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Chemometric Optimization, Development and Validation of RP-HPLC Method for Simultaneous Determination of Paracetamol, Chlorzoxazone, and Aceclofenac in Bulk and in Pharmaceutical Dosage Forms Using Experimental Design Methodology

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ABSTRACT

In the present study, an isocratic RP-HPLC method was investigated for the simultaneous determination of Paracetamol, Chlorzoxazone, and Aceclofenac in bulk and in tablet dosage form using statistical experimental designs. Three independent factors methanol content in the mobile phase composition, buffer pH and flow rate were used to design mathematical models. Here central composite design (CCD) was used to study the response surface technique and to study in depth the effects of these independent factors. The three responses were simultaneously optimized by using Derringer's desirability function. The predicted optimum assay condition consisted of methanol and phosphate buffer (pH 5.8) in a proportion of 43.80:56.20% v/v respectively, as a mobile phase at a flow rate of 0.8ml/min. The optimized assay condition was validated according to ICH guidelines to confirm, specificity, linearity, accuracy, and precision.

INTRODUCTION

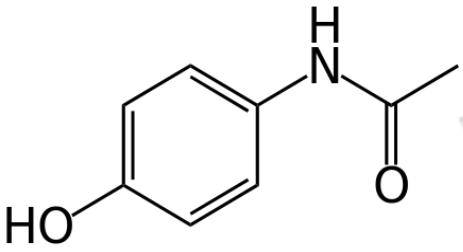
Reversed-phase liquid chromatography is the most versatile and analytical tool in the pharma industry. RP-HPLC methods are mainly used to estimate the purity of the drug substance and pharmaceutical formulations. In the present study, the experimental design approach was used to optimize the separation and to help out in the development of better understanding of the interaction of several chromatographic factors on separation quality. The three optimization steps involved in the method were identification of the goal of the intended method that depends on the types of method developed, such as for routine quality control assay method should be, fast, accurate and specific, then assessment of the factors that effects on the critical quality attributes like in HPLC resolution, retention & tailing factor etc., and also development of experimental design and mathematical model that expresses the relation between the factor and response.

In the present work, the important chromatographic factors were selected based on preliminary knowledge from the literature and optimized by a central composite design (CCD) experiment. A CCD design was used to locate the optimum flow rate, mobile phase, pH, % of organic modifier for separation by mapping the chromatographic response surface. Three chromatographic factors and levels were selected, in which experimental condition was optimized. To provide a CCD for three independent variables, a partial factorial design was combined with five replicates of center points and five axial points at an extreme level. The second order model was fitted to the experimental results. The qualities of the fitted polynomial models were examined on the basis of the coefficient of determination R^2. The position of the true optimum condition was recognized by applying Derringer's desirability function, where responses were simultaneously optimized [1].

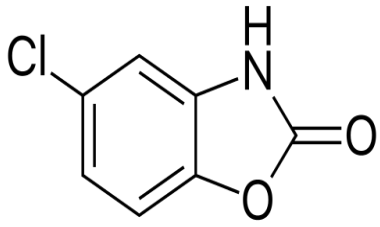
Chemometric technique is the best choice to optimize more than one response selection of multicriteria decision making at a time. However, this method optimizes only one response by targeting all other responses to appropriate constraints. When there is a mix of linear and non- linear responses or when all responses models are of linear or non –linear, Pareto-optimality, utility function or Derringer's desirability function can be used. There are many ways in which the individual desirability can be combined. If the combined criteria are a simple arithmetic average it is called a utility function and if it is a geometric mean it is referred to as designing desirability function. Further, Derringer's method offers the user flexibility in the definition of desirability functions. Derringer's desirability function was

introduced in chromatography by Deming, implementing resolution and analysis time as objective functions to improve separation quality. The Derringer's desirability function was applied to explore the user flexibility to this technique in selecting optimum chromatographic conditions for the determination of drugs in a variety of sample matrices [2].

