IJPSR (2017), Vol. 8, Issue 4 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 19 September, 2016; received in revised form, 09 November, 2016; accepted, 25 November, 2016; published 01 April, 2017

DESIGN OF EXPERIMENT UTILIZATION TO DEVELOP AND VALIDATE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TECHNIQUE FOR ESTIMATION OF PURE DRUG AND MARKETED FORMULATIONS OF ATORVASTATIN IN SPIKED RAT PLASMA SAMPLES

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Keywords:

Atorvastatin, Protein precipitation, Perchloric Acid, Potassium Carbonate, Potassium Hydroxide

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ABSTRACT: A novel method of estimation and validation of Atorvastatin by Reverse Phase-High Performance Liquid Chromatography coupled with Ultra-violet detection was developed which had high potential in determining drug concentration in rat plasma samples during preclinical studies. Atorvastatin being >99% protein bound, exhibits great challenge in getting extracted from plasma samples. Hence, by treating them with strong protein precipitating agents, that is, initially with 10% w/v perchloric acid and further treating its supernatant with mixture of 2M potassium carbonate and 3M of potassium hydroxide, drug extraction was facilitated. Diclofanac sodium was the selected internal standard. Process of elution was conducted using Phenomenex C_{18} column and mobile phase comprising of a mixture of methanol: water in 70:30 ratio adjusted to pH 5.5. This precise method was linear between a range of 10 to 1000 ng/ml with limit of detection and quantification as 10ng/ml and 15ng/ml respectively. 2-factor 3-level face centred Central Composite Design was employed using Design Expert Software ver. 8.0.0 to examine effect of independent chromatographic factors like pH of the mobile and methanol: water ratio on the dependent factors like retention time, theoretical plates and tailing factor. The ANOVA studies proved that the model employed for this study was significant. Further, to improve applicability, marketed drug formulation was spiked in rat plasma and developed method was applied for drug detection.

INTRODUCTION: Abnormally excessive concentrations of lipid and/or lipoproteins in the bloodstream cause a globally widespread condition called Hyperlipidemia. A major chunk of population in the developed countries suffers from this disease. According to World Health Organization (WHO), one-third of the cardiovascular disorders are attributable to high cholesterol, which causes many deaths and several disability adjusted life years globally.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.8(4).1708-16

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

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.8 (4).1708-16

The statistical figures showing the prevalence of this ailment keeps getting elevated every year, however, there is no recent information pertaining to it. This disease can occur due to inefficient lipid metabolism, genetic defects and environmental factors like having a fat-rich diet and sedentary lifestyle.[1]

Several classes of drugs play a significant role to maintain the normal levels of lipids and reduce the incidence of this condition and the complexities pertaining to it, of which statins form a major and effective group. Atorvastatin (ATV) belongs to this class of drugs, which selectively and competitively inhibits HMG-CoA reductase enzyme, a rate-limiting enzyme in cholesterol biosynthesis. [2] The drug happens to be a key component in majority of the treatment regimens as it not only helps in

lowering serum total and LDL cholesterol while increasing HDL cholesterol, but also diminishes the risk of secondary disorders; thereby reduces cardiovascular morbidity and mortality. [3]

However, due to several limitations and shortcomings related to ATV and its current dosage forms, development of advanced drug delivery system which can overcome these problems is the need of the hour. Hence, efficient methods which can monitor the plasma concentrations of the drug for its quantification are required to be established. Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) is the most sensitive, selective and simplified way of estimating ATV with minimum time and expense, and an improved industrial applicability. [4] Bahrami G et al (2005) developed a rapid and sensitive HPLC method for ATV determination in human serum through liquid–liquid extraction of drug and an internal standard (IS).

The authors used C_{18} analytical column and a mobile phase consisting of sodium phosphate buffer (0.05 M, pH 4.0) and methanol (33:67, v/v). The drug was detected by ultraviolet absorbance at 247 nm. [5] Iqbal Z et al (2011) developed and validated an isocratic reversed-phase high performance liquid chromatographic/ ultraviolet (RP-HPLC/UV) method for simultaneous determination of Rosuvastatin (RST) and ATV in human serum using naproxen sodium as an IS which showed an adequate separation and resolution for RST and ATV. [6] Baghdady YZ et al (2013) developed a method for simultaneous determination of Ezetimibe and ATV calcium by HPLC and was validated according to ICH guidelines and successfully applied for analysis of bulk powder, pharmaceutical formulations and spiked human plasma. [7]

