Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences
ISSN- 0975-1491 Vol 6, Issue 9, 2014

Original Article

# RP-UFLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF CLOPIDOGREL, PANTOPRAZOLE AND ROSUVASTATIN IN HUMAN PLASMA: DRUG INTERACTION STUDIES

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*Received: 16 Jun 2014 Revised and Accepted: 04 Sep 2014*

**ABSTRACT**

**Objectives:** Objective of our present study was to develop a novel ultra fast liquid chromatographic method for quantitative simultaneous estimation of Clopidogrel, Pantoprazole & Rosuvastatin in human plasma and to validate the developed method following USFDA guidelines.

**Methods:** In the current study, the analysis was performed on phenomenex C8 (250 × 4.6 mm, 5µm) column using phosphate buffer (pH-2.5) and acetonitrile (45: 55 v/v) as mobile phase at flow rate of 1.2 mL/min. The system consisted of a pump (Shimadzu, prominence, UFLC), with 20 µl sample injector, along with a PDA detector at a wavelength of 254, 243 nm and 220 nm for Clopidogrel, Pantoprazole and Rosuvastatin respectively. Data was compiled using Shimadzu LC Solution software.

**Results:** In this developed method Clopidogrel, Pantoprazole & Rosuvastatin, eluted at a retention time of 2.566, 5.002 and 9.301 min respectively. The proposed method is having linearity in the concentration range from 5 to 50µg/mL of Clopidogrel, Pantoprazole & Rosuvastatin. The current method was validated with respect to accuracy, linearity; precision, lowest limit of quantification (LLOQ) and recovery according to the USFDA guidelines. A good linear relationship over the concentration range of 5-50µg/ml was shown. Validation of the method was carried out as per the USFDA draft guidelines.

**Conclusion:** A novel specific, accurate, precise UFLC method was developed for quantitative simultaneous estimation of Clopidogrel, Pantoprazole & Rosuvastatin in human plasma and validated. The developed method is suitable and economic for routine analysis and pharmacokinetic studies of Clopidogrel, Pantoprazole & Rosuvastatin simultaneously. The method developed was found to be precise, accurate, specific, linear and sensitive. Statistical analysis shows that the method is reproducible and selective for the estimation of Clopidogrel, Pantoprazole & Rosuvastatin in dosage form of patient plasma.

**Keywords:** Bioanalytical, Clopidogrel, Pantoprazole & Rosuvastatin, RP-UFLC, USFDA.

## 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) superfamily 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, Pantoprazole & Rosuvastatin, 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).

Pantoprazole**,** (*RS*)-6-(Difluoromethoxy)-2-[(3, 4-dimethoxypyridin-2-yl) methylsulfinyl]-1*H*-benzo[d] imidazole (Fig. 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 the 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. Rosuvastatin, (3R, 5S, 6E)-7-[4-(4-fluorophenyl)-2-(N-methylmethanesulfonamido)-6-(propan-2-yl) pyrimidin-5-yl]-3, 5-dihydroxyhept-6-enoic acid (Fig. 1C) is a 3-hydroxy-3-methylglutaryl coenzyme A-reductase inhibitor, or statin, that has been developed for the treatment of dyslipidemia, atherosclerosis, high cholesterol, hyperlipoproteinemia, elevated LDL, Prevention of Cardiovascular Disease.

Rosuvastatin, a new statin, has been shown to possess a number of advantageous pharmacological properties, including enhanced HMG-CoA reductase binding characteristics, relative hydrophilicity, and selective uptake activity in hepatic cells. Cytochrome p450 (CYP) metabolism of Rosuvastatin appears to be principally mediated by the 2C9 enzyme, with little involvement of 3A4; this finding is consistent with the absence of clinically significant pharmacokinetic drug-drug interactions between Rosuvastatin, Clopidogrel & Pantoprazole known to inhibit CYP enzymes.

Literature survey reveals that few analytical methods have been reported for Clopidogrel, Pantoprazole & Rosuvastatin including 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].

Studies have shown that genetic polymorphisms in the hepatic cytochrome P450 (CYP2C19) influence the antiplatelet effects of

Clopidogrel. Moreover the same cytochrome partially metabolizes Rosuvastatin. Literature survey reveals that few analytical methods have been reported for Rosuvastatin include has been estimated by colorimetry [12], Spectrophotometric methods [13, 14], LC-MS/MS [15], RP-HPLC [16-21].