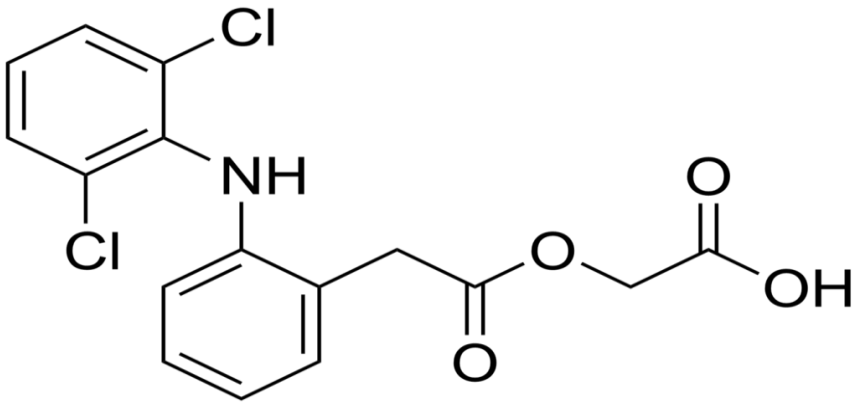
Paracetamol, also known as acetaminophen is a medicine used to treat pain and fever. Paracetamol is typically used for mild to moderate pain relief. It is chemically known as N-acetyl-para-aminophenol (APAP) [3]. Chlorzoxazone is a centrally acting muscle relaxant used to treat muscle spasm and the resulting pain or discomfort. It acts on the spinal cord by depressing reflexes. Chlorzoxazone is chemically known as 5-Chloro-2-benzoxazolinone [4]. Aceclofenac is a nonsteroidal anti-inflammatory drug (NSAID) analog of diclofenac. It is used for the relief of pain and inflammation in rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis. Chemically it is known as [(2-{2, 6-dichlorophenyl) amino} phenyl acetoxy acetic acid] [5].



Paracetamol



Chlorzoxazone



Aceclofenac

Figure: 1 Chemical Structure of Analytes

The literature survey reveals that few analytical methods are available for the simultaneous estimation of paracetamol, chlorzoxazone and aceclofenac by spectrophotometric method [6, 7] and HPLC method [8-14]. But there is not even a single method reported for the simultaneous determination of Paracetamol, Chlorzoxazone, and Aceclofenac using central composite design. Hence in the current work, an attempt was made to develop, optimize and validate an accurate and sensitive and robust HPLC method for the simultaneous determination of Paracetamol, Chlorzoxazone, and Aceclofenac in tablet dosage form using response surface methodology.

MATERIALS AND METHODS

Chemicals and reagents

Active pharmaceutical ingredient (API) standard of Paracetamol, Chlorzoxazone, and Aceclofenac was donated by Surien pharmaceuticals, Chennai, India. Methanol (MeOH) and water (HPLC grade) were purchased from E-Merck (India) Ltd, Mumbai. Dipotassium hydrogen phosphate and orthophosphoric acid (analytical-reagent grade) were supplied by GlaxoSmithKline Pharmaceuticals Limited, Mumbai, India. The pharmaceutical tablet formulation ACENOL-MR (Zorion drugs and herbs Pvt ltd., Haryana, India) which contains Paracetamol 325 mg, Aceclofenac 100 mg, and Chlorzoxazone 250 mg were purchased from local market.

Instrumentation

Chromatographic measurements were made on a Shimadzu (Tokyo, Japan) model which consisted of an LC10AD and LC10 ADvp solvent delivery module, SPD 10A UV-Visible detector, a Rheodyne injector (model 7125, USA) valve fitted with a 20µl loop, and UV detector (SPD-10A). 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 by using Ultrasonicator (3.5L100) PCI Analytics private ltd., Mumbai. UV spectrum was recorded using an UV-Visible spectrophotometer (Model Shimadzu, Japan (Model UV 1800) - software UV probe 2.32 version.

Software

Experimental design (Central Composite design), desirability function data and data analysis were performed by using Design Expert trial version 8.0.4.1.