However, a precise method for estimation of ATV has not been established in an animal model. This drug, being a highly protein bound drug (>99%), makes drug extraction from plasma very tedious. Hence, the present article focuses on the development of an efficient method of protein precipitation for complete extraction of drug from plasma samples of animals, progressing it to the development and validation of a simple, economical and convenient HPLC procedure with UV detection for determination of ATV in rat plasma samples after efficient by applying 3^2 face centred central composite design and exploiting the possibilities of different independent process variables on various dependent responses. Further, the suitability of this method for industrial adaptation by spiking the rat plasma with the samples of a marketed formulation Lipikind (10mg, Mankind Pharmaceuticals) was also examined.

MATERIALS AND METHODS:

Materials: ATV and Diclofenac sodium were gift samples from Cipla Pvt. Ltd. (Mumbai) and Aarti Drugs Ltd (Mumbai), which were found to be >99% pure. HPLC grade Methanol was purchased from SD fine-chem limited (Mumbai, India). Perchloric acid, Potassium Carbonate and Potassium Hydroxide was obtained from Sigma-Aldrich Pvt. Ltd, Mumbai. Lipikind tablets (10mg) were product of Mankind Pharmaceuticals, New Delhi. Other chemicals such as pH adjusting agents like glacial acetic acid and triethanolamine (TEA) were of HPLC grade. HPLC grade water was used for the preparation of aqueous mobile phase in all experiments. Rat blood was collected from healthy male Albino Wistar rats (200–250 g). The blood withdrawal was approved by the Institutional Animal Ethical Committee (Reg No: 83/PO/c/1999/CPCSEA).

Methodology:

Equipment: Sophisticated equipment, HPLC LC-2010 HT (Shimadzu, Japan) equipped with a Serial dual plunger and autosampler was used for chromatographic separation. Phenomenex C18 column (250 mm × 4.6 mm, 5 μm) was employed for the analysis. The detection was carried out with the UV-Visible SPD M20A detector. LC solutions software was used for the interpretation of the results.

Experimental Design for developed method of HPLC: Optimization techniques help in designing the experiments by using an appropriate model. It is beneficial to evaluate and identify the most imperative parameters with a minimum number of runs. During the optimization steps, area of the peak, theoretical plates and tailing factor were the responses which were screened in order to minimize the analysis time and maximize the peak resolution and optimal peak asymmetry of the

developed method. [8] Furthermore, face centered 3^2 central composite design was selected to determine the best experimental conditions in RP-HPLC. Nine experiments were conducted using the levels described in **Table 1**. Levels of methanol: water ratio were selected as 60:40, 70:30, 80:20. Similarly, the levels of the pH of the mobile phase were selected as 4.5, 5.5 and 6.5. Peak area ratio (Y1), which indicates the area of the peak, theoretical plates (Y2), and tailing factor (Y3) were the responses for these studies. [9]

TABLE 1: INDEPENDENT VARIABLES, DEPENDENT VARIABLES, AND LEVELS OF FACE-CENTERED 3^2 CENTRAL COMPOSITE DESIGN

Factors		Levels		
Independent	**Symbol**	**-1**	**0**	**+1**
Methanol: Water ratio	A	60:40	70:30	80:20
pH of the mobile phase	B	4.5	5.5	6.5
Dependent				
Peak area ratio			Y1	
Theoretical plates			Y2	
Tailing factor			Y3	

Preparation of Calibration Curve (CC): A stock standard solution (1 mg/mL) of ATV was prepared in methanol (HPLC grade). Primary working standard solution (10µg/ml) was prepared by serial dilution of the stock standard solution with the mobile phase. Working standard solutions (1µg/ml) of the selected Internal Standard (IS), Diclofenac Sodium were prepared by making appropriate serial dilutions of the solution in mobile phase. CC was prepared by serial dilution of ATV stock solution (10μg/ml) in the range of 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 ng/ml. A 50 µl aliquot of drug-free rat plasma was measured to an Eppendorf tube followed by spiking with 200μL of certain concentration of ATV standard solutions which ranged 50-1000ng/ml, as mentioned above. 200 µl of 1µg/ml Diclofenac sodium standard solution (IS) was added to these eppendroff tubes. After mixing, 500µl of 10% Perchloric acid was added, vortex-mixed for 1 min and centrifuged for 20 min at 4^{o}C at 10,000 rpm.

