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EFFECT OF PANTOPRAZOLE ON THE BINDING OF ATORVASTATIN TO BOVINE SERUM ALBUMIN BY VALIDATED HPTLC METHOD – AN *IN VITRO* METHOD

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ABSTRACT

The HPTLC method for the simultaneous determination of Atorvastatin and Pantoprazole was developed by using the solvent system composition made up of Toluene: Dichloromethane: Tetrahydrofuran in the ratio of 2:2:6 v/v/v with 0.1 ml glacial acetic acid with a chamber saturation time of 20 minutes travelled till 8 cm on the plate. The band width applied was 6 mm with detection wavelength of 286nm. The method developed was validated for the parameters as per the ICH Q2(R1) guidelines for specificity, linearity, range, accuracy, precision, limit of detection, limit of quantification, robustness and ruggedness. The R value of the drug Atorvastatin was found tobe 0.73±0.04 andforPantoprazole was found to be 0.57 ± 0.03. The peak areas of Atorvastatin and Pantoprazole were found tobe linear in the concentration range of 100-600 ng/band and between 50-300 ng/band respectively with >0.99 as the correlation coefficient value. The LOD and LOQ of Atorvastatin was found to be 40 ng/band and 60 ng/band. The detectable limit of Pantoprazole spotted was found to be 10 ng/band whereas the quantifiablelimit is 40 ng/band. When both the drugs are present together in the reaction pool the % protein binding was found to be 84.36 and 66.15 for the drug Atorvastatin and Pantoprazole respectively. The increase in the %displacement of the drugs in the mixture when compared with the individual drug binding is an indication to monitor both the drugs whenco administered.

KEYWORDS: Atorvastatin, Pantoprazole, HPTLC Method, Simultaneous Determination, Protein Binding Studies.

INTRODUCTION

Atorvastatin calcium is used in the treatment of hyperlipidemia with a molecular weight of 1209.42g/mol and IUPAC name of [R- R*, R*)]-2-(4- fluorophenyl) – β, δ- dihydroxy – 5 – (1-methyl ethyl) – 3 phenyl – 4 –[(phenylamino) carbonyl]- 1H pyrrole -1- heptanoic acid, calcium salt(2:1) trihydrate. The interacting drug Pantoprazole sodium sesquihydrateis a commonly used antacid with a formula of $C_{16}H_{14}F_2N_3NaO_4S$. 1.5H $_2$Oand 432.4g/mol weight chemically sodium – [5- (difluoromethoxy) – 2- [[(3,4 dimethoxy – 2 – pyridinyl) – methyl] - sulfinyl] – 1H – benzimidazolide sesquihydrate.drugs are shown in fig 1 and 3.[1-2]

The Chemical structure of the

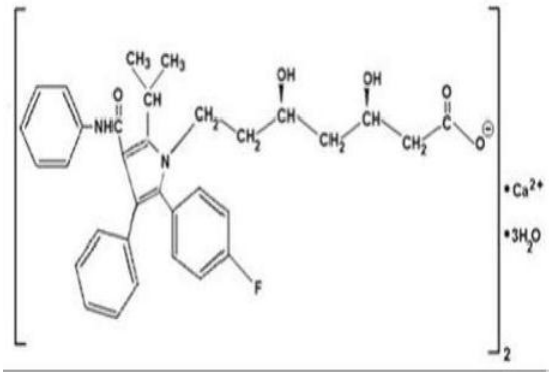

Fig. 1: Atorvastatin calcium.

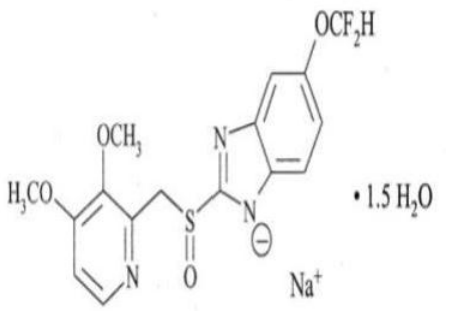

Fig. 2: Pantoprazole sodium sesquihydrate.