Preparation of standard solution

Accurately weighed the amount of Paracetamol 162.5 mg, chlorzoxazone 125 mg and aceclofenac 50 mg were transferred into in a separate 50 ml volumetric flask, 25 ml of mobile phase was added to dissolve and the volume was made up to the mark with mobile phase. Then the solution was sonicated for 10 minutes. From the above solution, 5 ml was diluted to 50 ml with the mobile phase. The concentration of the solution contains paracetamol 325 µg/ml, chlorzoxazone 250 µg/ml and aceclofenac 100 µg/ml.

Preparation of sample solution

Twenty tablets of Acenol-MR marketed tablet formulation were weighed and ground to fine powder. An accurately weighed powder sample equivalent to Paracetamol 162.5 mg, chlorzoxazone 125 mg and aceclofenac 50 mg was taken in a separate 50 ml volumetric flask, 25 ml of mobile phase was added to dissolve and the volume was made up to the mark with mobile phase. Then the solution was sonicated for 10 minutes and filtered. From the filtrate, 5 ml was transferred into a 50 ml volumetric flask and the volume was made with mobile phase. The concentration of the solution contains paracetamol 325 µg/ml, chlorzoxazone 250 µg/ml and aceclofenac 100 µg/ml.

Method validation

The proposed method was validated according to ICH guidelines [15, 16] for system suitability study, System suitability parameters were measured so as to verify the system performance. System precision was determined on six replicate injections of a standard preparation. All important characteristics, including tailing factor, theoretical plate number were measured.

The linearity study was established at the concentration range of 162.5-487.5µg/ml for paracetamol, 125-375 µg/ml for chlorzoxazone and 50- 150 µg/ml for aceclofenac. The standard stock solutions were diluted with mobile phase to get the above said the range of concentration for each drug. Each concentration was analyzed in three replicates. Peak areas

of drugs were plotted with their respective concentrations and chromatograms were recorded. The LOD and LOQ were calculated by using the standard deviation of the y-intercept and slope of the calibration curve constructed at the concentration levels of the three drugs.

Precision was determined by studying the intermediate precision and repeatability. Repeatability expresses the precision under the same operating condition over a short interval of time. The accuracy of the method was determined by performing the recovery experiment at 75%, 100% and 125% of the assay concentration. Each concentration was analyzed by the proposed method in 3 replicates. 20µl solutions were injected and the chromatograms were recorded. The proposed assay method was applied to marketed tablet product by injecting the sample solution and assayed by the proposed HPLC method.

RESULTS AND DISCUSSION

Optimization design and Analysis

In the present work chemometric optimization was conducted to reduce the overall assay development time and to obtain essential information regarding the sensitivity of different chromatographic separation attributes. One of the major challenges in the development of simultaneous HPLC method for the determination of drugs in the multicomponent formulation is early elution or co-elution or late elution of component drugs. Hence, optimization of drug separation is essential. There is a need to optimize runtime without losing resolution i.e., global optimization of the chromatographic response.

In this study, the factors that had significant effects on the chromatographic responses were identified and the chromatographic factors that had significant effects on separation attributes were optimized using central composite design and response surface methodology. An optimization procedure it is important to investigate the curvature term using factorial design with center points. ANOVA produced 2^K factorial design showed that curvature was significant for all responses (k_1, $Rs_{2,3}$, tR_2) since p-value was less than 0.05. This implied that the quadratic model should be considered to model the separation process. The selection of factors for optimization was based on a preliminary experiment and prior knowledge from literature as well as instrumental limitations.

The factors selected for optimization process were methanol concentration (A), buffer pH (B) and flow rate (C). The ranges of factors used were MeOH concentration (30 – 50%), buffer

pH (5.8 – 6.2) and flow rate (0.8 – 1.2 ml/min). Eventually, the three responses are a capacity factor of the first peak (K_1), the resolution between second and third peak ($Rs_{2,3}$) and retention time of second peak tR2, each having different targets. The levels of each factor studied for finding out the optimum values and responses were shown in Table 1. For an experimental design with the three factors, including linear, quadratic and cross terms, the model can be expressed as $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{33} X_{32}$ where Y is the response to be modeled, β is the regression coefficient and X_1, X_2 and X_3 represent factors A, B and C respectively. Statistical parameters obtained from ANOVA for 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 [16]. The adjusted R^2values were well within the acceptable limits of $R^2 \geq 0.80$ [17], which revealed that the experimental data showed a good fit with second order polynomial equations. For all the reduced model's p-value < 0.05 was obtained, implying these models were significant. The adequate precision value is a measure of the signal (response) to noise (deviation) ratio. A ratio greater than 4 is desirable [18, 19].

