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Development and Validation of RP-HPLC-PDA Method for Estimation of Mometasone Furoate, Salicylic Acid, Methyl Paraben and Propyl Paraben in Combined Topical Formulation by Using Design of Experiment

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ABSTRACT

Objective: To develop simple, rapid and precise liquid chromatographic method for simultaneous determination of Mometasone furoate (MF), Salicylic acid (SA), Methylparaben (MP) and Propylparaben (PP) form pharmaceutical preparation.

Methods: Isocratic separation of MF, SA, MP and PP was achieved using Kromasil C_{18} (250 × 4.6 mm, 5 µm) analytical column, mobile phase used was acetonitrile: 0.1% acetic acid (60:40% v/v) at flow rate of 0.8 ml/min. Column was maintained at 30°C and detector was set at 264 nm. The analytes concentrations in sample were measured on weight basis to avoid the internal standard. The method is validated as per ICH analytical method validation guidelines.

Results: Developed method was linear with correlation coefficient >0.998 for all the analytes. The recovery values for MF, SA, MP and PP ranged from 98.56-101.70%, and %RSD was always less than 1.17. The ranges of the independent variables used for the optimization were ACN: 60-90%, Temp: 30-50°C and flow rate: 0.8-1.0 ml/min. The influences of these independent variables on the tailing factor (T_f) and resolutions (Rs) were evaluated. Using this strategy, mathematical model were defined and response surface were derived for the separation, responses were simultaneously optimized by using DOE.

Conclusion: Optimised RP-HPLC-PDA method found to be accurate, precise, specific and economic with short run time. The method would be useful for both qualitative and quantative analysis of commercial formulations in pharmaceutical industry and research laboratories.

INTRODUCTION

The present work describes a simple reverse phase, isocratic LC method for the determination of MF, SA, MP and PP in an ointment. Mometasone furoate (MF) is chemically (11β, 16α)-9,21-dichloro-11-hydroxy-16-methyl-3,20-dioxopregna-1,4-dien-17-yl 2-furoate, it is a topical corticosteroid; it has anti-inflammatory, anti-pruritic, and vasoconstrictive activities [1]. Salicylic acid (SA) is chemically 2-hydroxybenzoic acid, it has bacteriostatic, fungicidal, and keratolytic actions [2]. Methyl paraben is a preservative with the chemical formula $CH_3(C_6H_4(OH)COO)$, and is the methyl ester of *p*-hydroxybenzoic acid [3]. Propyl paraben, is n-propyl ester of p-hydroxybenzoic acid and chemically it is Na $[C_3H_7(C_6H_4COO)O]$ and is used as a food additive and as an antifungal preservation

[4]. MF is reported in USP [5] and EU [6], literature survey revealed that there are stability indicating-HPLC [7,8] and LC-MS [9], HPLC assay methods available for MF determination in combination with other analytes in topical [10], metered dosage formulations [11] and TLC methods for its determination in topical preparations [12,13]. There are various methods reported for SA which includes UV Spectroscopic [14]. HPLC [15] methods for its determination in combination with other analytes in topical formulations. HPTLC [16] methods for its determination in topical preparations is reported. There are various methods reported for MP and PP which includes UV Spectroscopic [17], and liquid chromatographic methods for its individual determination or in combination with other analytes in topical formulations and food stuffs [18-21].

Literature survey reveals that there is no method available for determination of these four analytes in combined formulations which are present in the commercial market. Further estimation of the preservatives is essential to control the quality of the formulations as per ICH Q 1 and Q 8 guidelines [22,23]. Therefore it was felt necessary to develop HPLC method to analyze the drugs and preservatives simultaneously form commercial preparations. HPLC is the widely used, well accepted and versatile tool for analysis of food and drugs these days. Our aim was to develop a method which estimates these analytes in a shorter time and to develop low cost method. Therefore the aim of the study was to develop and validate sensitive, precise, accurate and specific HPLC method for the determination of MF, SA, PP and MP simultaneously in formulation as per ICH analytical method validation guidelines [24].

MATERIALS AND METHODS

Materials and Reagents

MF (% purity, 99.6) was gifted by Avik Pharmaceuticals Ltd., Vapi, SA (% purity, 99.8), MP and PP were gift by Nulife Pharmaceutical Pvt., Ltd., Pune. HPLC grade Acetonitrile (ACN), methanol and analytical reagent grade acetic acid were purchased from Research Lab., Mumbai, Double distilled water was made available at lab scale. Ointment formulation HH SALIC (10 g) manufactured by M/s HeSa Pharmaceutica, Baddi, Solan (HP) containing, MF 0.1% w/w, SA 3.5% w/w, MP 0.2% w/w and PP 0.02% w/w was purchased from local market and used for analysis.

Instrumentation and Chromatographic Conditions

The HPLC system consisted of a binary pump (model Waters 515 HPLC pump), auto sampler (model 717 plus Auto sampler), column heater (Model CHM, Sr. No. A08CHM 289M) and PDA detector (Waters 2998). Data collection and analysis was performed using Empower - version 2 software (Waters Inc.). Separation was achieved on Kromasil C_{18} column (250 mm × 4.6 mm, 5.0 µ) maintained at 30 ˚C. Isocratic elution with ACN: 0.1% GAA (60:40% v/v) as mobile phase at the flow rate 0.8 ml/min was carried out. The detection was monitored at 264 nm and injection volume was 20 µl.