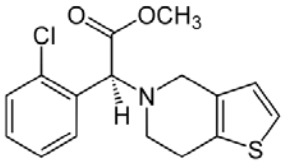

**Fig. 1(A): Structure of Clopidogrel**

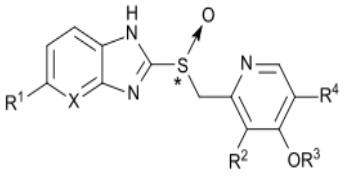

**Fig. 1(B): Structure of Pantoprazole**

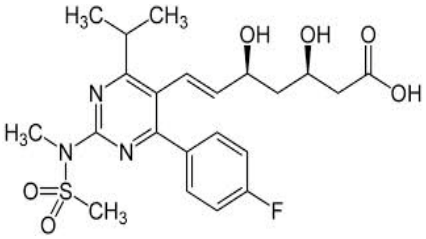

**Fig. 1(C): Structure of Rosuvastatin**

## MATERIALS AND METHODS

### Chemical and Reagents

Samples of Clopidogrel, Pantoprazole & Rosuvastatin were received from Wintac Limited, Bangalore, Karnataka, India. 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.

### Instrumentation

The present study was carried out on UFLC (SHIMADZU) equipped with LC solution software with PDA detector. Separation was attained using phenomenex C8 column.

The mobile phase was a mixture of potassium dihydrogen orthophosphate buffer (pH-2.5) and acetonitrile (45:55 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.

### Preparation of Mobile Phase

Mobile phase is prepared by adding 4.08g potassium dihydrogen orthophosphate in 250 ml of Millipore water, dissolve and adjust the pH to 2.5 using ortho phosphoric acid and made upto 1000 ml (0.03M) using Millipore water. Potassium dihydrogen orthophosphate buffer and acetonitrile in the ratio of 45: 55 (v/v).

### Preparation of Standard Solutions

Stock solution of Clopidogrel, Pantoprazole & Rosuvastatin were prepared by dissolving 10 mg of drugs Clopidogrel, Pantoprazole & Rosuvastatin in 50 ml of methanol in 100 ml volumetric flask dissolved and volume was made up to 100 ml using the methanol to get the standard stock solutions of concentration 0.1 mg/mL (100 µg/ml) for Clopidogrel, Pantoprazole & Rosuvastatin. Different working standard solutions were prepared from the above solution.

**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 | 254, 243 nm and 220 nm for Clopidogrel, Pantoprazole & Rosuvastatin respectively |
| Injection Volume | 20µL |
| Detector | PDA Detector |
| Elution | Isocratic |
| Mobile Phase | potassium dihydrogen orthophosphate buffer (pH-2.5) and acetonitrile (45:55 v/v) |
| Column oven temperature | 25 ± 5ºC |

### Method Development

### Selection of mobile phase

Mobile phases were tried in various ratios for selection of solvents of the desired polarity. The drugs Clopidogrel, Pantoprazole & Rosuvastatin 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 2.5), and acetonitrile in the ratio 45:55(v/v) (Fig 2).

### Stock and standard solution

The stock solution of Clopidogrel, Pantoprazole & Rosuvastatin were prepared by dissolving 10mg of each drug separately into methanol and volume was made up to 100 ml with same solvent. From stock solutions (100 µg/ml of each) 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 µg/ml concentration were prepared separately using methanol as solvent. Equal volumes of all the three drugs concentrations were mixed and used as standard solutions.

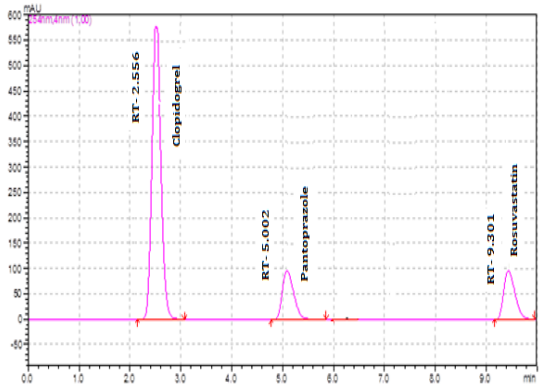


**Fig. 2: Chromatogram for Simultaneous estimation of Clopidogrel, Pantoprazole and Rosuvastatin (50µg/ml)**

**Preparation of Calibration Curve**

From the stock solution (100 µg/mL) aliquots of Clopidogrel, Pantoprazole & Rosuvastatin were pipette into a series of 10 mL volumetric flask. The final volume was made up to the mark by using HPLC grade methanol. 20µL 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, Pantoprazole & Rosuvastatin and are described by regression equation. The Beer's law was obeyed in the concentration range of 5 – 50 µg/mL (Figure 3).

