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APPLICATION OF DOE AND STATISTICAL ANALYSIS FOR DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR CHLROHEXIDINE GLUCONATE AND CETRIMIDE IN ITS BULK AND PHARMACEUTICAL DOSAGE FORMS

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Keywords:

Chlorhexidine gluconate, Cetrimide, RP-HPLC method, DOE, Anderson - Darling normality test

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ABSTRACT: The present work includes development of analytical method for simultaneous estimation of Chlorhexidine gluconate and Cetrimide by RP-HPLC method. The chromatographic conditions were successfully optimised for the separation of Chlorhexidine gluconate and Cetrimide by using Hypersil BDS C18 column (4.6 × 150 mm) 5µ, flow rate of 0.8 ml/min, mobile phase ratio of (30:55:15 v/v/v) ACN: methanol: phosphate buffer (KH_2PO_4 and K_2HPO_4) phosphate pH 3 (pH was adjusted with orthophosphoric acid) and detection wavelength used was 210 nm. The retention times were found to be 3.10 min and 3.9 min for Cetrimide and Chlorhexidine gluconate respectively. The % purity of Chlorhexidine gluconate and Cetrimide was found to be 99.92% and 100.45% respectively. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1). The linearity study of Chlorhexidine gluconate and Cetrimide was found in concentration range of 3 µg - 18 µg and 30 µg - 180 µg. DOE was applied in validation for checking robustness of the developed method using Box-Behnken design and also normal distribution of data was verified by Anderson-Darling normality test. The method developed was found to be specific, selective, and robust and can be applied for routine analysis of marketed formulation in laboratory premises.

INTRODUCTION: Chlorhexidine gluconate is an antiseptic and antibacterial drug with molecular weight of 897.75716 g/mol and pKa value of 10.3.[1] It was approved by USFDA on 19 December 2003. A RP - HPLC analytical method along with its impurity Para chloroaniline is available in literature for its estimation [2]. Cetrimide (Cetrimonium bromide) is a local infective agent with molecular weight 364.44 g/mol [3]. It was approved by USFDA on 30 June 2006. Due to absence of any significant chromophoric group in its structure till date no analytical method is available for its estimation. Also no analytical method is available for estimation of Chlorhexidine gluconate and Cetrimide in combination which is found in various marketed formulations like Savlon antiseptic solution [4] and Aceptic Lotion.

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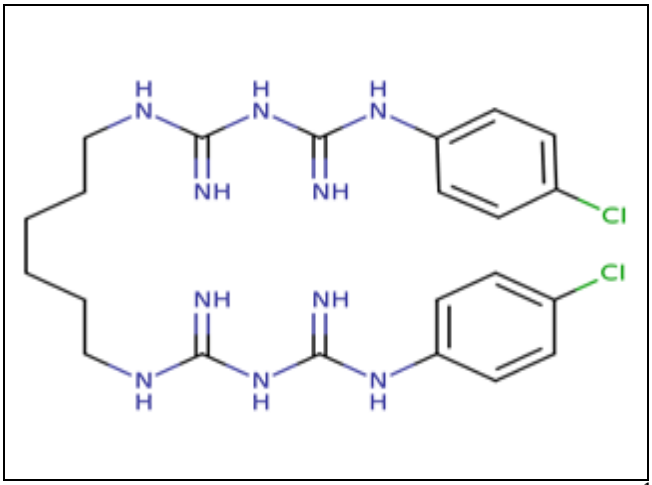

FIG. 1: STRUCTURE OF CHLORHEXIDINE GLUCONATE [1]

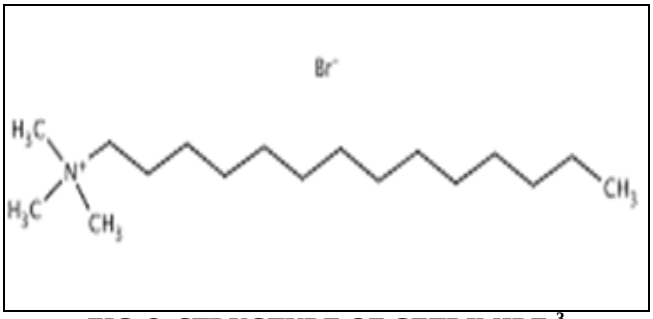

FIG. 2: STRUCTURE OF CETRIMIDE [3]