Preparation of Standard solutions and Calibration Curve

Standard stock solutions of MF, SA, MP and PP containing 1000 µg/ml in acetonitrile was prepared separately by dissolving appropriate amount of each standards separately. To study the linearity and range of each analyte, working solutions of mixed standards containing MF, SA, MP and PP were prepared from 1-10 µg/ml, 35-350 µg/ml, 2-20 µg/ml and 0.2-2 µg/ml, respectively in mobile phase and injected on to column. Calibration curves were plotted as concentration of drugs versus peak area response for each analyte.

System suitability test (SST) and Formulation Analysis

For System suitability test (SST), standard solution containing MF, SA, MP and PP, in the concentration 5, 175, 10 and 1 µg/m, respectively were prepared by mixing and diluting stock solutions with the mobile phase. System suitability was determined from six replicate injections of the SST standard before sample analysis. For estimation of analytes form the ointment, 2 g of formulation equivalent to 2 mg of MF (70 mg of SA, 4 mg of MP and 0.4 mg of PP) was weighed and transferred to 100 ml volumetric flask containing 60 ml of acetonitrile and heated on hot water bath for 5-10 min. Further solution was sonicated for 20 min. and volume was made up to 100 ml with mobile phase and it was filtered through 0.45 micron membrane filter. Filtrate was suitably diluted with mobile phase to get a solution of 5 µg/ml of MF (175 µg/ml of SA, 10 µg/ml of MP and 1 µg/ml of PP) and injected in to the column to get the chromatogram.

Method validation

The HPLC method was validated in terms of precision, accuracy, specificity, sensitivity, robustness, solution stability and linearity according to ICH guidelines.

Assay method precision of repeatability was studied by injection target analyte concentration six times and %RDS was calculated. Inter-day and intra-day method precision was determined using three concentrations and three replicates. The assay method was evaluated for recovery of the standards from excipients. To test method recovery three different quantities (80, 100, and 120%) of the standards were added to preanalysed formulation and analysed using the developed HPLC method. Limit of detection (LOD) and Limit of quantitation (LOQ) values were calculated by using σ (SD of response) and b (slope of the calibration curve) using equations, LOD=(3.3 × σ)/b and LOQ=(10 × σ)/b. Calculated method sensitivity values were confirmed by repeated

injection of samples containing amounts of analyte in the range of the LOD and LOQ. To determine the robustness of the method, the final experimental conditions were intentionally altered, and the results were examined. The flow rate was varied by ±5%; column temperature by ±2°C; the effect of columns from different suppliers was studied; the measurement wavelength was varied by ±1 nm; the injection volume was changed by ±2 µl, and the organic solvent content changed by ±5%. The short-term stability of the drug solution was determined by storing it at room temperature for 12 h and then analysing; the long-term stability was determined by storing it at 4°C for 30 days. Autosampler/mobile phase stability was determined by keeping the samples for 24 h in the autosampler.

RESULTS AND DISCUSSION

Method Development and Optimization of Chromatographic Condition by DOE

Well-defined symmetrical peaks were obtained upon measuring the response of eluent under the optimized conditions after thorough experimental trials that can be summarized. Two columns were used for performance investigations, including Kromasil C_{18} (4.6 × 250 mm, 5 µ) and Waters, symmetry C_{18} (4.6 × 250 mm, 5 µ) columns, the first column was the most suitable one since it produced symmetrical peaks with high resolution.

Trial-1 development was initiated with mobile phase based on the literature information with ACN: Methanol: Water (25:25:50% v/v/v) at ambient temperature. Further, several modifications in the mobile phase composition were tried in order to study the possibilities of changing the selectivity of the chromatographic system. These modifications included the change in the ratio of the organic solvents, pH (0.3-6.5), flow rate (0.7-1.3 ml/min.), temperature (ambient to 35 °C), and use of different columns. It was observed that acetonitrile as organic solvent and Kromasil C_{18} column gave better elution with respect to peak shape and system suitability parameters. Therefore acetonitrile as organic phase and Kromasil C_{18} column were considered for further development trials.

Further, the effect of changing the ratio of ACN (40-80%) on the selectivity and retention times was investigated. **Figure 1A** shows representative chromatograms obtained during optimization trials 1. During trials 2, 60% ACN was found to be most suitable, giving well resolved peaks and high theoretical plates. The effect of flow rate on the formation and separation of peaks was studied by varying the flow rate from 0.5-1.0 ml/min; a flow rate of 0.8 ml/min was optional for good separation and resolution of peaks in a reasonable time. Initially the SA showed peak asymmetry (T_f 1.5-1.8), with adding 0.1% glacial acetic acid (GAA) in mobile phase, symmetric peak was observed with tailing, 1.0-1.2 without affecting other peaks **(Figure 1B)**. The UV detector response of MF, SA, MP and PP were studied and the best wavelength was found to be 264 nm **(Figure 2)** showing highest peak sensitivities without any baseline disturbances.

