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RP-HPLC Estimation of Clobetasol Propionate and Salicylic Acid using Quality by Design Approach

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The RP-HPLC method for CLOP and SA estimation from bulk and pharmaceutical dosage from has been developed and validated. For analytical methods to be robust, current ICH guidelines, Q8 to Q11 were suggested use of analytical quality by design (AQbD) includes adoption of current systematic approaches. The proposed method was optimized and developed using Taguchi orthogonal design. The RP-HPLC method parameters were optimized by box-Behnken design. The stationary phase used C18 Princeton column (150mm × 4.6mm × 5μm) with acetonitrile: 0.05M phosphate buffer (pH 2.5, adjustment with by ortho - phosphoric acid) as mobile phase at ratio of 60:40v/v, 1.0 ml/min of flow rate along with UV-Visible wavelength of detection 240 nm. The linearity over concentration 5-15 μg/ml for CLOP and 600-1500 μg/ml for SA (r2 =0.9969 for CLOP and 0.9943 for SA) was found. The retention time for SA was 2.2 min. and CLOP 7.0 minute. The % recovery was found to be 98.0.3 SA and 97.84 or CLOP. As per ICH analytical method validation guidelines [Q2 (R1)], the RP-HPLC method was validated.

Keywords: Clobetasol propionate, Ointment analysis, RP-HPLC Salicylic acid.

Clobetasol propionate is clobetasol's 17-O-propionate ester. It's a powerful corticosteroid that's used in treatment of eczema and psoriasis, among other skin conditions. It works as an anti-inflammatory agent. Clobetasol propionate exerts its action by binding to cytoplasmic glucocorticoid receptors and then stimulates glucocorticoid receptor mediated expression of genes (Figure 01). This causes the stopping of inflammatory mediator's production to reduce while anti-inflammatory proteins production to rise. CLOP produces phospholipase A2 inhibitory proteins which causes stoppage of anti-inflammatory precursors release like arachidonic acid from membrane phospholipids [1-3].

Salicylic acid, also known as 2-hydroxy benzoic acid, is an antibacterial, antifungal, and keratolytic agent. Warts, psoriasis, corns, and other skin problems are treated with it. It softens and loosens dry, scaly, or thickened skin, allowing it to slip off or be readily removed.

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Salicylic acid permanently blocks the activity of COX-1 and COX-2, which results in reduced production of prostaglandins and thromboxanes from arachidonic acid. Because of its analgesic and anti-inflammatory properties, salicylate is used to treat rheumatic disorders due to their analgesic and anti-inflammatory properties[4,5].

As per the Literature, many analytical methods like RP-HPLC, UV-Visible Spectrophotometry, Colorimetry, GC, etc. had found for the SAL and CLO determination in combination with many other drugs[2,6-11]. The RP-HPLC methods reported earlier for this combination were gradient, longer Rt for CLO as 18.70 min.[12] and require column temperature 45°C with short Rt for SAL as 2.25 min[2]. Also, there is no analytical method with analytical quality by design approach has been reported for SAL and CLO combination. The 6% SAL and 0.0.5 % CLO formulation (Manufactured by Besto chem. Ltd. Delhi) marketed by Liva Healthcare Ltd. available to treat eczema, psoriasis with other skin condition issues.

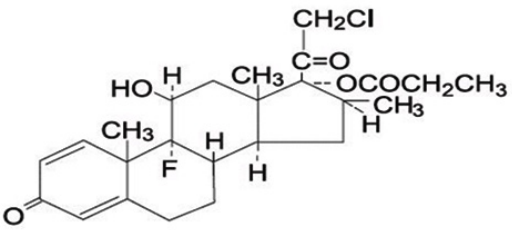

Fig. 1. Clobetasol propionate

MATERIAL AND METHODS

Chemicals and reagents

Clobetasol propionate a gift sample obtained from Orbicular Pharmaceutical Ltd, Hyderabad. Salicylic Acid a laboratory sample obtained from research-lab fine chem industries, Mumbai.

The (HPLC grade) solvents used were methanol, acetonitrile, water whereas chemicals were of analytical grade like sodium dihydrogen phosphate monohydrate (Merck), and sodium hydroxide (Research fine lab).

Instrumentation

Chromatographic analysis was carried out on Agilent (1200) Series) with EZ Chrome Elite 3.3.2 software. The stationary phase was C18 (Princeton) (4.6 mm x 150 mm x 5 mm), acetonitrile: Phosphate buffer (pH 2.5) as mobile phase with detection at 240 nm. The DOE software used was Design-Expert 13.

