"Development and Validation of HPTLC Method for the Determination of Clopidogrel bisulphate in Pharmaceutical Dosage Form and Simulated Biofluids"

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KEYWORDS
Clopidogrel bisulphate, Simulated biofluids, HPTLC technique, ICH guidelines (Q2R1).

ABSTRACT:
A simple, rapid, and sensitive high performance thin layer chromatographic method has been developed for the quantification of clopidogrel in simulated biofluids and pharmaceutical dosage form and validated according to standard guidelines. The separation of clopidogrel bisulphate was achieved using aluminium-backed layer of silica gel 60 F_{254}. The toluene: acetonitrile: methanol: acetone (5.5:3:1:0.5 v/v/v/v) as the mobile phase used. Densitometric analysis was carried out in the absorbance mode at 230 nm. The system was found to give compact spots for clopidogrel bisulphate (Rf value of 0.81). Sample preparation of drug from simulated biofluids was carried out with 0.45 µm nylon syringe filter. The limit of detection was 7.36 ng/band and limit of quantification was 22.89 ng/band. The linear regression analysis data for the calibration plots showed good linear relationship with r^2 = 0.9996 in the concentration range 25-250 ng per spot for both standard solutions and spiked simulated biofluids. The intra-day and inter-days precision (RSDs) was less than 2 % and the accuracy was more than 99%. The method could be applied for the estimation of clopidogrel bisulphate in simulated biofluids as well as to pharmaceutical dosage forms.
***Abbreviations** – Clopidogrel bisulphate (CLB), Simulated biofluids (SBFs)*

1. Introduction

Clopidogrel bisulphate (CLB) (S-methyl 2-(2-chlorophenyl-6,7-dihydrothieno [3,2-c] pyridine-5(4)-acetatesulfate) is a thienopyridine antiplatelet drug (1). This drug successfully used to treat a variety of cardiovascular conditions, such as atherothrombosis, unstable angina, or myocardial infarction (2). It inhibits adenosine diphosphate's (ADP) platelet receptor binding selectively and irreversibly, as well as ADP-mediated glycoprotein GPIIb/IIIa complex activation after biotransformation into a thiolic active metabolite via CYP3A4 and CYP2C19 cytochrome P450 isoforms (3,4).

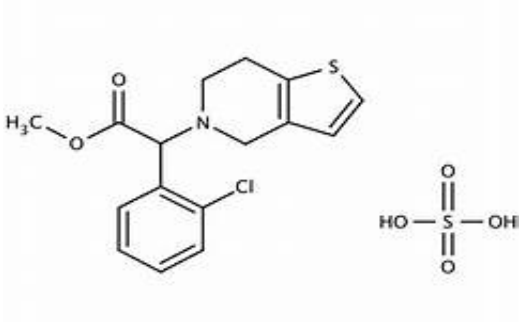

Fig 1. Structure of Clopidogrel bisulphate

Pharmaceutical dosage forms are predicted to function as intended in vivo through the use of simulated biofluids (SBFs). Correlation with in vivo data is not possible using the medium commonly employed for quality control dissolving testing, as they do not accurately reflect all physiological circumstances of the most commonly used routes of administration. A sufficient simulation of the in vivo conditions is necessary to predict the performance of the dosage form in the location where the majority of absorption takes place (5).

There have been numerous reported methods for the quantification of CLB in pharmaceutical dosage forms and plasma sample using chromatographic techniques. CLB has been examined in pharmaceutical dosage form by HPLC technique (6–12) and UV (13-14). However, ultra-fast liquid chromatography (UFLC) has only been used to quantify the parent medication and its active metabolite in plasma samples (15). CLB

has been quantified inpharmaceutical formulations and human, rat and dog plasma samplesusing HPLC MS/MS (16), tandem mass spectroscopy (17,18), HPLC (19–21) and UPLC (22).All of these methods involve time-consuming separation, which complicates routine analysis. It is of interest to mention, no HPTLC-based technique has been reported to quantify clopidogrel bisulphate in simulated biofluids which is useful for pharmaceutical industries and research laboratories

The purpose of this study was to develop a versatile and economical HPTLC method for the estimation of clopidogrel bisulphate both in pharmaceutical dosage forms and simulated biofluids. The method has been validated in accordance with ICH guideline (23) to assess its specificity, sensitivity, accuracy, precision, and robustness. This estimation method requires 1-3 minute per sample as compared to HPLC and UPLC methods that requires 2- 60 minutes per sample averagely. Further, this paper contains sections of material and methods, results and discussion. At last, it is concluded with successful results.