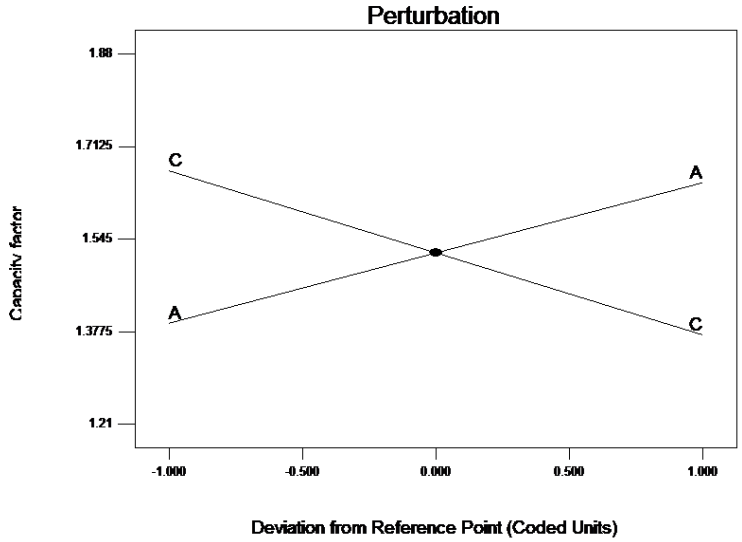
Table 1 Central composite arrangement and responses

Run	Type	Methanol concentration A (%v/v)	Buffer B (pH)	Flow rate C (ml/min)	Capacity factor ($k_{1)}$	Resolution ($Rs_{2,3}$)	Retention time (tR2)
16	Center	40.00	6.00	1.00	0.967	7.232	6.165
5	Center	40.00	6.00	1.00	0.967	7.232	6.165
12	Center	40.00	6.00	1.00	0.967	7.232	6.165
2	Center	40.00	6.00	1.00	0.967	7.232	6.165
17	Center	40.00	6.00	1.00	0.967	7.232	6.165
1	Center	40.00	6.00	1.00	0.967	7.232	6.165
14	Axial	23.18	6.00	1.00	1.129	8.43	7.015
13	Axial	56.82	6.00	1.00	1.164	7.298	6.565
15	Axial	40.00	5.66	1.00	0.942	7.437	5.369
7	Axial	40.00	6.34	1.00	1.232	7.456	6.231
8	Axial	40.00	6.00	0.66	1.076	7.738	6.735
19	Axial	40.00	6.00	1.34	0.963	7.41	5.438
3	Fact	30.00	5.80	0.80	1.005	7.253	5.279
18	Fact	50.00	5.80	0.80	1.016	7.682	6.8
11	Fact	30.00	6.20	0.80	0.972	7.648	5.56
10	Fact	50.00	6.20	0.80	1.264	6.126	6.077
6	Fact	30.00	5.80	1.20	1.045	7.678	6.652
9	Fact	50.00	5.80	1.20	1.262	8.411	6.065
20	Fact	30.00	6.20	1.20	0.975	7.532	5.102
4	Fact	50.00	6.20	1.20	0.96	7.075	4.895

