Research Article

Bio-Analytical UFLC Method Development and Validation for Simultaneous Estimation of Clopidogrel and Pantoprazole in Human Plasma

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ABSTRACT

A simple, sensitive, robust and specific Ultra fast liquid chromatographic (UFLC) method was developed and validated for the simultaneous determination Clopidogrel and Pantoprazole in human plasma. In the current study, the analysis was performed on phenomenex C8 (250 × 4.6mm, 5µm) column using potassium dihydrogen orthophosphate buffer (pH-3.0) and acetonitrile (40: 60 v/v) as mobile phase at flow rate 1.2 mL/min. The analyte were monitored with PDA detector at 252nm. In this developed method Clopidogrel and Pantoprazole elutes at a retention time of 5.14 and 2.62 min respectively. The proposed method is having linearity in the concentration range from 5 to 50µg/mL of Clopidogrel and Pantoprazole. The current method was validated with respect to linearity; precision, lowest limit of detection (LOD) and lowest limit of quantification (LOQ), accuracy and recovery according to the USP guidelines. The system consisted of a pump (Shimadzu, prominence, UFLC), with 20µl sample injector, along with a PDA ( ) detector at a wavelength of 252nm. Data was compiled using Shimadzu LC Solution software. A good linear relationship over the concentration range of 5-50µg/ml was shown. Validation of the method was carried out as per the USP. The method developed was found to be precise, accurate, specific and selective. Statistical analysis shows that the method is reproducible and selective for the estimation of Clopidogrel and Pantoprazole in dosage form.

Keywords: Bioanalytical, Clopidogrel, Pantoprazole, UFLC, USP.

INTRODUCTION

Clopidogrel**,** (CPG) (+)-(S)-methyl 2-(2-chlorophenyl)-2-(6, 7-dihydrothieno [3, 2-c] pyridin-5(4H)-yl) acetate (Fig 1A) is a prodrug that is converted in the liver to an active thiol metabolite, which irreversibly inhibits the platelet P2Y12 adenosine diphosphate receptor. This bioactivation is mediated by hepatic cytochrome P450 isoenzymes, with cytochrome P450 2C19 playing a major role. The cytochrome P450 (CYP) super family of heme enzymes plays an important role in the metabolism of a large number of endogenous and exogenous compounds, including most of the drugs currently on the market. Inhibitors of CYP enzymes have important roles in the treatment of several disease conditions such as numerous cancers and fungal infections in addition to their critical role in drug-drug interactions Given the important role of cytochrome P450 2C19 in the bioactivation of Clopidogrel, drugs that inhibit this enzyme may reduce the antiplatelet effect of Clopidogrel. It is used in the Prevention of vascular ischemic events in patients with symptomatic atherosclerosis, acute coronary syndrome without ST-segment elevation (NSTEMI), ST elevation MI (STEMI).

Literature survey reveals that few analytical methods have been reported for Clopidogrel include RP-HPLC methods[1-4], HPTLC method[5,6] , UV method[7], normal phase HPLC[8], GC method[9], LC-MS method[10], capillary electrophoresis method.[11]

Pantoprazole**,** (*RS*)-6-(Difluoromethoxy)-2-[(3, 4-dimethoxypyridin-2-yl) methylsulfinyl]-1*H*-benzo[d] imidazole (Figure 1B) is a proton pump inhibitor drug that inhibits gastric acid secretion. Pantoprazole is metabolized in the liver by the cytochrome P450 system. Metabolism mainly consists of demethylation by CYP2C19 followed by sulfation. Another metabolic pathway is oxidation by CYP3A4. Pantoprazole metabolites are not thought to have any pharmacological significance. Generally inactive at acidic pH of stomach, thus it is usually given with a pro kinetic drug. Pantoprazole binds irreversibly to H+K+ATPase (Proton pumps) and suppresses the secretion of acid. As it binds irreversibly to the pumps, new pumps have to be made before acid production could be resumed. The drug's plasma half-life is about 2 hours. Pantoprazole is used for short-term treatment of erosion and ulceration of the esophagus caused by gastro esophageal reflux disease. Initial treatment is generally of eight weeks' duration, after which another eight week course of treatment may be considered if necessary. It can be used as a maintenance therapy for long term use after initial response is obtained.