The reported methods for Atorvastatin calcium are UV Spectrophotometric Assay of Atorvastatin API Formulation and their Comparative Study,[3]

Validated UV-Visible

Spectrophotometric Method for the Estimation of Atorvastatin in Pure and Pharmaceutical Dosage Form Using Methyl Orange Reagent,[2] Validated spectrofluorimetric method for the determination of atorvastatin in pharmaceutical preparations,[4] Simultaneous Estimation of Ezetimibe and Atorvastatin in Pharmaceutical Formulations by RP-HPLC,[5] Simultaneous Estimation of Atorvastatin, Clopidogrel and Aspirin in Capsule Dosage forms using UV-Spectroscopy,[6]

Spectrophotometric and TLC-densitometric methods for the simultaneous determination of Ezetimibe and Atorvastatin calcium.[7]

There are no reported methods for simultaneous determination of the drug Atorvastatin belonging to the class of lipid lowering agents and co-prescribed drug Pantoprazole coming under the class of Proton pump inhibitors.

The present study focuses on the development and validation of HPTLC method for the simultaneous determination of Atorvastatin and Pantoprazoleas per the ICH guidelines of Q2(R1). The validated method is applied for the *invitro* drug-drug interaction studies between Atorvastatin and the co-administered drug Pantoprazole.

MATERIALS AND METHODS

Linomat 5 Applicator, CAMAG TLC Scanner 3 controlled by WinCATS-planar chromatography manager, version 1.2.6 (Camag, Muttenz, Switzerland) has been used for work. The solvents and chemicals used were procured from s d Fine Chemicals Ltd, Mumbai, India.

Merck TLC plates coated with silica gel $60F_{254}$ on aluminium sheet were used as stationary phase (Merck chemicals Ltd., Darmstadt, Germany). The solution pH was checked with Elico pH meter (Elico Ltd., Hyderabad, India) and weighing was carriedout withShimadzu Electronic Balance (Shimadzu Corporation, Kyoto, Japan).

Optimization of experimental conditionsSelection of solvent

The solubility was checked with various aqueous and organic solvents like water, acetonitrile, methanol, tetrahydrofuran and chloroform. Atorvastatin and Pantoprazole were found to be completely soluble in methanol which was selected as the solvent to carry out the work.

Selection of wavelength

The sensitivity of the HPTLC method depends upon the proper selection of wavelength for UV detection. So, the ideal wavelength was selected withmaximum absorbance and acceptable peak area for the drug to be detected at lower concentration. The solution in the concentration of 10µg/mlwas scanned in the UV region. The spectrum of Atorvastatin and Pantoprazole showed acceptable absorbance at 286nm. Hence it was fixed as the detection wavelength for the study.

Selection of chromatographic plate

The TLC plate which was used for the study was made up of silica gel 60 F_{254} coated on a aluminium sheet with a particle size of 2. The size of plate ranges in 20 x 20 cm which was

cut into appropriate size for the method development and optimisation.

Selection of mobile phase

The mobile phase was selected based on the acceptable peak characteristics of Atorvastatin and Pantoprazole such as the retardation factor, resolution between both the peaks, tailing factor, asymmetric factor and peak area. The mobile phase of different composition was tried in increasing propositions of tetrahydrofuran and glacial acetic acid. The mobile system optimized for the study is made up of Toluene: Dichloromethane: Tetrahydrofuran in the ratio of 2:2:6 %v/v/v of glacial acetic acid.

Selection of chamber saturation

The saturation of the chamber used for the plate development affects the R_f value. The chamber saturation of 10 to 30 minutes was tried to avoid edge effects and fluctuations in R_f value. The suitable saturation time of 20 minutes wasfixed for the study.

Selection of distance travelled by the solvent front

The sample spotted on the plate travels with the mobile phase which affects the R_f value. So, the distance of the solvent front was varied between 7.5 to 8.5 and optimized. The solvent front distance of 8cm was selected for the studies.

Preparation of standard stock solutionsPreparation of atorvastatin solution

The stock solution of atorvastatin and pantoprazole in the concentration of 1000 mcg/ml was prepared individually with solvent methanol and diluted further to prepare the working standard solution.

Preparation of working standard solutions

From the stock solution of Atorvastatin a volume of 2 ml was withdrawn and from the stock solution 1ml was pipetted for Pantoprazole, individually from the solution with the concentration of 1000 µg/ml and transferred into 10 ml volumetric flask. The solvent methanol was used to makeup the volume and this solution was used for the sampling.

Validation of the method

The validation of the developed method was carried out in terms of specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), inter and intraday precision and stability studies as per ICH guidelines.

Fixed chromatographic conditions

Table 1: Fixed experimental conditions.

