Journal of Advances in Biology & Biotechnology
4(3): 1-10, 2015; Article no.JABB.17269
ISSN: 2394-1081

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Chemometric Assisted HPLC Method for the Simultaneous Estimation of Aspirin, Atorvastatin and Clopidogrel in Biological Matrix

Ramalingam Sathiyasundar[1*] and Kannappan Valliappan[1]

[1]Department of Pharmacy, Faculty of Engineering and Technology, Annamalai University, Annamalainagar, Tamilnadu - 608002, India.

Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2015/17269
Editor(s):
(1) James W. Lee, Department of Chemistry and Biochemistry, Old Dominion University, USA.
Reviewers:
(1) Anonymous, Iuliu Hatieganu University of Medecine and Pharmacy, Romania.
(2) Anonymous, Indukaka Ipcowala College of Pharmacy, India.
(3) Justin Kabera, School of Chinese Materia Medica, Pharmacology, Tianjin University of TCM, China.
Complete Peer review History: http://sciencedomain.org/review-history/10128

Original Research Article

Received 6th March 2015
Accepted 16th June 2015
Published 10th July 2015

ABSTRACT

Aim: A new efficient simple and robust liquid chromatographic method has been developed and validated for the simultaneous estimation of aspirin, atrovastatin and clopidogrel in human plasma sample.
Study Design: The investigation was focused on studying the influence of organic phase modifier, pH and flow rate, which modify the separation of chromatographic behavior of these compounds.
Methodology: To optimize the chromatographic factors that had a significant effect on separation attributes central composite design was applied. The global optimization of the chromatographic responses such as capacity factor, resolution and analysis time optimized derringer's desirability function was employed.
Results: The optimized chromatographic condition was Acetonitrile: Methanol: 0.1% Triethylamine (pH 3.0 adjusted with Ortho phosphoric acid) (53.55: 05: 41.45 % v/v), 1.14 ml/min flow rate and measurement was performed PDA at 220 nm. The spiked plasma sample was extracted by a simple protein precipitation method and then the sample was extracted with cold methanol and solvents was evaporated by the dry air method.

**Corresponding author: E-mail: sundaranalysis@gmail.com;*

Conclusion: This method can be useful for the computable determination of aspirin, atrovastatin and clopidogrel in human plasma suitable for bioequivalence and pharmacokinetic studies in healthy human volunteers.

Keywords: Central composite design (CCD); HPLC; aspirin; atrovastatin and clopidogrel.

1. INTRODUCTION

Atrovastatin (ATV) is chemically known as [R-(R, R)] -2-(4-fluorophenyl) -β, -dihydroxy-5-(1-methylethyl) –3-phenyl-4 [(phenylamino) carbonyl] -LH-pyrrole-1-heptanoic acid. Atrovastatin is a statine derivative, and it clinically used in the treatment of antihyperlipidemics. It does inhibit the HMG-CoA reductase, an enzyme found in liver tissue that play a key role in the production of cholesterol in the body, and efficiently used for coronary heart disease and myocardial infarction stroke, unstable angina and revascularization [1]. Clopidogrel bisulfate (CLP), is chemically (+) -(S) -(2-chlorophenyl)-6,7- dihydrothieno [3,2-c] pyridine- 5 (4H) acetic acid methyl ester sulphate, it's a pro-drug activated in microenzyme (i.e. CYP 450 and CYP_2C_{19}), CLP is irreversible inhibiting of P2Y12 receptor on platelet cell membranes, and preventing adenosine diphosphate (ADP) from activating platelets and eventual cross-linking by the protein fibrin. It is used in the treatment of coronary artery disease, cerebrovascular disease [2]. Aspirin (ASP) is chemically known as acetyl salicylic acid, its anti-plateleting agent, aspirin is adhering and aggregating platelets secrete TXA-2, which leads to further platelet recruitment and activation. TXA-2 formation is catalyzed by the enzyme Cyclo-oxygenase. This anti-aggregatory effect is considered as the main mechanism for the protection against thrombotic events. Additional proposed protective effects of aspirin include anti-inflammatory properties and anti-thrombin actions [3].