Literature survey reveals that few analytical methods have been reported for pantoprazole include has been estimated by colorimetry[12], Spectrophotometric methods[13, 14], LC-MS/MS[15], RP-HPLC.[16-21]

MATERIALS AND METHODS

Chemical and Reagents

Pure sample of Clopidogrel and pantoprazole were received from Wintac Limited, Bangalore. The human plasma was received from JSS Hospital, Mysore, Karnataka, India. All the chemicals and reagents used

were of analytical grade only. Milli-Q-water was used throughout the process, methanol, acetonitrile of HPLC grade were procured from Merck Chemical Laboratories, Bangalore, India.

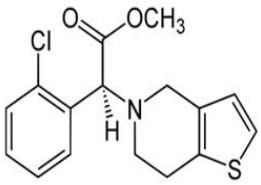

Figure 1(A): Structure of Clopidogrel

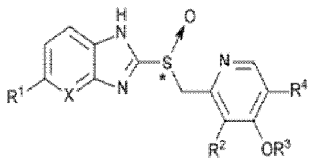

Figure 1(B): Structure of Pantoprazole

Instrumentation

The present research was carried on UFLC (SHIMADZU) equipped with PDA detector with LC solution software. Separation was attained using phenomenex C8 column. The mobile phase was a mixture of potassium dihydrogen orthophosphate buffer (pH-3.0) and acetonitrile (40:60 v/v) at flow rate 1.2 mL/min. The contents of mobile phase were filtered before use through membrane filter (0.45 µ). The optimized chromatographic conditions are shown in Table 1.

Table 1: Optimized chromatographic conditions

Chromatographic Conditions	
Column	C8 (250 x 4.6 mm. 5 µ) phenomenex
Flow rate	1.2 mL/min
Run time	10 min
Wavelength	252 nm
Injection Volume	10µL
Detector	PDA Detector
Elution	Isocratic
Mobile Phase	potassium dihydrogen orthophosphate buffer (pH-3.0) and acetonitrile (40:60 v/v)
Column oven temperature	25 ± 5°C

Preparation of Mobile Phase

Mobile phase is prepared by adding 4.08g potassium dihydrogen orthophosphate in 250ml of Millipore water, dissolve and adjust the pH to 3.0 using ortho phosphoric acid and made up to 1000ml (0.03M) using Millipore water and acetonitrile were used in the ratio of 40: 60 (v/v).

Preparation of Standard Solutions

Stock solution of Clopidogrel and pantoprazole was prepared by dissolving 100 mg of drugs Clopidogrel and pantoprazole in 50 mL of methanol in 100mL volumetric flask dissolved and volume was made up to 100 mL using the methanol to get the standard stock solutions of concentration 1 mg/mL (1000 µg/mL) for both Clopidogrel and pantoprazole. Different working standard solutions were prepared from the above solution.

Method Development

Selection of mobile phase

Different mobile phases were tried in various ratios for selection of solvents of desired polarity. The drugs Clopidogrel and pantoprazole were injected with different mobile phases at different ratios and flow rates till a sharp peak, without any interference was obtained. The mobile phase selected with good resolution was phosphate buffer (pH 3), and acetonitrile in the ratio 40:60(v/v).

Stock and standard solution

The stock solution of Clopidogrel and pantoprazole were prepared by dissolving 10mg of each separately into methanol and volume was made up to 100ml with same solvent. From stock solutions (100 µg/ml of each) 5, 10, 20, 30, 40, 50 µg/ml concentration were prepared separately using methanol as solvent. Equal volumes of both concentrations were mixed and used as standard solutions.

Preparation of Calibration Curve

From the stock solution (1000 µg/mL) aliquots of Clopidogrel and pantoprazole were pipette into a series of 10 mL volumetric flask. The final volume was made up to the mark by using HPLC grade methanol. 10µL solution was injected to the column and peak areas were measured and the calibration curve was obtained.

