

Original Article

SIMULTANEOUS ESTIMATION OF CLOPIDOGREL AND ATORVASTATIN IN HUMAN PLASMA USING BIO-ANALYTICAL RP-ULTRA FAST LIQUID CHROMATOGRAPHIC METHOD

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ABSTRACT

This manuscript depicts a simple, accurate, sensitive, precise and robust Ultra fast liquid chromatographic (UFLC) method developed and validated for the simultaneous determination Clopidogrel and Atorvastatin in human plasma as per USFDA draft guidelines.

In the current study, the analysis was performed on phenomenex C8 (250 × 4.6 mm, 5 μ m) column using phosphate buffer (pH-2.5) and acetonitrile (50: 50 v/v) as mobile phase at flow rate of 1.3 mL/min. In the current developed method, Clopidogrel and Atorvastatin eluted at a retention time of 4.06 and 10.31 min respectively. The proposed method is having linearity in the concentration range from 10 to 50 μ g/mL of Clopidogrel and Atorvastatin. The current method was validated with respect to linearity; precision, lowest limit of detection (LLOD), accuracy and recovery according to the USFDA guidelines.

The system consisted of a pump (Shimadzu, prominence, UFLC), with 20 μ l sample injector, along with a PDA detector at a wavelength of 243 nm and 220 nm for Atorvastatin and Clopidogrel respectively. Data was compiled using Shimadzu LC Solution software.

A good linear relationship over the concentration range of 10-50 μ g/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 selective and reproducible for the estimation of Clopidogrel and Atorvastatin in combined dosage forms.

Keywords: Bioanalytical, Clopidogrel, Atorvastatin, 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) 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 method⁷, normal phase HPLC⁸, GC method⁹, LC-MS method[10], capillary electrophoresis method[11].

Atorvastatin, (3R, 5S, 6E)-7-[4-(4-fluorophenyl)-2-(N-methyl methane sulfonamido)-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, hyper lipoproteinemia, elevated LDL, Prevention of Cardiovascular Disease.

Atorvastatin 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 Atorvastatin 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 Atorvastatin and clopidogrel known to inhibit CYP enzymes. 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 Atorvastatin. Literature survey reveals that few analytical methods have been reported for Atorvastatin include has been estimated by colorimetry[12], Spectrophotometric methods[13,14], LC-MS/MS[15], RP-HPLC[16].

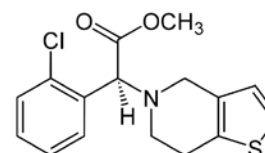


Fig. 1(A): Structure of Clopidogrel

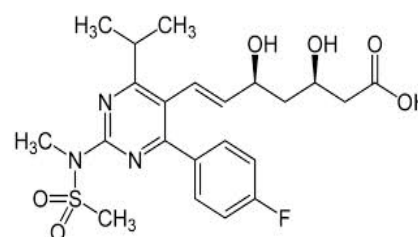


Fig. 1(B): Structure of Atorvastatin

MATERIALS AND METHODS

Chemical and reagents

Samples of Clopidogrel and Atorvastatin 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 current study was carried out on UFLC (SHIMADZU) equipped with LC solution software with PDA detector. Separation was attained using phenomenon 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) phenomenon
Flow rate	1.3 mL/min
Run time	15 min
Wavelength	243 nm and 220 nm for Atorvastatin and Clopidogrel respectively
Injection Volume	20μL
Detector	PDA Detector
Elution	Isocratic
Mobile Phase	potassium dihydrogen orthophosphate buffer (pH-2.5) and acetonitrile (50:50 v/v)
Column oven temperature	25 ± 5°C

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 and acetonitrile in the ratio of 50: 50 (v/v).

Preparation of standard solutions

Stock solution of Clopidogrel and Atorvastatin was prepared by dissolving 100 mg of drugs Clopidogrel and Atorvastatin 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 1 mg/mL (1000 μg/ml) for both Clopidogrel and Atorvastatin. 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 atorvastatin 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 50:50 (v/v) (fig. 3).

Stock and standard solution

The stock solution of clopidogrel and Atorvastatin were prepared by dissolving 10mg of each separately into methanol and volume was made up to 100 ml 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 Atorvastatin 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 Atorvastatin and are described by regression equation. The Beer's law was obeyed in the concentration range of 10 – 50 μg/mL (fig. 2).