Aspirin, atorvastatin and clopidogrel are one of the most preferred and prescribed for those have a severe heart problem. This combo drug therapy, mostly applied in such cases like bypass surgery or have been placed a stent in their arteries needs to undergo treatment comprising both of antilipidemics and anticlotting agents. There are many methods have been reported in various literatures for aspirin, atorvastatin, clopidogrel individually, only a few methods are available for simultaneous estimation of aspirin, atorvastatin [4,5], aspirin, clopidogrel [6,7] and atorvastatin, clopidogrel [8,9] in human plasma sample. Through a literature search there is no method concern for the simultaneous separation of aspirin, atorvastatin, and clopidogrel in biological matrix. The main objective of the present study was to develop and optimize high performance liquid chromatography method for the simultaneous estimation of aspirin, atrovastatin and clopidogrel in human plasma sample.

2. EXPERIMENTAL

2.1 Apparatus

The study was performed by using Shimadzu (Japan) chromatography equipped with an LC-20 AD and LC-20 AD vp solvent-delivery module, an SPD-20A PDA detector, rheodyne model 7125 injector valve fitted with a 20 µL sample loop. The system was controlled through a system controller (SCL-10A) and a personal computer using a Shimadzu chromatographic software (LC Solution, Release 1.11SP1) installed on it. The mobile phase was degassed using a sonicator (Branson Ultrasonics Corporation, USA). Absorbance spectra were recorded using a UV-Visible spectrophotometer (Model UV-1601PC, Japan) employing quartz cell of 1 cm path length. The chromatography analyses were done on an Phenomenex® analytical column Gemini C18 (150 mm × 4.6 mm I.D and 5 µm particle size).

2.2 Materials and Reagents

Reference standards of aspirin, atrovastatin, and clopidogrel were gifts from Ranbaxy Laboratories Ltd., New Delhi, India. Acetonitrile (MeCN), Methanol (MeOH) are HPLC grade and Triethylamine (TEA) other reagents of analytical grade were from SD Fine Chemicals (Mumbai, India). The HPLC-grade water was collected by using Milli-Q water system (Millipore Academic, Bangalore, India). The human plasma was gifted by the Raja Muthiah Medical College and Hospital (RMMCH), Annamalai Nagar, India.

2.3 Standard Solutions

Standard stock solutions of ASP, ATV and CLP (1 mg/ml) were prepared in the mobile phase.

Working standard solutions were freshly obtained by diluting the standard stock solutions with mobile phase during the analysis time. Calibration curves showing peak area ratios of ASP, ATV and CLP to that of the internal standard versus drug concentrations were established in the range of 2-10 µg/mL for ASP, CLP, and 1-5 µg/mL for ATV, in presence of warfarin (5 µg/mL) as an internal standard. Standard solution prepared for the optimization procedure assay constituted 4 µg/ml of ASP, CLP, and ATV for 2 µg/ml respectively.

2.4 Chromatography Procedure

Chromatographic separations were conceded on a Phenomenex® C18 analytical column (150 mm × 4.6 mm i.d., 5 *µ*m) connected with a Phenomenex® C18 guard cartridge (4 mm × 3 mm i.d., 5 *µ*m). The mobile phase consisted of MeCN: MeOH: 0.1% TEA in a ratio of 53.55: 05: 41.45 % (v/v/v) and pH of 0.1% TEA was adjusted to 3.0 with 10% orthophosphoric acid. In order to increase the sensitivity for the less concentrated compound and to decrease the background from mobile phase a wavelength of 220 nm was selected for detection. An injection volume of the sample was 20 *µ*l. The HPLC system was used in an air-conditioned laboratory atmosphere (25 ± 2°C).