Linear correlations were found between peak ratios of Clopidogrel and pantoprazole and are described by regression equation. The Beer's law was obeyed in the concentration range of 5 – 50 µg/mL (Figure 2).

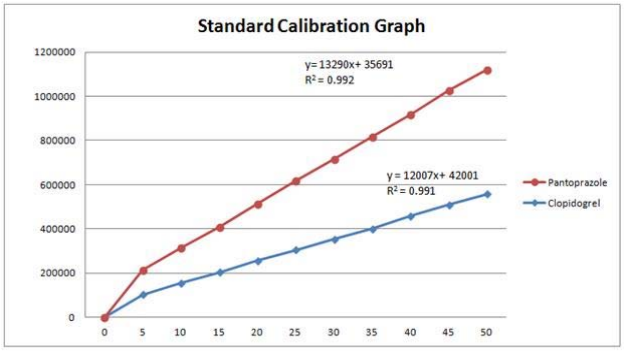

Figure 2: Standard calibration graph of Clopidogrel and Pantoprazole

The regression parameters and system suitability of the method were shown in Table 2.

Determination of drugs in plasma (spiking method)

0.1 ml of drug is added to 0.1 ml of plasma (obtained by centrifuging the blood samples at 10,000 rpm for 10 minutes) in appendroff tubes and made up to the volume (1.8 ml) with acetonitrile for the precipitation of proteins. It is further centrifuged at 10,000 rpm for 10 minutes. Supernatant fluid is decanted into vial by filtering with syringe filters of 0.45µ size.

The obtained chromatograms are shown in Figure 3 (A and B).

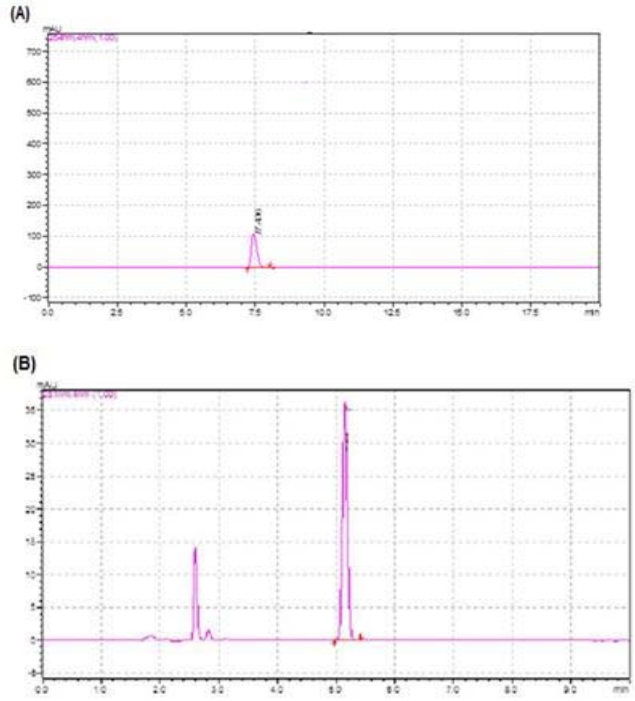

Figure 3: Chromatogram of **(A)** Blank, **(B)** Clopidogrel and pantoprazole in plasma

RESULTS AND DISCUSSION

Method Validation

Since the UFLC method was developed, validation of the method by using various parameters was performed to ensure that the accomplishment of the method meets the requirements of the described bioanalytical applications.

Following parameters were performed for method validation:

1. System suitability
2. Specificity
3. Detection Limit (LOD)
4. Quantification Limit (LOQ)
5. Linearity
6. Precision
7. Accuracy

Linearity

From the experimental conditions described above, linear calibration curves of Clopidogrel and pantoprazole were obtained for ten different concentrations level for both. The r^2 for Clopidogrel was 0.991 and for pantoprazole was 0.990. Linear correlations were found between peak area of Clopidogrel and pantoprazole concentration and are described by the regression equation. The linearity range for Clopidogrel and pantoprazole is 5-50 µg/ml. Results are specified in Table 2.