Methods

Preparation of Mobile phase and physical standard stock solutions

It was found that acetonitrile, and sodium dihydrogen phosphate buffer (pH 2.5) satisfactory resolution as compared to other mobile phases. The

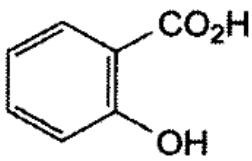

Fig. 2. Salicylic acid

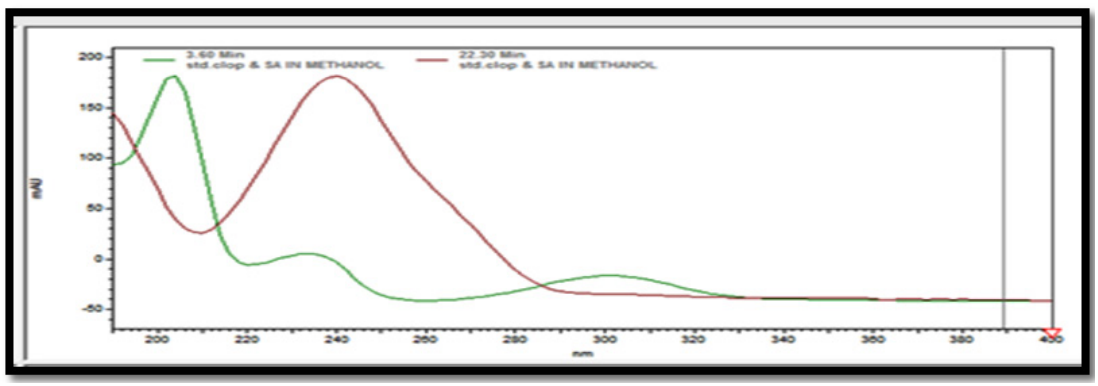

Fig. 3. Overlay spectrum of UV spectrum of SA and CLOP

optimum composition of mobile phase utilized was acetonitrile, 0.05 M sodium dihydrogen phosphate buffer (pH 2.5) (60:40) with flow rate 1ml/min, wavelength of detection 240 nm at room temperature. The mobile phase was filtered to remove particulate matter and sonicated to degas before its use. The standard stock solution of SA and CLOP were prepared using methanol. The mix working physical standard solution containing 10 ìg/ml CLOP and 100 ìg/ml of SA was prepared with appropriate dilution.

Linearity study

The aliquots solution of (0.05-0.15ml) of standard stock solution of clop and (0.6-1.5 ml) of SA was diluted to 10 ml of mobile phase. The last concentration in range of (5-15ìg/ml) for CLOP and (600-1500 ìg/ml) for SA solution was obtained by dilution with acetonitrile. Each concentration was analyzed in triplicate.

Pharmaceutical formulation analysis

The 2 gm of ointment has been weighed that contain (120 mg of SA and 1 mg of CLOP) and add 30 ml n-hexane solution and add 5 ml methanol two times and shake continuously 15-20 min. and separate the two layers in separator. After lower layer withdrawn, sonicated and diluted to 25.0 ml with mobile phase. Then 2.5 ml withdrawn diluted with mobile phase to 10.0 ml which is equivalent to (1200 ìg/ml) SA and (10 ìg/ml) CLOP.

RESULTS AND DISCUSSION

Wavelength selection

The appropriate selection of wavelength of detection in HPLC-UV analysis improves sensitivity of method. The wavelength at which both drugs show good response is called an ideal wavelength for the analysis drugs. The SA and CLOP standard solutions scanned over range of 200-400 nm. For simultaneous determination of both drugs from ointment dosage form, the wavelength 240 nm was used because both drugs had appropriate absorbance at 240 nm. (Figure 03).

Optimization of chromatographic parameters by using experimental design

Screening by Taguchi orthogonal model

Taguchi orthogonal model was used to screen the effect of chromatographic parameters

Table 1. Experimental factors for Taguchi orthogonal model

Experimental factors	Code	level low	High
Organic content of mobile phase (%)	A	40	60
salt of concentration	B	10	50
pH of aqueous phase	C	2.5	5.5
flow rate	D	0.8	1.2
column type	E	C8	C18
solvent type	F	Methanol	Acetonitrile
pH modifier	G	Sodium	Potassium

