IJPSR (2015), Vol. 6, Issue 1 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 02 June 2014; received in revised form, 12 August, 2014; accepted, 22 September, 2014; published 01 January, 2015

DEVELOPMENT AND VALIDATION OF BIO-ANALYTICAL RP-ULTRA FAST LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF CLOPIDOGREL AND ROSUVASTATIN IN HUMAN PLASMA

Jinesh Bahubali Nagavi * and Bannimath Gurupadayya

Department of Pharmaceutical Analysis, JSS College of Pharmacy, JSS University, Mysore 570015, Karnataka, India.

Keywords:

Bioanalytical, Clopidogrel, Rosuvastatin, RP-UFLC, USFDA.

Correspondence to Author:

Jinesh Bahubali Nagavi

Ph.D Research Scholar, Department of Pharmaceutical Analysis, JSS College of Pharmacy, JSS University, Mysore 570015, Karnataka, India.

E-mail: nagavi.jinesh@gmail.com

ABSTRACT: This manuscript describes a simple, sensitive, rapid and precise Ultra fast liquid chromatographic (UFLC) method was developed and validated for the simultaneous determination Clopidogrel and Rosuvastatin in human plasma according to USFDA draft guidelines. In the current study, the analysis was performed on phenomenex C8 (250 × 4.6mm, 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. In this developed method Clopidogrel and Rosuvastatin eluted at a retention time of 4.697 and 3.337 min respectively. The proposed method is having linearity in the concentration range from 10 to 50µg/mL of Clopidogrel and Rosuvastatin. The current method was validated with respect to linearity; precision, lowest limit of detection (LOD), accuracy and recovery according to the USFDA guidelines. The system consisted of a pump (Shimadzu, prominence, UFLC), with 20µl sample injector, along with a PDA detector at a wavelength of 243 nm and 220 nm for Rosuvastatin and Clopidogrel respectively. Data was compiled using Shimadzu LC Solution software. A good linear relationship over the concentration range of 10-50µg/ml was shown. Validation of the method was carried out as per the USFDA draft guidelines. The method developed was found to be precise, accurate, specific, linear and selective. Statistical analysis shows that the method is reproducible and selective for the estimation of Clopidogrel and Rosuvastatin in dosage form.

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.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(1).219-25

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(1).219-25

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].

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 1B)** 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 and clopidogrel known to inhibit CYP enzymes.

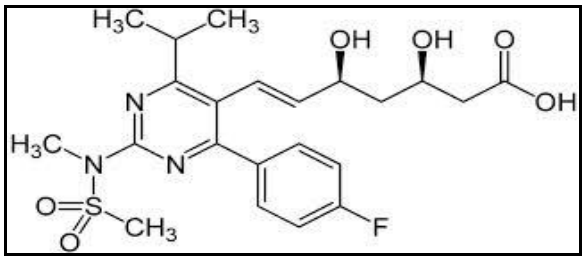

FIGURE 1(B): STRUCTURE OF ROSUVASTATIN

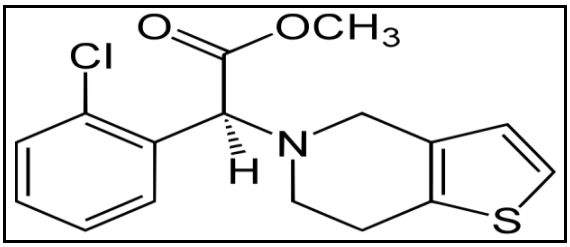

FIGURE 1(A): STRUCTURE OF CLOPIDOGREL

Studies have shown that the 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].

MATERIALS & METHODS:

Chemical and Reagents:

Samples of Clopidogrel and Rosuvastatin 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.

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-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	243 nm and 220 nm for Rosuvastatin and Clopidogrel respectively
Injection Volume	20µL
Detector	PDA Detector
Elution	Isocratic
Mobile Phase	potassium dihydrogen orthophosphate buffer (pH-3.0) and acetonitrile (45:55 v/v)
Column oven temperature	25 ± 5ºC

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 upto 1000ml

(0.03M) using Millipore water and acetonitrile in the ratio of 45: 55 (v/v).