MATERIALS AND METHODS

Instrumentation

Precoated Silica Gel G 60 F254 aluminium sheets (20 cm × 10 cm, 200µm thickness, E. Merck, Germany) were used as the stationary phase for the HPTLC study of CLB. The samples were spotted using a Linomat V (CAMAG, Switzerland) sample applicator and a CAMAG syringe, with each band measuring 8 mm in width and 10 mm apart. Constant application rate of 160 nL/s, slit dimension of 5 mm × 0.45 mm, and scanning speed of 20 mm/s are among the equipment specifications. Chromatography plates were developed starting at a 70.00 mm solvent front position. The plates were allowed to air dry before densitometric scanning was carried out using Vision CATS software (Version 3.2) and a CAMAG TLC scanner III set to UV mode at 230 nm.

Chromatographic procedure

The stationary phase for the chromatographic separation was 20 x 10 cm precoated Silica Gel G 60 F254 (Merck). Using nitrogen gas spraying technique, 8 mm bands of the standard and sample solutions from a 100 µl CAMAG syringe were sprayed to the HPTLC plates. Using a CAMAG Linomat V sample applicator, this was carried out at a consistent application rate of 160 nL/s. The bands remained two to three minutes air drying. Before that, the chambers in the 20 x 10 cm CAMAG twin trough chambers were saturated with mobile phase for 20 minutes at ambient temperature (25°C). After achieving a solvent front at 70 mm of the plate length, the plates were taken out of the chamber. The plates were left to air dry for five to ten minutes after the solvent front was marked. For CLB, densitometric analysis was performed at 230 nm using the CAMAG TLC Scanner 3. Vision CATS (version 3.2) software was used to combine the chromatograms, and the outcomes were assessed.

Chemicals and Reagents

Reference standard of CLB were donated by Sun Pharmaceutical Industries Limited Gujarat, India. Commercially available clopidogrel bisulphate formulation Clopitab tablets, was purchased from local pharmacy store. Sodium chloride, Potassium chloride, Potassium phosphate dibasic trihydrate, Sodium hydroxide, Magnesium chloride hexahydrate, Sodium sulphate (Loba Chemie Pvt. Ltd.), Sodium bicarbonate, Hydrochloric acid (99.5%, Finar Limited), 1 M hydrochloric acid, Sodium oleate, Calcium chloride, Tris(hydroxymethyl) aminomethane, Pepsin, Maleic acid (Central Drug House (P) Ltd.), Sodium taurocholate (Sigma-Aldrich), Lecithin (30% Himedia), Glyceryl monooleate (95%, Merck), Toluene, Acetonitrile, Methanol, Chloroform, Acetone (HPLC grade, Central Drug House (P) Ltd.).

Preparation of standard solution

A stock solution of clopidogrel bisulphate was prepared by dissolving 10 mg of clopidogrel bisulphate in 10 ml of volumetric flask with methanol. The stock solution was further diluted with methanol to obtain working solutions in the concentration 0.05 mg/ml.

Sample Preparation

The tablet brand name Clopitab that contain 75 mg of CLB according to the label. Accurately weight and powder twenty tablets of clopidogrel bisulphate, amount equivalent to 39.4 mg of powder containing 10 mg of drug. The powder was then carefully transferred into separate 10 ml of volumetric flasks. The CLB solution was then dissolved using HPLC grade methanol, sonicated, and filtered. These solutions were further diluted with methanol, and Linomat V was used to apply 2 µl of each formulation upon an HPTLC plate.

Preparation of simulated human blood plasma pH 7.4 (24)

The simulated human blood plasma pH 7.4 was prepared by dissolving 8.035 g of sodium chloride, 0.355 g of sodium bicarbonate, 0.225 g of potassium chloride, 0.231 g of potassium phosphate dibasic trihydrate, 0.311 g of magnesium chloride hexahydrate, 39 ml of 1 M hydrochloric acid, 0.292 g of calcium chloride, 0.072 g of sodium sulphate and 6.118 g of tris(hydroxymethyl) aminomethane and distilled water was added 1000 ml until a final volume was achieved.