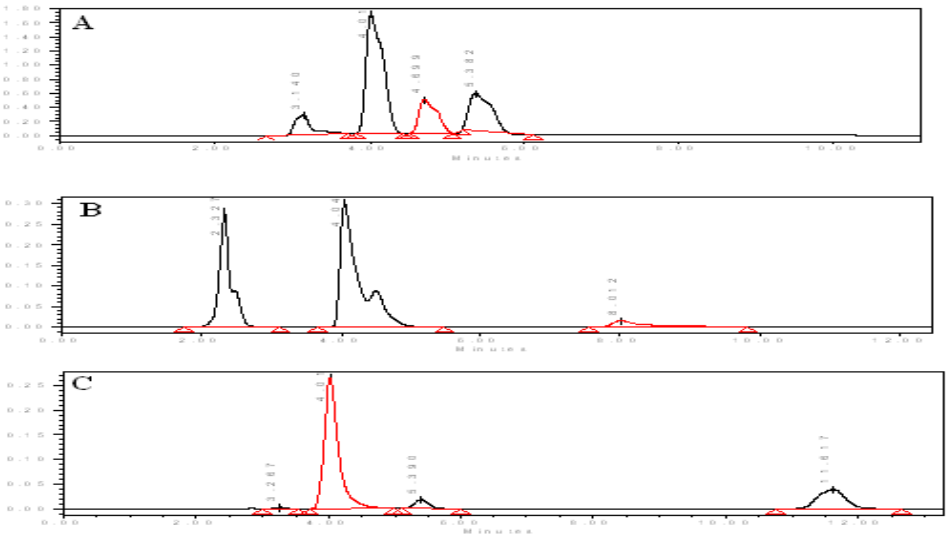

Figure 1. Representative chromatograms obtained during trials A, B and C.

Therefore trial 3 was performed using kromasil C_{18} column (250 mm × 4.6 mm, 5.0 µ), ACN: 0.1% GAA (60: 40% v/v) as mobile phase at 0.8 ml flow rate and column maintained at 30°C. Sufficient separation was achieved with the chromatographic conditions and same conditions were considered to study the effect of various parameters and for further development using scientific approaches and to optimize chromatographic method **(Figure 1C)**. Chromatographic data form trial 3 was feed in DOE software, factors selected with level are shown in **Table 1** which was feed in software, and software predicated 19 runs with various parameter levels **(Table 2)**.

All experiments were conducted in randomized order to minimize the effects of uncontrolled variables. Replicates (n=6) of the central points were performed to estimate the experimental error. Chromatographic data for 19 set of parameters was generated as suggested by DOE software. Experiments were carried out as per run, data generated was analysed and results of experimental trials conducted were compiled **(Table 2)**.

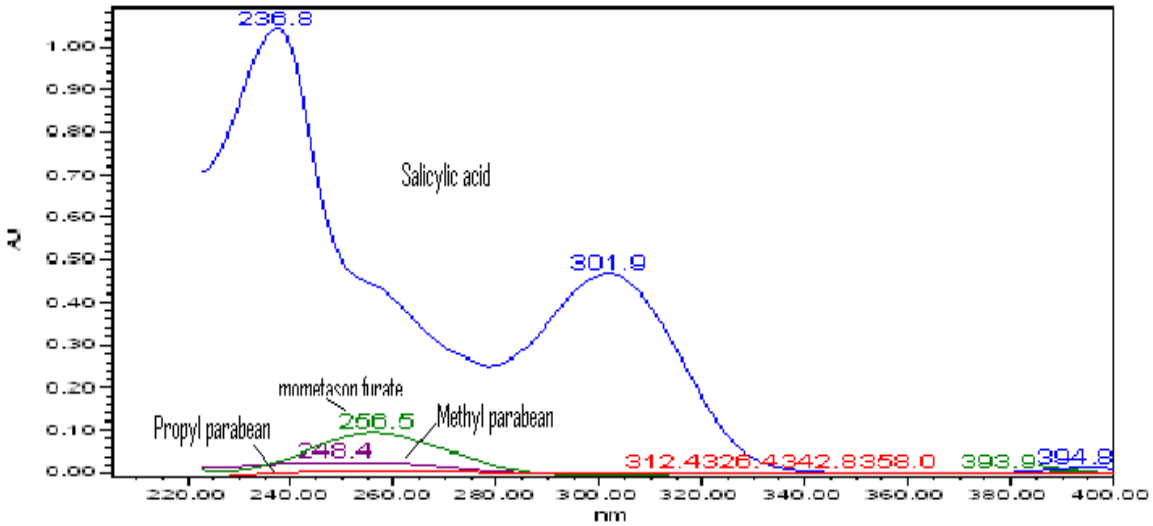

Figure 2. Online overlain spectra of analytes.

Table 1. Design factor.

Name	Units	Level	
		Low	High
Flow rate	ml/min	0.8	1.0
Concentration of Acetonitrile	v/v	60	90
Temperature	˚C	30	50

Table 2. Central composite design, responses and software validation data.

Factor*				Experimental Responses#						
Run	A	B	Temp. (°C)	T_f PP	R_s PP and SA	T_f SA	R_s SA and MP	T_f M.P	R_s MP and MF	T_f MF
1	0.80	80.00	30	5.31	1.035	4.12	1.913	4.23	3.521	5.2
2	0.90	70.00	30	2.9	1.352	2.65	2.084	2.51	4.416	2.42
3	1.00	70.00	32	3.4	1.428	2.9	2.171	2.7	3.825	2.72
4	0.90	60.00	32	2.6	1.725	2.4	2.436	2.5	5.175	2.4
5	0.90	80.00	30	5.52	1.004	4.62	1.845	4.92	3.647	5.84
6	1.00	70.00	28	2.9	1.697	2.6	2.299	2.3	4.867	2.3
7	0.80	60.00	30	1.5	1.119	1.61	2.705	1.42	5.011	1.31
8	1.00	60.00	32	2.8	1.792	2.7	2.421	2.5	5.231	2.3
9	0.90	60.00	28	1.6	2.101	1.59	2.813	1.61	6.026	1.54
10	0.80	80.00	28	4.96	1.011	4.52	2.152	4.87	3.592	5.63
11	1.00	80.00	30	5.75	1.105	4.75	1.654	5.32	3.304	6.23
12	1.00	80.00	32	5.81	1.002	5.21	1.521	5.62	3.162	5.1
13	0.90	60.00	30	2.3	2.025	2.2	2.612	2.1	5.725	2.1
14	0.90	70.00	28	2.4	1.063	2.41	2.131	2.6	5.326	2.26
15	0.80	60.00	30	2.5	1.756	1.9	2.135	1.8	5.531	1.7
16	0.80	60.00	32	1.7	1.910	1.82	2.525	1.7	4.803	1.54
17	0.80	60.00	28	1.1	2.394	1.31	2.998	1	5.946	1.1
18	1.00	60.00	28	1.7	2.016	1.73	2.524	1.85	5.792	1.72
19	0.80	70.00	28	2.1	1.305	2.21	2.326	2.24	5.411	1.9