The 200µl of supernatant was transferred to another Eppendorf vial and approximately 25µl of mixture 2 M K_2CO_3/6 M KOH was added to obtain the sample pH 6.0–6.5. After centrifugation under the same conditions, the obtained supernatant (approx 60µl) was injected into the chromatographic system for analysis. [10] All these solutions were stable for seven days when stored at room temperature (20–25 ^{o}C) and were used within one week. The bulk spiked CC samples were stored at −20^{o}C and brought to room temperature before use.

Validation of the developed method: Validation of the developed method was carried out as per ICH guidelines [Q2 (R1)] which include various parameters like selectivity, specificity, system suitability, linearity, accuracy, precision, recovery, ruggedness, limits of detection and quantification. This included the examination of the injections of six consecutive replicate of the standard sample solutions. Selectivity is a tool to determine the ability of the analytical method to differentiate and quantify the analyte in the presence of some components expected in the sample. Sensitivity was determined by analyzing control rat plasma in replicates (n = 6) spiked with the analyte at the lowest level of the calibration standard, that is 0.01µg/ml. [11]

System Suitability Tests: The system suitability parameters were determined by injecting six times the standard solution containing ATV at concentration of 600 ng/mL and Diclofenac sodium (IS) at a concentration of 1µg/ml. The retention time (R_t), Area (A), height (H), tailing factor (T) and theoretical plate number (N) were the various parameters which were tested on the sample containing the combination solution of 600 ng/mL of ATV and 1 µg/mL of IS.[12]

Linearity: Calibration curves were constructed with eleven standard solutions, containing the two compounds simultaneously, ranging from 50-1000 ng/ml. Linearity was determined through the calculation of a regression line by the method of least squares, representing the peak areas a function of the standard concentration. [13]

Precision and accuracy: Precision was determined by repeatability (intraday estimation of drug) and intermediate precision (accuracy) for three consecutive days. The intra-day assay precision and accuracy were estimated by analyzing six replicates containing ATV at three different concentrations i.e., 10, 100 and 1000 ng/ml. The inter-day assay precision was determined by analyzing the three concentrations on six different runs. The criteria for acceptability of the data included accuracy within ±2% deviation (DEV) from the nominal values and precision within 2% relative standard deviation (RSD). For intra-day, accuracy and precision at each concentration were assayed on the same day. The inter-day accuracy and precision were evaluated for three subsequent days. [14]

Extraction recovery: The recovery of ATV was determined at concentration of 10, 100 and 1000 ng/ml. Six replicates at each concentration level with peak area response from non-extracted control samples at the same concentration level were prepared and injected into the HPLC system. [13]

Ruggedness: From the stock solution, sample solutions of ATV (10ng/ml, 100ng/ml and 1000ng/ml) were equipped and analyzed by two different analysts employing analogous operational and environmental surroundings. The peak area was calculated for identical concentration solutions six times. [15, 16]

Limit of Detection and Limit of Quantification: Five standard solutions were prepared by serial dilution of ATV stock solution (10μg/ml) in the range of 5, 7, 10, 12, and 15ng/ml in order to determine the Limit of detection (LOD) and Limit of Quantification (LOQ). LOD and LOQ were calculated according to LOD = 3.3 σ/S and LOQ = 10 σ /S, where σ is the standard deviation of the response and S is the slope of the calibration curve. [17]

Estimation of ATV marketed formulation spiked in rat plasma: To further improve the applicability of the method, a marketed formulation of high dose ATV (Lipikind (10mg, Mankind Pharmaceuticals)) was spiked in rat plasma and the developed method was applied for the detection of ATV. Standard stock solution (1 mg/ml) of Lipikind (10mg, Mankind Pharmaceuticals) was prepared in methanol and subsequent dilutions with mobile phase were carried out to obtain a concentration (100ng/ml). The final sample was prepared as described in the previous section.