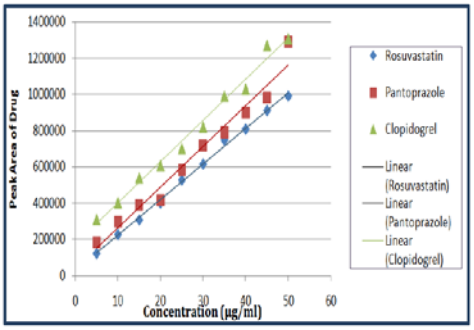


**Fig. 3: Standard calibration graph of Clopidogrel, Pantoprazole and Rosuvastatin**

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 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.

The obtained chromatograms are shown in Figure 4.

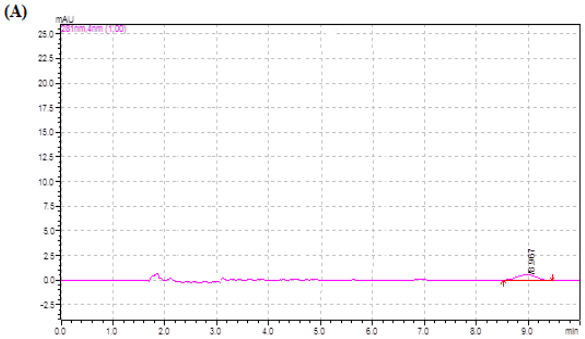


**Fig. 4(A): Chromatogram of Blank.**

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

| Parameter | Clopidogrel | Pantoprazole | Rosuvastatin |
|---|---|---|---|
| Linearity (µg/ml) | 5-50 | 5-50 | 5-50 |
| Regression Equation | 21909x + 106284 | 13290x + 35691 | 19969x + 79109 |
| Regression coefficient (R2) | 0.994 | 0.9924 | 0.9973 |
| Slope | 97774 | 85001 | 85001 |
| Intercept | 458786 | 583384 | 583384 |
| Retention Time (Rt) | 2.566 | 5.002 | 9.301 |
| LLOQ (µg/ml) | 5.193 | 5.264 | 5.920 |
| Resolution (RS) | 2.58 | 2.33 | 2.63 |
| Capacity Factor (K) | 2.76 | 3.06 | 2.11 |
| Tailing Factor (T) | 1.037 | 1.265 | 1.61 |
| Theoretical Plates | 3486 | 5401 | 3942 |