2.5 Plasma Sample Extraction Technique

The 1 ml blank plasma and equal quantity of cold-Methonal in a glass-stoppered 15 ml centrifuge tube were spiked with the working solutions of ASP, ATV and CLP and IS_1 achieve a concentration of 500 µg ml^{-1} each. The samples were gently shaken for 5 min and centrifuging on a laboratory centrifuge (Remi®, R&C, Remi Equipment, Mumbai, India) at 4500 RPM for 5 min. The supernantant organic layer was transferred to Petri-dish and the contents were evaporated into the air-dry method. The residue was reconstituted in 100 µL of mobile phase and vortex mixed for 60 seconds. Aliquots of 20 µL were injected into the chromatographic system. The same procedure was carried out for blank plasma samples to check the cleanness of the extracts. To assess the efficiency of the extraction procedure, the spiked plasma sample was extracted according to the above procedure, but the addition of IS_1 after extraction. The recoveries of each drug and IS from spiked plasma were determined by comparing the peak area of each analyte after extraction with the respective non-extracted standard solution at the same concentration in both low and high concentrations of each compound was checked. The concentration of the IS were established in 5000 ng/ml. The mean, % recoveries achieved when analysed plasma samples were, 90, 92 and 89 % for ASP, CLP and ATV with the values within parenthesis being the % CV of the six replicates. The % CV of the assay results were <15, indicating the precision of the analytical methodology.

3. RESULTS AND DISCUSSION

3.1 Central Composite Design and Analysis

In order to obtain a second order predictive model, rotatable central composite design (RCCD) is employed, which is a design type under RSM. CCD is chosen due to its flexibility and can be applied to optimize an HPLC separation by gaining better understanding of factor's main and interaction effects [10-12]. The selection of key factors examined for optimization was based on preliminary experiments. The factors selected for optimization process were (*A*) MeCN concentration (45-55), (*B*) flow rate (1.0- 1.4) and (*C*) pH of 0.1% TEA (3.0 - 4.0) and MeOH concentration was fixed at 5.0% v/v, therefore no significant variations in MeOH concentration but decrease the peak asymmetric factor and peak broadening. Then 0.1% TEA was added for enhancing the resolution and shape of the peak. The capacity factor for the first eluted peak (k_1), the resolution of the critical separation of ATV-IS ($Rs_{2,3}$) and retention time of CLP (tR_4) were selected as responses. In the optimum design, additional experiments (axial points) were incorporated into the two-level factorial design to obtain a CCD. All experiments were conducted in randomized order to minimize the effects of uncontrolled variables that may introduce a bias on the measurements. Replicates (*n*=6) of the central points were performed to estimate the experimental error. The range of the factors, the experimental design matrix and the results are presented in Table 1. The experimental results of the CCD were fitted with a second-order polynomial expression.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

Where, Y is the response to be modeled, β is the regression coefficient and X1, X2 and X3 represents factors A, B and C respectively.

Statistical parameters obtained from ANOVA and the reduced models are given in Table 2.

The insignificant terms (P > 0.05) were eliminated from the model through a backward elimination process to obtain a simple and realistic model. Since R^2 always decreases when a regressor variable is eliminated from a regression model, in statistical modeling the adjusted R^2 which takes the number of regressor variables into account, is usually selected [13]. In the present study, the adjusted R^2 were well within the acceptable limits of $R^2 \geq 0.80$ which revealed that the experimental data shows a good fit with the second-order polynomial equations [14].

For all the reduced models, P value of < 0.05 is obtained, implying these models are significant. The adequate precision value is a measure of the signal (response) to noise (deviation) ratio". A ratio greater than 4 is desirable [15]. In this study, the ratio was found to be in the range of 10.04 – 44.35, which indicates an adequate signal and therefore the model is significant for the separation process. The coefficient of variation (CV) is a measure of reproducibility of this model because the % CV was found to be less than 10% [16].