*A-Flow rate, B-concentration of methanol, C- pH and #T_f: tailing factor and R_s: Resolution.

Response Surface Plots

Before starting an optimization procedure, it is important to investigate the term using factorial design with centre point. ANOVA generation for 3^2 factorial design shows that curvature is significant for all the responses (Rs) and tailing factor (T_f) since p-value is less than 0.05 [25]. This implies that a quadratic model should be considered to model the separation process. In order to obtain second order predictive model, central composite design (CCD) is employed, which is a design type under responses surface model. CCD is chosen due to its flexibility and can be applied to optimize an HPLC separation by understanding of factor's main and interaction effects. The selection of key factors examined for optimization was based on preliminary experiments and prior knowledge from DOE literature **(Table 3)**.

Response surface methodology as experimental design was used to determine the effect of independent variables on all possible dependent variables. In this experimental design the three factors viz, flow rate, concentration of acetonitrile and temperature were considered to find out their effects on tailing and resolution.

Table 3. ANOVA used to generate statistical models.

Response model	SS	df	Ms	F value	P value	R^2	Precision
Tailing (PP)	43.65	9	4.85	357.23	<0.0001	0.9972	56.262
Resolution (between PP and SA)	1.43	3	0.48	34.61	<0.0001	0.8738	13.391
Tailing (SA)	27.36	9	3.04	138.98	<0.0001	0.9929	34.179
Rs (between SA and MP)	0.50	3	0.17	43.45	<0.0001	0.8968	20.337
Tailing (MP)	35.80	9	3.98	76.77	<0.0001	0.9871	24.913
Rs (between MP and MF)	0.78	3	0.26	76.68	<0.0001	0.9388	24.719
Tailing (MF)	52.37	9	5.82	100.25	<0.0001	0.9901	28.211

ss: Sum of Square, df: Degree of Freedom, Ms: Means Square.

The effect of the variables on the tailing and resolution in formulations are shown in following equations (eq. 1-7)-

$R1=2.95+0.38A+1.72B+0.28C-0.12AB+0.04AC-0.13BC-0.12A^2+0.94B^2-0.13C^2$ (1)

$R2=0.34+(4.299E-004A) -0.33B-0.03C$ (2)

$R3=2.55+0.34A+1.38B+0.12C+0.08AB +0.13AC-0.25BC-0.16A^2+0.81B^2+0.06C^2$ (3)

$R4=0.77-0.06A-0.17B-0.05C$ (4)

$R5=2.44+0.37A+1.61B+0.07C+0.08AB+0.18AC-0.24BC-0.29A^2+1.06B^2+0.17C^2$ (5)

$R6=1.48+(2.711E-003A)-0.2B-0.1C$ (6)

$R7=2.45+0.36A+1.92B-0.28C+0.04AB+0.07AC-0.44BC-0.29A^2+1.47B^2-0.08C^2$ (7)

In above equations parameters A, B and C are flow rate, concentration of acetonitrile and temperature, respectively.

From the equations 1, 3, 5 and 7 the factors A, B and C shows positive effect on tailing individually. In combination AB and AC shows positive effect except in eq.1, in this eq. BC shows negative effect. When parameter A, B and C were increased (doubled) A shows negative effect, B shows positive effect and C shows positive effect for eq.1 and eq.3 and negative effect for eq.7 **(Figures 3-9)**.

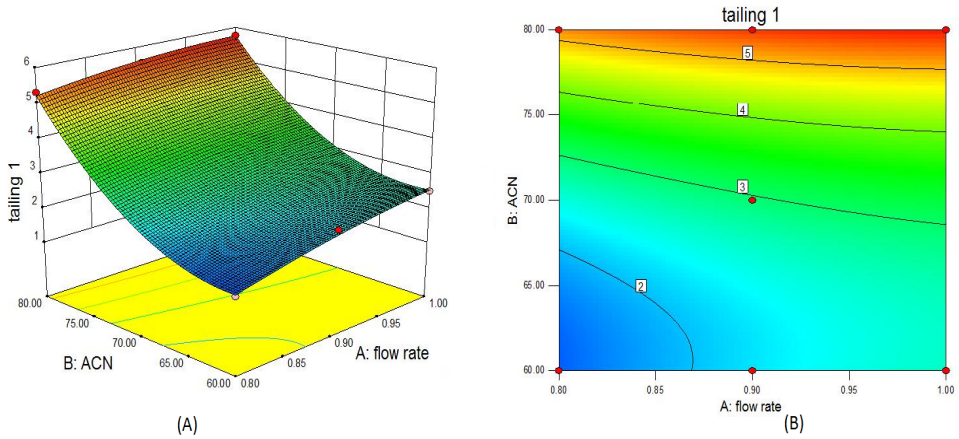

Figure 3. (A) Response surface plot showing the influence of flow rate and concentration of acetonitrile on tailing (PP) and (B) Counter plot showing the relationship between various levels of flow rate and concentration of acetonitrile.

