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Research Article

BIO-ANALYTICAL DETERMINATION OF CLOPIDOGREL AND PANTOPRAZOLE BY RP-HPLC METHOD IN RAT PLASMA: APPLICATION TO DRUG INTERACTION STUDY

B.M. GURUPADAYYA*, SINDHURA SAMA

Department of Pharmaceutical Analysis, JSS College of Pharmacy, JSS University, Mysore – 570 015, Karnataka, India.
Email: bm_guru2004@yahoo.co.in

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ABSTRACT

Objective: To develop simple, sensitive, rapid, robust and reproducible method for the bioanalytical determination of clopidogrel and pantoprazole in rat plasma using reverse phase high performance liquid chromatographic method.
Method: The analysis was performed on C_8 (250 × 4.6mm, 5µm) column with a mobile phase consisting of 0.03M potassium dihydrogen ortho phosphate buffer (pH 3), acetonitrile in the ratio of 40:60 (v/v) with a flow rate of 1.2 ml/min. The analyte was monitored with UV detector at 240 nm. In the developed method pantaprazole elutes at retention time of 2.6 min and clopidogrel at 8.2 min.
Results: The proposed method is having linearity in the concentration range 10-50 µg/ml for both clopidogrel and pantoprazole. The method was validated with respect to system suitability, linearity, precision, limit of detection (LOD) and limit of quantification (LOQ), accuracy (recovery), ruggedness, robustness, stability, forced degradation studies (specificity). The method is extended to animal study. The pharmacokinetic parameters (AUC,C_{max},T_{max}) was found statistically significant($p<0.05$).
Conclusion: The results suggest that concomitant use of clopidogrel and pantoprazole alters pharmacokinetics of clopidogrel.

Keywords: Bio-Analytical, Clopidogrel, Pantoprazole, RP-HPLC Method, Rat Plasma, Drug Interaction Study

INTRODUCTION

Clopidogrel, (CPG) (+)-(S)-methyl 2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)acetate (Fig 1) 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. 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], normal phase HPLC[7], GC method[8], LC-MS method[9], capillary electrophoresis method[10], UV method[11].

Pantoprazole, (*RS*)-6-(Difluoromethoxy)-2-[(3,4-dimethoxypyridin-2-yl)methylsulfinyl]-1*H*-benzo[*d*] imidazole (Fig 2) 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. Pantoprazole is relatively free of drug interactions; however, it may alter the absorption of other medications that depend on the amount of acid in the stomach, such as ketoconazole or digoxin. 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 gastroesophageal 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.

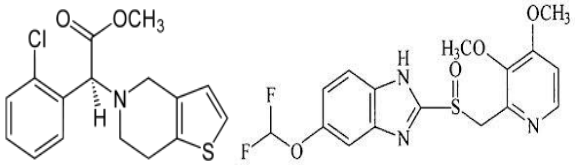

Figure 1: Structure of clopidogrel
Figure 2: Structure of pantoprazole

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].

Proton pump inhibitors are among the most widely prescribed medications worldwide. Emerging evidence suggests that some proton pump inhibitors can inhibit cytochrome P450 2C19, possibly altering clopidogrel's pharmacokinetics and potentially leading to an increased risk of adverse cardiac outcomes·In the present study, we developed simultaneous determination two drugs clopidogrel and pantaprazole in rat plasma by reverse phase HPLC method.

MATERIALS AND METHODS

Chemicals and Reagents

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

INSTRUMENTATION AND ANALYTICAL CONDITIONS

A HPLC equipped with UV detector was used for the present research work. The separation was achieved using C-8 column. The mobile phase was a mixture of phosphate Buffer, and acetonitrile (40:60) v/v. The contents of mobile phase was filtered before use through 0.45 µ membrane filter, degassed with a helium spurge for 15min was used at flow rate of 1.2mL/min. The column temperature was maintained at 20±10^{0}C.The injection volume of samples was 10 µL. The analyte was monitored at wavelength of 240nm.

Animal study

The study adheres to principles of laboratory animal care and is approved by the animal care committee IAEC/CPSEA-institutional animal ethics/committee for the purpose of control and supervision of experiments on animals [22, 23].

Healthy Albino rats (both sex) of weight 150-250g were taken and grouped. The rats were taken from animal house of JSS College of Pharmacy, which were quarantined a week before. The rats were housed with free access to food and water, except for the final 12 h before experimentation. Rats were weighed and grouped such that each group contained rats which were weighing approximately equal amount of weight. Four groups of rats were made. Each group was found to consist of six rats. First group was treated as control. Second group was treated with clopidogrel alone. Third group was treated with pantoprazole alone. Fourth group was treated with both clopidogrel and pantoprazole. Rats were orally treated with a dose of 5mg/kg of clopidogrel and 3mg/kg of pantoprazole using a gauge. Blood samples were collected for every 0 hr, 0.5 hr, 1hr, 3hr, 5hr, 7hr, 9hr and 12 hr. 2ml of blood sample was collected from retro orbital vein after oral administration of drugs to each of six rats. The blood samples were collected into centrifuge tubes. The plasma was obtained by centrifuging the blood samples at 10,000 rpm for 10 minutes; transferring the supernatant fluid into the clean, dry centrifuge tube and stored at -20ºC until analyzed.

