 pump and a Rheodyne model 7725i injector with a 5 μl loop, which were coupled to a GBC model LC1210 UV–vis detector operated at 235 nm. Data acquisition was performed using WinChrom chromatography software package: ChemWin, version 1.2. All pH measurements were performed on a pH meter (Metrohm, model 654 Herisau).

2.2. Materials and reagents

Solvents were of HPLC grade and were purchased from Lab-Scan Science Ltd., Ireland. Sodium dihydrogen phosphate monohydrate, sodium hydroxide, hydrochloric acid and hydrogen peroxide (30% v/v) all of analytical-reagent grade were purchased from E. Merck, Darmstadt, Germany; *ortho*-Phosphoric acid (85% v/v) for analysis was purchased from Panreac-Quimica SA, Barcelona, Spain. Water was deionised and further purified by means of a Milli-Q Plus water purification system, Millipore Ltd. Clopidogrel hydrogen sulphate of pharmaceutical purity grade was kindly provided by Sanofi Winthrop Industrie, Cedex, France, while naproxen of pharmaceutical purity grade was kindly provided by Minerva Hellas. All substances were used without any further purification. Plavix tablets are products of Sanofi Winthrop AE; each tablet was labelled to contain 75.0 mg of clopidogrel. The excipients present in tablets are: starch, lactose, castor oil, cellulose, magrocol 6000, iron oxide (E172), titanium dioxide (E171) and carnauba wax.

2.3. Chromatographic conditions and measurement procedure

Chromatographic separation was performed on a reversed phase BDS C8 column (250 × 2.1 mm i.d., 5 μm particle size) Shandon Scientific Ltd., (Chesire, U.K.). The mobile phase consisted of 0.010 M disodium hydrogen phosphate, adjusted to pH 3.0 with *ortho*-Phosphoric acid, and acetonitrile (35:65, v/v). The mobile phase was filtered through a 0.2 μm nylon-membrane filter (Gelman-Sciences Ltd.) and degassed under vacuum prior to use. A flow rate of 0.30 ml min^{-1} with a column inlet pressure of 1150 psi was used in order to separate clopidogrel and the internal standard naproxen. Peak areas were measured and HPLC analysis was conducted at ambient temperature.

2.4. Stock and working standard solutions

Stock standard solutions of clopidogrel, Clp, 1.0 mg ml^{-1}, and naproxen, Np, 1.0 mg ml^{-1} were prepared by dissolving appropriate amounts of the compounds in methanol. These solutions were stored in the dark under refrigeration at 4 °C and were found to be stable for several weeks.

A series of working standard solutions of Clp were prepared by the appropriate dilution of the above mentioned stock standard solution in the mobile phase to reach concentration ranges of 1.00–3.00 μg ml^{-1} for Clp. In each sample 100 ng ml^{-1} of the internal standard Np was added. Standard solutions were found to be stable during the analysis time.

2.5. Assay of pharmaceutical preparations

Twenty tablets were weighed and finely pulverised. An appropriate portion of this powder, equivalent to 75.0 mg of Clp was placed in a 50 ml volumetric flask with 40 ml of acetonitrile. The solution was sonicated for 20 min and diluted to volume with acetonitrile. A portion of this solution was centrifuged at 4000 rpm (2890 × *g*) for 5 min. A 1-ml aliquot was transferred to a 100 ml volumetric flask and diluted to volume with water. Consequently a 1-ml aliquot of this solution was further diluted to 10 ml mobile phase containing 100 ng ml^{-1} of the internal standard Np; 5 μl sample was injected into the HPLC system. Peak area ratios of clopidogrel to that of the internal standard were then measured for the determinations. The same procedure was followed for the content uniformity test, using one tablet per sample.

2.6. Calibration procedure

Calibration curve of Clp was conducted using the series of working standard solutions described previously. The concentration range of Clp was 1.00–3.00 μg ml^{-1}. All solutions were analysed immediately after their preparation. Triplicate 5 μl injections were made of each solution and the peak area ratio of Clp to that of the internal standard was plotted against the corresponding concentration to obtain the calibration graph.

The over-all precision and accuracy of the HPLC assay was evaluated by analysing four series of standard solutions of Clp, at concentrations of 1.00, 1.50 and 3.00 μg ml^{-1}. The relative standard deviation (% RSD) was determined in order to assess the precision of the assay, while the accuracy was expressed by the relative percentage error (E_r %).