The main aim of the study was to develop a RP-HPLC method for the determination of Chlorhexidine gluconate and Cetrimide bulk and its formulation as no such method for is available in literature till date. The robustness of the developed method is then validated using DOE approach using Box-Behnken design of Response surface methodology and also normal distribution of the data is checked by application of Anderson-Darling normality test for statistical analysis.

MATERIALS AND METHODS:

Apparatus and Software: The liquid chromato-graphic system was of Waters, Ahmedabad and consisting of following components a gradient pump, PDA detector, a manual injection facility with 20 µl fixed loop. The chromatographic analysis was performed using Empower 3 software on a Hypersil BDS C18 column (250 × 4.6 mm, 5 µm particle size).

Materials: Chlorhexidine gluconate and Cetrimide was kindly supplied as a gift sample by Mil Laboratories Pvt. Ltd., Vadodara.

Reagents and Chemicals: Methanol and Acetonitrile used were of HPLC grade and were purchased from Fisher Scientific Pvt. Ltd., Double distilled water was prepared at the laboratory premises. All other reagents and chemicals used were of analytical grade.

Preparation of Mobile Phase Buffer: 20 mM phosphate buffer was prepared by dissolving 0.272 g of potassium dihydrogen orthophosphate in sufficient water to produce 100 ml. The pH was adjusted to 3 using orthophosphoric acid. The buffer was filtered through 0.22 µ membrane filter, stored at ambient temperature.

Preparation of Mobile Phase: (Phosphate Buffer (pH 3): ACN: Methanol (15:30:55) v/v/v): The appropriate volumes of phosphate buffer, acetonitrile and methanol were transferred into a reagent bottle, mixed thoroughly, sonicated for 10 min and filtered through 0.22 µm membrane filter and used as mobile phase.

Preparation of Stock Solutions (1000 ppm): 10 mg each of CH and CET were weighed accurately and transferred into a 10 ml volumetric flask containing 1 ml Acetonitrile. DDW was added up to the mark to produce a stock solution containing 1000 µl/ml of CH and CET respectively.

Preparation of Working Standards and Calibration Curve Solutions: For preparation of working standard solution, 2.5 ml each of CH and CET transferred into a 25 ml volumetric flask containing 2.5 ml Acetonitrile. DDW was added up to the mark to produce a stock solution containing 100 µl/ml of CH and CET respectively. Considering the ratio of CH and CET in commercial formulation to be 1:10 appropriate aliquots of CH and CET working standard solutions were taken in different 6 ml volumetric flasks each and diluted up to the mark with mobile phase to obtain final concentrations of 3 - 18 µl/ml and 30 - 180 µl/ml respectively.

Method Development: For development of liquid chromatographic method, various parameters were considered like 1) Selection of appropriate λ_{max} for detection. For this parameter, whole UV range was scanned by PDA detector [2]) Mobile phase selection was based on one factor at a time method (on trial and error basis). The pH of buffer was selected based on pKa values of drugs [3]) For selection of diluents of final samples, various solvents were tried [4]) The flow rate was also selected based on one factor at a time method (on trial and error basis).

Applicability of the Method: The developed HPLC method was applied for analysis of its formulation available in market. "Savlon antiseptic solution" manufactured by ITC was procured from local pharmacy. 0.5 ml of the sample formulation was withdrawn in a 50 ml volumetric flask and diluted up to the mark using Acetonitrile and DDW to produce a clear solution. The resulting solution was again diluted by withdrawing 1 ml and making up to 10 ml to give the final solution for analysis.

The final solution was analyzed and chromatogram was recorded. Concentrations of both analytes were then calculated from the calibration graph. Six replicate samples were used for analysis.