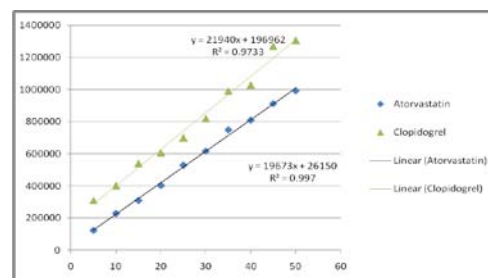


Fig. 2: Standard calibration graph of Clopidogrel and Atorvastatin

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	Atorvastatin
Linearity (μg/ml)	10-50	10-50
Regression Equation	21940x + 196962	19673x + 26150
Regression coefficient (R ²)	0.9733	0.9974
Slope	97049	89201
Intercept	560286	596201
Retention Time (Rt)	4.059	10.311
LLOQ (μg/ml)	10.11	10.41
Resolution factor (RS)	7.2	7.2
Capacity Factor (K')	4.9	5.0
Tailing Factor (T)	1.0	0.9
Theoretical Plates	4110.51	6941.79
HETP	91.0	80.0

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 0.45 μ size.

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

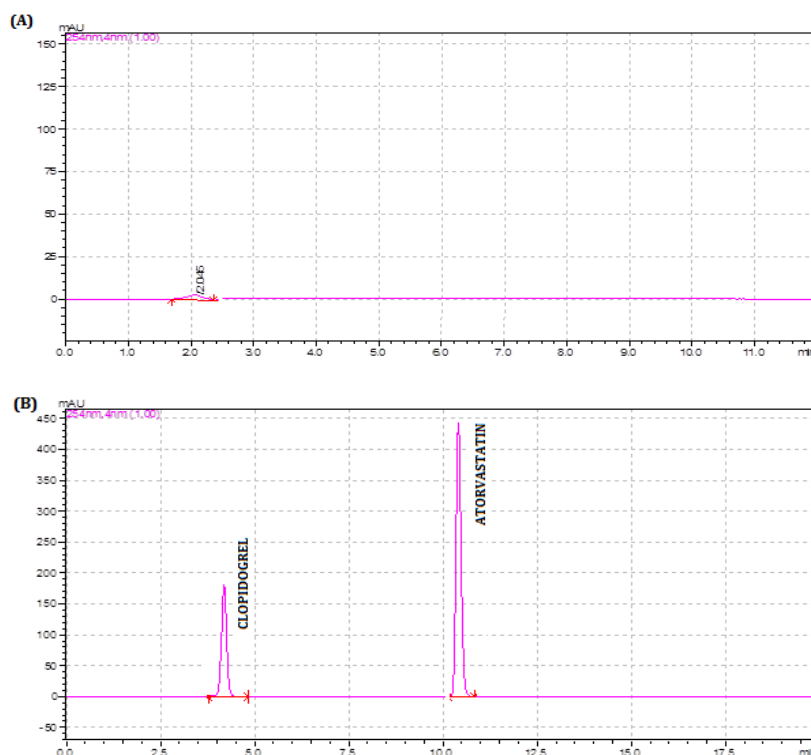


Fig. 3: Chromatogram of (A) Blank, (B) Clopidogrel and Atorvastatin in plasma

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

1. System suitability
2. Specificity
3. Quantification Lower Limit (LLOQ)
4. Linearity
5. Precision
6. Accuracy

Linearity

From the experimental conditions described above, linear calibration curves of Clopidogrel and Atorvastatin were obtained for ten different concentrations level for both. The r^2 for clopidogrel was 0.994 and for Atorvastatin was 0.9973. Linear correlations were found between peak area of Clopidogrel and Atorvastatin concentration and are described by the regression equation. The linearity range for Clopidogrel and Atorvastatin is 10-50 μ g/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 μ l of the blank was injected in duplicate to the UPLC system and chromatographed.

20 μ l of Clopidogrel and Atorvastatin standard solutions were injected in duplicate to the UPLC system. Standard chromatograms obtained are presented in fig. 4(A, B and C).

Precision and accuracy

The accuracy of an analytical method is the percentage of relatedness 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 Atorvastatin are presented in (Table 3A). The Between-run precision and accuracy of the method for clopidogrel and Atorvastatin are presented in (Table 3B). All values for accuracy and precision were within the recommended limits.