Table 1. Experimental design and results of a rotatable central composite design

Std	Run	Point type	A (%,v/v)	B (ml/min)	C	k_1	tR_4	$Rs_{2,3}$
20	1	Center	50.00	1.20	3.50	0.898	14.908	3.686
7	2	Fact	45.00	1.40	4.00	0.723	22.618	7.065
10	3	Axial	58.41	1.20	3.50	0.700	8.514	0.573
9	4	Axial	41.59	1.20	3.50	1.216	28.640	10.311
18	5	Center	50.00	1.20	3.50	0.895	14.818	3.454
12	6	Axial	50.00	1.54	3.50	0.458	11.672	3.561
16	7	Center	50.00	1.20	3.50	0.891	14.898	3.648
13	8	Axial	50.00	1.20	2.66	0.899	6.372	3.431
6	9	Fact	55.00	1.00	4.00	0.915	13.895	2.835
14	10	Axial	50.00	1.20	4.34	0.998	20.338	4.624
17	11	Center	50.00	1.20	3.50	0.926	15.584	3.308
15	12	Center	50.00	1.20	3.50	0.910	15.764	3.698
11	13	Axial	50.00	0.86	3.50	1.002	20.649	4.053
3	14	Fact	45.00	1.40	3.00	0.698	12.286	6.519
1	15	Fact	45.00	1.00	3.00	1.014	16.875	6.577
19	16	Center	50.00	1.20	3.50	0.901	15.764	3.698
5	17	Fact	45.00	1.00	4.00	0.983	31.41	8.298
8	18	Fact	55.00	1.40	4.00	0.595	10.088	2.217
2	19	Fact	55.00	1.00	3.00	2.017	13.646	1.688
4	20	Fact	55.00	1.40	3.00	0.551	7.475	1.593

Table 2. Response models and statistical parameters obtained from ANOVA for CCD (After backward elimination)

Responses	Regression model	Model P-value	% CV	Adequate precision	Adjusted R^2
K_1	+ 0.90-0.15A-0.16B + 0.029C +0.032A^2	<0.0001	4.95	39.886	0.9798
$Rs_{(1,2)}$	+3.58-2.67A-0.21B+0.44C+0.072AB	<0.0001	6.76	44.352	0.9857
tR_4	+15.29-5.27A-2.82B+3.75C+0.43AB +1.15A2	<0.0001	7.26	19.786	0.9272

Only significant coefficients with P < 0.05 are included. Factors are in coded levels

As can be seen in Table 2, the interaction term with the largest absolute coefficients between the fitted models is *AB* (+ 0.43) of tR_4 model. The positive interaction between *A* and *B* is statistically significant (< 0.0001) for tR_4. The non-parallel lines obtained from the AB interaction plot (Fig. 1) support this observation. The study reveals that changing the fraction of MeCN from low (−1) to high (+1) results in a rapid decline in *tR4* of CLP both at the low (−1) and high level (+1) pH of the 0.1% TEA. Further, at low level of factor A, an increase in the flow rate results in a marginal decrease in the last eluted peak *(tR_4).*

Therefore, when the MeCN concentration is set at its lowest level, the flow rate has to be at its highest level to shorten the last eluted peak (tR4) at the same time decreased the capacity factor and resolution. Especially this interaction is synergistic, as it led to a decrease in analysis time. The existence of such interactions emphasizes the necessity to carry out active multi factor experiments for optimization of the chromatographic separation. In (Fig. 2) Perturbation plots are presented for predicted models in order to addition a better understanding of the investigated procedure. This type of plots shows the effect of an independent factor on a specific response, with all other factors held constant at a reference point [17]. A steepest slope or curvature indicates sensitiveness of the response to a specific factor. The above figure showed that MeCN (factor *A*) had the most important effect on capacity factor k_1 followed by factor *B* and then *C*.

Response surface plots for k_1, $Rs_{2,3}$ and tR_4 are illustrated in (Fig. 3a, b, c) (% of acetonitrile concentration is plotted against the flow rate with pH of 0.1% TEA held constant at the center value for $Rs_{2,3}$ plot. For k_1 and tR_4, percentage of MeCN concentration is plotted against the flow rate with pH of 0.1% TEA held constant). Analysis of the perturbation plots and response plots of optimization models revealed that factor *A* and *B* had the significant effect on separation of the analytes, whereas the factor *C* is the least significant.

3.2 Multi Response Optimization

When more than one response need to be optimized simultaneously with different targets, Derringer's desirability function can be applied in liquid chromatographic method development [18-21]. Derringer's desirability function (di) was employed for overall optimization of three responses and to select optimal conditions for the analysis of the human plasma sample. The criteria for the optimization of each individual responses are resolution and a capacity factor which is measuring the quality of the separation and the analysis time as a measure of economy. Our goal was to maximize capacity factor, resolution and minimize analysis time.