METHOD DEVELOPMENT

Selection of mobile phase

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

Preparation of mobile phase

Phosphate buffer was prepared by dissolving 0.9 gm of sodium dihydrogen orthophosphate and 1.2 gm of citric acid in 1000 mL water and sonicated for 5 minutes then the pH was adjusted to 3 using orthophosphoric acid. Then it was filtered by vaccum filtration. The mobile phase was then prepared by mixing phosphate buffer and acetonitrile which were of HPLC grade in the ratio 40:60(v/v).

Stock and standard solution

The stock solution of clopidogrel and pantoprazole was 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) 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.

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 upto 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.

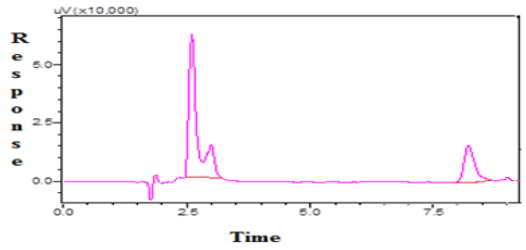

Figure 3: Chromatogram of clopidogrel and pantoprazole in rat plasma

Preparation of calibration curve

The calibration curve was established by plotting the ratio of the peak areas of clopidogrel and pantoprazole versus the concentrations of samples. Linear correlations were found between peak ratio and concentration and are described by the regression equation. The Beer's law is obeyed in the concentration range of 10-50µg/ml for both drugs. The regression parameters and system suitability of the method were shown in Table 1 and 2 respectively.

Table 1: The regression parameters of the method

Parameter	Clopidogrel	Pantoprazole
Range (µg/ml)	10-50	10-50
Regression Equation	97774x+458786	85001x+583384
Regression coefficient (r^2)	0.9969	0.9981
Slope	97774	85001
Intercept	458786	583384

Table 2: System suitability parameters of the method

Parameters	Clopidogrel	Pantoprazole
Retention Time (Rt)	8.2	2.6
LLOQ (µg/ml)	4.71	6.374
Resolution factor (R_S)	6.4	6.4
Capacity Factor (K')	5.4	5.4
Tailing Factor (T)	1.4	1.5
Theoretical Plates	4436.43	7746.8
HETP	75.0	85.0

METHOD VALIDATION

Precision and accuracy

Accuracy and precision were determined by replicate analysis of samples with known content. The mean value should be within 15% of the actual value. 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 1 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 3). The inter-day precision and accuracy of the method for clopidogrel and pantoprazole are presented in (Table 4). All values for accuracy and precision were within the recommended limits.

Table 3: Intraday Precision of clopidogrel

	Concentration (µg/ml)	Mean (µg/ml)		%RSD	
		Clopidogrel	Pentoprazole	Clopidogrel	Pentaprazole
Low(n=3)	10	10.11	10.25	0.07	0.06
Medium (n=3)	30	30.5	31.6	0.08	0.06
High(n=3)	50	51.31	50.11	0.06	0.07

Table 4: Interday Precision of clopidogrel and pentoprazole

	Concentration (mg/ml)	Mean (µg/ml)		%RSD	
		Clopidogrel	Pantoprazole	Clopidogrel	Pantoprazole
Low(n=3)	10	10.23	10.54	0.06	0.07
Medium (n=3)	30	31.2	30.76	0.07	0.06
High(n=3)	50	50.31	51.22	0.07	0.05

Recovery

Recovery of the method was performed comparing the three quality control (QC) samples at low, medium and high concentrations (10, 30, 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. The results obtained from the proposed method are recorded in Table 5.

Table 5: Percent recovery studies of clopidogrel and pantoprazole

Level	Concentration (µg/ml)	%Recovery Clopidogrel	%Recovery Pantoprazole
Low	10	96.5	97.7
Medium	30	97.8	98.4
High	50	98.8	97.6

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 LQCand HQC samples. The freeze–thaw stability was determined over three freeze–thaw cycles within 3 days. Spiked plasma samples were frozen at -22 ^{0}C for 24 h and thawed at room temperature in each freeze–thaw cycle. To study short-term stability, the frozen (-22 ^{o}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 ^{o}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^{o}C for 1 month. The results obtained from assessment of stability are given in Table 6 and 7. Three freeze–thaw cycles of the quality control samples did not seem to affect quantification. Quality-control samples stored in a freezer at -22^{o}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^{o}C.