Method Validation: [5]

Linearity and Range: The proposed RP-HPLC method showed good linearity in the concentration range of 3 - 18 µg/ml for CH and 30 - 180 µg/ml for CET.

Precision: Inter-day and intra-day precision for the method were measured in terms of % RSD. The experiment was repeated 3 times in a day (Intraday precision) and the average % RSD values of the results were calculated. Similarly the experiment was repeated on 3 different days (Inter day precision) and the average % RSD value for absorbance of CH and CET were calculated. The low value of SD obtained confirms the precision of the method.

LOD and LOQ: Calibration curve was repeated for 9 times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were measured as follows. LOD = 3.3 * SD/slope of calibration curve, LOQ = 10 * SD/slope of calibration curve where SD = Standard deviation of intercepts

Accuracy: Accuracy of the method was confirmed by recovery study from marketed formulation at 3 level of standard addition (80%, 100%, and 120%) of label claim. Recovery greater than 98% with low SD justified the accuracy of the method

Robustness: The robustness of the method was determined by using DOE approach. In it Box-Behnken design of response surface methodology was utilized. The factors considered for development of design were flow rate, λ_{max} and mobile phase ratio whereas the responses considered for the study were retention time, theoretical plates and resolution between the peaks of CH and CET. The plots and the statistical analysis implicate the robustness of the method.

Statistical Analysis: [6, 11] In order to verify whether a statistical procedure follows a normal distribution or not, 3 common types of Normality tests are performed namely 1) Anderson-Darling Test 2) Ryan-Joiner Test 3) Kolmogorov-Smirnov Test. As Anderson-Darling Test is especially effective in detecting departure from normality in low and high values of distribution it is used in our study to verify the normality of data distribution.

RESULTS AND DISCUSSION: The present investigation is aimed to develop a new method for the simultaneous estimation of Chlorhexidine gluconate and Cetrimide by RP-HPLC method, its validation by application of DOE approach and verifying the normality of data by statistical Anderson Darling normality test. Literature reveals that there is no analytical method reported for the simultaneous estimation Chlorhexidine gluconate and Cetrimide by RP-HPLC method. Hence, it was felt that, there is a need of new analytical method development for the simultaneous estimation of Chlorhexidine gluconate and Cetrimide in pharmaceutical dosage form.