Table 2. Design matrix of Taguchi orthogonal model

Run	Organic content of mobile phase (%)	salt of concentration	pH of aqueous phase	flow rate	column type	solvent type	pH modifier
1	40	50	5.5	0.8	C8	Acetonitrile	Na^+ salt
2	60	10	5.5	0.8	C18	Methanol	Na^+ salt
3	40	10	2.5	1.2	C18	Acetonitrile	Na^+ salt
4	60	50	2.5	0.8	C18	Acetonitrile	K^+ salt
5	60	50	2.5	1.2	C8	Methanol	Na^+ salt
6	40	50	5.5	1.2	C18	Methanol	K^+ salt
7	60	10	5.5	1.2	C8	Acetonitrile	K^+ salt
8	40	10	2.5	0.8	C8	Methanol	K^+ salt

organic content of mobile phase (A), salt of concentration (B), pH of aqueous phase (C), flow usrate (D), column type (E), solvent type (F), pH modifier (G). On the basis of initial experiments, the range of value used in the design was A: 40-60 v/v, B:10-50, C:2.5-5.5pH, D: 0.8-1.2ml/min, E: C8-C18 F: acetonitrile and methanol F:10-50mM as shown in Table No.1. The experiments were run and responses were number of theoretical plates, retention time, and tailing factor.

The experimental general in Taguchi orthogonal model

The method variables were screened by Taguchi orthogonal design as shown in Table No. 2. The main goal of this study was to pinpoint the key factors that have a significant impact on method performance using a smaller number of experiments.

This design was used to evaluate the retention time, tailing factor, resolution,

Table 3. Responses Taguchi orthogonal model

Run	Response 1 Retention time		Response 2 Tailing factor		Response 3 Resolution		Response 4 No. of theoretical plates	
	SA	CLOP	SA	CLOP	SA	CLOP	SA	CLOP
1	2.30	14.3	1.45	2.27	0	3.90	2569	93
2	5.64	50	1.79	5	0	30	2436	2000
3	3.5	20.4	1.57	1.23	0	31.75	2280	11465
4	0.9	4.0	0.90	1.43	0	13.22	479	3262
5	5.6	50	1.29	5	0	30	1527	2000
6	1.68	50	1.73	5	0	30	1177	2000
7	2.12	6.98	1.20	1.19	0	15.81	814	7722
8	2.32	12.60	1.68	1.33	0	15.25	1394	2102

Table 4. Design matrix of BBD model for optimizing chromatographic variables

Run No.	Factor 1 Organic phase content (%)	Factor 2 pH	Factor 3 Flow rate	Responses Retention time		Tailing factor		Resolution	No.of theoretical plates	
				SA	CLOP	SA	CLOP	CLOP	SA	CLOP
1	50	4	1.2	1.4	1.88	1.07	1.88	12.52	2177	1943
2	50	5.5	1	1.6	5.4	2.91	1.65	0	191	2355
3	60	4	1	1.6	4.5	2.1	1.5	0	214	2237
4	70	4	1.2	1.2	2.4	2.5	1.5	7.89	2572	2535
5	60	5.5	1.2	1.16	3.1	1.2	1.5	9.32	721	2655
6	60	5.5	0.8	1.7	4.4	1.15	1.41	9.52	847	2984
7	60	2.5	0.8	2	5.2	1.13	1.5	0	2151	2944
8	60	4	1	1.6	4.5	2.1	1.5	0	214	2237
9	70	5.5	1	1.5	2.7	2.15	1.51	6	1027	2827
10	60	2.5	1.2	1.4	2.9	2.02	1.58	5.68	329	2661
11	60	4	1	1.6	4.4	2.13	1.71	8.15	397	2414
12	50	4	0.8	30	10	5	1.89	0	300	1998
13	60	4	1	1.6	4.4	2.13	1.71	8.15	397	2414
14	60	4	1	1.6	4.4	2.13	1.71	8.15	397	2414
15	50	2.5	1	1.8	7.1	2.5	1.7	10.71	485	1836
16	70	4	0.8	1.9	3.7	1.97	1.5	8.12	1861	3076
17	70	2.5	1	1.7	3.1	0.93	1.43	5.04	399	3068

no. theoretical plates responses as shown in Table No. 3.

The method variables were screened by Taguchi orthogonal design. The half normal and Pareto chart results shows solvent type, flow rate, pH and organic phase composition in mobile phase expressively affects critical quality attributes. In solvent acetonitrile, retention time of SA was 1.7-2.5 min. as compared to methanol. The overall performance of all design only affects the solvent factor.

Box-Behnken design

The critical method variables which affects critical method attributes were optimized using Box-Behnken design. A three level box-Behnken design with five center replication was applied for optimization of the organic phase content of mobile phase (A), pH of aqueous phase (B), flow rate (C) as the Table No. 4 show parameters and responses[13-14].

We used statistical multiple linear regression analysis to optimize the analytical data. The ANOVA and F test were used to select the best fitting second-order quadratic polynomial equation. In Table No. 5, the value of regression coefficient and their associated p- values are shown.

It was observed that pH of aqueous phase significantly affects the response (p- value <0.005).