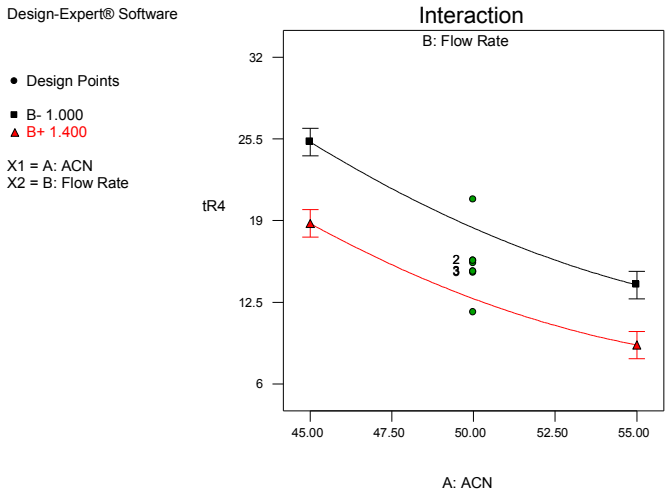


Fig. 1. AB interaction plot for tR4 Response

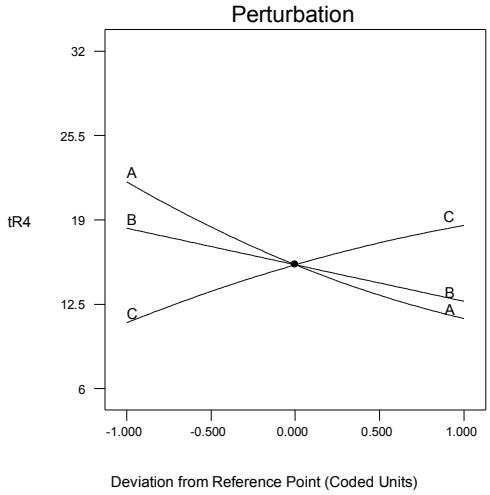
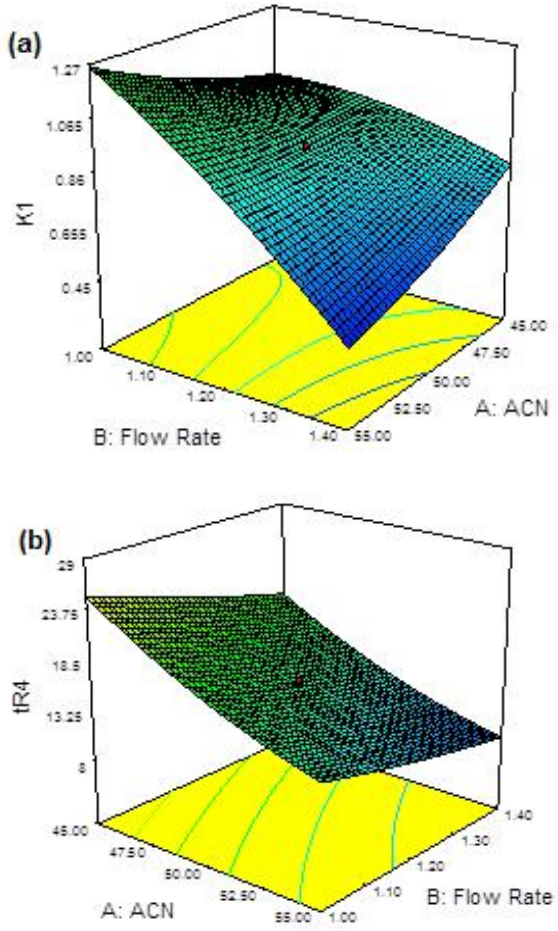


Fig. 2. Perturbation plots for tR4 responses, shows the effect of each of the independent variables on tR4