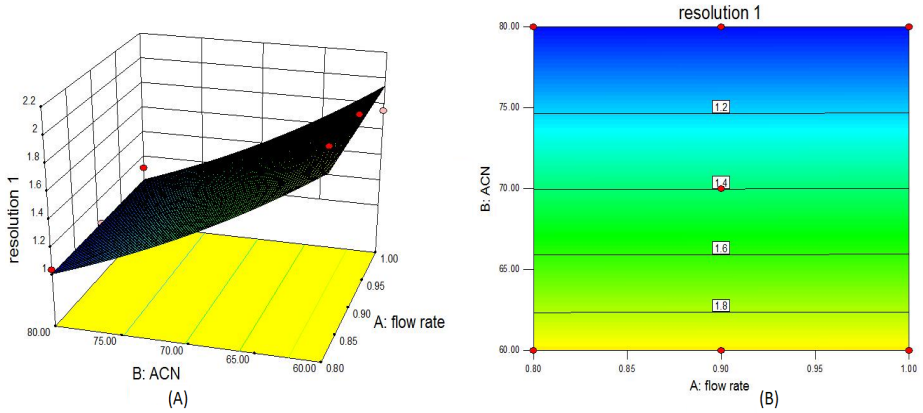

Figure 4. (A) Response surface plot showing the influence of flow rate and concentration of acetonitrile on resolution {between PP and SA} and (B) Counter plot showing the relationship between various levels of flow rate and concentration of acetonitrile.

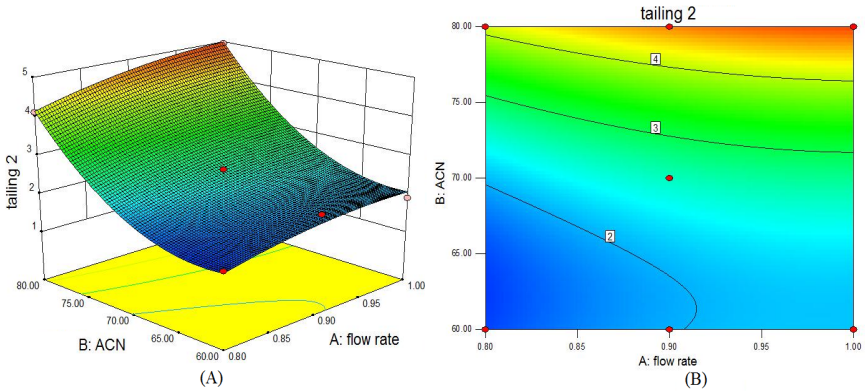

Figure 5. (A) Response surface plot showing the influence of flow rate and concentration of acetonitrile on tailing (SA) and (B) Counter plot showing the relationship between various levels of Flow rate and Concentration of acetonitrile.

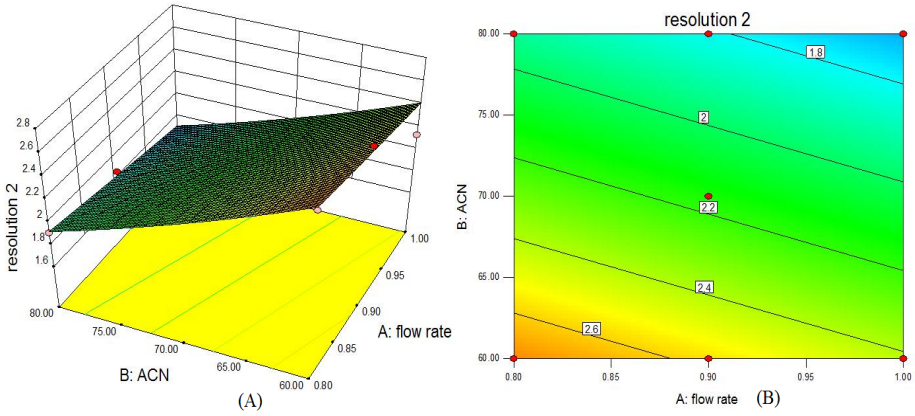

Figure 6. (A) Response surface plot showing the influence of Flow rate and Concentration of acetonitrile on resolution {between SA 7 MP} and (B) Counter plot showing the relationship between various levels of Flow rate and Concentration of acetonitrile.

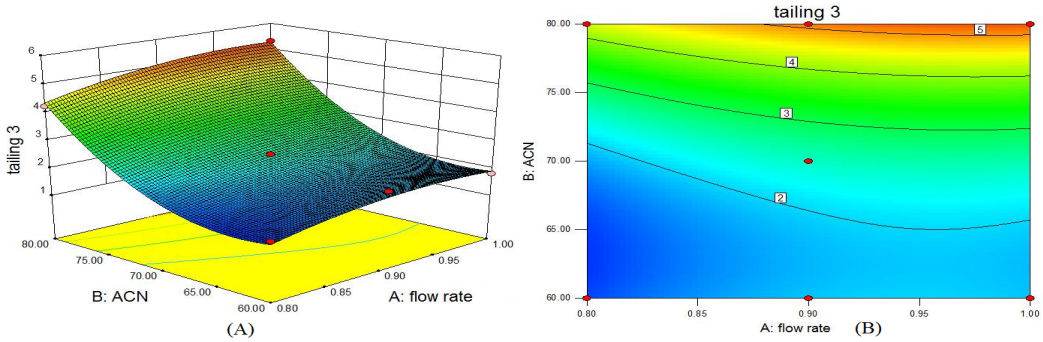

Figure 7. (A) Response surface plot showing the influence of Flow rate and Concentration of acetonitrile on tailing (MP) and (B) Counter plot showing the relationship between various levels of Flow rate and Concentration of acetonitrile.