The main effect of organic content, pH of aqueous phase and flow rate

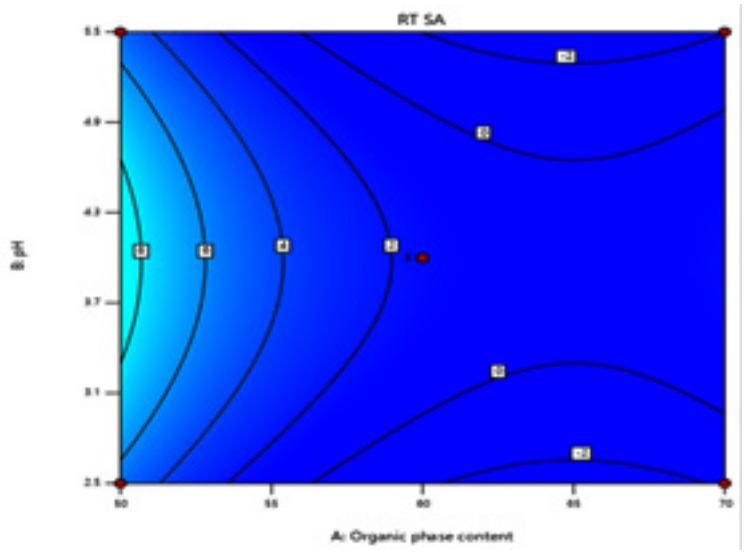

Fig. 4. Surface plot of RT vs organic content with aqueous phase pH

Table 5. Analysis of variance for response

Source	Sum of square	Df	Mean square	F – value	P – value	
Model	53.87	6	8.98	15.56	0.0002	Significant
A-Organic phase content	19.47	1	19.47	35.75	0.0002	
B -pH	0.9112	1	0.912	1.58	0.2374	
C- flow rate	21.19	1	21.19	36.73	0.0001	
AB	0.42	1	0.42	0.73	0.4122	
AC	11.63	1	11.63	20.16	0.0012	
BC	0.2500	1	0.25	0.43	0.5252	
Residual	5.77	10	0.57			
Lack of fit	5.76	6	0.95	319.84	< 0.0001	Significant
Pure error	0.0120	4	0.0030			
Cor total	59.64	16				

From the plots observed, the 60 % organic content, pH 2.5, flow rate 1 ml/min was investigated to obtain maximum responses.

Interaction plot of responses

The main effect plot for each factor was different for response. The % of organic content was increases and pH of aqueous phase 2.5 till the responses i.e. retention time of salicylic acid to be increase as shown in Figure No.4. The % of organic content was increases and pH of aqueous phase 2.5 till the responses i.e. tailing factor of salicylic acid to be increase. Figure No.5.

The % of organic content was 60% increases, aqueous phase pH 2.5 till the responses i.e. tailing factor of salicylic acid to be increase as shown in Figure No.6. The % of organic content was 60% increases & pH of aqueous phase 2.5 till the responses i.e. retention time clobetasol propionate to be increase (4-5min.) as shown in Figure No.7.