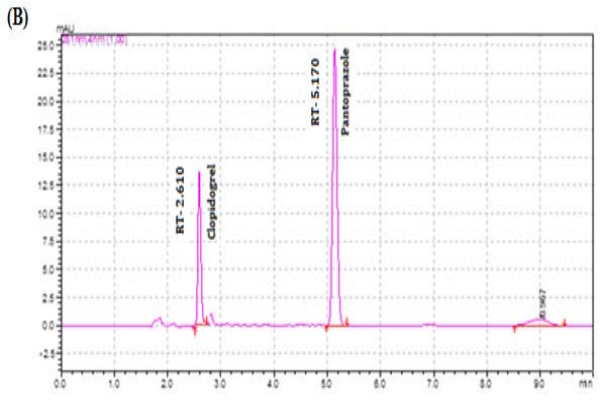


**Fig. 4(B): Chromatogram of Clopidogrel & Pantoprazole,**

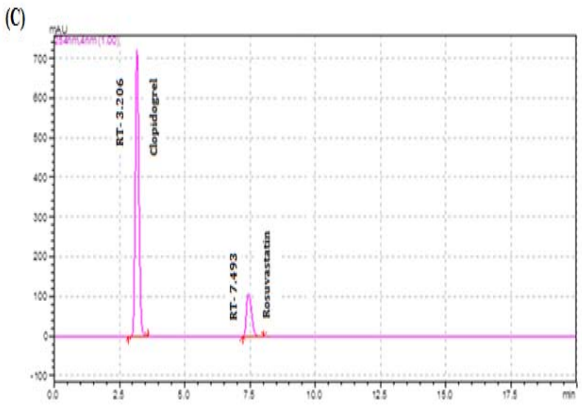


**Fig. 4(C): Chromatogram of Clopidogrel & Rosuvastatin,**

**Protein precipitation**

The protein precipitation method was used for extraction of Clopidogrel, Pantoprazole and Rosuvastatin from plasma individually using acetonitrile as protein precipitant. 100 µL of blank plasma was spiked with 100.0 µL of standard Clopidogrel, Pantoprazole and Rosuvastatin from 100 µg/mL dilution of Clopidogrel, Pantoprazole and Rosuvastatin separately. This spiked plasma was vortexed for 2 min.

The mixture was further vortexed for 2 min and centrifuged at 10000 rpm for 10 min. After centrifugation, 100 µL of the supernatant was collected and 100 µL of Internal Standard nimesulide was added of required concentration and diluted to 1.0 mL with acetonitrile. A 20.0 µL aliquot of final preparation was injected into the HPLC system.

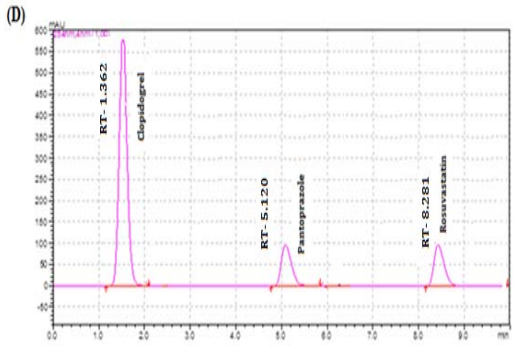


**Fig. 4(D): Chromatogram of Clopidogrel, Pantoprazole & Rosuvastatin in plasma.**

## 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. Quantification Lower Limit (LLOQ)
4. Linearity
5. Precision
6. Accuracy

### System suitability parameters

The system suitability parameters such as asymmetric factor, tailing factor, theoretical plates and plate numbers were measured. The values found for these parameters are described in Table 3. All the system suitability parameters found to be according to the acceptable limits of the bio -analytical methods.

### Linearity

From the experimental conditions described above, linear calibration curves of Clopidogrel, Pantoprazole & Rosuvastatin were obtained for ten different concentrations level for both. The r2 for Clopidogrel was 0.994 and for Rosuvastatin was 0.9973.

Linear correlations were found between peak area of Clopidogrel, Pantoprazole & Rosuvastatin concentration and are described by the regression equation. The linearity range for Clopidogrel, Pantoprazole & Rosuvastatin is 10-50 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, Pantoprazole & Rosuvastatin standard solutions were injected in duplicate to the UPLC system. Standard chromatograms obtained are presented in Fig 5 (A, B, C and D).

**Table 3: System suitability parameters of bio-analytical method**

| Parameters | Results | | | | | | Acceptable limits |
|---|---|---|---|---|---|---|---|
| | Clopidogrel | IS | Pantoprazole | IS | Rosuvastatin | IS | |
| Asymmetry | 1.02 | 0.99 | 1.09 | 0.97 | 1.22 | 0.97 | < 1.5 |
| Tailing Factor | 1.037 | 1.032 | 1.265 | 1.201 | 1.61 | 1.54 | < 2 |
| Plate no. | 3486 | 3393 | 5401 | 5011 | 3942 | 3301 | > 2000 |
| Resolution | 2.58 | 1.98 | 2.33 | 2.01 | 2.63 | 1.80 | > 1.5 |
| Capacity factor | 2.76 | 2.12 | 3.06 | 2.09 | 2.11 | 2.22 | > 2 |