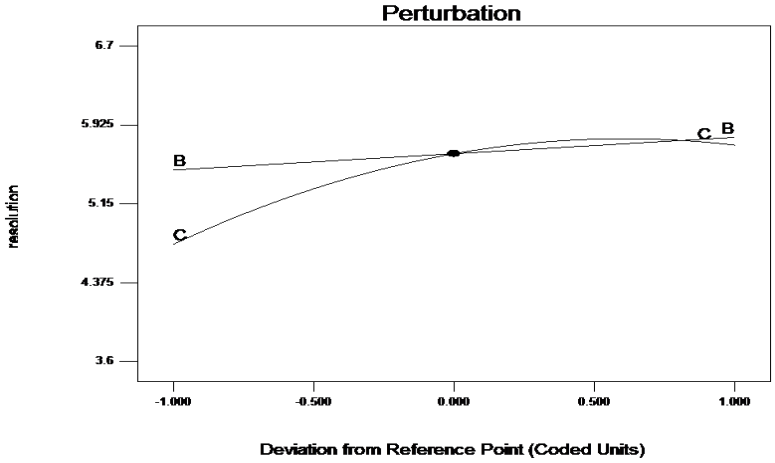
Table 2 Reduced response models and statistical parameters obtained from ANOVA for CCD

Responses	Regression model	Adjusted R^2	Model p value	C.V (%)	Adequate Precision
K_1	+1.00+0.041*A+0.024*B-0.015*C-0.073AC+0.053A^2	0.9563	<0.0001	8.40	6.059
$Rs_{2,3}$	+7.31-0.020*A-0.19*B+0.39BC+0.17A^2	0.9162	<0.0001	4.49	8.526
tR_2	+6.04+0.036*A-0.23*A-0.35*AC	0.9292	<0.0001	9.11	4.771

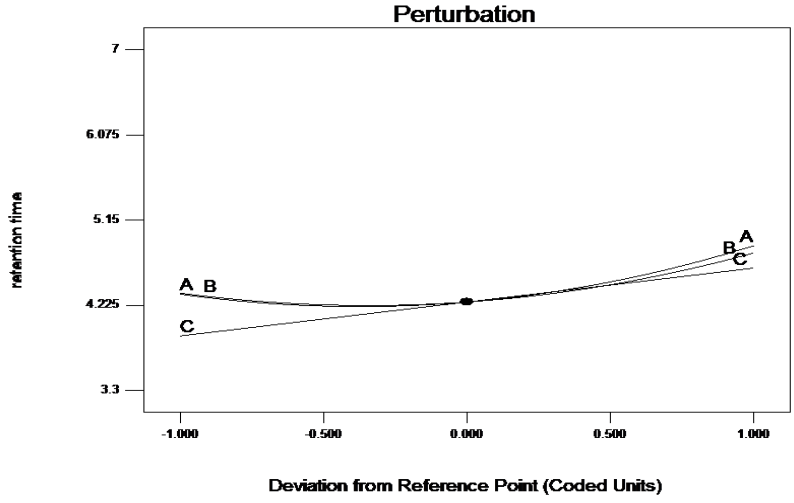
The ratio was found to be in the range from 4.771 to 8.526 which indicated an adequate signal and therefore the model was significant for the separation process. The coefficient of variation (C.V% - 9.11%) is a measure of reproducibility of the model and as a general rule, a model can be considered reasonably reproducible if it is less than 10%. In table 2 the interaction terms with the largest term coefficient among the fitted model were BC (+0.39) of the $Rs_{2,3}$ model. The positive interaction between B and C was statistically significant (< 0.0001) for$Rs_{2,3}$. The existence of such interactions emphasizes the necessity to carry out active multifactor experiments for the optimization of chromatographic separation. In order to gain a better understanding of the results the predicted models were presented in the form of perturbation plot figure 2 and 3D response surface plot figure 3. Variables giving quadratic and interaction terms with the largest absolute coefficients in the fitted models were chosen for the axes of the response surface plots. Consequently, factors A and C were selected for the response plots of k1, $Rs_{2,3}$ and tR_2 with factor B held constant usually at the central value of phosphate buffer pH 6.00. All these three-dimensional plots were beneficial to gain an overall understanding of the influence of phosphate buffer pH and flow rate on analysis time ($Rs_{2,3}$). Perturbation plots provide silhouette views of the response surface plots, where it shows how the response changes as each factor move from a chosen reference point, with all other factors, held constant at the reference value. The steepest slope or curvature indicates the sensitiveness of the response to a specific factor. Figure 2b showed that phosphate buffer pH (factor B) had a most important effect on a resolution between paracetamol (chlorzoxazone) and aceclofenac $Rs_{2,3}$ followed by factor C and then factor A. The rest of the factors (Methanol concentration and flow rate) had a significant effect on tR3 and k1. When k1 and tR_2 values were increased, the level of methanol concentration (factor A) increased and when k1 and tR_3 values decreased, the level of flow rate (factor C) was increased.