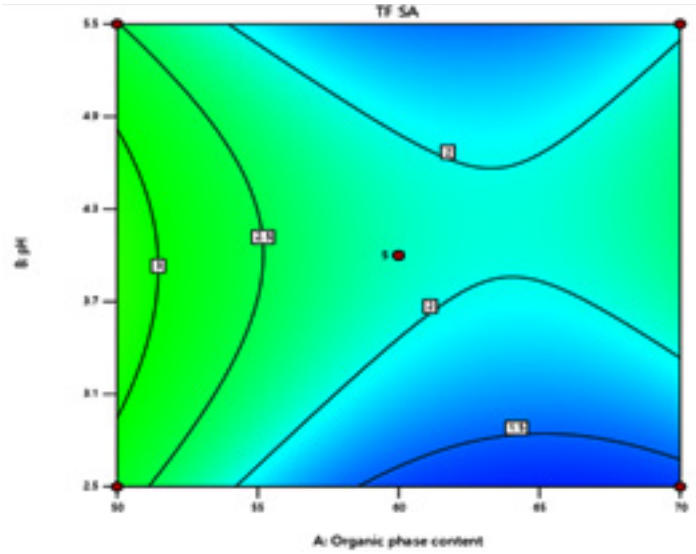

Fig. 5. Surface plot of Tf vs organic content with aqueous phase pH

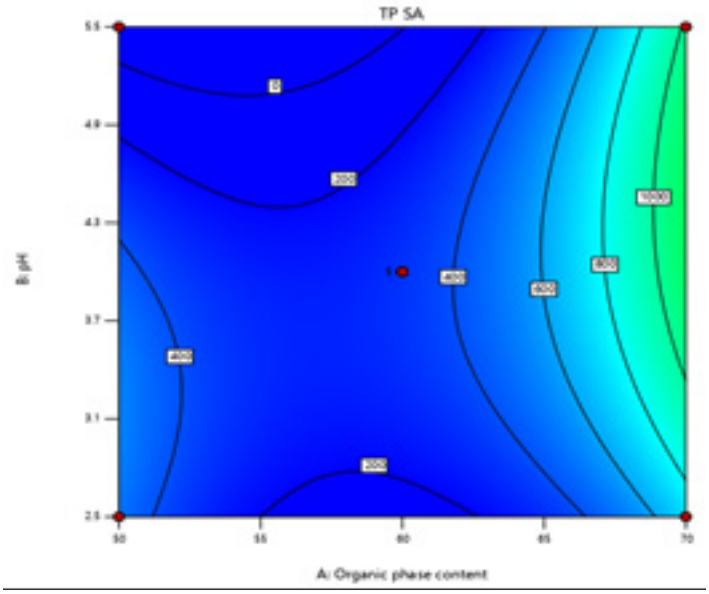

Fig. 6. Surface plot of TP vs organic content with aqueous phase pH

The % of organic content was 60% increases with aqueous phase pH 2.5 till the responses i.e. resolution for CLOP to be increase as shown in Figure No.8. The % of organic content was 60% increases and pH of aqueous phase 2.5 till the responses i.e. tailing factor clobetasol propionate to be less than 2.0% as shown in Figure No.9.

The % of organic content was 60% increases and pH of aqueous phase 2.5 till the responses i.e. theoretical plates clobetasol propionate to more than 2300 as shown in Figure No. 10.

The effect of variables like A, B and C on Rt was shown with the help of 2D response surface plots.

Optimized method parameters for experimentation

From the surface plot and interaction plots method parameters which makes the rugged method were chosen. The method to be considered rugged with respect to method parameters: organic

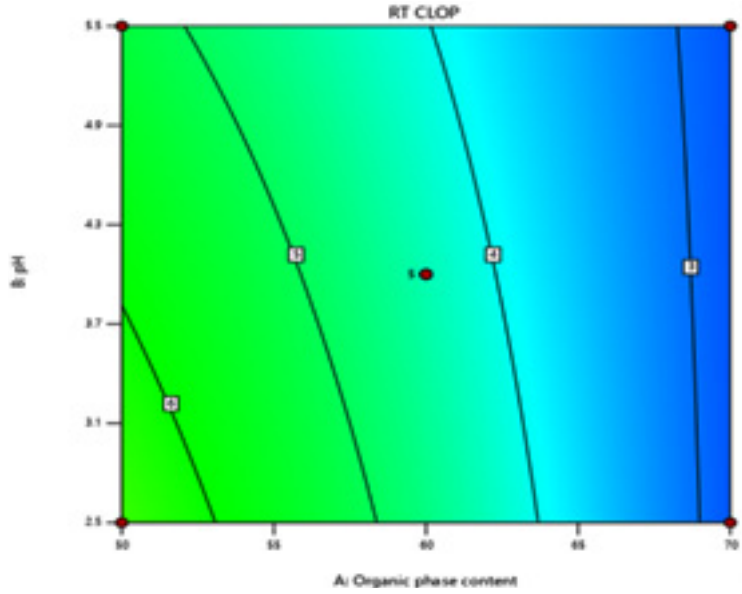

Fig. 7. Surface plot of RT vs organic content with aqueous phase pH

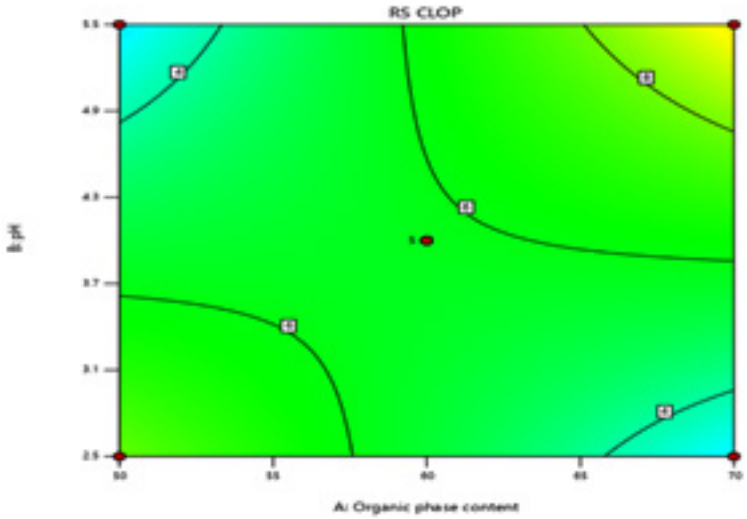

Fig. 8. Surface plot of RS vs organic content with aqueous phase pH

content of mobile phase (A) (50-70) %, pH of aqueous phase (B) (2.5 - 5.5) and flow rate (C) (0.8 -1.0 ml/minutes.) with the resolution more than 3, tailing factor less than 2.0% and retention time to be acceptable within 8 minutes as design space.