RESULTS AND DISCUSSION:

Optimization: Response Surface Based on 3^2Central Composite Design: A 3^2 central composite design (CCD) was utilized to obtain the surface response graphs in order to determine the optimum conditions and to examine the interactions between factors employed for the design. This design allowed the response surface to be modelled by performing the number of experiments equal to 2k+2k+1, where k is the number of variables (k=3), which makes a total of 13 experiments to be executed as per CCD design. [18]

Three-dimensional response surface plots are given in **Fig. 1** and are highly imperative in order to study the effects of the factors and their interaction on the selected responses. The peak area ratio indicates the amount of area covered by the peak on the graph. The intermediate values of the factors were found to be the best as the peak area ratio was found to be maximum, which was desirable, when the values of methanol: water ratio was 70:30 and the pH of the mobile phase was 5.5. This is represented in **Fig. 1 (a).** Similarly, theoretical plates were found to be the maximum at middle values of the selected factors. It is understood that more number of theoretical plates indicates better resolution of the peaks. The maximum value of the theoretical plates was found when the methanol: water ratio was 70:30 and the pH of the mobile phase was 5.5. This relation has been clearly indicated in the **fig. 1(b).**

Tailing factor is an important criterion in the selection of the best peaks of HPLC. The desirable tailing factor should be as low as possible so that the symmetry of the peak is maintained and the effect of the other factors can be neglected. It was observed that tailing factor was improved at an intermediate level of the selected pH of the mobile and selected ratios of methanol:water, as shown in **Fig. 1(c).** The tailing factor was found to be least when the pH of the mobile phase was 5.5 and methanol: water concentration is 70:30, which are the intermediate values. [19]

The model was authenticated by analysis of variance (ANOVA) employing Design Expert software version 8.0.0. The ANOVA tests demonstrated that the models materialized to be adequate, with significant lack of fit (P< 0.0001) and with a satisfactory coefficient of correlation (r). It was found that the final optimized mobile phase comprised of methanol: water ratio as 70:30 and pH of this mobile phase as 5.5. [20]

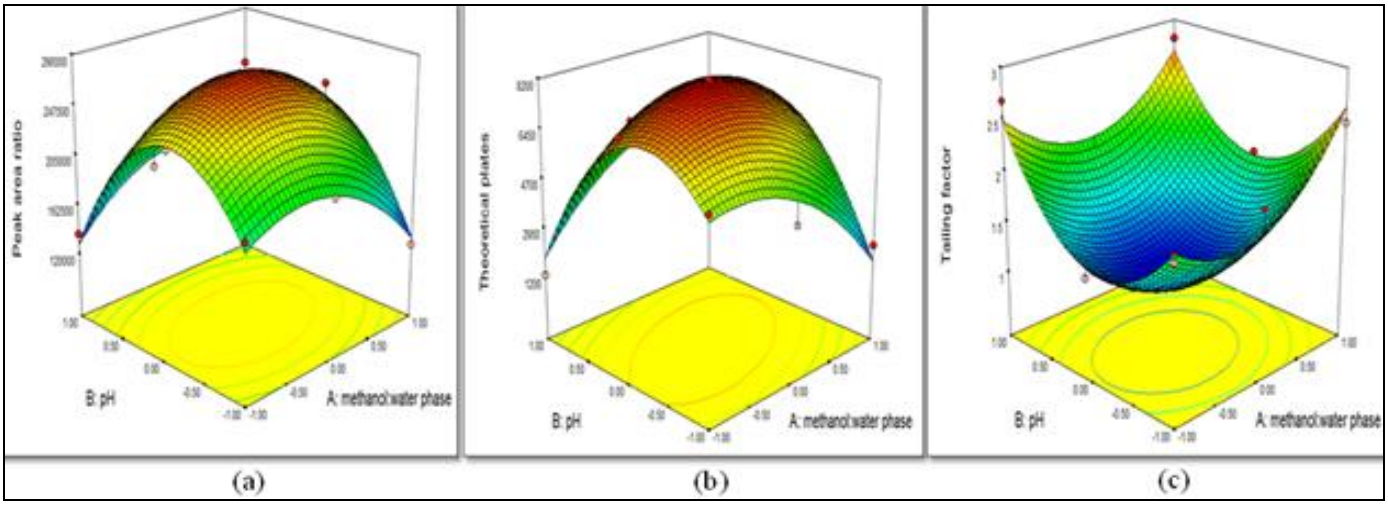

FIG. 1: THREE-DIMENSIONAL SURFACE RESPONSE GRAPHS SHOWING (A) THE EFFECT OF RATIOS OF METHANOL: WATER CONTENT IN MOBILE PHASE AND ITS PH ON THE PEAK AREA RATIO (B) THE EFFECT OF RATIOS OF METHANOL: WATER CONTENT IN MOBILE PHASE AND ITS PH ON THE THEORETICAL PLATES (C) THE EFFECT OF RATIOS OF METHANOL: WATER CONTENT IN MOBILE PHASE AND ITS PH ON THE TAILING FACTOR