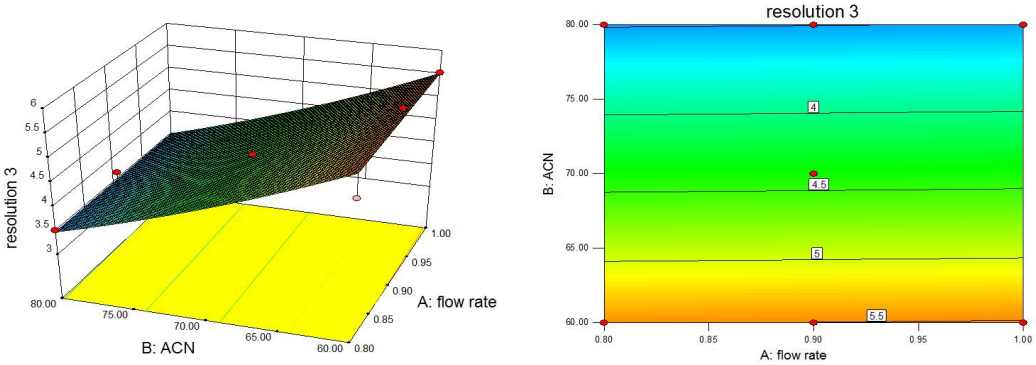

Figure 8. (A) Response surface plot showing the influence of Flow rate and Concentration of acetonitrile on resolution {between MP and MF} and (B) Counter plot showing the relationship between various levels of Flow rate and Concentration of acetonitrile.

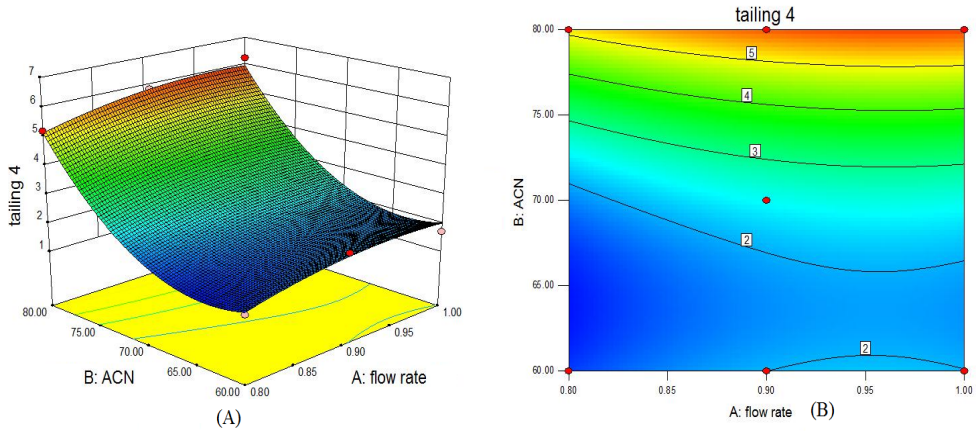

Figure 9. (A) Response surface plot showing the influence of flow rate and concentration of acetonitrile on tailing (MF) and (B) Counter plot showing the relationship between various levels of flow rate and concentration of acetonitrile.

The adequate precision value is measure of the signal to noise ratio and ratio greater than 4 is desirable [26]. The study reveals that changing the fraction of ACN, TEMP and flow rate form low to high shows effect on retention time and resolution of analytes. Counter plot and response surface plot for tailing and resolution factor are illustrated in **(Figures 3-9)**. Counter plot explain the relationship between various levels of flow rate and concentration of methanol. Response surface plot represents influence of flow rate and concentration of methanol on tailing and resolution of drug (% acetonitrile concentration plotted vs. flow rate when temp. held at constant at the centre value). Counter and response plots generated with the optimization models revealed that factor A, B and C have significant effect on separations of analytes.

Validation of design expert software

After statistical analysis by design expert software, predicted optimized results shown by design expert was recorded and which is batch 17. Predicted results (software generated) were compared with observed results (experimental values) and experiments were validated as shown in **Table 4**. Chromatogram obtained by using optimised conditions for standard is presented in **Figure 10** and typical chromatogram of ointment formulation is presented in **Figure 11**.

Table 4. Validation of design experiment software.

Sr. No.	Batch. 17	Predicted value		Observed value		SEM *
		Tailing	Resolution	Tailing	Resolution	
1	PP	1.04	-------	1.04	--------	0.0866
2	SA	1.4	2.01	1.08	2.181	0.1099
3	MP	1.28	2.88	1.21	3.3047	0.1692
4	MF	1.02	6.09	1.05	5.923	0.1791

*SEM: Standard Error of Mean.