Preparation of Standard Solutions

Stock solution of Clopidogrel and Rosuvastatin was prepared by dissolving 100 mg of drugs Clopidogrel and Rosuvastatin 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 Rosuvastatin. Different working standard solutions were prepared from the above solution.

METHOD DEVELOPMENT:

Selection of mobile phase

Mobile phases were tried in various ratios for selection of solvents of desired polarity. The drugs clopidogrel and 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 3), and acetonitrile in the ratio 45:55(v/v) **(Fig 3)**.

Stock and standard solution

The stock solution of clopidogrel and Rosuvastatin 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) 10, 15, 20, 25, 30, 35, 40, 45, 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 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 and Rosuvastatin and are described by regression equation. The Beer's law was obeyed in the concentration range of 10 – 50 µg/mL **(Figure 2).**

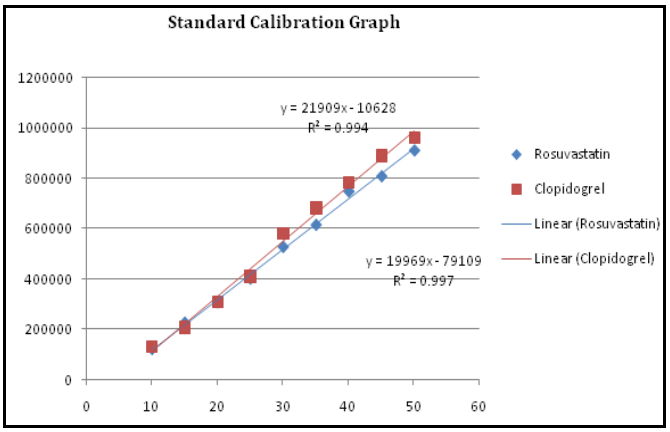

FIGURE 2: STANDARD CALIBRATION GRAPH OF CLOPIDOGREL AND ROSUVASTATIN

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

TABLE 2: THE REGRESSION AND SYSTEM SUITABILITY PARAMETERS OF THE METHOD

Parameter	Clopidogrel	Rosuvastatin
Linearity (µg/ml)	10-50	10-50
Regression Equation	21909x + 106284	19969x + 79109
Regression coefficient (R^2)	0.994	0.9973
Slope	97774	85001
Intercept	458786	583384
Retention Time (Rt)	3.592	2.433
LLOQ (µg/ml)	10.71	10.67
Resolution factor (RS)	6.7	6.7
Capacity Factor (K')	5.2	5.2
Tailing Factor (T)	1.1	1.7
Theoretical Plates	4573.51	7923.79
HETP	81.0	90.0

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 3 (A** and **B).**

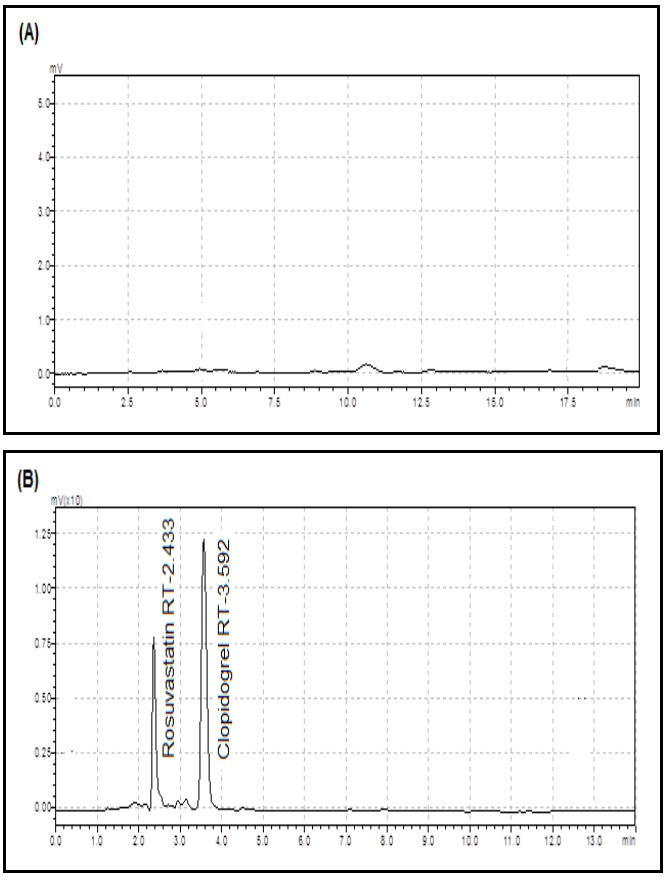

FIGURE 3: CHROMATOGRAM OF (A) BLANK, (B) CLOPIDOGREL AND ROSUVASTATIN IN PLASMA.