Experimental design was used to optimize the specific method variables and effect of variables on the method attributes. The Table No. 6 has been shown the chromatographic condition and selection of stationary phase, mobile phase and all the parameters which was very critical to the method development for clobetasol propionate and salicylic acid estimation by using Box- Behnken design.

Table 6. Optimized chromatographic condition by Box-Behnken design

Parameters	Chromatographic condition
Stationary phase	C18 Princeton (150 mm x 4.6mm x 5 µm)
Mobile phase	Acetonitrile, phosphate buffer pH 2.5 (60:40)
Wavelength of analysis	240 nm
Flow rate	1 ml/min
Loop capacity	20 µl

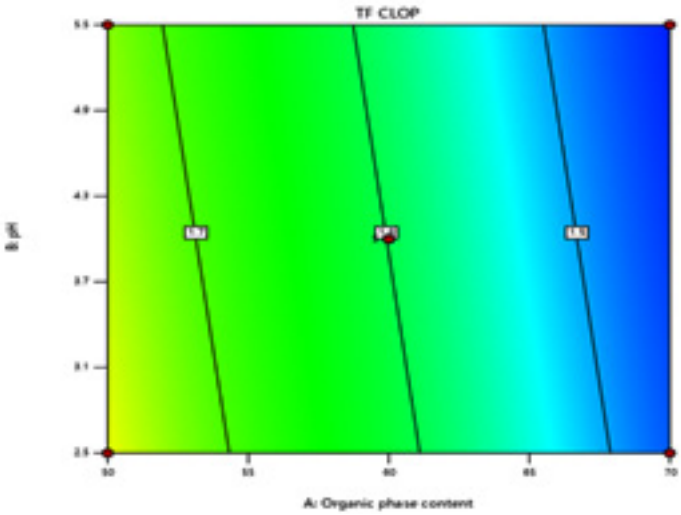

Fig. 9. Surface plot of Tf vs organic content with aqueous phase pH

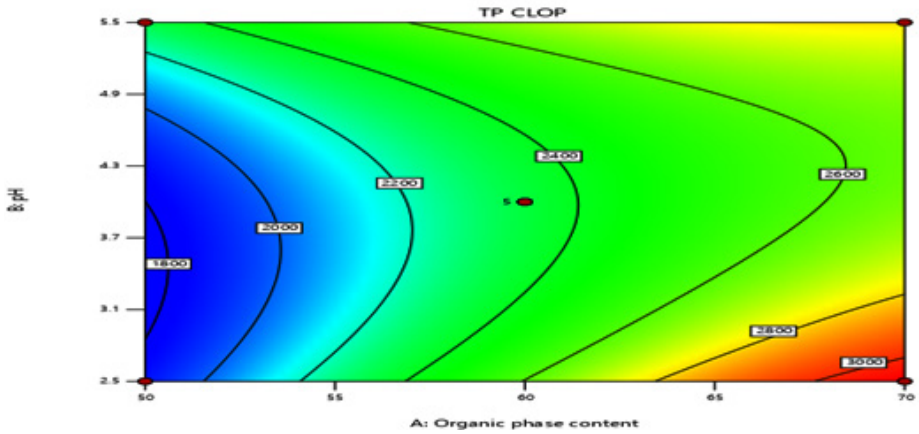

Fig. 10. Surface plot of RT vs organic content, and aqueous phase pH

By using optimized chromatographic condition from above Table No.6, the sample analysis of 10 ìg/ml of CLOP and (10 ìg/ml) of SA (10 ìg/ml) was performed. The chromatogram was obtained is shown in below Figure No.11.

Method validation

As per ICH Analytical method validation guidelines [Q2 (R1)], the proposed method was validated for parameters like accuracy, precision, linearity and range, LOD, LOQ, robustness, specificity etc. (ICH guidelines 2005).

System suitability

Using optimized chromatographic conditions, mix working standard solutions were analyzed. Each sample was analyzed five times. Table No.7 displays the results of system suitability.