In order to determine the effect of the excipients used in the formulation of tablets on the determination of Clp, the standard addition method was used. Therefore, five equal amounts of powdered tablets equivalent to 75.0 mg of clopidogrel, were spiked with different amounts of the reference standard of Clp. The samples were analysed as mentioned in the assay procedure, while in each sample, 100 ng ml^{-1} of the internal standard Np was added. Peak area ratios of Clp to that of the internal standard were measured for the quantitative determination of Clp.

To assess the proposed HPLC method as a stability indicator for Clp, chromatograms were recorded under various stress conditions where degradation was stimulated by heat, acidic or basic environment.

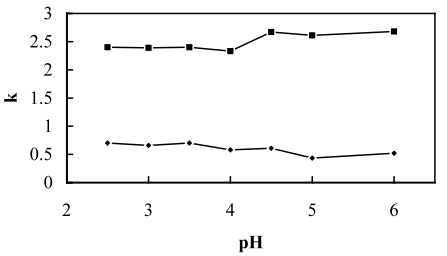

Fig. 2. Plots of the capacity factor, k, vs. pH of the phosphate buffer in the mobile phase of naproxen (◆) and clopidogrel (■).

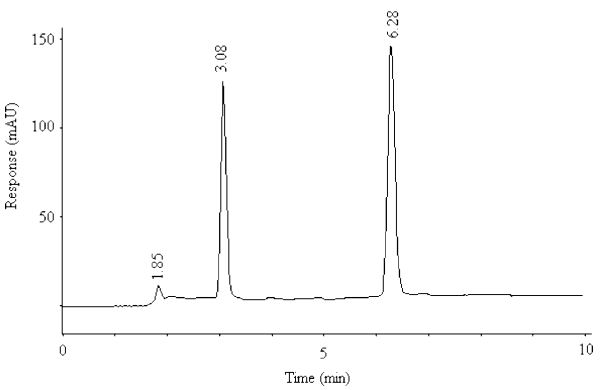

Fig. 3. Representative chromatogram of a mixture of the internal standard, naproxen (100 ng ml^{-1}) and clopidogrel (2.5 μg ml^{-1}) at retention times 3.08 and 6.28 min, respectively. Chromatographic conditions: reversed-phase HPLC on a small-bore C8 BDS column; mobile phase: 0.010 M sodium dihydrogen phosphate (pH 3.0) and acetonitrile (35: 65, v/v); flow rate 0.30 ml min^{-1} and a UV detector at 235 nm.

3. Results and discussion

3.1. Chromatographic characteristics

The chromatographic separations were performed on a semi-micro BDS C8 column (2.1 mm i.d.), offering the advantage of lower solvent consumption and increased mass sensitivity [16]. The effect of composition and pH of the mobile phase on the retention time of Clp and the internal standard, Np, was investigated. Results of the effect of acetonitrile in the mobile phase are presented in Fig. 1. An increase in the percentage of acetonitrile decreases the retention of compounds; Clp and Np. Increasing acetonitrile concentration to more than 75% Np peak is eluted with the solvent front, while at acetonitrile concentration lower than 60% the elution of Clp peak is seriously delayed. The optimum acetonitrile concentration was found to be 65%. The effect of pH in the chromatographic elution of both compounds was also investigated by changes the pH values of the aqueous component of the mobile phase from 6.0 to 2.5 using disodium hydrogen phosphate buffer (0.010 M). For all experimental pH values, the drugs are eluted in order of Np and Clp (Fig. 2). The effect of pH in the retention of both compounds is related to the degree of ionisation. Naproxen is an acidic compound (pK_α 4.2) and owing to ionization the retention is lower for pH values above 4.5, where Np is eluted close to solvent front. On the other hand, clopidogrel is a weak base (pK_α 4.5), thereby at pH values below 4.5 its retention is decreased due to protonization. A pH value of 3.0 was chosen for the optimum separation of the compounds, as at this pH the analyte peaks were well defined and resolved. The optimum wavelength for detection was at 235 nm, at which the best detector responses for all substances were obtained.

The specificity of the HPLC method is illustrated in Fig. 3 where complete separation of the compounds was observed. Clp was eluted at 6.28 min, while the internal standard Np was eluted at 3.08 min.