Stationary phase	Pre coated silica gel 60 F_{254} aluminum sheets
Mobile phase	TOL: DCM: THF (2:2:6v/v/v) with 0.1ml of glacial acetic acid
Chamber saturation time	20 minutes
Migration distance	80 mm
Band width	6 mm
Slit dimension	5 x 0.45 mm
Source of radiation	Deuterium lamp (D2)
Scanning wavelength	286 nm
R_f value	Atorvastatin: 0.73 ± 0.04 Pantoprazole: 0.57 ± 0.03

1. Specificity

The excipients mixture for Atorvastatin was prepared according to the composition mentionedin the Lipitor 40 mg tablet monograph. For the drug Pantoprazole the monograph of Protonix 20 mg composition of excipients was included for this specificity studies. The triplicate spots were applied and the chromatograms were evaluated for their peak characteristics.

2. Linearity and Range

The linearity of response was assessed by applying different volumes from the working standard solutions of 1,2,3,4,5and6 µl spotted on TLC plate to obtain linear concentration range of 100-600 ng/band of Atorvastatin and 50-300 ng/band of Pantoprazole. The spots were developed and evaluated densitometrically using CAMAG HPTLC system.

3. Precision

Intra-day precision

The spotting volume of 4 µl was injected from the drug solution containing 100 µg/ml of Atorvastatin and 50µg/ml of Pantoprazole. The concentration of Atorvastatin and Pantoprazole was 400ng/band and 200ng/band. The studies were carried out at 3 different time intervals on the same day with 6 determination at each time. The % RSD for values obtained were calculated.

Inter-day precision

The spotting volume of 4µl was injected from the drug solution containing 100 µg/ml of Atorvastatin and 50µg/ml of Pantoprazole. The concentration of Atorvastatin and

Pantoprazole was 400ng/band and 200ng/band. The studies were carried out on 3consecutive days with 6 determinations at each time. The % RSD for values obtained werecalculated.

4. Accuracy

The tablet powder equivalent to 10 mg was weighed and transferred into the 10 ml volumetricflask. The accuracy of the method was studied by adding known concentration of the standard drug and calculating the % recovery. It was carried out by mixing known quantity ofstandard drug with the assay sample at three different levels of 50%, 100%, 150% respectively. The %RSD for the values obtained were calculated and tabulated.

5. Limit of detection (LOD) and Limit of quantification (LOQ)

LOD & LOQ of Atorvastatin and Pantoprazole were analysed by spotting the volume of the standard drug solution below the established linearity range using the drug solution of 100 µg/mland 50µg/ml respectively.

6. Robustness and Ruggedness

The robustness for the method developed was carried out by deliberate variations in the chamber saturation time of ± 2 mins and the mobile phase composition with the difference of ± 0.1 ml in the volume for the solvent tetrahydrofuran. The ruggedness was carried out by the procedure with two different analyst using similar experimental conditions.

7. Stability studies

The stability of the analytes on the developed plate is essential to assess the rate of conversion into degradants within specified period. It was studied by scanning the plate at different time intervals and the peak areas were compared with the peak area of freshly scanned plate. The % change in the peak areas was calculated.

8. Application of the developed method for the analysis of tablet formulation

Preparation of Atorvastatin tablet formulation

20 individual tablets of Atorvastatin were weighed accurately and average weight was calculated. Tablet powder equivalent to 10 mg of Atorvastatin was weighed and added to a 10 ml of methanol and sonicated for 10 minutes. The solution was filtered using Whatmann filter paper and further diluted to give a concentration of 200µg/ml in the mixture solution.

Preparation of pantoprazole tablet formulation

20 individual tablets of Pantoprazole were weighed accurately and average weight was

calculated. A quantity of 10 mg equivalent of tablet powder was weighed and same procedure repeated to give a concentration of 100 µg/ml of Pantoprazolein the mixture solution.

Effect of pantoprazoleon the binding of atorvastatin to bovine serum albuminActivation of dialysis membrane

The dialysis membrane tubes were cut into each 13 cm length and its activation had been done by boiling the membrane for 2hrs in 250ml of distilled water at 70ºC (±5ºC). The boiled membranes were washed thoroughly with fresh distilled water and utilized for the study.

Preparation of standard stock solutions

The stock solution in the concentration of 1000 µg/ml of Atorvastatin and Pantoprazole was prepared individually with solvent methanol.

Preparation of working standard solutions

The concentration of the drug solution employed was 400 µg/ml of Atorvastatin and 200 µg/ml of Pantoprazole.