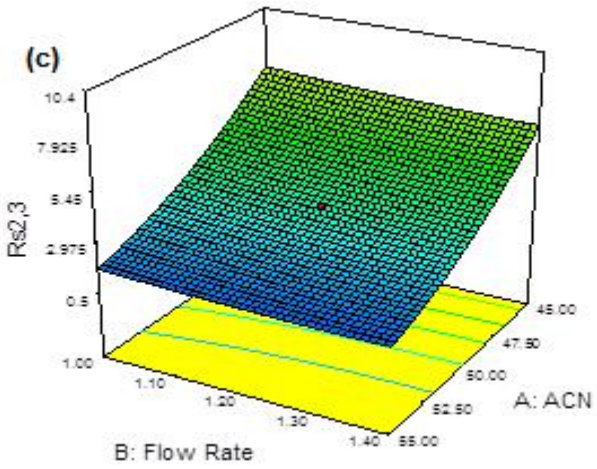


Fig. 3. Response surface plot for the responses (a) capacity factor of the first eluted peak (k1) (b) retention time of last eluted peak (tR4) (c) resolution of the critical pair (Rs 2,3).

Table 3. Criteria for the optimization of the individual responses for the analysis of plasma samples

Responses	Goal	Lower limit	Upper limit	Importance	Weight
K_1	In range	0.46	3.0	5	1
tR_4	Minimize	6.37	31.4	3	1
$Rs_{(2,3)}$	Maximize	0.57	10.3	3	1

Table 4. Comparison of observed and predicted values of different objective functions under optimal conditions

Optimum conditions	MeCN (%)	pH	Flow rate (ml/ min)	K_1	tR_4	$Rs_{(2,3)}$
Global Desirability value (D) = 0.921	53.55	3.0	1.14			
Predictive value				1.25	10.41	1.80
Experimental value				1.25	10.41	1.81
Experimental Error (%)				0.0	0.0	0.1

3.2.1 Optimal condition for plasma assay

Derringer's desirability function defined for the different responses are shown in above Table 3. For optimal condition for plasma assay were recognized by varying the response goals and their importance values, in order to search an optimum experimental condition for analyzing plasma samples. For instance, the high value of k_1 has to be selected for the separation of first eluated peak (ASP) from the initial disturbances of plasma matrix. Therefore, k_1 was assigned in the range of 1.25 to 3.0 and the importance value was given in 5. Following the response goals above, the optimization procedure was carried out in which the maximum desirability value (D = 0.921), and are presented in Fig. 4. The coordinates, producing the maximum value were MeCN concentration of 53.55%, pH 3.0 and flow rate of 1.14 mL^{-1}. The chromatogram from the above conditions were presented in Fig. 5. In order to investigate the predictability of the proposed model, the agreement between experimental and predicted responses are shown in above Table 4.

The Percentage of prediction error was calculated by Equation (2), the prediction efficiency of the model was confirmed by performing the experiment under the optimal condition the experimental error was acquired k_2 = 0.0, tR_4 = 0.04 and $Rs_{2,3}$= 0.06 % respectively.

Predicted Error = Experimental – Predicted / Predicted x 100 (2)

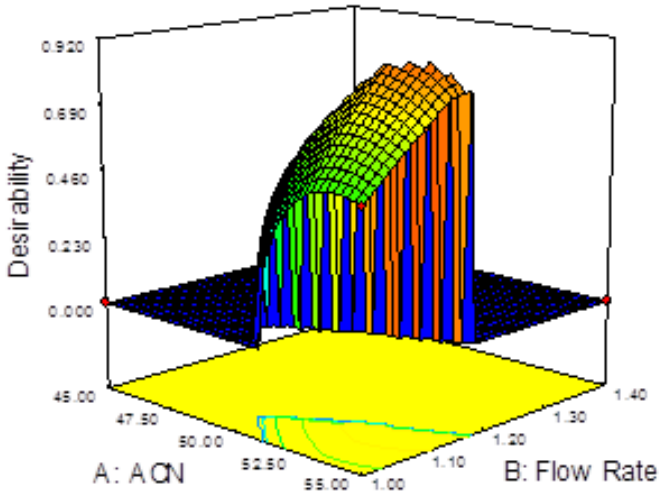


Fig. 4. Graphical representation of the maximum global desirability functions for optimal plasma condition. The best compromise is obtained at the top of the graph, D = 0.921