3.2. Linearity and reproducibility

Calibration graphs were constructed at six concentration levels in the range 1.00–3.00 μg ml^{-1} for Clp; three independent determinations were performed at each concentration ($n = 3$). Linear relationship was obtained between the peak area ratio of Clp to that of the internal standard Np and the corresponding concentration, as shown by the equation presented in Table 1. The correlation coefficient (r) and the standard error of the estimate (S_r) of the calibration line are also given, along with the standard deviations of the slope and intercept.

In order to further evaluate the linearity of the proposed method, five calibration equations were constructed over a period of 4 weeks. The average

Table 1
Calibration equations for the determination of clopidogrel by high-performance liquid chromatography

Sample (μg ml^{-1})[a]	Regression equations[b]	r[c]	S.D.[d]		S_r[e]
Clp			Slope	Intercept	
1.00–3.00	$R_{Clp} = 0.927C_{Clp} + 0.034$	0.9996	0.016	0.029	0.022
Mean of ten calibration curves over a period of 2 months					
1.00–3.00	$R_{Clp} = 0.964C_{Clp} - 0.041$	>0.9991	0.041	0.028	<0.032

[a] Clp, clopidogrel.
[b] Ratios of peak area of clopidogrel to that of the internal standard, R, vs. the appropriate concentration, C, in μg ml^{-1}; six standards.
[c] Correlation coefficient.
[d] Standard deviation of slope (μg ml^{-1}) and intercept.
[e] Standard error of the estimate.

regression equation is also presented in Table 1, along with the RSD values of the slopes and intercepts, the correlation coefficient invariably exceeded 0.9991.

Intra-day data for the precision and accuracy of the method given in Table 2, indicate RSD = 0.41–0.99% and $E_r = -0.7$–1.0%. Moreover, the inter-day RSD% values (Table 2) for the determination of Clp were ranged from 0.66 to 1.96%.

The limit of detection of Clp attained as defined by IUPAC [18], $LOD_{(k=3)} = k \times S_a/b$ (where b is the slope of the calibration graph and S_a is the standard deviation of the intercept), was found to be 0.12 μg ml^{-1}. The limit of quantitation LOQ was also attained according to the IUPAC definition, $LOQ_{(k=10)} = k \times S_a/b$, and was found to be 0.39 g ml^{-1}.

In order to evaluate the robustness of the proposed method [19,20], the influence of small deliberate variations of the method parameters in the retention times of Clp and Np was examined thoroughly. The factors selected to examine were the pH of the buffer, the flow rate and the percentage of acetonitrile in the mobile phase; each factor was charged at three levels (-1, 0 and 1). One factor at the time was changed to estimate the effect. Thus, replicate injections ($n = 3$) of a mixed standard solution containing 1.50 μg ml^{-1} of Clp and 100.0 ng ml^{-1} of Np were performed under small changes of three chromatographic parameters (factors). Results, presented in Table 6, indicate that the selected factors remained unaffected by small variations of these parameters.

The statistical evaluation of the proposed HPLC method revealed its good linearity and reproducibility and led us to the conclusion that it could be used for the rapid and reliable determination of Clp in tablets.

3.3. Label claim recoveries from clopidogrel tablets

The proposed method was evaluated in the assay of commercially available tablets containing 75.0 mg of clopidogrel. Ten replicate determinations were carried out on an accurately weighted

Table 2
Accuracy and precision of within and between run analysis for the determination of clopidogrel by high-performance liquid chromatography

Nominal concentration (μg ml^{-1})	Assayed concentration (μg ml^{-1})		
	Mean ± S.D.	RSD (%)[a]	E_r (%)[b]
Intra-day ($n = 5$)			
1.00	1.01 ± 0.01	0.99	1.0
1.50	1.49 ± 0.01	0.67	−0.6
3.00	2.98 ± 0.01	0.41	−0.7
Inter-day ($n = 5$)			
1.00	1.02 ± 0.02	1.96	2.0
3.00	3.05 ± 0.02	0.66	1.3

[a] Percentage relative standard deviation.
[b] Relative percentage error.