Specificity

Specificity is the capability to evaluate the analyte distinctly in the presence of expected impurities and degraded products.

20 µl of the blank was injected in duplicate to the UPLC system and chromatographed.

20 µl of Clopidogrel and pantoprazole standard solutions were injected in duplicate to the UPLC system. Standard chromatograms obtained are presented in Figure 4 (A, B and C).

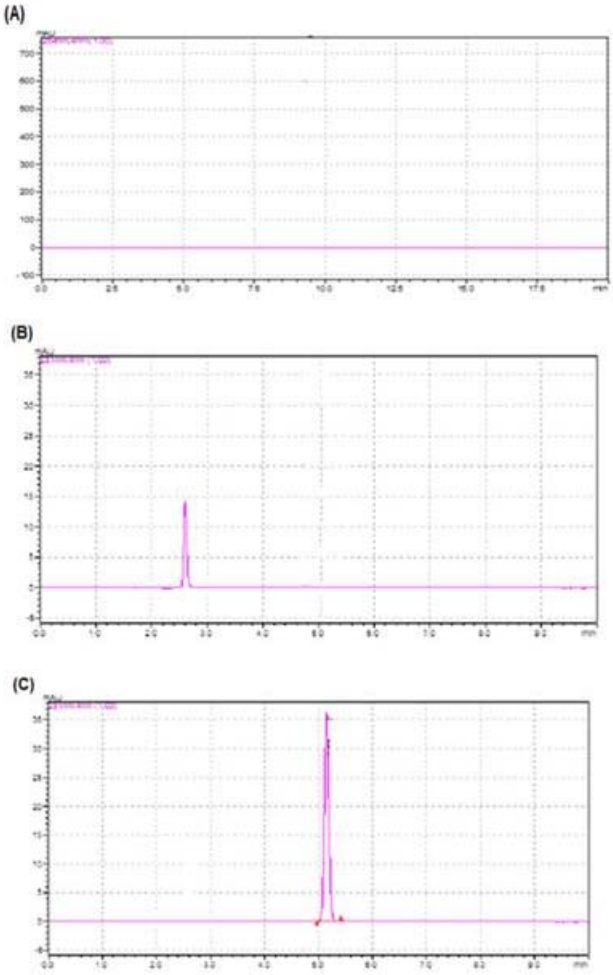

Figure 4: Chromatogram of **(A)** Blank, **(B)** Standard solution of Pantoprazole (50 µg/ml), **(C)** Standard solution of Clopidogrel (50 µg/ml).

Precision and accuracy

The accuracy of an analytical method is the percentage of relativeness between the conventional true value and the value obtained by that method.

Precision and Accuracy were determined by replicate analysis of known content of sample. The mean value should be within 15% of the actual value as per the acceptance criteria. The difference between mean amounts added and recovered (RE, %) serves as a measure of accuracy. The coefficient of variation (CV %), as a measure of precision at each concentration, should

not exceed 15%. Intra-day and inter-day accuracy and precision were evaluated by analysis of quality-control samples containing Clopidogrel at three different concentrations - a low concentration (LQC), a concentration near the centre of the calibration plot (MQC) and a concentration near the upper limit of the calibration plot (HQC). Intra-day accuracy and precision were evaluated by analysis of these QC samples prepared and analyzed on the same day (eight samples of each concentration; three replicate injections). Inter-day accuracy and precision were evaluated by analysis of these QC samples prepared and analyzed on five different days (three samples of each concentration; three replicate injections). The intra-day precision and accuracy of the method for Clopidogrel and pantoprazole are presented in (Table 3A). The inter-day precision and accuracy of the method for Clopidogrel and pantoprazole are presented in (Table 3B). All values for accuracy and precision were within the recommended limits.