Perturbation plot for Capacity factor

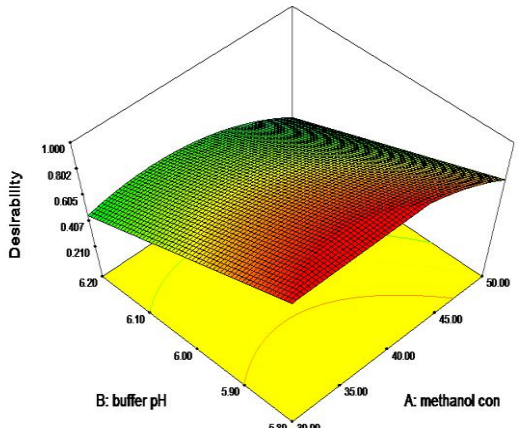


Perturbation plot for Resolution

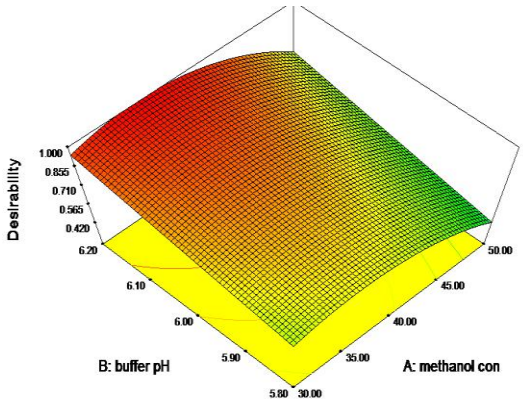


Perturbation plot for Retention time

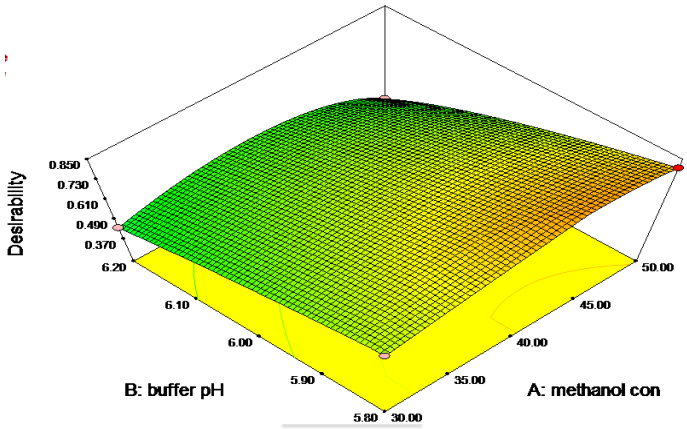
Figure: 2 Perturbation plots



Response surface plot capacity factor k_1



Response surface plot resolution $Rs_{2,3}$



Response surface plot Retention time tR_2

Figure: 3 Response Surface Plots

Analysis of the perturbation plot and response surface plot of optimization models revealed that factor B and C had a significant effect on the separation of analytes, whereas the factor A, Methanol concentration was of little significance. The criteria for the optimization of each individual response were shown in Table 3.