System suitability tests: System suitability tests were performed for the proposed method of estimating ATV and to check its applicability and commerciality. Also, many parameters like resolution (Rs), retention time (Rt), Area (A), height (H), tailing factor (T) and theoretical plate number (N), capacity factor (K') and asymmetry were checked to ensure column efficiency, selection of the chromatographic conditions and its repeatability. (**Table 2**). The capacity factor (k′) showed good resolution with respect to the void volume as the values were between 1 and 10. The Relative standard deviation (RSD) of peak areas of six consecutive injections was found to be less than 2%, which indicated good injection repeatability and adequate precision. The tailing factor (T) for the ATV was found to be close to 1, reflecting good peak asymmetry. Resolution between ATV and diclofenac sodium was found to be 5.32, which showed good separation of peaks. Higher values of theoretical plate number (N) demonstrated good column efficiency. **Fig. 2** depicts the HPLC chromatogram which was obtained when 100ng/ml of ATV and 1µg/ml of IS (Diclofenac Sodium) was spiked in the rat plasma and estimated for its concentration by the developed method of HPLC. [21]

TABLE 2: SYSTEM SUITABILITY PARAMETERS

Parameter	Compound	
	Atorvastatin	Diclofenac sodium
*Resolution (Rs)	5.32	
Retention Time (Rt)	4.905	7.02
Area (A)	63554	1496550
Height (H)	7579	81027
Tailing Factor (T)	1.192	1.023
Theoretical Plate number (N)	7634.339	9875.23
Capacity factor (K')	5.29	6.03
Asymmetry	1.32	1.63

*Resolution between Atorvastatin and IS.

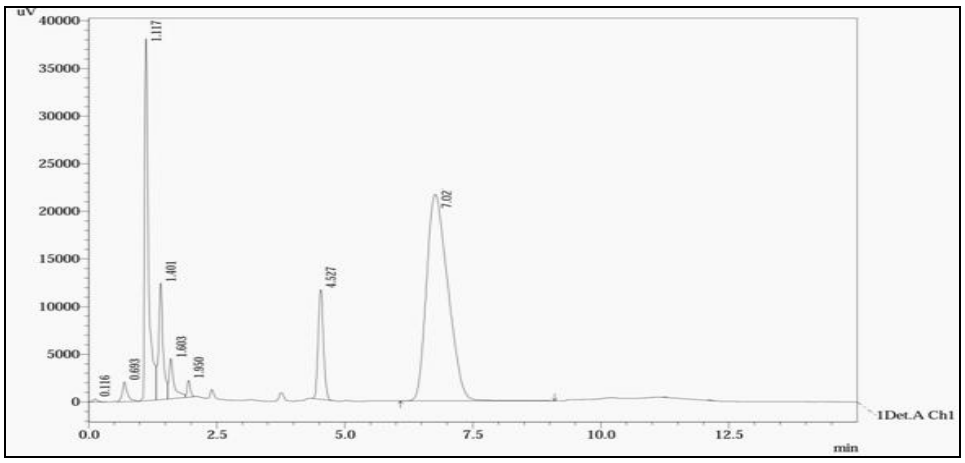

FIG. 2: HPLC CHROMATOGRAM OF RAT PLASMA SPIKED WITH ATV PURE DRUG AND IS

Linearity: The regression equation and determination coefficients were estimated by assessing the linearity of the developed method over a drug concentration range of 50-1000ng/ml. The coefficient was found to be 0.9973 and the regression equation which was generated was found to be y=593.48x. The linearity of the developed method to estimate ATV has been clearly shown in the **Fig. 3**.