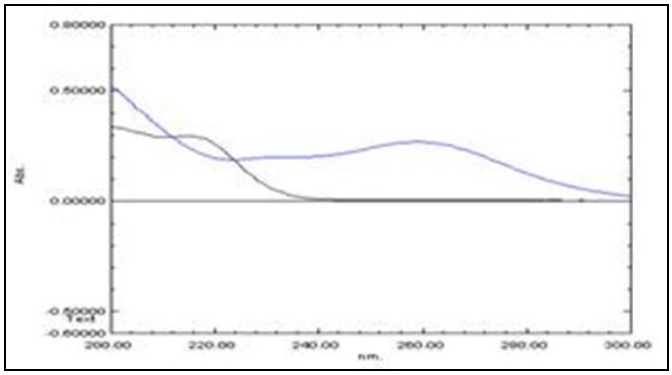

FIG. 3: SELECTION OF WAVELENGTH MAXIMA FOR COMBINATION DOSAGE FORM = 210 nm

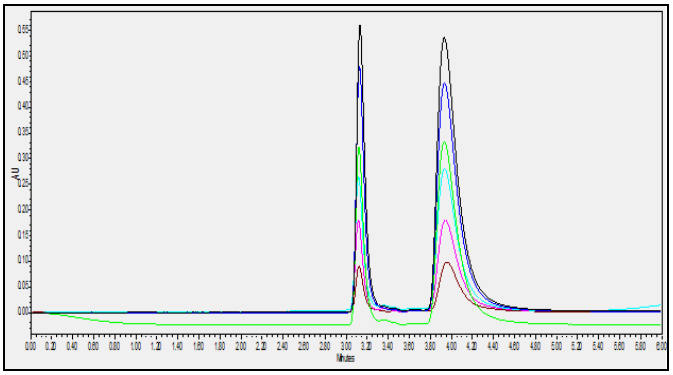

FIG. 4: OPTIMIZED PEAK AND CALIBRATION CURVE FOR CETRIMIDE AND CHLORHEXIDINE GLUCONATE

For optimization of chromatographic conditions for combined dosage form, various combinations of solvents, dilutions solvents, pH, λ_{max} were tried and finally chromatographic conditions selected for analysis of sample included detection wavelength of 210 nm, mobile phase ratio of (30:55:15 v/v/v)

ACN: methanol: phosphate buffer (KH_2PO_4 and K_2HPO_4) phosphate pH 3 (pH was adjusted with ortho-phosphoric acid) using Hypersil BDS C18 column (4.6 × 150 mm) 5µ, flow rate of 0.8 ml/min and diluents for sample preparation was selected to be mobile phase itself.

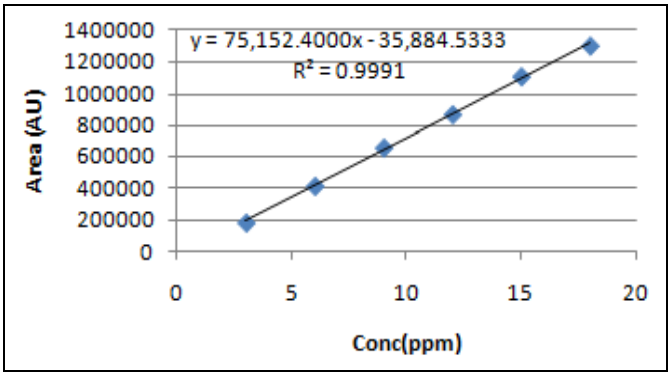

FIG. 5: CALIBRATION CURVE OF CHLORHEXIDINE GLUCONATE

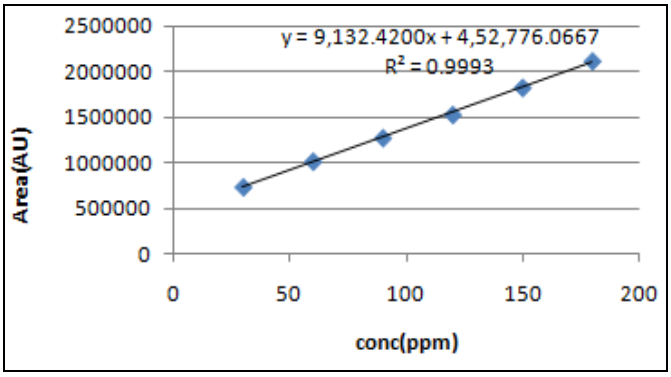

FIG. 6: CALIBRATION CURVE OF CETRIMIDE

TABLE 1: SUMMARY OF VALIDATION PARAMETERS OF RP-HPLC METHOD

Parameter	Chlorhexidine gluconate	Cetrimide
Analytical wavelength (nm)	210nm	210nm
Retention time (min)	3.8	3.2
Linearity range (µg/ml)	3-18	30-180
Regression equation	Y = 75,152.4000x – 35884.5333	Y= 9132.4200x+452776.0667
Correlation coefficient	0.9991	0.9993
Intraday precision (%RSD)	0.046%	0.893%
Inter day precision (%RSD)	1.06%	0.568%
LOD (µg/ml)	0.256	1.475
LOQ (µg/ml)	0.768	4.435
Accuracy (% Mean Recovery)	98-102%	98- 102 %

TABLE 2: SYSTEM SUITABILITY TEST (SST) PARAMETERS

Parameter	Data obtained for Cetrimide	Parameter	Data obtained for Chlorhexidine gluconate
Retention time (min) ± SD	3.2 ± 0.0784	Retention time (min) ± SD	3.8 ± 0.1893
Theoretical plate ± SD	8100 ± 228.492	Theoretical plate ± SD	2103 ± 103.380
Tailing factor ± SD	1.23 ± 0.29	Tailing factor ± SD	1.19 ± 0.11
Resolution	3.2 ± 0.264		