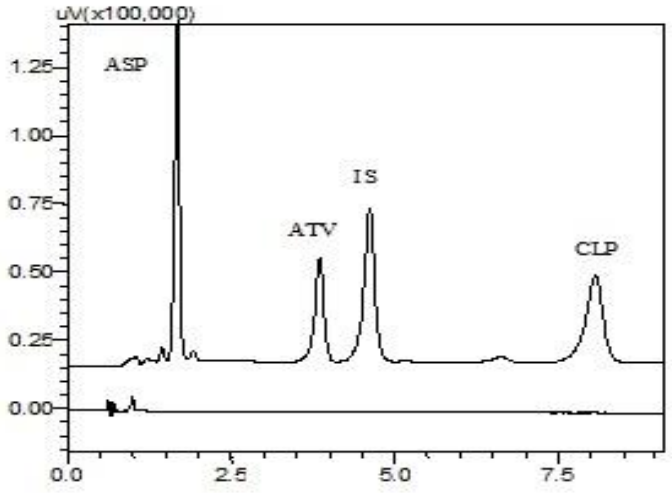


Fig. 5. Chromatogram of the Optimum conditions for the separation ASP, ATV, CLP in spiked human plasma

3.3 Validation of Plasma Assay Method

Linearity was established at five levels over the concentration ranges of 2.0-10 µg mL^{-1} for ASP, CLP and 1-5 µg mL^{-1} for ATV, in the presence of warfarin (5.0 µg mL^{-1}) as internal standard. Typically, the mean (*n* = 6) regression equations were: y = 0.55 x + 0.011 for ASP, y = 0.036 x + 0.002 for CLP and y = 0.052 x - 0.002 for an ATV with R^2 values more than 0.998 for all the analytes. Since the correlation coefficients are good indicators of linearity performance of an analytical procedure a one way ANOVA was performed. For all the analytes, the calculated F_{calc} values less than the F_{Crit} at 5% significance level, indicating that there was no significant difference between replicate determinations for each concentration level. The LOD and LOQ were estimated at 4.06, 12.32 ng $mL^{-1}$for ASP, 1.37, 4.17 ng $mL^{-1}$for CLP and 7.85, 23.78 ng mL^{-1} for ATV was founded. There was no plasma peaks co-eluted with the analytes and IS, indicating that the optimized assay method is selective and specific to the blank plasma used in this study. Accuracy, assessed by spike

recovery, in which the % recovery of analytes at each level (n = 3) and mean % recovery (n = 9) were determined. The recoveries of ASP, CLP, and ATV at each level were found well within the acceptable criteria of bias ± 2 %. The mean % recovery (n = 9) for each analyte was also tested for significance by using the Student t - test. Since the t_{Calc} is less than the theoretical t value (t_{Crit} = 2.306), at 5% significance level, the null hypothesis (the recovery is unity or 100%) were accepted. The intra and inter-day precision was confirmed since, the % CV were well within the target criteria [22].

4. CONCLUSION

New efficient, simple and robust reversed-phase high-performance liquid chromatography method was developed, optimized and validated for the simultaneous estimation of the aspirin, atrovastatin and clopidogrel in spiked human plasma sample using statistical experimental design. The significant factors were optimized by applying CCD and surface response methodology. The objective responses, capacity factor of the first eluted peak (k_1), resolution ($Rs_{2,3}$) and the analysis (retention) time (tR_4) were then simultaneously optimized by applying Derringer's desirability function. The method was validated and the validation study supported the selection of the assay conditions by confirming that the assay was specific, accurate, linear, precise, and robust. The validated assay condition may be employed for the simultaneous estimation of aspirin, atrovastatin and clopidogrel in human plasma sample.

ACKNOWLEDGEMENT

Author is grateful to University Grants Commission (UGC), New Delhi, India, for the financial assistance through UGC-BSR fellowship and to UGC SAP-DRS Phase II sponsored Department of Pharmacy, Annamalai University, Tamilnadu, India for providing the facilities to carry this research work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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