Preparation of phosphate buffer pH (7.4)

The buffer pH 7.4 was prepared by weighing accurately 0.6 g of potassium dihydrogen orthophosphate, 6.4 g of disodium hydrogen orthophosphate and 5.85 g of sodium chloride in1000 ml of water. The pH of 7.4 was adjusted using 0.1M HCl or 0.1 M NaOH, wherever necessary.

Preparation of bovine serum albumin

A quantity of 0.48 g of Bovine Serum Albumin was accurately weighed and transferred to 50ml of volumetric flask. Sufficient volume of distilled water was added and dissolved completely after which the volume was made up to 50 ml with the same.

Preparation of the reaction mixture

From the stock solution of Atorvastatin 4 ml was withdrawn and from the stock solution of Pantoprazole 2 ml was withdrawn. Both the solutions were added into 10 ml volumetric flask and final volume was made up with methanol. The solution was mixed well and from this a volume of 2.5 ml of drug solution and 2.5 ml of bovine serum albumin was added to the dialysis membrane

Protein binding study of atorvastatin

About 2.5ml of 400 µg/ml of Atorvastatin solution and 2.5 ml of 1.4 x 10^{-4} BSA solution was filled into the previously activated dialysis membrane tubes and sealed. A volume of 50ml of phosphate buffer was taken in measuring cylinders and tubes were immersed in the phosphate buffer solution. At regular intervals of 0,30,60,90 and 120 minutes. The samples were withdrawn from the measuring cylinders. The solution was mechanically stirred using a magnetic stirrer rotating at 40 rpm and analysed for the detector response using the method developed.

Protein binding study of pantoprazole

In previously activated dialysis membrane about 2.5ml of 200 µg/ml of Pantoprazole solution and 1.4 x 10^{-4} BSA solution was filled into the tubes and sealed. A volume of 50ml of phosphate buffer was taken in measuring cylinders and tubes were immersed in the phosphate buffer solution. The samples were withdrawn at regular intervals of 0,30,60,90 and 120 minutes, from the measuring cylinders. The solution was mechanically stirred using a magnetic stirrer rotating at 40 rpm and analysed for the detector response using the method developed.

Assessment of protein binding and *in vitro* drug-drug interaction study

The *in-vitro* drug-drug interaction study was carried out for the drug Atorvastatin and Pantoprazole. To the previously activated dialysis membrane, 2.5 ml of standard solution of mixture containing 400 µg/ml Atorvastatin and 200 µg/ml of Pantoprazole along with 2.5 ml of 1.4×10^{-4} M BSA were added into the tubes and sealed. A volume of 50ml of phosphate buffer was taken in measuring cylinders and the tubes were immersed in the phosphate buffer solution. The samples were withdrawn at regular intervals of 0,30,60,90 and 120 minutes, from the measuring cylinders. The mixture was mechanically stirred using a magnetic stirrer rotating at 40 rpm and analysed for the detector response. The chromatograms were recorded and the concentration of unbound drug was determined by calculating peak area ratios with the standards. The percentage of protein binding (F) was calculated as follows:

$F = (B - A) / B \times 100$

Where,

A = Concentration of free drug in buffer compartment B = Concentration of total drug in buffer compartment

The percentage of protein binding (F) was calculated and the difference in the percentage protein binding of Atorvastatin was calculated to find the percentage displacement of Atorvastatin due to interaction with Pantoprazole.

RESULTS AND DISCUSSION

HPTLC Method development and optimization

The HPTLC method was developed using different organic solvents based on the polarity and solubility of the drugs. So, the solvent system made up of Toluene: Dichloromethane: Tetrahydrofuran in the ratio of 2:2:6 v/v/v has been selected. The proportional change in the ratio of Tetrahydrofuran had a significant effect in the R_f value of the spots. The solvent composition carried the drug Atorvastatin and Pantoprazole to the same distance on the plate. So, to have acceptable separation between the two drugs selected 0.1 ml of glacial acetic acid was added which resulted in good resolution of both the drugs. The experimental criterion such as the distance travelled by the solvent front, chamber saturation time and the peak characteristics including the peak symmetry, area and R_f value were fixed after numerous trials.

The method developed wasvalidated forthe linearity, range, accuracy, inter day precision, intraday precision, ruggedness, robustness, limit of detection and limit of quantification.