Preparation of simulated gastric fluid pH 1.6 (25)

Hydrochloric acid was used to adjust the pH of the simulated gastric fluid to 1.6 after dissolving 80 (µM) of sodium taurocholate, 20 (µM) of lecithin, 0.1 (mg/ml) of pepsin, and 34.2 (mM) of sodium chloride in 1000 ml of distilled water.

Preparation of simulated intestinal fluid pH 6.5 (26)

The simulated intestinal fluid pH 6.5 was prepared by dissolving 10 (mM) of sodium taurocholate, 3 (mM) of lecithin, 28.6 (mM) of maleic acid, 52.5 (mM) of sodium hydroxide, 145.2 (mM) of sodium chloride, 6.5 (mM) of glyceryl monooleate, 40 (mM) of sodium oleate and 1000 ml distilled water was added until a final volume was achieved.

Simulated biofluids sample preparation

The sample was withdrawn from the dissolution media, and filtered through a 0.45µm nylon syringe filter. Then it was directly applied to a chromatographic plate using Linomat V.

Validation Studies

The chromatographic method was validated according to international guidelines with emphasis on specificity, linearity, recovery, precision (repeatability and intermediate precision), sensitivity, and robustness (23).

Linearity

The analyte concentration and the chromatogram's area under the curve were used for generating the linearity graph. Then, using least squares linear regression, the slope, intercept, and coefficient of determination were determined. The measurements were made three times. The required R-squared value is > 0.9990.

Sensitivity

The sensitivity of the method was assessed by determining the limit of detection (LOD), and limit of quantification (LOQ), using the signal-to-noise (S/N) ratio at a level of three and ten times, respectively. The LOD is the minimum concentration whose response is equal to three times the value of S/N while the LOQ is evaluated as the minimum concentration whose response is equal to ten times the S/N value

Accuracy/ Percent Recovery

Accuracy was determined by standard addition method. By adding standard drug solution to pre-analysed sample solution in three replicates at three distinct concentrations 80%, 100%, and 120% this parameter was investigated. Peak regions were evaluated and a chromatogram was obtained. The corresponding calibration curves were used to calculate each drug's concentration and, consequently, its recovery. All levels %RSD must be less than 2.0%.

Precision

Three replicates of the mixed standards of the analytes at three QC levels were analysed on the same day and on three successive days, respectively, to examine the intra-day and inter-day precision of the method. Every level's %RSD needs to be less than 2.0%.

Robustness

The robustness of this proposed method was determined by evaluating the influence of minor deliberate changes made in procedure variables like volume of the mobile phase (0.2 mL), composition of the mobile phase, chamber saturation time. (as shown in further section)

RESULTS AND DISCUSSION

Sample Preparation

Sample preparation, based on the filtration of the analytes from the tablet formulation with methanol, was found to be specific as no interfering peaks were observed in the chromatograms. As well as, the percentage recovery obtained is ~~also~~ more than 99% that gives us a favourable or positive environment/situation.

In simulated bio-fluids, the sample was filtered by 0.45µm nylon syringe filter. In This technique, there is no need of any sample extraction procedure.

Optimization of Chromatographic Conditions

The HPTLC procedure was optimized with a view to develop a sensitive and reproducible assay method for Clopidogrel bisulphate. The mobile phase consisting of toluene: acetonitrile: methanol: acetone (5.5:3:1:0.5 v/v/v/v) gave good resolution, sharp and symmetrical peaks with Rf value of 0.81 Clopidogrel bisulphate at 230 nm wavelength (Fig.2). Also, the spots were compact and not diffused. It was observed that prewashing of TLC plates with methanol followed by drying and activation, and pre-saturation of TLC chamber with mobile phase for 20 min ensured good reproducibility for peak shapes and areas of drugs.