Table 2: The regression and System suitability parameters of the method

Parameter	Clopidogrel	Pantoprazole
Linearity (µg/ml)	5-50	5-50
Regression Equation	12007x + 42001	13290x + 35691
Regression coefficient (R^2)	0.9913	0.9924
Slope	97774	85001
Intercept	458786	583384
Retention Time (Rt)	5.14	2.62
LLOQ (µg/ml)	5.71	2.67
Resolution factor (RS)	6.7	6.7
Capacity Factor (K')	5.2	5.2
Tailing Factor (T)	1.1	1.7
Theoretical Plates	4376.51	7810.79
HETP	81.0	90.0

Table 3: Intraday and Interday Precision of Clopidogrel and pantoprazole

(A) Intraday Precision						(B) Interday Precision					
Concentration (µg/ml)		Mean (µg/ml)		%RSD		Concentration (µg/ml)		Mean (µg/ml)		%RSD	
		Clopidogrel	Pantoprazole	Clopidogrel	Pantoprazole			Clopidogrel	Pantoprazole	Clopidogrel	Pantoprazole
Low (n=3)	5	5.11	5.25	0.07	0.06	Low (n=3)	5	5.21	5.30	0.06	0.08
Medium (n=3)	25	25.5	26.6	0.08	0.06	Medium (n=3)	25	25.7	25.96	0.07	0.05
High (n=3)	50	51.30	50.16	0.06	0.07	High (n=3)	50	51.30	50.35	0.05	0.06

Recovery

Recovery of the method was performed comparing the three quality control (QC) samples at low, medium and high concentrations (5, 25, 50 µg/ml) The recoveries of Clopidogrel and pantoprazole were determined by comparing peak area obtained for QC samples that were subjected to the extraction procedure with those obtained from blank plasma extracts that were spiked post extraction to the same nominal concentrations.

Stability studies

The stability in human plasma over three freeze–thaw cycles and during short-term, long-term, and post-preparative storage was tested by analysis of LQC and HQC samples. The freeze–thaw stability was determined over three freeze–thaw cycles within 3 days. Spiked plasma samples were frozen at -22°C for 24 h and thawed at room temperature in each freeze–thaw cycle. To study short-term stability, the frozen (-22°C) and then thawed plasma samples were kept at room temperature for 6 h before sample preparation. The results obtained from these test samples were compared with those from freshly thawed and processed samples (reference samples). Long-term stability was determined after keeping spiked plasma samples frozen at -22°C for 1 month. For this stability test the samples (test samples) were analyzed and the results were compared with those obtained from freshly prepared and processed samples (reference samples). The stability in stock solutions was studied after storage at 2°C for 1 month. Three freeze–thaw cycles of the quality control samples did not seem to affect quantification. Quality-control samples stored in a freezer at -22°C were stable for at least 1 month. Thawing of the frozen samples and keeping them at room temperature for 6 h had no effect on quantification. The stability in stock solutions was confirmed after storage for 29 days at 2°C.

Table 4: Summary of validation parameters data for Clopidogrel and Pantoprazole

Parameters		Clopidogrel	Pantoprazole	Acceptance criteria
Retention Time (min)		5.14	2.62	-
LOD (µg/ml)		5	5	-
LLOQ (µg/ml)		6.5	7.2	-
Linearity (µg/ml)		5-50	5-50	-
Accuracy (% Recovery)		96.7-98.2%	96.2-98.4	90 -110%
Precision (%RSD)	System	0.025	---	< 2%
	Method	0.0020	---	
	Intermediate precision	0.72	---	
Specificity		No peak of diluent, excipients and impurities were detected.		No peak should be detected
System Suitability Parameters	N	9772	---	>2000
	HETP	0.0021	---	-
	Asymmetry	1	---	~1
	Resolution	1.115	---	

CONCLUSION

The method involves simple and precise method for bioanalytical determination of Clopidogrel and pantoprazole in human plasma. This study showed that Clopidogrel along with pantoprazole significantly decreased plasma level of Clopidogrel.

Such a variation would lead to sub therapeutic concentration and a consequent lack of therapeutic efficacy of Clopidogrel. This consequence may be expected due to inhibition of enzyme cytochrome P450 2C19 which is responsible for bioactivation of Clopidogrel.

In conclusion, present study showed that pantoprazole can alter the pharmacokinetics of Clopidogrel to significant levels. Summary of validation parameters data for Clopidogrel and Pantoprazole is presented in table 4.

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