(*Data obtained from 6 replicate Injections)

TABLE 3: APPLICABILITY OF METHOD: FORMULATION ANALYSIS (SAVLON SOLUTION: 3% w/v OF CH AND 30% w/v IN 100 ml SOLUTION, MFG. BY: ITC)

Actual conc. (mg in 10 ml)	Amount of CH found (mg in 10 ml)	% Label claim	SD	% RSD	Actual conc. (mg in 10 ml)	Amount of CET found (mg in 10 ml)	% Label claim	SD	% RSD
3	3.018	100.600%	0.6751	0.6744%	30	30.002	100.006%	0.8318	0.8288%
3	3.029	100.966%			30	29.929	99.763%		
3	3.012	100.400%			30	30.129	100.430%		
3	2.978	99.266%			30	29.982	99.940%		
3	2.982	99.400%			30	30.600	102.000%		
3	3.001	100.033%			30	30.010	100.033%		

TABLE 4: APPLICATION OF DOE FOR ROBUSTNESS OF METHOD: BBD OF RSM [7, 9, 10]

Factors	Responses
Flow rate	RT of CET
Wavelength	RT of CH
pH	TP of CET
	TP of CH
	Resolution

TABLE 5: EXPERIMENTAL RUNS

Std.	Run	Block	Factor 1 A: Flow rate ml/min	Factor 2B: Wavelength nm	Factor 3 C: pH unit	Response 1 Rt of CET min	Response 2 Rt of CH min	Response 3 TP of CET	Response 4 TP of CH	Resolution
7	1	Block 1	0.7	210	3.2	3.21	3.9	2104	8102	3.25
9	2	Block 1	0.8	207	208	3.2	3.85	2202	8001	3.14
11	3	Block 1	0.8	207	3.2	3.2	3.8	2196	8110	3.09
6	4	Block 1	0.9	210	2.8	3.19	3.81	2099	8053	3.34
3	5	Block 1	0.7	213	3	3.2	3.86	2058	8201	3.01
8	6	Block 1	0.9	210	3.2	3.3	3.79	2150	8103	3.16
4	7	Block 1	0.9	213	3	3.1	3.8	2100	8194	3.12
5	8	Block 1	0.7	210	2.8	3.18	3.8	2083	8120	3.11
12	9	Block 1	0.8	213	3.2	3.2	3.82	2209	8102	3.06
10	10	Block 1	0.8	213	2.8	3.19	3.81	2100	8013	3.30
2	11	Block 1	0.9	207	3	3.21	3.82	2085	8038	3.28
1	12	Block 1	0.7	207	3	3.2	3.8	2108	8024	3.24

DOE PLOTS:

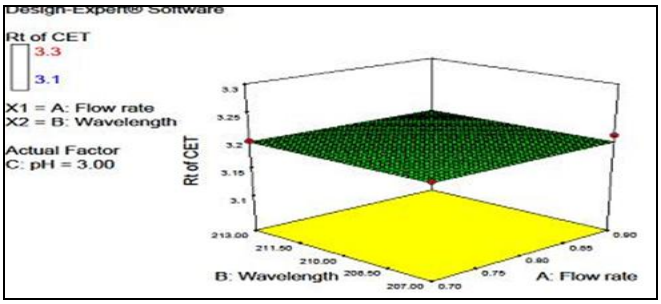

FIG. 7: 3D CONTOUR PLOT

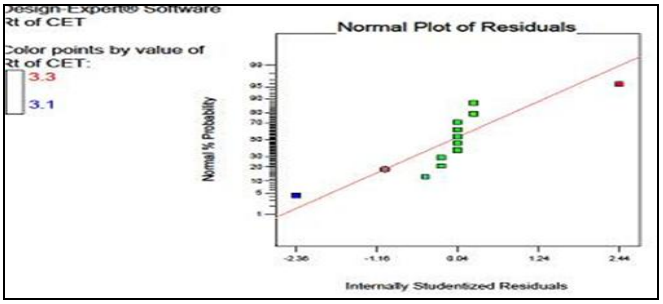

FIG. 8: NORMAL PLOT OF RESIDUAL

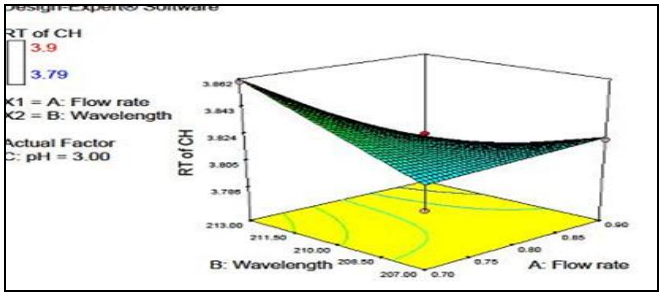

FIG. 9: 3D CONTOUR PLOT

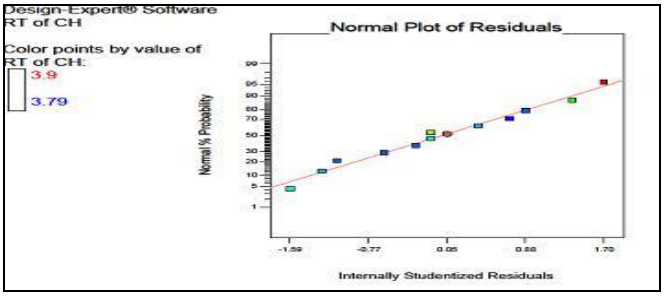

FIG. 10: NORMAL PLOT OF RESIDUAL

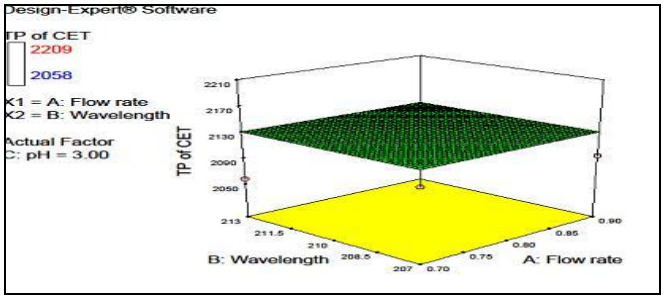

FIG. 11: 3D CONTOUR PLOT

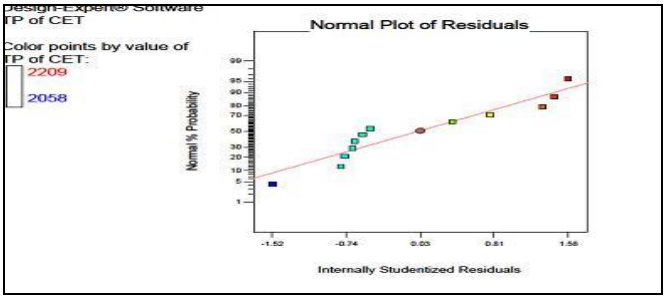

FIG. 12: NORMAL PLOT OF RESIDUAL

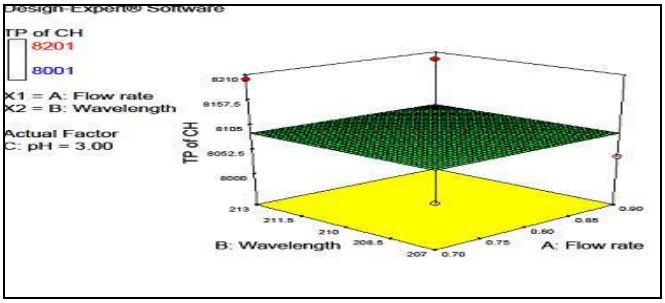

FIG. 13: 3D CONTOUR PLOT

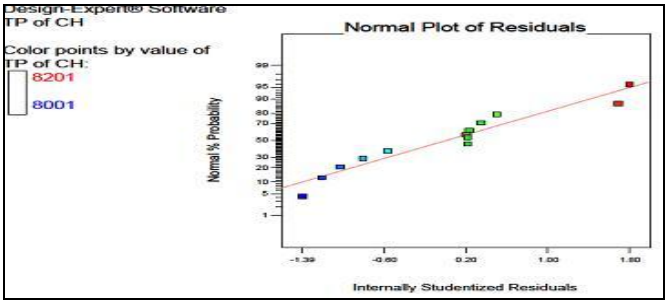

FIG. 14: NORMAL PLOT OF RESIDUAL

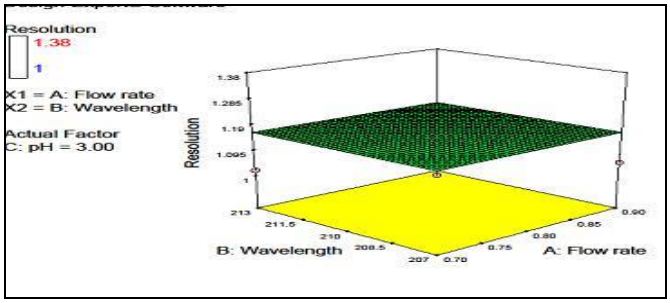

FIG. 15: 3D CONTOUR PLOT

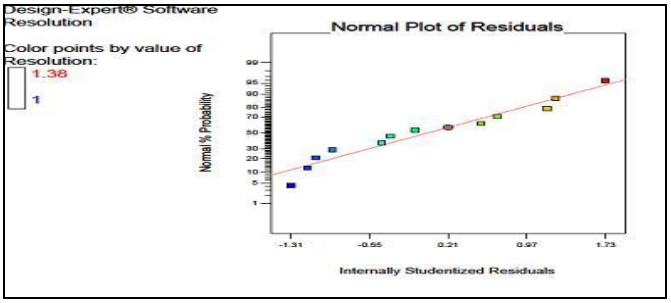

FIG. 16: NORMAL PLOT OF RESIDUA

TABLE 6: MODEL SUMMARY STATISTICS

Statistical parameters	R1	R2	R3	R4	R5
SD	0.034	0.071	50.86	65.47	0.12
Mean	3.2	3.83	2131.92	8088.42	1.18
CV%	1.06	1.86	2.39	0.81	9.79
R-Squared	0.0000	0.0000	0.0000	0.000	0.0000