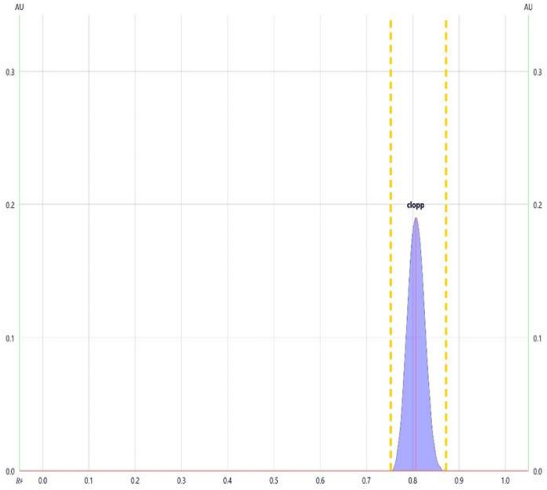

Fig 2. A typical chromatogram of Clopidogrel sulphate

Validation parameters

Method was validated as per ICH guideline with respect to linearity, range, accuracy precision, limit of detection, limit of quantification and results are shown ~~in Tables~~ as follows-

Linearity and range

Peak area ratio towards concentration fitted well to a straight line for calibration curves that demonstrated high linearity r^2 = 0.9996 for the concentration range of 25 – 250 ng/band and the regression equation: Y = (0.00004599 ± 0.0001227) X + (0.002898 ± 0.001581) was found of the tablet sample. The regression equations for the simulated intestinal fluid, simulated plasma, and simulated gastric fluid were Y = (0.00003946 ± 0.00047) X + (0.0012833 ± 0.00017), Y = (0.0000364 ± 0.000374) X + (0.001356 ± 0.00013), and Y = (0.00004924 ± 0.000702) X + (0.00137 ± 0.000458), respectively as indicated in Table I.

Table I. Linear regression and statisticaldata for the clopidogrel bisulphate (n=3)

Parameter	Clopidogrel bisulphate			
	Tablet sample	Simulated Gastric fluid	Simulated Plasma	Simulated Intestinal fluid
Linearity range (ng/band)	25 – 250	25 - 250	25 - 250	25 - 250
Correlation coefficient (r^2 ± SD)	0.9996407 ± 0.001339	0.9980 ± 0.008606	0.9983 ± 0.0009539	0.9974 ± 0.001357
Slope Mean ± SD	0.00004599 ± 0.0001227	0.00003946 ± 0.00047	0.00003649 ± 0.000374	0.00004924 ± 0.000702
Standard error	0.0000708	0.000018	0.0000647	0.0000405
Intercept Mean ± SD	0.002898 ± 0.001581	0.001283 ± 0.0001700	0.001356 ± 0.0001301	0.001378 ± 0.000458

LOD and LOQ

The LOD and LOQ for the analytes were calculated from the slope of the calibration lines and the standard deviation of the intercept. The results for tablet formulation and in SBFs are presented in Table II.

Table II. LOD and LOQ of clopidogrelbisulphate

Parameter	Tablet sample (ng/band)	Simulated Gastric fluid (ng/band)	Simulated Plasma (ng/band)	Simulated Intestinal fluid (ng/band)
LOD	7.36	7.25	7.36	7.78
LOQ	22.89	21.63	21.86	21.92

Accuracy

After standard clopidogrel bisulphate was added to the same amount of the sample solution 80%, 100% and 120%, the percentage of recovered clopidogrel bisulphate in the tablet sample was found to be between 100.88, in simulated gastric fluid 98.10, simulated plasma 98.32, and simulated intestinal fluid 99.527. An average percent recovery of 99.69 in an API, 98.71 in simulated gastric fluid, 98.05 in simulated plasma, and 99.02 in simulated intestinal fluid as shown in Table III.

Amount of sample taken (ng/band)	Standard added (ng/band)	% Recovery	Average recovery
Tablet sample 50	80	100.88	99.96
50	100	99.56	100.09
50	120	98.36	100.02
Simulated gastric fluid 50	80	98.35	97.79
50	100	100.16	100.05
50	120	98.32	98.51
Simulated Plasma 50	80	99.65	98.50
50	100	100.68	100.06
50	120	99.23	99.14
Simulated intestinal fluid 50	80	98.32	99.20
50	100	100.53	100.24
50	120	98.36	99.11

Precision

The results of the intraday and interday precision experiments are described in Table IV. The % CV (percent of coefficient of variation) of CLB was recorded to as 1.54 – 1.90 %, 1.2-1.8 %, 1.93 – 2.01 % and 1.83 – 2 % in tablet sample, simulated gastric fluid, simulated plasma and simulated intestinal fluid respectively at three different concentrations for intra – day precision. The % CV of clopidogrel was recorded to as 1.7 – 2 %, 1.5-1.96 %, 1.96 – 2.0 % and 1.65 – 1.98 % in tablet sample, simulated gastric fluid, simulated plasma and simulated intestinal fluid respectively at three different concentrations for inter – day precision.