Table 3
Determination of clopidogrel in commercial formulations by high-performance liquid chromatography

Commercial formulation: plavix (75 mg)	Clopidogrel found (mg/tablet)[a]	
Lot	Mean ± S.D. ($n = 10$)	Recovery (%)
1	74.75 ± 0.04	99.7
2	74.86 ± 0.19	99.8

[a] The indicated values are the mean of ten different analyses of the same commercial batch.

amount of the pulverized tablets equivalent to 75.0 mg of Clp. The percent label claim found was to be 99.93 ± 0.25 ($n = 10$, RSD = 0.25%) or 74.95 mg per tablet.

The method proved to be suitable for the content uniformity test, where a great number of assays on individual tablets are required. Two different lots of commercially available tablets containing Clp were analyzed using the proposed procedure and the results are summarized in Table 3. Recoveries achieved were in accordance with the actual content of Clp in tablets.

In order to assess the specificity of the proposed method, recoveries studies were also performed, by spiking sample powders with appropriate amounts of the reference standard of Clp. A calibration curve was constructed by plotting the amount of the drug found (mg) versus the amount of the drug added (mg). The following linear regression equation was obtained through regression analysis of data:

$$C^{f}_{Clp} = 0.996\ (\pm 0.007) \times C^{a}_{Clp} + 75.35\ (\pm 0.35),\quad r = 0.9998$$

where C^{f}_{Clp} and C^{a}_{Clp} are the amounts in (mg) found and added, respectively, for Clp; r is the correlation coefficient of the regression equation.

The y-axis intercept of the above mentioned linear regression equation indicate the amount (mg) of the drug found in the powdered tablets, while the percentage recoveries were calculated as: % recovery = slope × 100. The results presented in Table 4 indicate that there is no interference from the excipients used in the formulation of the tablets.

3.4. Degradation studies

In order to assure the selectivity and provide an indication of the stability-indicating properties of the proposed method, forced degradation studies were performed under various stress conditions. Thus, appropriate amounts of powdered tablets equivalent to the average tablet weight (approximately 248.3 mg), were stressed with 1.0 N HCl, 1.0 N NaOH and 5%v/v H_2O_2 at room temperature. Moreover, samples of powdered tablets were exposed to daylight for a period of 15 days. After the degradation treatments were completed, the samples were analysed according to assay sample preparation, after being neutralized with acid/base, when necessary.

The base stressed samples, in 1.0 N NaOH, showed approximately 89.6% degradation in less than 5 min. One unknown degradation peak (t_r = 2.18 min) appeared in the first 2 min of degradation and further increased till 10 min

Table 4
Recoveries of clopidogrel in spiked commercial samples

Drug	Amount added (mg)	Amount found (mg)	m[a]	Recovery[b] (%)
Clopidogrel	12.5	87.4	0.9962	99.62
	25.0	100.4		
	37.5	113.1		
	50.0	125.3		
	75.0	149.8		

[a] m is the slope the linear regression analysis of the amount found vs. the amount added.
[b] Recovery (%) = $m \times 100$.

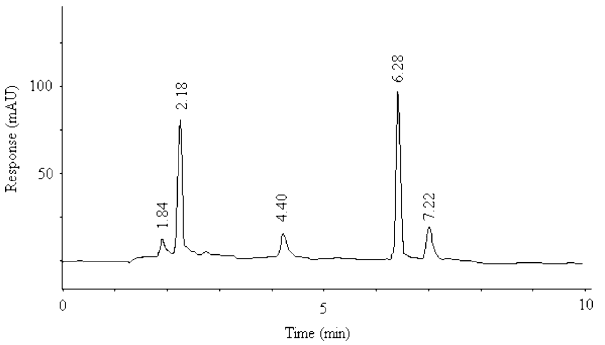

Fig. 4. A typical chromatogram obtained from the analysis of blistered tablets of clopidogrel after storage at 50 °C under heat dry conditions for 4 weeks; Chromatographic conditions: reversed-phase HPLC on a small-bore C8 BDS column; mobile phase: 0.010 M sodium dihydrogen phosphate (pH 3.0) and acetonitrile (35: 65, v/v); flow rate 0.30 ml min^{-1} and a UV detector at 235 nm.

where almost complete degradation of Clp occurred.

On the other hand, in the acid stressed samples, degradation proceeded very slowly, the sample showed 3.5% degradation in 24 h, while almost 95.0% degradation was observed after 4 days, one degradation peak ($t_r = 2.18$ min) was also appeared after 24 h of degradation in acid media.