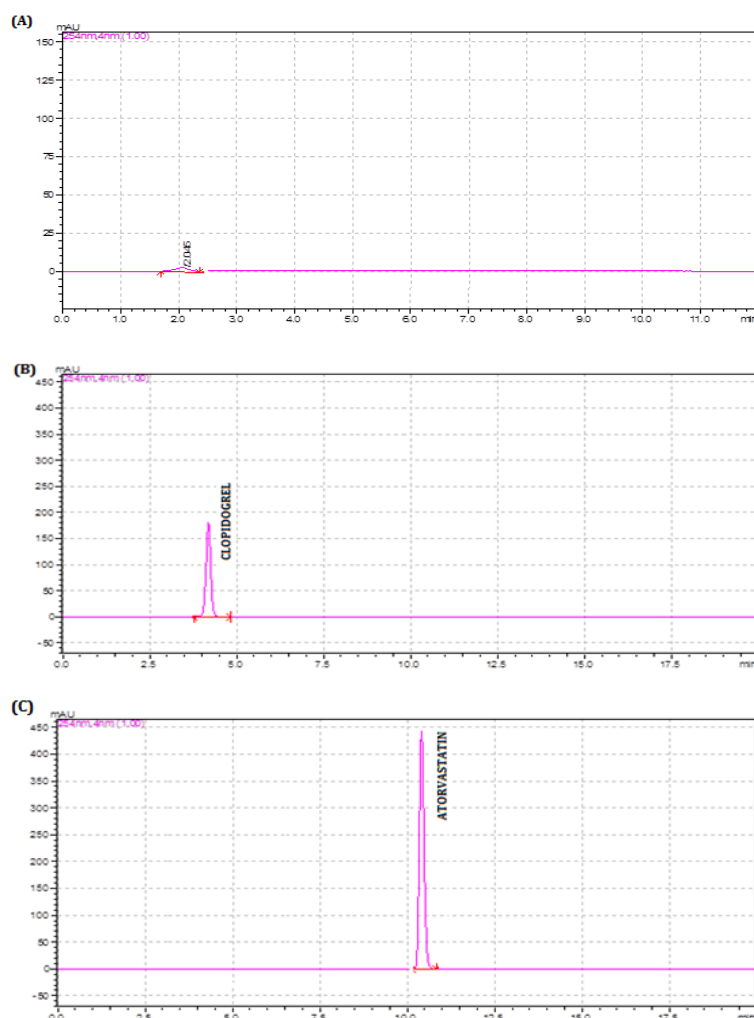


Fig. 4: Chromatogram of (A) Blank, (B) Standard solution of Clopidogrel (50µg/ml), (C) Standard solution of Atorvastatin (50µg/ml)

Table 3: Within-run and between-run precision of clopidogrel and atorvastatin

(A) Within-run Precision					(B) Between-run Precision						
Concentration (µg/ml)	Mean (µg/ml)		%RSD		Concentration (µg/ml)	Mean (µg/ml)		%RSD			
	Clopidogrel	Atorvastatin	Clopidogrel	Atorvastatin		Clopidogrel	Atorvastatin	Clopidogrel	Atorvastatin		
Low (n=3)	10	9.30	10.31	0.07	0.08	Low (n=3)	10	9.81	10.29	0.07	0.09
Medium (n=3)	30	31.40	31.3	0.08	0.11	Medium (n=3)	30	30.2	31.61	0.08	0.07
High (n=3)	50	49.77	50.28	0.07	0.08	High (n=3)	50	50.12	50.42	0.08	0.08

Table 4: Percent recovery studies of clopidogrel and atorvastatin

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

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 Atorvastatin 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.

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. 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°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 5: Freeze thaw stability of clopidogrel and atorvastatin

Stability	Concentration of Clopidogrel		Concentration of Atorvastatin	
	(10µg/ml)	(50 µg/ml)	(10µg/ml)	(50 µg/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

CONCLUSION

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

Table 6: Summary of validation parameters data for clopidogrel and atorvastatin

Parameters		Clopidogrel	Atorvastatin	Acceptance criteria
Retention Time (min)		4.06	10.31	-
LLQ (µg/ml)		10.071	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	91.0	80.0	-
	Asymmetry	1.1	1.7	~1
	Resolution	1.115		

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