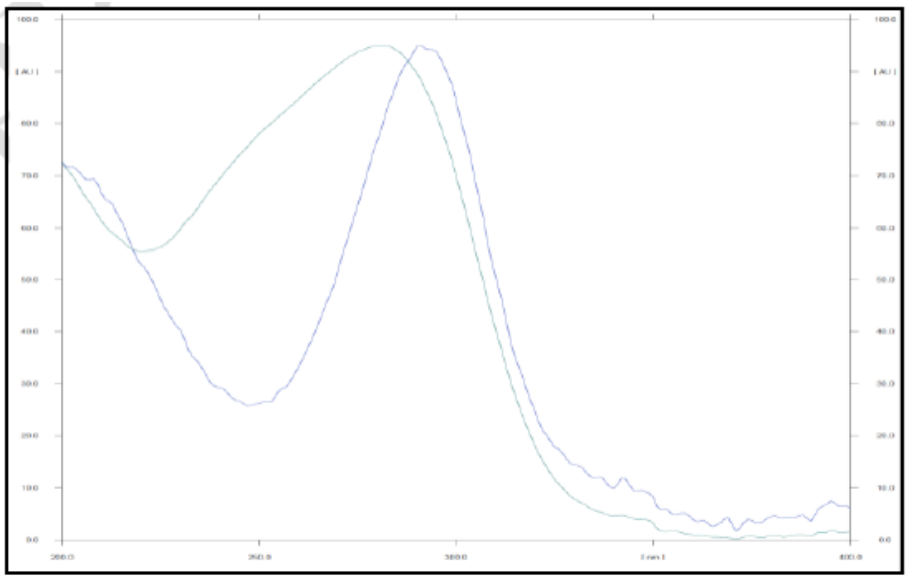

Fig. 3: Overlay UV spectrum of Atorvastatin and Pantoprazole.

The wavelength of 286 nm was selected for the study from the scanned plate in the HPTLC (CAMAG) scanner, as both drugs gave good detection response with an acceptable peak area

and peak shape having a resolution more than 2 (Fig 3).

Method validation

1. Specificity

The excipients present in Pantoprazole tablet for 20 mg dosage (Protonix) was found to have the inactive ingredients composition of sodium carbonate, hydroxyl propyl methyl cellulose, polyethylene glycol, talc, tween 80 and mannitol as per the monograph. The mixture of excipients was prepared according to the formula mentioned in the product monograph and the solutions was prepared with methanol as a solvent. It was further sonicated for 10 mins to ensure the solubility of excipients and filtered using Whatman filter paper. The samples were spotted and it was found that were no absorbable peaks for the excipients solution.

For the drug Atorvastatin the excipients listed as per the monograph of Lipitor of 40 mg tablet consist of calcium carbonate, lactose, magnesium stearate and cellulose. The solution of excipients was filtered to get a clear solution. The excipients solutions were spotted and the chromatogram recorded was checked for the peaks and there were no peaks identified for the excipients solution.

2. Linearity and Range

Different volumes of working standard solutions (1,2,3,4,5, and 6µl) were spotted with the help of Linomat V automatic sample applicator on the TLC plates. The peak areas of Atorvastatin and Pantoprazole were noted with $R_f$respectively (Fig 5). value 0.73 ± 0.04 and 0.57± 0.03.

The linear regression data showed a good linear relationship over a concentration range of 100-600 ng/band of Atorvastatin and 50-300 ng/band of Pantoprazole. The calibration data and regression data are shown in Table 2 and 3. The calibration graph of Atorvastatin and Pantoprazole was shown in Fig 6 and the overlain densitogram of Atorvastatin and Pantoprazole is shown in Fig 4.

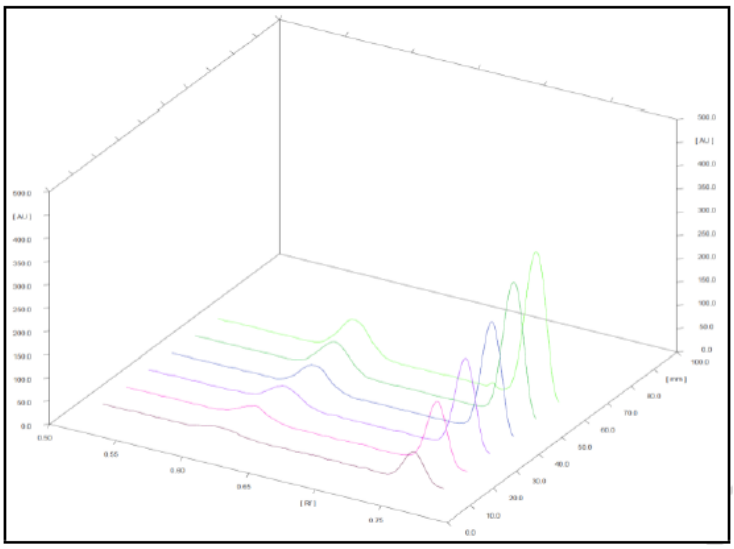

Fig. 4: Overlay densitogram of Atorvastatin and Pantoprazole.