Powdered tablets of clopidogrel in (5% v/v) sodium peroxide solution showed 23.5% degradation in 24 h and approximately 94.9% degradation occurred within 3 days. One degradation peak ($t_r = 2.18$ min) was appeared after 24 h of degradation. No significant degradation was observed in samples of powdered tablets of clp that were left in daylight for 15 days.

Moreover, blistered tablets of Clp stored for 4 weeks at 50 °C under heat dry and heat wet conditions showed significant degradation; approximately 83.6 and 85.4% of Clp recovered under heat dry and heat wet conditions, respectively. In both cases, three degradation peaks were observed and eluted at 2.18, 4.40 and 7.22 min. A typical chromatogram obtained from the analysis of blistered tablets of Clp after storage at 50 °C under heat dry conditions for 4 weeks is presented in Fig. 4. The percentage recoveries of clopidogrel, along with the retention times of the degradation peaks are presented in Table 5.

4. Conclusions

The proposed high-performance liquid chromatographic method has been evaluated over the linearity, precision, accuracy and specificity and proved to be convenient and effective for the quality control of clopidogrel in pharmaceutical dosage forms. The measured signal was shown to be precise, accurate and linear over the concentration range tested (1.0–3.0 μg ml^{-1}) with a corre-

Table 5
Degradation of clopidogrel in 75 mg plavix tablets

Storage conditions	Time	% Recovered	Retention time of degradation products
Acid 1.0 N HCl, 25 °C	24 h	96.5	2.18
	4 days	2.5	2.18
Base 1.0 N NaOH, 25 °C	5 min	67.5	2.18
	10 min	1.5	2.18
H_2O_2 5% v/v, 25 °C	24 h	76.5	2.18
	2 days	30.9	2.18
	3 days	5.1	2.18
Heat dry, 50 °C	2 days	98.9	2.18
	21 days	92.4	2.18, 4.40, 7.22
	28 days	83.6	2.18, 4.40, 7.22
Heat wet, 50 °C	2 days	98.6	2.18
	21 days	93.3	2.18, 4.40, 7.22
	28 days	85.4	2.18, 4.40, 7.22
Daylight, 25 °C	24 h	99.5	–
	15 days	99.4	–

Table 6
Robustness evaluation of the high-performance liquid chromatographic method

Chromatographic changes		Clopidogrel			Naproxen		
Factor[a]	Level	t_r[b]	k'[c]	T[d]	t_r[b]	k'[c]	T[d]
A: pH of the buffer							
2.90	−1	6.23	2.40	1.15	3.02	0.65	1.26
3.00	0	6.28	2.39	1.15	3.08	0.66	1.27
3.10	1	6.29	2.40	1.16	3.08	0.67	1.30
Mean ± S.D. ($n=3$)		6.27 ± 0.03	2.40 ± 0.01	1.15 ± 0.01	3.06 ± 0.03	0.66 ± 0.01	1.28 ± 0.02
B: Flow rate (ml min^{-1})							
0.28	−1	6.37	2.34	1.17	3.17	0.64	1.30
0.30	0	6.28	2.39	1.15	3.08	0.66	1.27
0.32	1	6.14	2.41	1.19	3.01	0.65	1.27
Mean ± S.D. ($n=3$)		6.26 ± 0.11	2.38 ± 0.04	1.17 ± 0.02	3.09 ± 0.08	0.65 ± 0.01	1.28 ± 0.02
C: % of acetonitrile in the mobile phase (v/v)							
64	−1	6.32	2.35	1.17	3.11	0.67	1.29
65	0	6.28	2.39	1.15	3.08	0.66	1.27
66	1	6.19	2.41	1.14	3.01	0.65	1.24
Mean ± S.D. ($n=3$)		6.26 ± 0.07	2.38 ± 0.03	1.15 ± 0.02	3.07 ± 0.05	0.66 ± 0.01	1.27 ± 0.02

[a] Three factors (A, B and C) were slightly changed at three levels (1, 0, −1); each time a factor was changed from level (0) the other factors remained at level (0).
[b] Retention time.
[c] Capacity factor.
[d] Tailining factor.

lation coefficient better than 0.9991. Moreover, the lower solvent consumption along with the short analytical run time of 7.0 min leads to an environmentally friendly chromatographic procedure. Stressed degradation studies on sample preparations did not exhibit any degradation peaks that could interfere with the elution of clopidogrel.

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