Table 3 Criteria for the Optimization of the Individual Responses

Response	Lower limit	Upper limit	Criteria/Goal
k_1	0.942	1.264	Minimize
$Rs_{2,3}$	6.126	8.63	is in range
tR_2	5.43	6.65	Minimize

From the above table, it could be seen under the column criteria that the response of tR_2 was minimized in order to shorten the analysis time and the response of $Rs_{2,3}$ was in range to allow the baseline separation of paracetamol (chlorzoxazone) and aceclofenac. In order to separate the first eluting peak of chlorzoxazone (paracetamol) from the solvent front, K_1 was minimized. Importance could range from 1 to 5 which gave emphasis to a target value. Following the conditions and restrictions above, the optimization procedure was carried out. The response surface obtained for the global desirability function was presented in figure 4.

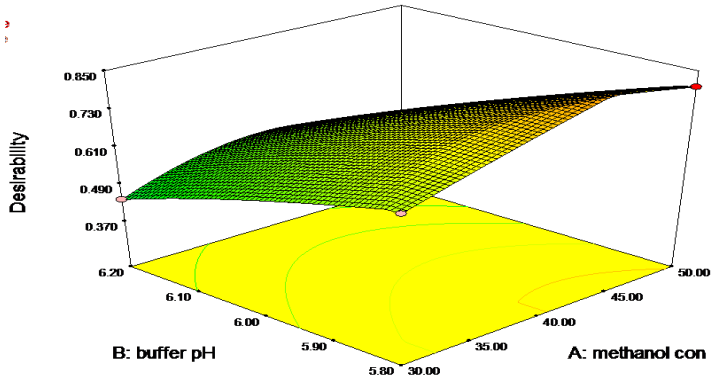


Figure: 4 Graphical Representation of global desirability function (D=0.844)

From the figure 4, it could be concluded that there was a set of coordinates producing high desirability value (D = 0.844), Methanol concentration 43.80.7%, buffer pH 5.80, and flow rate of 0.8 ml/min. The optimized formulation assay conditions were using Gemini C_{18} column with Methanol: phosphate buffer pH 5.80 (43.80:66.20%v/v) as mobile phase at a flow rate of 0.8 ml/min and UV detection at 276 nm. The predicted response values of K_1, $Rs_{2,3}$ and tR_2 corresponding to the latter value of D were found to be 0.96, 7.06, and 6.08 minutes. The agreement between experimental and predicted responses under optimal conditions was shown in table 4 and the corresponding chromatograms were shown in figure 5.

Table 4 Comparison of experimental and predictive values of different objective functions under optimal conditions

Optimum conditions	**Methanol (%v/v)**	**Buffer pH**	**Flow rate (ml/min)**	**K_1**	**$Rs_{2,3}$**	**tR_2**
Predictive	43.80	5.80	0.80	0.97	6.55	5.86
Experimental	43.80	5.80	0.80	0.99	6.65	6.02
Average error				2.08	1.50	2.65
Desirability Value (D) = 0.844						

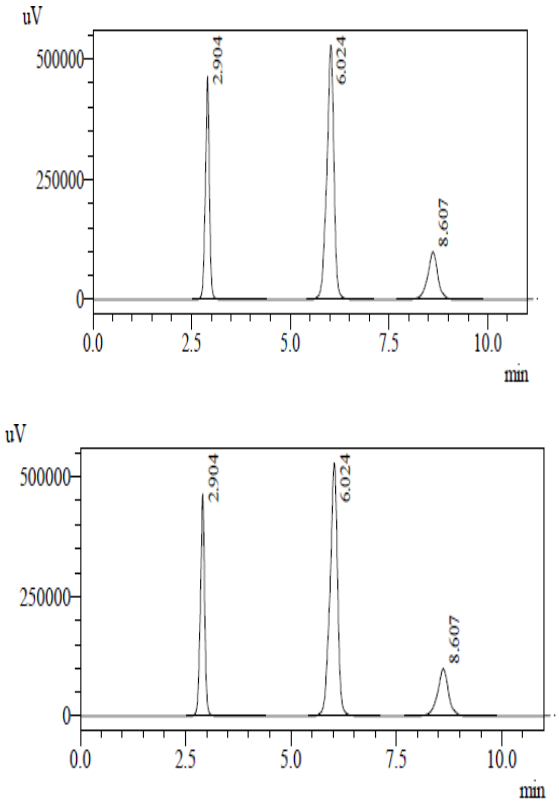


Figure: 5 Chromatograms for comparison of Experimental and Predictive Values of different functions

Method Validation

The developed method was validated to check system suitability, linearity, precision (method precision and system precision), intermediate precision and accuracy.