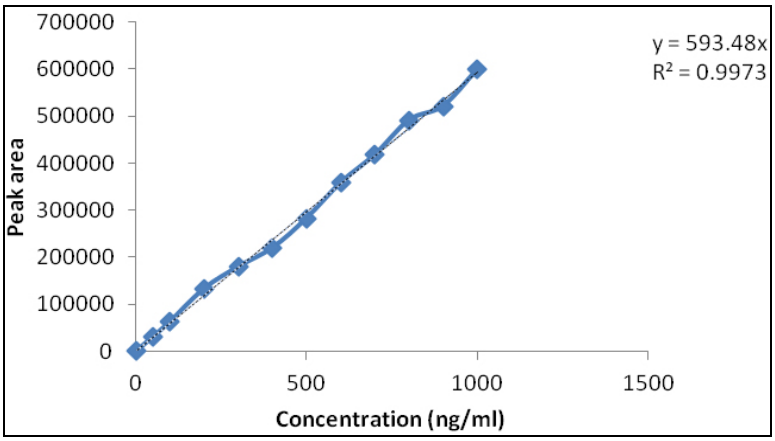

FIG. 3: LINEAR STANDARD CURVE DETERMINATION OF ATV IN SERUM (CONCENTRATION RANGE 10-1000 ng/ml)

Precision and Accuracy: Samples containing drug in concentrations of 10, 100 and 1000 ng/ml were estimated intra-day and inter-day to confirm the precision and accuracy of the developed method. The results of the conducted study are given in **Table 3.** All the data obtained fulfil the acceptance criteria. Intra-day and inter-day precision (%R.S.D.) of the methods were lower than 2% and were within the acceptable limits to be in concurrence with the guidelines for United States Pharmacopeial norms method validation.[18] Accuracy was with the deviation between the nominal concentration and calculated concentration for ATV well below the limit of ±2%. The results obtained for the detremination of precision and accuracy were reproducible and robust. [22]

TABLE 3: INTRA- AND INTER-DAY PRECISION AND ACCURACY DETERMINATION OF ATV CONCENTRATION IN SPIKED RAT PLASMA SAMPLES

Spiked Concentration* (ng/ml)	Mean Measured concentration (ng/ml± SD)		Precision (%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
10	10.06±0.136	9.733±0.28	1.35	2.88	0.66	1.33
100	100.833±1.47	101.833±1.60	1.45	1.57	0.83	1.83
1000	1008.667±2.61	1014.167±2.136	1.25	0.21	0.86	1.41

*(*n* = 6 at each concentration for intra-day and *n*= 6 for interday precision).

Extraction recovery: The comparison of peak area obtained from extracts of spiked plasma samples with the peak area obtained from the direct injection of known amounts of standard solutions of ATV lead to the determination of absolute extraction recovery of ATV from plasma. This study was conducted over the samples containing 10, 100 and 1000 ng/ml ATV in plasma along with 1µg/ml of IS (Diclofenac sodium). The extraction recovery for all the samples were found to be between 93% to 95% as given in **Table 4.**

TABLE 4: RECOVERY OF ATV AND IS FROM SPIKED RAT SAMPLES

Plasma Concentration of ATV (ng/ml)*	Mean recovery of extraction (%± SD)
10	94.39±3.045
100	93.01±0.622
1000	93.25±0.623

*(n = 6)

Ruggedness: Ruggedness is assessed when two different analysts perform the same analysis under the same experimental conditions. As seen in **Table 5**, the contents of the drug were not greatly affected by these changes as the values of % RSD did not vary much when the drug estimation was carried out by the different analysts.

TABLE 5: VALUES OF RUGGEDNESS STUDIES FOR THE DEVELOPED METHOD

*Concentration (ng/ml)	Amount determined by analyst 1 (%±SD)	RSD (%)	Amount determined by analyst 2 (%±SD)	RSD (%)
10	10.09±0.13	1.37	10.03±0.1	1.006
100	101.185±1.27	1.25	101.56±1.11	1.09
1000	1003.483±6.483	0.64	1009.805±11.55	1.14

*(n=6)

Limit of Detection (LOD) and Quantitation (LOQ): The LOD and LOQ for ATV were found to be 10 ng/ml and 15 ng/ml respectively.

Detection of ATV in plasma samples: The developed method was found suitable for the detection of ATV marketed formulation (Lipikind (10mg, Mankind Pharmaceuticals)) in the plasma sample. ATV gave a sharp peak at 4.782 min with a good resolution. The outcome of the study was that no interfering peaks of any of the excipients were observed as shown in **Fig. 4**. Hence, the presently developed method show a potential of detecting ATV in the rat plasma samples which is an important animal model for preclinical studies. Further, the method also has applicability to quantify the drug in a marketed formulation in the presence of rat plasma which makes the method more commercially acceptable.