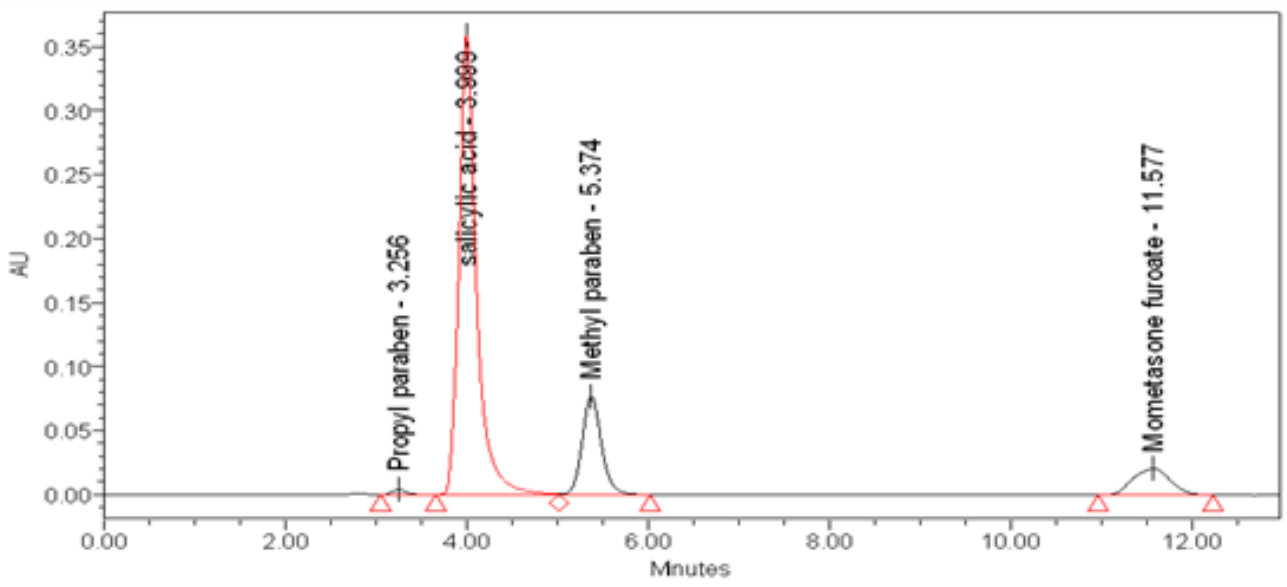

Figure 10. Typical chromatogram of standards using optimized conditions.

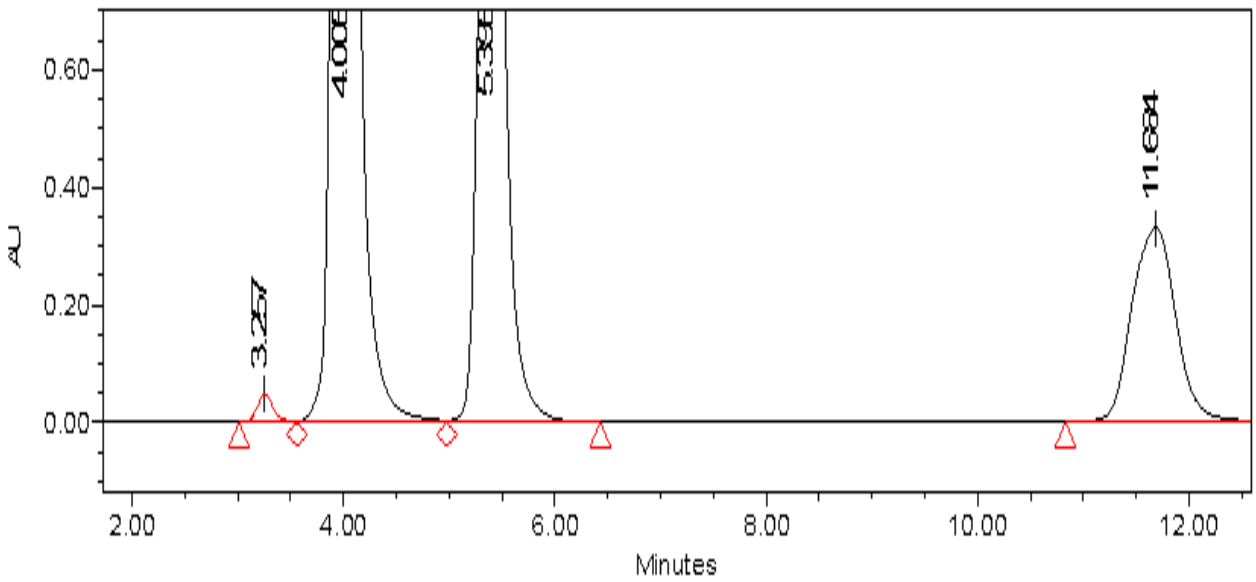

Figure 11. Typical chromatogram of the ointment formulation.

Method Validation

The method was validated in accordance with ICH guidelines for linearity, range, accuracy, precision, LOD and LOQ, specificity, solution stability and robustness.

Linearity, Range and Method Sensitivity: Linearity was determined for MF in the range of 1-10 µg/ml, for SA 35-350 µg/ml, for MP 2-20 µg/ml and for PP 0.2-2 µg/ml. The correlation coefficient values were >0.9988 (n=5). The regression equations data for the linearity and range is presented in **Table 1**. Excellent correlation exists between response factor and concentration of drugs within the concentration range indicated above. Low values of LOD and LOQ indicate sensitivity of method. Results of linearity, range and method sensitivity are presented in **Table 5**.

Table 5. Linearity, range and method sensitivity data.