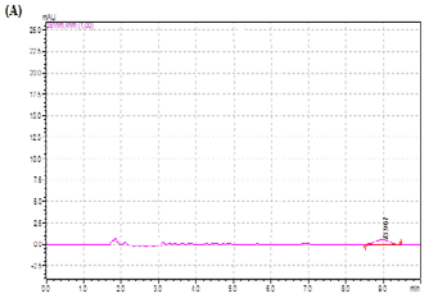


**Fig. 5(A): Chromatogram of Blank,**

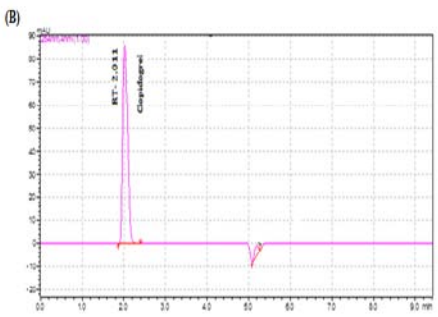


**Fig. 5(B): Chromatogram of Standard solution of Clopidogrel (50µg/ml),**

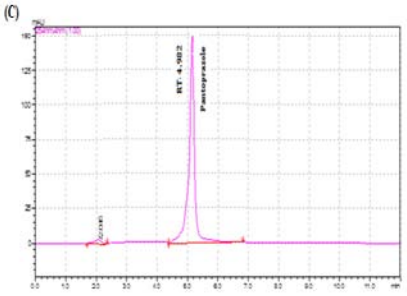


**Fig. 5(C): Chromatogram of Standard solution of Pantoprazole**

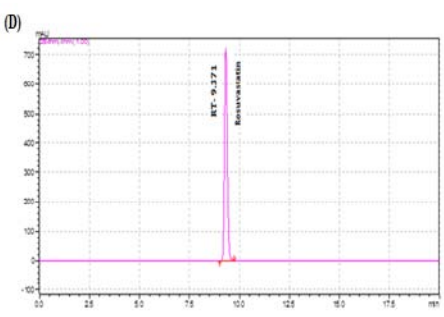


**Fig. 5(D): Chromatogram of Standard solution of Rosuvastatin (50µg/ml).**

**Precision and accuracy**

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

Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy was measured using a minimum of five determinations per concentration. The mean value was found to be within 20% of the actual value except at LLOQ, where it should not deviate by more than 25%.

The precision was measured using a minimum of five determinations per concentration. The precision determined at each concentration level did not exceed 20% of the CV except for the LLOQ, where it should not exceed 25% of the CV.

Precision was further subdivided into within-run and between-run precision. Within-run (also known as intra-batch precision or repeatability) is an assessment of the precision during a single analytical run. Between-run precision (also known as inter batch precision or repeatability), is a measurement of the precision with time, and may involve different analysts, equipment, reagents, and laboratories. Samples with concentrations over the ULOQ were diluted with the same matrix as used for the study samples, and accuracy and precision was determined.

The Within-run precision and accuracy of the method for Clopidogrel, Pantoprazole & Rosuvastatin are presented in (Table 4A). The Between-run precision and accuracy of the method for Clopidogrel, Pantoprazole & Rosuvastatin are presented in (Table 4B). All values for accuracy and precision were within the recommended limits.

**Table 4: Within-run and Between-run Precision of Clopidogrel, Pantoprazole & Rosuvastatin**

| **(A) Within-run Precision** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Concentration (µg/ml)** | | **Mean (µg/ml)** | | | **%RSD** | | |
| | | **Clopidogrel** | **Pantoprazole** | **Rosuvastatin** | **Clopidogrel** | **Pantoprazole** | **Rosuvastatin** |
| Low (n=3) | 5 | 5.21 | 5.26 | 5.25 | 0.06 | 0.03 | 0.07 |
| Medium (n=3) | 30 | 30.11 | 29.98 | 30.6 | 0.07 | 0.05 | 0.08 |
| High (n=3) | 50 | 49.30 | 50.30 | 50.16 | 0.06 | 0.09 | 0.07 |
| **(B)** Between-run Precision | | | | | | | |
| Concentration | | Mean (µg/ml) | | | %RSD | | |
| (µg/ml) | | Clopidogrel | Pantoprazole | Rosuvastatin | Clopidogrel | Pantoprazole | Rosuvastatin |
| Low (n=3) | | 5.12 | 5.44 | 5.30 | 0.06 | 0.10 | 0.08 |
| Medium (n=3) | | 30.7 | 29.92 | 30.16 | 0.07 | 0.08 | 0.05 |
| High (n=3) | | 49.21 | 50.19 | 50.35 | 0.05 | 0.03 | 0.06 |