Table IV. Intra-day and inter-day precision data for clopidogrel bisulphate (n=3)

Intraday Precision				Interday Precision		
Actual concentration (ng/band)	Mean ± SD	Standard error	% CV	Mean ± SD	Standard error	% CV
Tablet sample 50	0.002253 ± 0.0000351188	0.0000202	1.54	0.002277 ± 0.0000404	0.00002333	1.7
100	0.004747 ± 0.0000945163	0.0000545	1.90	0.004773 ± 0.0000960	0.0000554	2
150	0.006257 ± 0.0000122202	0.0000705	1.83	0.006197 ± 0.000100167	0.0000578	1.99
Simulated gastric fluid 50	0.002293333 ± 0.0000251	0.0000145	1.2	0.002306667 ± 0.0000305	0.0000176	1.5
100	0.004746667 ± 0.0000505	0.0000315	1.26	0.00482 ± 0.0000888	0.0000513	1.96
150	0.00626 ± 0.000108167	0.0000624	1.8	0.0062833 ± 0.0001006	0.0000581	1.77
Simulated plasma 75	0.002256667 ± 0.0000450	0.0000260	2	0.0023 ± 0.0000458	0.0000264	2
100	0.004753333 ± 0.0000975	0.0000545	2.01	0.0047366 ± 0.0000901	0.000156205	1.99
125	0.006436667 ± 0.0001193	0.0000688	1.93	0.0063666 ± 0.0001171	0.0000676	1.96
Simulated intestinal fluid 50	0.00233 ± 0.0000456	0.0000496	1.83	0.00238 ± 0.0000458	0.0000264	1.98
100	0.00454 ± 0.0000963	0.0000783	2	0.004756 ± 0.0000907	0.0000523	1.96
150	0.00635 ± 0.000117	0.0000423	1.9	0.00655 ± 0.0000953	0.0000550	1.65

Robustness

The robustness of the proposed method was determined by evaluating the influence of minor deliberate changes in procedure variables like volume of the mobile phase (0.2 mL), composition of the mobile phase, chamber saturation time resulted in a negligible change in the retention time and peak area of all analytes.

APPLICATION OF THE METHOD

Analysis of marketed formulation

The applicability of the method was verified by determination of clopidogrel bisulphate in pharmaceutical preparation. The percent recovery of the proposed method ranges from 99.50 to 99.84% averaging to 99.67 %.

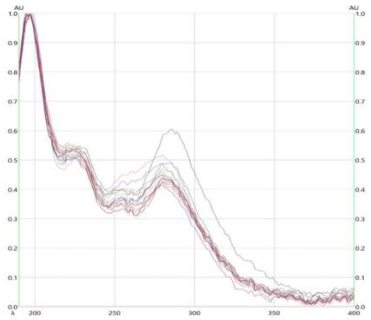

Fig 3. Overlay spectra of sample and standard of clopidogrel bisulphate

This simple bioanalytical method also allowed quantification of clopidogrel in simulated biofluids. This reported HPTLC method provides an efficient technique for quality control of oral dosage form of clopidogrel in pharmaceutical industry as well as a handy economic research tool in drug testing laboratory. This method may also be applied for the evaluation of drug-drug and drug-food interactions studies in the clinical setup.

CONCLUSION

A rapid, sensitive and specific HPTLC method was developed for the determination of clopidogrel bisulphate in simulated biofluids and pharmaceutical dosage form. No significant interferences caused by the endogenous compoundswere observed. Compared to the traditional HPLC has a very short single run time of 1.0 min per sample, which makes it an attractive procedure in high throughput bioanalysis of clopidogrel bisulphate.This method showed adequate sensitivity, linearity, precision and accuracy.

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Conflict of Interest

Authors report no conflict of interest concerning this research article.

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