Linearity study

The calibration curve was created by graphing the drug concentration against the peak area of SA and CLOP, respectively. The calibration curves are shown in Figure No.12 for SA and Figure No.13 for CLOP**.**

Table 7. Results of system of suitability

Sr.No	Peak area		Asymmetry		Retention time		RS	Theoretical plates	
	SA	CLOP	SA	CLOP	SA	CLOP	CLOP	SA	CLOP
1	128995	931198	1.94	1.51	1.74	4.45	9.33	1745	2345
2	123385	944235	1.93	1.54	1.7	4.43	9.19	1756	2384
3	130121	945224	1.85	1.56	1.69	4.28	8.96	1845	2436
4	128552	962541	1.86	1.49	1.69	4.23	9.23	1823	2311
5	130020	965414	1.89	1.52	1.74	4.33	9.45	1745	2345
Mean	128214.6	949722.4	1.894	1.524	1.712	4.344	9.232	1896	2364.2
SD	2487.639	12681.04	0.036111	0.024166	0.023152	0.084758	0.163021	42.56947	42.69614
%RSD	1.940215	1.335236	1.906597	1.585702	1.352317	1.951162	1.76583	2.245225	1.805944

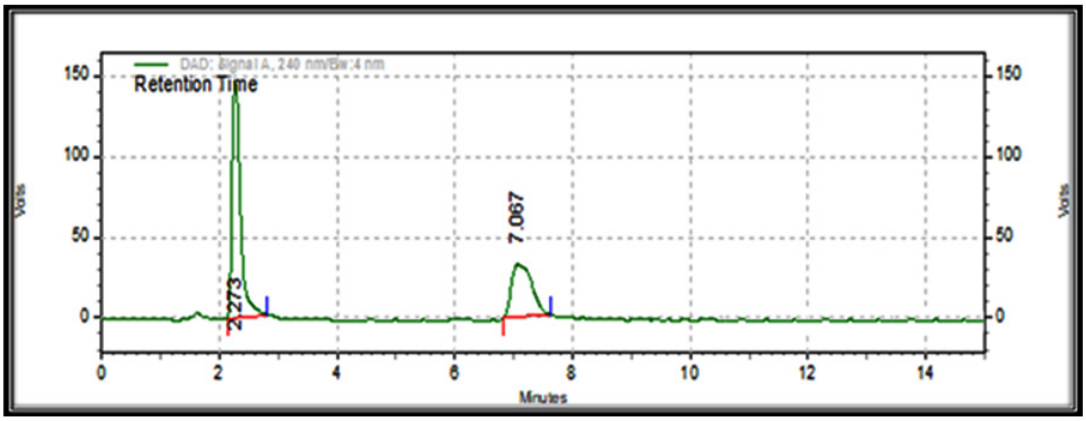

Fig. 11. HPLC Chromatogram of standard SA and CLOP

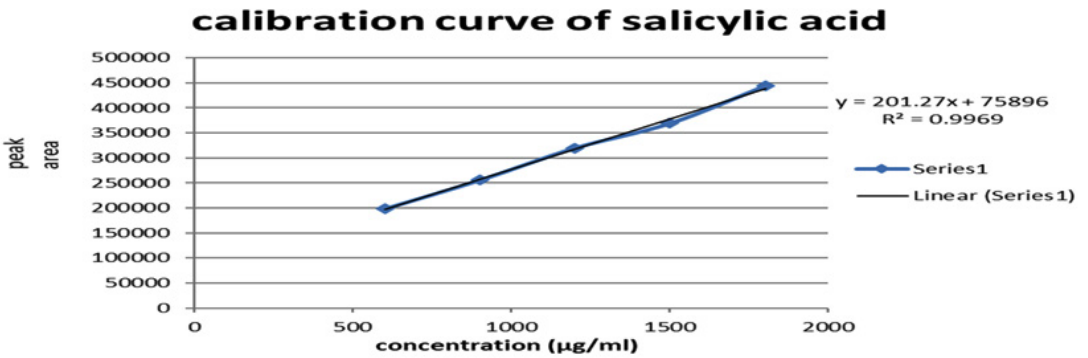

Fig. 12. Salicylic acid calibration curve

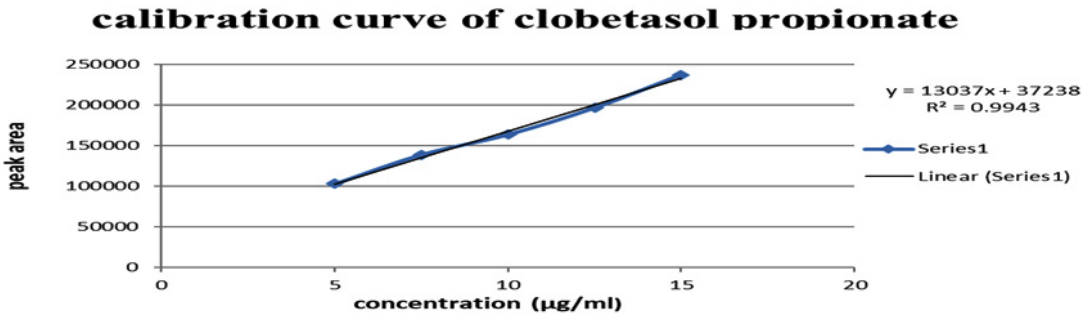

Fig. 13. Clobetasol propionate calibration curve

Table 8. Results of ointment analysis

Drug	Amount taken (µg/ml)	% Amount found ±SD	% RSD
SA	1200	99.56 ± 0.36	0.36
CLOP	10	95.78 ± 0.56	0.61

Table 9. Summary of validation parameters for HPLC method

Parameters	Observations	
	SA	CLOP
Linearity range (µg/ml)	600-1500	5-15
Regression equation	Y = 201.27x +75896	Y = 13037x + 37238
Correlation coefficient	0.9969	0.9943
% recovery ± SD	98.03 ± 0.77	97.84 ± 0.56
LOD	40.79	3.20
LOQ	123.59	9.72
Intra-day precision (% RSD)	0.65	0.89
Inter-day precision (% RSD)	1.05	0.69
Specificity	Specific	Specific
Robustness	Robust	Robust

The SA and CLOP were found linear with concentration range of 600-1500ig/ml with r^2 value 0.9969 and concentration range of 5-15ig/ml with r^2 value 0.9943 respectively.

Application of proposed method to laboratory mixture

Laboratory mixture analysis was conducted to see the feasibility of the optimized method for quantitative analysis. The % purity was found 98.41 ± 1.21 for SA with % RSD value 1.23 whereas 99.88 ± 1.40 for CLOP with % RSD value 1.43.

Application of proposed method to ointment analysis

The SA and CLOP from pharmaceutical ointment dosage form were analyzed by developed analytical method. The ointment formulation analysis results are depicted in the following Table No. 8.

Accuracy (% recovery)

The accuracy of analytical method for CLOP and SA estimation was determined at 80 %, 100 % and 120 % of the label claim. The accuracy of analytical method was determined by calculating the % recovery. The peak area was recorded of each analysis. The % recovery was found 98.03 ± 0.77 for SA with % RSD 0.78 and 97.84 ± 0.56 for CLOP with % RSD 0.58 respectively. The results shows the method to be accurate one reflecting with low % RSD values.