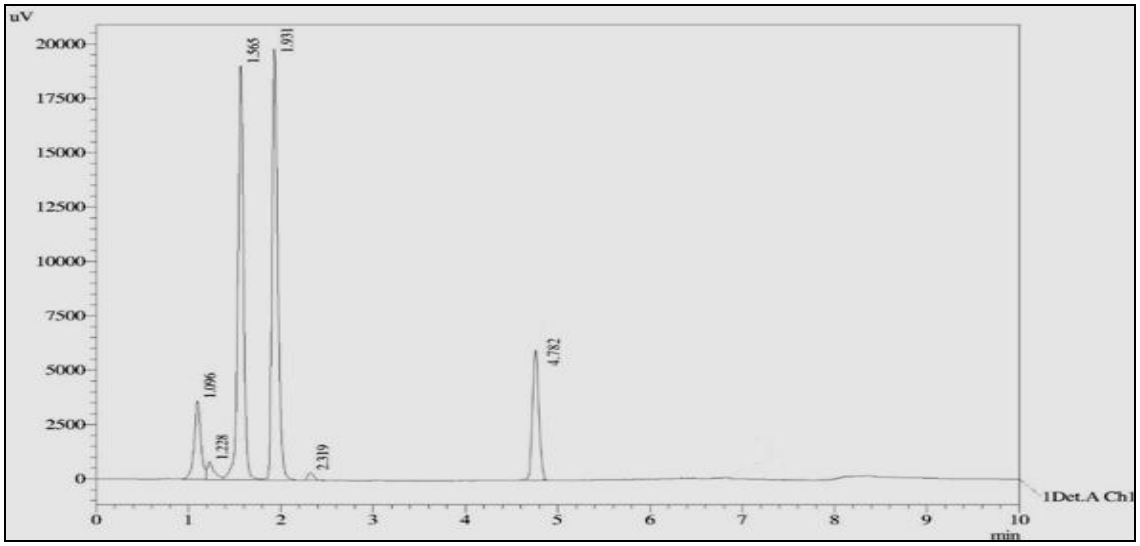

FIG. 4: HPLC CHROMATOGRAM OF RAT PLASMA SPIKED WITH ATV MARKETED FORMULATION (LIPIKIND (10mg, MANKIND PHARMACEUTICALS))

CONCLUSION: A simple, sensitive and robust method for the determination of ATV, an anti-hyperlipidemic agent, in spiked rat plasma by HPLC was developed and validated. A unique protein precipitation method was employed for sample preparation followed by chromatographic separation and UV detection. Diclofenac sodium was employed as an IS. No interfering peaks were observed at the elution times of ATV and IS. System suitability parameters like linearity, precision, accuracy, resolution, theoretical plates, retention times etc of the proposed method were checked and were found to be in appropriate. Linearity was demonstrated over the concentration range of 10 to 1000 ng/ml. LOD and LOQ were found to be 10ng/ml and 15ng/ml. The method was accurate, reproducible, specific, and provided excellent separation and enable the quantification of ATV in rat plasma. Face centred 3^2 central composite design was applied to study and understand the effect of various independent factors like pH of the mobile phase and methanol:water ratio on the dependent factors like peak area ratio, theoretical plates and tailing factor. These effects were found to be noteworthy and the p-values were below 0.001, which proved that the model employed here was highly significant. The study was also extrapolated for determination of ATV in tablet formulation which would improve the industrial applicability as well as detection of ATV levels after administering dose of the drug.

ACKNOWLEDGEMENTS: The authors are thankful to Al-Ameen College of Pharmacy, Bangalore, India for providing the necessary facilities to carry out the research work. Authors are also grateful to Cipla Pvt. Ltd, Mumbai, India for providing the gift sample of Atorvastatin.

DISCLOSURE STATEMENT: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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How to cite this article:
Mathur M and Devi VK: Design of experiment utilization to develop and validate high performance liquid chromatography technique for estimation of pure drug and marketed formulations of atorvastatin in spiked rat plasma samples. Int J Pharm Sci Res 2017; 8(4): 1708-16.doi: 10.13040/IJPSR.0975-8232.8(4).1708-16.