Table 6: Freeze Thaw Stability of clopidogrel and pantoprazole

Stability	Concentration of clopidogrel		Concentration of pantoprazole	
	(10 µg/ml)	(50 µg/ml)	(10µg/ml)	(50 µg/ml)
Initial	10.21	50.23	10.32	50.24
Final	10.11	50.32	10.12	50.33
Deviation	-0.10	0.90	-0.20	0.90
%RSD	0.06	0.07	0.06	0.07
Accuracy (%)	99.02	100.1	99.02	100.1

Table 7: Short Term Stability of Clopidogrel

Stability	Concentration of clopidogrel		Concentration of pantoprazole	
	(10 µg/ml)	(50 µg/ml)	(10 µg/ml)	(50 µg/ml)
Initial	9.91	49.95	10.45	50.55
Final	9.99	49.99	10.57	50.45
Deviation	0.8	0.04	0.12	-0.10
%RSD	0.07	0.06	0.07	0.06
Accuracy (%)	100.8	100.08	101.1	99.8

Pharmacokinetic study

The proposed HPLC method was successfully applied to monitor quantitatively for the determination of plasma ceftrixone and vancomycin in plasma concentrations to either sex of rats with dose of 5mg/kg of clopidogrel and 3mg/kg of pantoprazole. The mean plasma drug concentration-time profile observed in these pharmacokinetics studies is shown in Table 8. The values of all major pharmacokinetic parameters like maximum plasma concentration (Cmax), time required for maximum plasma concentration (Tmax), area under curve (AUC0-∞), area under first moment curve (AUMC0-∞), terminal half life (t1/2), mean residence time (MRT) and clearance have been summarized in Table 9.

Table 8: Pharmacokinetic study of clopidogrel and pentoprazole

Time (hr)	Clopidogrel Conc(µg/ml)	Pantoprazole Conc(µg/ml)	Clopidogrel in combination Conc (µg/ml)
0.5hr	0.68	0.93	0.26
1hr	0.82	1.50	0.67
3hr	1.17	0.62	0.93
5hr	1.23	0.20	1.12
7hr	1.39	0	1.19
9hr	0.93	0	0.3
12hr	0.11	0	0.09

Table 9: Pharmacokinetic parameters of clopidogrel and pantoprazole

Parameters	Clopidogrel only	Pantoprazole only	Clopidogrel in combination	Pantoprazole in combination
C_{max}(µg/ml)	1.4	1.6	1.32	1.35
T_{max} (h)	1.2	1.3	1	1.1
AUC_0^t (ng·h/ml)	6.972	7.2	6.453	6.95
AUC^{∞}_t (ng·h^2/ml)	0.412	0.2	0.321	0.25
AUC_0^{∞} (ng·h/ml)	7.384	7.4	6.772	7.2

RESULT AND DISCUSSION

In the present work, developed a simple and accurate HPLC method for the bioanalytical determination of clopidogrel and pentaprazole in plasma. The method was validated according to ICH guidelines. From the chromatogram good separation of clopidogrel and pantoprazole was observed at retention time of pantoprazole 2.6 min and clopidogrel at 8.2 min with a correlation coefficient (r^2) 0.9969 for clopidogrel and (r^2) 0.9981 for pantoprazole. The lower limit of quantification (LLOQ) was calculated and found to be 4.71 µg/ml and 6.3µg/ml for clopidogrel pantoprazole respectively. Intraday and intraday precision values % RSD values of clopidogrel and pantoprazole were summarized in table 3 and 4 respectively. Recovery values of clopidogrel and pantoprazole were shown in table 5. Stability studies values of clopidogrel and pantoprazole were depicted in table 6 and 7.

The mean plasma levels were evaluated for clopidogrel alone and in combination with pantoprazole. The plasma level of clopidogrel was significantly decreased in pantoprazole combination group. All the pharmacokinetic parameters were summarized in table 8 and 9. Statistical analysis was done and results were statistically significant ($p<0.05$).

CONCLUSION

The method involves simple and precise method for bioanalytical determination of clopidogrel and pantoprazole inn rat plasma. This study showed that concomitant use of clopidogrel along with pantoprazole significantly decreased plasma level of clopidogrel. Such a variation would lead to subtherapeutic concentration and a consequent lack of therapeutic efficacy of clopidogrel. This consequence is expected due to inhibition of enzyme cytochrome P450 2C19 which is responsible for bioactivation of clopidogrel.

In conclusion, present study showed that pantaprazole can alter the pharmacokinetics of clopidogrel to significant levels.

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