The system suitability tests ensured the validity of the analytical procedure as well as confirmed the resolution between different peaks of interest. Acceptance criteria for system suitability were, asymmetry factor should not be not more than 2.0. Theoretical plates should be not less than 2000 and %RSD of peak area should not be more than 2.0[15]. The reports were shown in Table 5.

Table 5 System suitability parameters

Parameters	Compound		
	Paracetamol	Chlorzoxazone	Aceclofenac
Capacity factor (k)	0.897	0.966	0.907
Retention time (Rt) in min	6.087	2.905	8.845
Theoretical plates (N	995978.742	993323.815	995100.071
Resolution (Rs)		12.322	7.182

An excellent linearity was established of five levels in the range of 162.5-487.5µg/ml for paracetamol, 125-375 µg/ml for chlorzoxazone and 50- 150 µg/ml for aceclofenac respectively. The correlation coefficient values were found to be more than 0.999 for all the analytes. It indicates that the proposed method is linear. The LOD values were found to be 0.516 µg/ml, 0.380 µg/ml, 0.921 µg/ml for the analytes paracetamol, chlorzoxazone and aceclofenac respectively. The LOQ values were found to be 1.56 µg/ml for paracetamol, 1.150µg/ml for chlorzoxazone and 2.97 µg/ml for aceclofenac respectively. The method and system precision (n=6) was confirmed since the %RSD was well within the acceptance criteria (less than 2%). Accuracy (n=9) assessed by spike recovery, were found to be 99.79% for paracetamol, 99.59% for chlorzoxazone and 99.44% for aceclofenac respectively, which were within acceptable ranges of 100±2%. Good agreement was found between the assay results and the label claim of the product. The %RSD for the tablets was found to be less than 2% indicating the precision of the analytical methodology [15]. The reports indicated the method was accurate and precise. Finally, commercial tablet product Acenol-MR was assayed by the proposed HPLC method. The percentage purity of the assay was found to be 99.85% for paracetamol, 100.15% for chlorzoxazone and 99.98% for aceclofenac respectively. The results of validation parameters are shown in Table -6.

Table 6 Reports for validation parameters

Parameters	**Paracetamol**	**Chlorzoxazone**	**Aceclofenac**
Linearity range (µg/ml)	162.5-487.5	125-375	50-150
Slope	19315	12698	16879
Intercept	103150	38131	7632.4
Correlation coefficient	0.9990	0.9996	0.9997
Accuracy (%Recovery)	99.79	99.59	99.44
Precision System and Method (%RSD)	0.026, 0.054	0.074, 0.054	0.0057, 0.0048
Intermediate precision	0.025	0.062	0.83
LOD & LOQ (µg/ml)	0.516, 1.56	0.380, 1.15	0.921, 2.79
Assay sample (%)	99.85	100.15	99.98

CONCLUSION

An efficient isocratic RP-HPLC method was developed, optimized and validated for the simultaneous estimation of the analytes in API and pharmaceutical formulation (tablet). The developed HPLC method could be immense relevance. This method reduces overall assay development time and provides essential information regarding the sensitivity of various chromatographic factors and their interaction effects on the attributes of separation. Time of analysis, resolution, and quality of the peaks was simultaneously optimized by applying useful tools of chemometrics, central composite design and Derringer's desirability function. The validation study supported the selection of the assay conditions by confirming that the assay was specific, accurate, linear, precise, and robust. Therefore, this HPLC method can be used as a routine quality control analysis for simultaneous estimation of paracetamol, chlorzoxazone, and aceclofenac in bulk and tablet dosage form in a pharmaceutical environment. The results of the study demonstrate the benefit of applying this approach in selecting optimum conditions for the determination of drugs in a pharmaceutical formulation.

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