RESULTS & 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:

- System suitability
- Specificity
- Quantification Lower Limit (LLOQ)
- Linearity
- Precision
- Accuracy

Linearity

From the experimental conditions described above, linear calibration curves of Clopidogrel and Rosuvastatin were obtained for ten different concentrations level for both. The r^2 for clopidogrel was 0.994 and for Rosuvastatin was 0.9973. Linear correlations were found between peak area of Clopidogrel and Rosuvastatin concentration and are described by the regression equation. The linearity range for Clopidogrel and 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 and Rosuvastatin standard solutions were injected in duplicate to the UPLC system. Standard chromatograms obtained are presented in **Fig 4 (A, B** and **C)**.

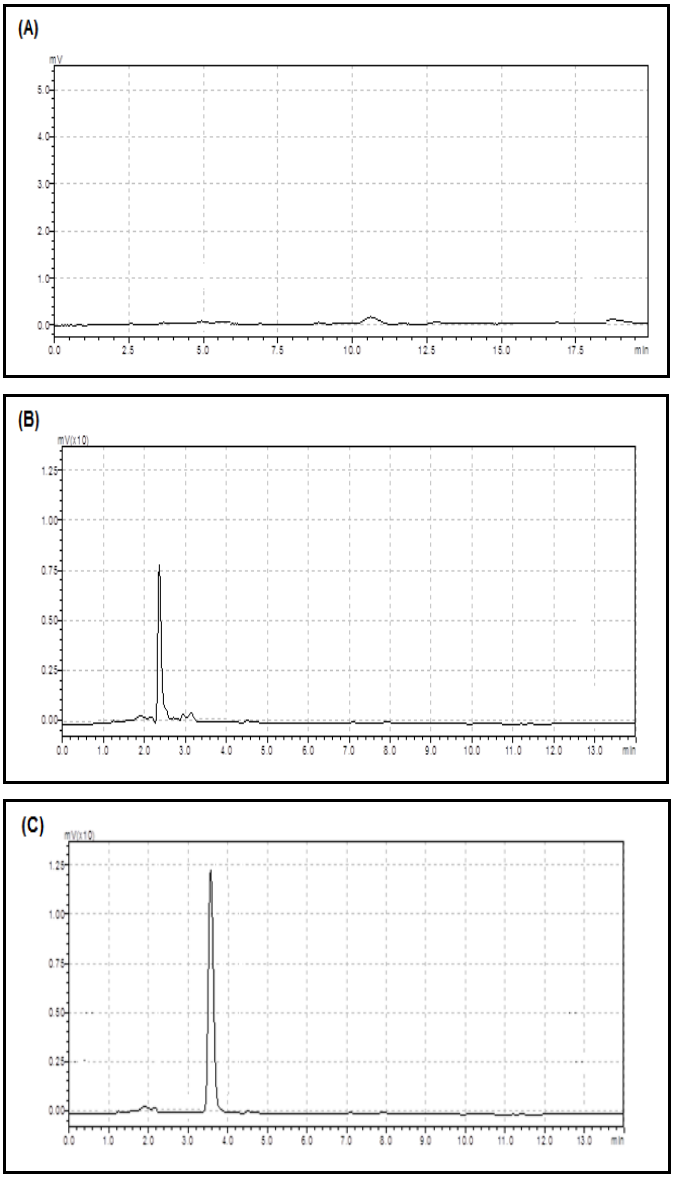

FIGURE 4: CHROMATOGRAM OF (A) BLANK, (B) STANDARD SOLUTION OF ROSUVASTATIN (50µg/ml), (C) STANDARD SOLUTION OF CLOPIDOGREL (50µg/ml).