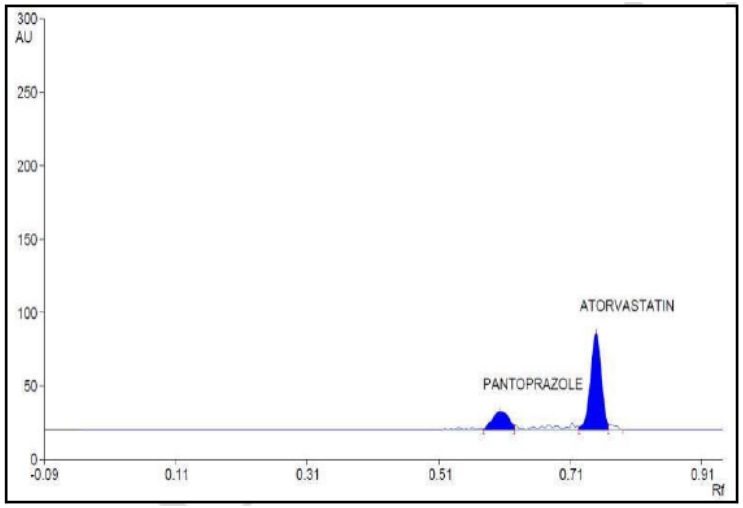

Fig. 5: Densitogramof Atorvastatin 100 ng/band and Pantoprazole50 ng/band.

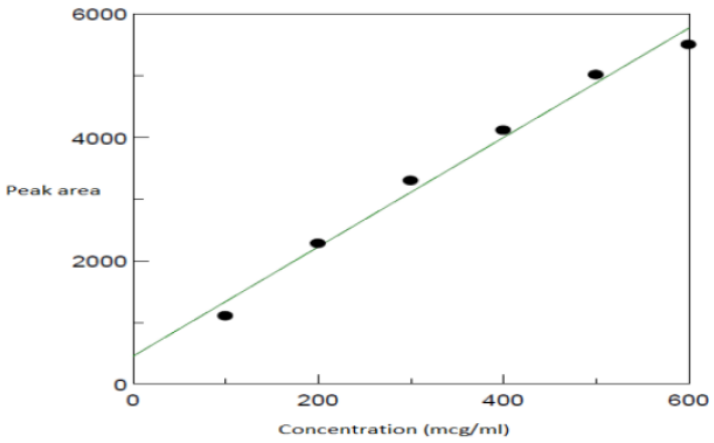

Fig. 6: Calibration graph of atorvastatin.

Table 2: Calibration data for atorvastatin.

Concentration (ng/band)	Peak area(AU)
100	1108
200	2281
300	3299
400	4118
500	5015
600	5502

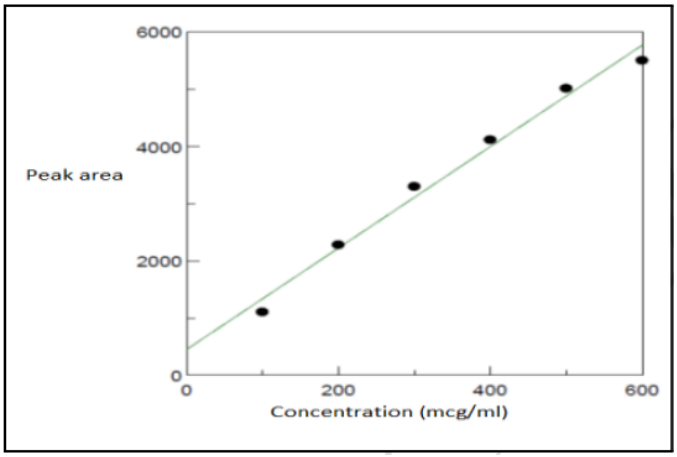

Fig. 7: Calibration graph of pantoprazole.

Table 3: Calibration data for pantoprazole.