**Table 5: Percent recovery studies of Clopidogrel, Pantoprazole & Rosuvastatin and Rosuvastatin.**

| **Level** | **Concentration (µg/ml)** | **%Recovery of Clopidogrel** | **%Recovery of Pantoprazole** | **%Recovery of Rosuvastatin** |
|---|---|---|---|---|
| Low | 5 | 97.6 | 98.1 | 96.8 |
| Medium | 30 | 98.2 | 97.0 | 98.4 |
| High | 50 | 96.7 | 97.9 | 98.2 |

**Table 6: Freeze Thaw Stability of Clopidogrel, Pantoprazole & Rosuvastatin**

| **Level/ Time (hr)** | **Clopidogrel (%RSD)** | | | | | **Pantoprazole (%RSD)** | | | | | **Rosuvastatin (%RSD)** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **0** | **3** | **6** | **12** | **24** | **0** | **3** | **6** | **12** | **24** | **0** | **3** | **6** | **12** | **24** |
| LQC | 3.54 | 2.18 | 3.46 | 2.97 | 2.41 | 5.01 | 3.44 | 2.01 | 3.91 | 2.21 | 3.90 | 2.91 | 2.68 | 4.03 | 3.90 |
| MQC | 2.84 | 3.26 | 4.19 | 2.99 | 2.75 | 5.55 | 2.11 | 3.04 | 3.98 | 3.00 | 3.66 | 4.01 | 4.81 | 5.20 | 3.90 |
| HQC | 3.39 | 2.88 | 3.41 | 2.04 | 2.94 | 6.10 | 5.11 | 2.05 | 2.99 | 1.94 | 5.21 | 4.99 | 5.01 | 5.66 | 4.91 |

**Table 7: Summary of validation parameters data for Clopidogrel, Pantoprazole & Rosuvastatin**

| **Parameters** | | **Clopidogrel** | **Pantoprazole** | **Rosuvastatin** | **Acceptance criteria** |
|---|---|---|---|---|---|
| Retention Time (min) | | 2.566 | 5.002 | 9.301 | - |
| LLOQ (µg/ml) | | 5.193 | 5.264 | 5.920 | - |
| Linearity (µg/ml) | | 5-50 | 5 - 50 | 5-50 | - |
| Accuracy (% Recovery) | | 96.7-98.2% | 97.0 – 98.1 | 96.8-98.2 | 80 -120% |
| Precision (%RSD) | Within-run | 0.065 | 0.060 | 0.075 | < 2% |
| | Between-run | 0.060 | 0.070 | 0.065 | |
| Specificity | | No peak of diluent, excipients and impurities were detected. | | | No peak should be detected |
| | N | 4573.51 | 5903.52 | 7923.79 | >2000 |
| System Suitability Parameters | HETP | 81.0 | 88.0 | 90.0 | - |
| | Asymmetry | 1.02 | 1.09 | 1.20 | ~1 |
| | Resolution | 2.513 | | | |

**Recovery**

Recovery of the method was performed comparing the three quality control (QC) samples at low, medium and high concentrations (5, 30, 50 µg/ml) The recoveries of Clopidogrel, Pantoprazole & Rosuvastatin and Rosuvastatin 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.

**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 -22oC for 24 h and thawed at room temperature in each freeze–thaw cycle. To study short-term stability, the frozen (-22oC) 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 -22oC 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 2oC for 1 month. The results obtained from assessment of stability are given in Table 6. Three freeze–thaw cycles of the quality control samples did not seem to affect quantification. Quality-control samples stored in a freezer at -22oC 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 2oC.

## CONCLUSION

The developed and validated method involves simple and precise method for bioanalytical determination of Clopidogrel, Pantoprazole & Rosuvastatin in human plasma. This study showed that Clopidogrel along with Pantoprazole & Rosuvastatin 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 and Rosuvastatin can alter the pharmacokinetics of Clopidogrel to significant levels. Summary of validation parameters data for Clopidogrel, Pantoprazole & Rosuvastatin is presented in table 7.

## CONFLICT OF INTEREST

None

## ACKNOWLEDGEMENTS

The authors express their sincere thanks to the Head of the Department, Dept. of Pharmaceutical Chemistry and Principal, JSS College of Pharmacy, JSS University, Mysore for providing the necessary facilities and encouragement to carry out the research work.

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