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. 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 and Rosuvastatin are presented in **(Table 3A)**. The Between-run precision and accuracy of the method for clopidogrel and Rosuvastatin are presented in **(Table 3B).** All values for accuracy and precision were within the recommended limits.

TABLE 3: WITHIN-RUN AND BETWEEN-RUN PRECISION OF CLOPIDOGREL AND ROSUVASTATIN

(A) Within-run Precision						(B) Between-run Precision					
Concentration (µg/ml)		Mean (µg/ml) Clopidogrel	Rosuvastatin	%RSD Clopidogrel	Rosuvastatin	Concentration (µg/ml)		Mean (µg/ml) Clopidogrel	Rosuvastatin	%RSD Clopidogrel	Rosuvastatin
Low (n=3)	10	9.21	10.25	0.06	0.07	Low (n=3)	10	9.12	10.30	0.06	0.08
Medium (n=3)	30	30.11	30.6	0.07	0.08	Medium (n=3)	30	30.7	30.16	0.07	0.05
High (n=3)	50	49.30	50.16	0.06	0.07	High (n=3)	50	49.21	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 (10, 30, 50 µg/ml) The recoveries of clopidogrel 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 4**.

TABLE 4: PERCENT RECOVERY STUDIES OF CLOPIDOGREL AND ROSUVASTATIN.

Level	Concentration (µg/ml)	%Recovery Clopidogrel	%Recovery Rosuvastatin
Low	10	97.6	96.8
Medium	30	98.2	98.4
High	50	96.7	98.2

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^{o}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 5**. 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 5: FREEZE THAW STABILITY OF CLOPIDOGREL AND ROSUVASTATIN

Stability	Concentration of clopidogrel		Concentration of Rosuvastatin	
	(10µg/ml)	(50 µg/ml)	(10µg/ml)	(50µg/ml)
Initial	10.21	50.23	10.32	50.24
Final	9.11	50.32	9.12	50.33
Deviation	-0.10	0.90	-0.20	0.90
%RSD	0.06	0.07	0.06	0.07

CONCLUSIONS: The developed and validated method involves simple and precise method for bioanalytical determination of Clopidogrel and Rosuvastatin in human plasma. This study showed that clopidogrel along with 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 Rosuvastatin can alter the pharmacokinetics of clopidogrel to significant levels. Summary of validation parameters data for Clopidogrel and Rosuvastatin is presented in **Table 6**.

TABLE 6: SUMMARY OF VALIDATION PARAMETERS DATA FOR CLOPIDOGREL AND ROSUVASTATIN

Parameters		Clopidogrel	Rosuvastatin	Acceptance criteria
Retention Time (min)		3.592	2.433	-
LLOQ (µg/ml)		10.71	10.67	-
Linearity (µg/ml)		10-50	10-50	-
Accuracy (% Recovery)		96.7-98.2%	96.8-98.2	80 -120%
Precision (%RSD)	Within-run	0.065	0.075	< 2%
	Between-run	0.06	0.065	
Specificity		No peak of diluent, excipients and impurities were detected.		No peak should be detected
System Suitability Parameters	N	4573.51	7923.79	>2000
	HETP	81.0	90.0	-
	Asymmetry	1.1	1.7	~1
	Resolution	1.115		

ACKNOWLEDGEMENTS: The authors express their sincere thanks to the Principal, JSS College of Pharmacy, Mysore and JSS University, Mysore for providing the necessary facilities to carry out the research work.

REFERENCES:

1. Panda SS, Ion-pairing RP-HPLC method for simultaneous determination of aspirin and clopidogrel bisulphate in tablet and capsule dosage form. International J Pharm Tech Research, 2010; 2(1): 269-273.
2. Patel RB, Shankar MB, Patel MR, Bhatt K.K. Simultaneous estimation of acetylsalicylic acid and clopidogrel bisulfate in pure powder and tablet formulations by high-performance column liquid chromatography and high-performance thin-layer chromatography. J AOAC Int, 2008; 91(4): 750-755.
3. Anandakumar T, Ayyappan V, Raghu Raman, Vetrichelvan T, Sankar ASK, Nagavalli D, RP-HPLC analysis of aspirin and clopidogrel bisulphate in combination. Indian J Pharm Sci, 2011; 69: 597-599.
4.
5. Londhe SV, Mulgund SV, Deshmukh RS, Jain K SSimultaneous HPTLC analysis of aspirin, atorvastatin calcium and clopidogrel bisulphate in the bulk drug and in capsules, Acta Chromatogr, 2010; 22(2): 297-305.
6. Durga Rao D, Kalyanaraman LS, Sait S, Venkata Rao PA, A validated stability-indicating normal phase LC method for clopidogrel bisulfate and its impurities in bulk drug and pharmaceutical dosage form. J Pharm Biomed Anal, 2010; 52(1):160-165
7. Fayed AS, Weshahy SA, Shehata MA, Hassan NY, Pauwels J, Hoogmartens J, Van Schepdael A, Separation and determination of clopidogrel and its impurities by capillary electrophoresis, J Pharmaceut Biomed, 2009; 49(2): 193-200.
8. Kalaichelvi R, Fatima Rose M, Vadivel K, Jayachandran E, Simple extractivecolorimetric determination of Rosuvastatin sodium by aciddye complexation method in solid dosage form, Int J Chem Res, 2010; 1(1): 6-8.
9. Kakde RB, Gedam SN, Chaudhary NK, Barsagade AG, Kale DL Kasture AV, Three-wavelength spectrophotometric method for simultaneous estimation of Rosuvastatin and domperidone in pharmaceutical preparations, Inter J Pharm Tech Research, 2009; 1(2): 386-389.

10. Pimpodkar NV, Nalawade RS,. Kuchekar BS, Mahajan NS and Jadhav RL, New spectrophotometric method for the estimation of Rosuvastatin in bulk and pharmaceutical formulation, Inter J Chem Sci, 2011; 6(2): 993-999.
11. Challa BR, Boddu SH, Awen BZ, Chandu BR, Bannoth CK, Khagga M, Kanala K, Shaik RP, Development and validation of a sensitive bioanalytical method for the quantitative estimation of Rosuvastatin in human plasma samples by LC-MS/MS: application to bioequivalence study. J Chromatogr B, 2010; 878(19):1499-1505.
12. Prasanna Reddy B and Kiran Kumar Reddy N, Development and validation of rp-hplc for the Rosuvastatin sodium sesquihydrate in pharmaceutical dosage forms and human plasma, Inter J Chem Tech Research, 2011;1(2): 195-198.
13. Rajnish Kumar, Pinderjit Singh and Harinder Singh, Development of UV Spectrophotometric method for estimation of Rosuvastatin in pharmaceutical dosage forms, Inter J Pharm Research & Development, 2011; 3(2):113-117.
14. Prasanna Kumar Reddy B, Ramanjaneya Reddy Y and Ramachandra D, Determination of Rosuvastatin sodium and lansoprazole in individual tablet dosage forms by RP-HPLC using single mobile phase. E-Journal of Chemistry, 2011; 6(2): 489-494.
15. Gupta KR, Chawla RB and Wadodka SG, Spectrophotometric methods for simultaneous estimation of Rosuvastatin and itopride hydrochloride in capsules Orbital the Electro J Chem, 2010; 2(2):181-188.
16. Sivakumar T, Manavalan R and Valliappan K, Development and validation of a RP-HPLC method for simultaneous determination of domperidone and Rosuvastatin in pharmaceutical dosage forms. Acta Chromatogr, 2007; 18:130-142.
17. FDA Guidance for Industry, Bioanalytical Method Validation, Biopharmaceutics, September 2013.
18. United States Pharmacopoeia (USP31-2009)
19. British Pharmacopoeia, 2009.

How to cite this article:
Nagavi JB and Gurupadayya B: Development and Validation of Bio-Analytical RP-Ultra Fast Liquid Chromatographic Method for Simultaneous Estimation of Clopidogrel and Rosuvastatin in Human Plasma. Int J Pharm Sci Res 2015; 6(1): 219-25.doi: 10.13040/IJPSR.0975-8232.6 (1).219-25.