Concentration (ng/band)	Peak area(AU)
50	326
100	680
150	952
200	1208
250	1533
300	1811

Table 4: Regression data of Atorvastatin and Pantoprazole.

Regression data	Atorvastatin	Pantoprazole
Slope	7.857	5.8514
Intercept	720	61
Correlation coefficient	0.9929	0.9990

3. Precision Intraday precision

The intraday precision studies were carried out using the solution of drug mixture in the concentration of 400:200 ng/band of Atorvastatin and Pantoprazole. The intraday precision was studied on the same day at three time intervals. The intraday precision studied on the 3 different days at three-time intervals. The values were statistically evaluated for the % RSD value was calculated for mean determinations and found to be below 2.

4. Accuracy

The recovery of the standard drug added to the assay solution at three different levels of 50%, 100%, 150% were studied. The % recovery for Atorvastatin and Pantoprazole at all the three different levels were calculated. The %RSD for the three determinations at each level was found to be below 2.

5. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ of the drug Atorvastatin was found to be 40ng/band and 60ng/band. The detectable and quantifiable concentration of Pantoprazole was found to be 10ng/band and 40 ng/band.

6. Robustness and Ruggedness

The robustness studies carried out with slight modifications in the chamber saturation time and mobile phase composition. The method was found to be robust. The method was found to be rugged with the results obtained from the two analyst under similar experimental conditions.

7. Stability studies of the developed plates

The developed plates for the sample of Atorvastatin and Pantoprazole were checked for its stability at room temperature. The plate was scanned at regular time intervals. The drug Atorvastatin was found to be stable with peak area change less than 10%. The drug Pantoprazole was found to be more than 10% change in peak area.

8. Application of the developed method for the analysis formulation Preparation of atorvastatin tablet formulation

An average of twenty tablets containing 10 mg of Atorvastatin were weighed individually and taken into a 10 ml volumetric flask. The filtrate which has a concentration range of 200 µg/ml was taken. The % label claim was calculated and the estimated amount was close to the labelled value.

Preparation of pantoprazole tablet formulation

An average of twenty tablets containing 10mg of Pantoprazole were weighed individually and taken in a 10 ml volumetric flask. The filtrate which has a concentration range of 100µg/ml was taken. The % label claim was calculated and the estimated amount was close to the labelled value.

Effect of pantoprazole on the binding of atorvastatin to bovine serum albumin

Chromatogram of phosphate buffer pH 7.4

The buffer solution prepared was diluted and spotted separately to identify for any peaks at the Rf value of both the drugs Atorvastatin and Pantoprazole. There were no peaks detected for the buffer solution.

Protein binding study of atorvastatin

The drug solution of Atorvastatin in the concentration of 400µg/ml was prepared and the protein binding studies was carried out.

The samples were collected at 0, 30, 60, 90 and 120 minutes were spotted on the plate and scanned to detect the peaks. The difference in peaks areas at each time intervals were substituted and the concentration of the drug were calculated as shown in the table 5.

Table 5: Results of protein binding study of atorvastatin (individual).

Time (Min)	Peak area(AU)	Concentration (µg/ml)
0	0	0
30	388	11.76
60	462	14.00
90	531	16.04
120	684	20.73

Protein binding study of pantoprazole

The drug Pantoprazole was prepared in the concentration of 200µg/ml in methanol and the protein binding was evaluated.

An aliquot was collected at different time intervals at 0, 30, 60, 90 and 120 minutes and scanned to detect the peaks. The peak area difference at each time intervals were used to find the concentration of the drug which is shown in table 6.

Table 6: Results of protein binding study of pantoprazole (individual).

Time (Min)	Peak area(AU)	Concentration (µg/ml)
0	0	0
30	493	24.49
60	654	32.48
90	756	37.55
120	823	40.88

***In-vitro* drug-drug interaction studies of Atorvastatin and Pantoprazole by protein binding studies**

The mixture solutions of Atorvastatin and Pantoprazole was prepared with the same concentration of 400 µg/ml and 200 µg/ml and it is used for the interaction studies.

The mixture of Atorvastatin and Pantoprazole was prepared with the concentration of 400 µg/ml of Atorvastatin and 200 µg/ml of Pantoprazole placed in the dialysis membrane along with bovine serum albumin were studied for their displacement property at regular time intervals. The representative densitograms given in fig 8 and 9 and the results of the bovine serum albumin binding was given in table7.