Adjusted R^2	0.0000	0.0000	0.0000	0.0000	0.0000
Predicted R^2	-0.1901	-0.1901	-0.1901	-0.1901	-0.1901
F value	1.15	5.06	2586.99	4285.72	0.013
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
PRESS	0.015	0.066	33866	5603.97	0.18

$$AD = -N - \frac{2i-1}{N}\left(\ln(F(Y_i)) + \ln(1 - F(Y_{N+1-i}))\right)$$

$$AD^* = AD\left(1 + \frac{0.75}{N} + \frac{2.25}{N^2}\right)$$

TABLE 7: ANDERSON DARLING NORMALITY TEST FOR CHECKING NORMAL DISTRIBUTION OF DATA FOR CHLORHEXIDINE GLUCONATE

Data	Sorted	Count	F1i	1-F1i	F2i	Si	N Plt Line	N Plt Line
363678	363678	1	0.09323358	0.90676642	0.0922127	-4.7563052	377662.5	0.1
545997	545997	2	0.2102753	0.7897247	0.202243	-9.4728691	603319.8	0.26
728291	728291	3	0.38594605	0.61405395	0.399137	-9.3525411	759408.7	0.42
921163	921163	4	0.60086299	0.39913701	0.614054	-6.9794258	902180	0.58
1125552	1125552	5	0.79775701	0.20224299	0.7897247	-4.158199	1058269	0.74
1300085	1300085	6	0.90778734	0.09221266	0.9067664	-2.1407708	1283926	0.9