Precision, Repeatability study

The repeatability and intermediate

precision study was used to determine precision study of method. The intermediate precision and repeatability study was executed by analyzing physical mixture. The inter-day precision % RSD for SA was 0.65 whereas 0.89 for CLOP. The intra-day precision % RSD for SA was 1.05 whereas 0.69 for CLOP. In repeatability study, % RSD values were 0.83 for SA whereas 0.48 for CLOP. The % RSD value less than two signifies the method is precise.

Specificity and Selectivity

The developed method was found to be selective and specific as no other component eluting and interfering at SA and CLOP retention time. There was no any interference from formulation components and both drugs and resolved properly. The base line also did not shown any significant noise. No any co-elution observed during analysis while studying with photo-diode array detector. The photodiode array detector was used to assess and confirm the peak purity of SA and CLOP. The summary of validation parameters for HPLC method as shown in Table No. 9.

CONCLUSION

The proposed RP-HPLC method was simple, accurate, reproducible and robust for estimation of salicylic acid (SA) and (CLOP) clobetasol propionate in bulk as well as ointment dosage form. The SA and CLOP % recovery values were found 98.29 and 98.46 % signifies accuracy of the developed method. The method showed high precision as evidenced by the low relative standard deviations at each level of the recovery experiment. The proposed method was validated, developed and optimized as per ICH Q2 (R1) guidelines and using Taguchi orthogonal design respectively. Box- Behnken design was used to optimize HPLC method parameters. Clobetasol Propionate and Salicylic Acid analysis from pharmaceutical dosage forms, the developed and optimized method is effective and is a valuable quality control tool.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Funding Sources

Nil.

Authors' Contribution

K. S. Bagad, K. Bacchao, Dipak D. Patil were involved in study conception and design, data collection, S. B. Bagade, Dipak D. Patil in analysis and interpretation of results. R. D. Amrutkar, Dipak D. Patil in manuscript preparation along with all authors where all equally contributed.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

Ethics Approval Statement

No any studies on human or animal was conducted hence it is not applicable.

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