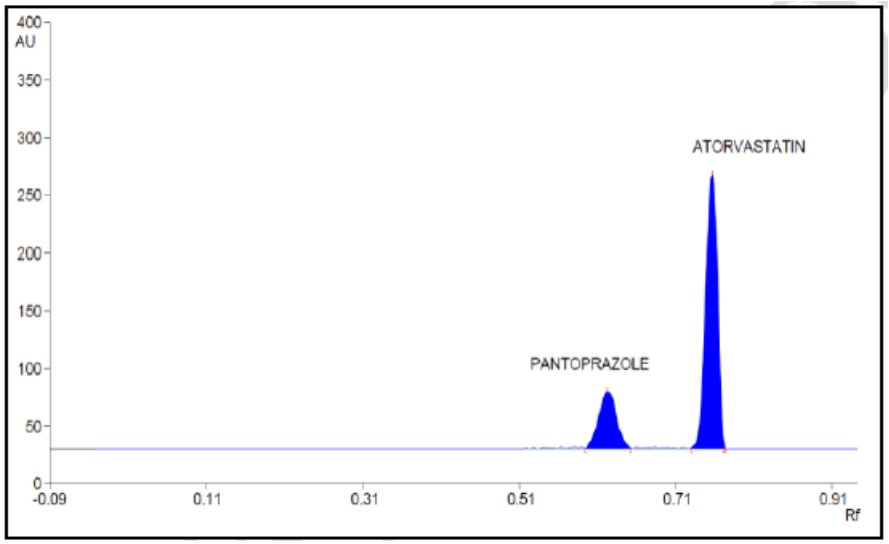

Fig. 8: Densitogram of unbound Atorvastatin and Pantoprazole admixtureat 0 min.

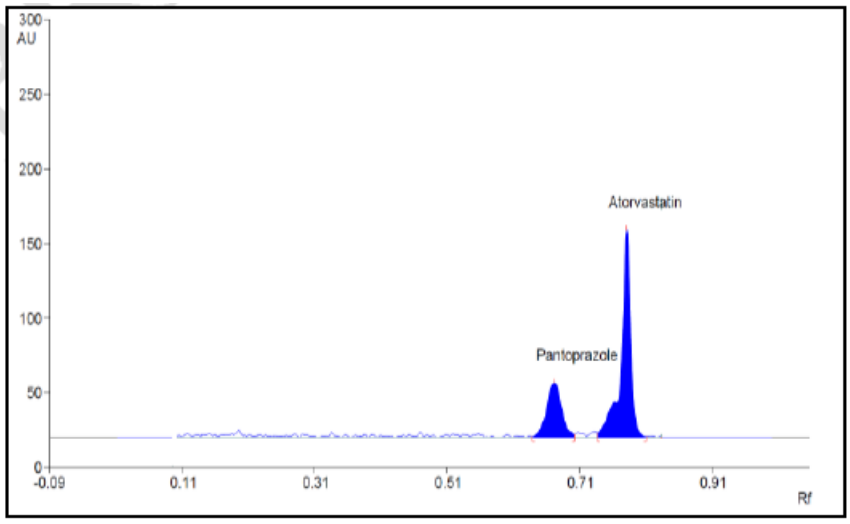

Fig. 9: Densitogram of Atorvastatin and Pantoprazole admixture after equilibrium at 120 min.

Table 7: Results of *in-vitro* drug-drug interaction study of Atorvastatin and Pantoprazole.

Concentration (µg/ml)		Concentration of unbound drug (µg/ml)		% protein binding		% Displacement	
ATV	PAN	ATV	PAN	ATV	PAN	ATV	PAN
400	200	18.64	36.42	84.36	66.15	15.64	33.85

CONCLUSION

The chromatographic method developed for the simultaneous determination of Atorvastatin and Pantoprazole by using the automated sampler and detection by CAMAG scanner was found to be very sensitive technique with sample requirement in ng level.

The method was found to have acceptable resolution between the peaks with acceptable peak characteristics.

The ICH validation parameters specifies that it can used for the routine analysis of both the drugs in the bulk drugs and formulations. The *in vitro* drug-drug interaction studies is an insight into the changes of the drug concentration of Atorvastatin and Pantoprazole when present together at a biological pH of 7.4. The increase in the %displacement of the drugs in the mixture when compared with the individual drug binding is an indication to monitor both the drugs when co-administered.

The studies has to be further investigated for its pharmacokinetics parameters and *invivo* studies for both the drugs Atorvastatin and Pantoprazole.

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