TABLE 8: TEST STATISTICS

0.1434	AD test statistic
0.17023	AD* test statistic
0.932945	P-value

Inference: p- value greater that alpha value of 0.05 signifies normal distribution of data

TABLE 9: ANDERSON DARLING NORMALITY TEST FOR CHECKING NORMAL DISTRIBUTION OF DATA FOR CETRIMIDE

Data	Sorted	Count	F1i	1-F1i	F2i	Si	N Plt Line	N Plt Line
730687	730687	1	0.09206744	0.90793256	0.0860661	-4.8378735	754570.3	0.1
1011844	1011844	2	0.21775591	0.78224409	0.2121202	-9.2249475	1081808	0.26
1269729	1269729	3	0.39094947	0.60905053	0.4128188	-9.1196179	1308160	0.42
1524638	1524638	4	0.58718123	0.41281877	0.6090505	-7.1979306	1515200	0.58
1821408	1821408	5	0.78787976	0.21212024	0.7822441	-4.3559842	1741553	0.74
2111775	2111775	6	0.91393389	0.08606611	0.9079326	-2.0524044	2068790	0.9

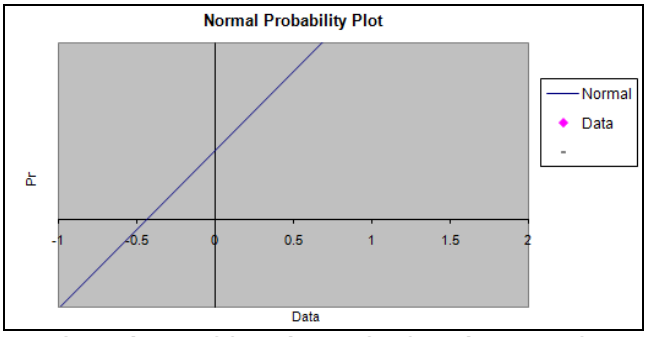

FIG. 17: ANDERSON DARLING NORMALITY PLOT

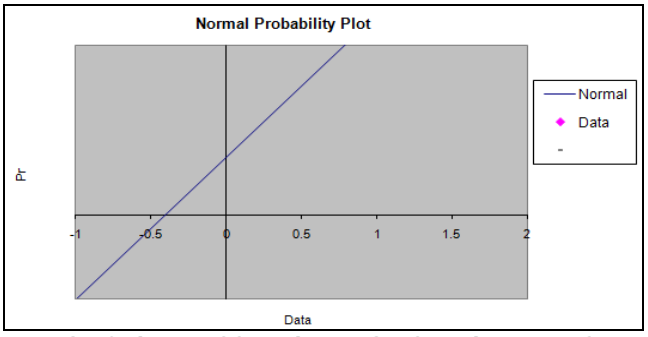

FIG. 18: ANDERSON DARLING NORMALITY PLOT

TABLE 10: TEST STATISTICS

0.1315	AD test statistic
0.156108	AD* test statistic
0.954926	P-value

Inference: p- value greater that alpha value of 0.05 signifies normal distribution of data

CONCLUSION: A simple RP-HPLC method was developed for the combined dosage form of Chlorhexidine gluconate and Cetrimide. The retention times were found to be 3.10 min and 3.9 min for Cetrimide and Chlorhexidine gluconate respectively. The % purity of Chlorhexidine gluconate and Cetrimide was found to be 99.92% and 100.45%. It was validated as per ICH guidelines and robustness study was done using DOE approach.

The only significant factor affecting the robustness of method was pH of mobile phase having impact on response of Retention time of Chlorhexidine gluconate. All other parameters were robust. Normal distribution of data was inferred by application of Anderson darling normality test.

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COMPETING INTERESTS: We hereby declare that the work submitted in this manuscript has not been published or under consideration in any other journal.

CONFLICT OF INTEREST: Nil

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