Parameter/Analyte Name		PP	SA	MP	MF
Range (µg/ml)		0.2-2	35-350	2-20	1-10
Regression y=mx+c	Intercept(c)	5940.4	4234.1	9010.2	5723.1
	Slope (m)	33698	249012	409213	9061
	r	0.9988	0.9992	0.9989	0.9998
Method sensitivity	LOD (µg/ml)	0.00197	0.00681	0.4901	0.0472
	LOQ (µg/ml)	0.0596	0.02065	1.4852	0.1425

Formulation analysis, system suitability test (SST), accuracy and precision study: Commercial ointment formulation as described in materials and reagents sections was used for the study. Assay of the analytes was always in the range of 100 ± 1.45%.

RSD was always <1.5. Values of SST parameters for analytes were within the limit. Results for the accuracy of analytes tested in drug products by the technique of standard addition, recovery for all the analytes was in the rage of 100 ± 1.7% and %RSD was always <1.17. Formulation analysis, system suitability, accuracy and precision study data is presented in **Table 6**.

Table 6. Formulation analysis, system suitability, accuracy and precision study data.

Parameter/Analyte Name	PP	SA	MP	MF
Formulation Assay, % RSD	101.45, 1.5	100.65, 0.3	99.93, 0.89	100.33,0.88
System suitability data				
Retention time (t_R) in min.	3.26	4.03	5.39	11.68
USP Resolution (R_S)	---	2.18	3.30	5.92
Tailing factor (T_f)	1.040	1.08	1.21	1.05
No. of theoretical plates (N)	2268.97	2493.88	3113.71	3968.27
Capacity factor (k)	2.25	3.03	4.39	1.06
% Recovery, % RSD at selected recovery levels				
80%	99.78, 0.62	98.56, 0.52	98.80, 1.57	101.70, 0.76
100%	100.78, 1.02	101.48, 0.84	100.21, 1.17	98.73, 0.49
120%	99.01, 0.32	100.63,0.32	100.46, 0.65	99.18, 0.881
Precision, % RSD				
Repeatability, n=6	1.4	0.5	0.7	0.9
Intra-day precision, n=3x3 times	1.12, 0.45	262.12 ,0.45	15.12, 0.45	7.79, 0.82
Inter-day precision, n=3x5 days	1.87,0.82	262.27,0.82	15.27,0.82	7.07,0.86

Robustness

The study was performed as per procedure described above. The solutions containing 5 µg/ml of MF, 175 µg/ml of SA, 10 µg/ml of MP and 1 µg/ml of PP were injected in the column. A number of replicate analyses (n=3) were conducted at 3 levels of the factor (-, 0, +). Results of the robustness study for flow rate (t_R) and column oven temperature are presented in **Table 7**. Results of the robustness study for other parameters i.e. columns from different suppliers; change in measurement wavelength, changed in injection volume and change in organic solvent content were studied. Assay values and %RSD determined for each of the parameter change level were always within the limit.

Table 7. Results of robustness study.

Factor (Limit)	Level	Analyte Name	System Suitability Parameters (SD) n=3				% Assay, % RSD, n=3
			t_R*	N	Rs	k*	
Flow rate (ml/min) (±0.05 ml)	(-) 0.75	PP	3.258	2268	-	1.07	100.16, 0.24
		SA	4.033	2493	2.18	3.03	98.97, 0.16
		MP	5.394	3113	3.30	4.39	99.78, 0.25
		MF	11.64	3968	2.22	2.26	101.04, 0.27
	(+) 0.85	PP	3.165	2212	-	1.07	100.15, 0.78
		SA	3.989	2433	2.154	3.06	98.98, 0.24
		MP	5.298	3102	3.312	4.98	99.54, 0.59
		MF	11.45	3945	2.35	2.14	101.41, 0.49
Column oven Temperature (°C) (±2°C)	(-) 28	PP	3.243	2257	-	1.05	101.3, 0.29
		SA	4.00	2453	2.14	3.01	101.15, 0.72
		MP	5.28	3121	3.32	4.23	99.89, 0.47
		MF	11.54	3945	2.09	2.22	99.75, 0.55
	(+) 32	PP	3.21	2257	-	1.05	101.3, 0.29
		SA	3.956	2486	2.21	3.03	101.12, 1.27
		MP	5.198	3143	3.33	4.98	99.92, 0.83
		MF	11.25	3923	2.12	2.17	101.6, 0.95

* tR=Retention Time N: Number of Theoretical Plates, R: Resolution and k: Capacity Factor.

Specificity, Solution Stability and Mobile Phase Stability

The specificity of the method was determined by peak purity test. The peak purity was determined with the PDA detector, where values of peak angle less than peak threshold indicate peak purity.

Result of short-term, long-term and the auto sampler stability of the MF, SA, MP and PP solutions were calculated from nominal concentrations and found conc. Results of the stability studies were within the acceptable limit (98–102%). The RSD of assay of analytes during solution stability and mobile phase stability experiments was within 1.8%. No significant changes were observed in the content of analytes during solution stability and mobile phase stability experiments. Results of the peak purity and method stability study are resented in **Table 8**.

Table 8. Results of peak purity and method stability study.

Parameter/Analyte Name		PP	SA	MP	MF
Method Specificity/Peak purity	Peak Angle	0.46	0.77	0.35	0.21
	Peak threshold	0.67	1.02	0.56	0.53
Method stability (% RSD) n=4	Short term	1.6	0.8	1.3	0.8
	Long term	1.8	0.6	1.4	0.7
	Mobile phase	1.1	1.0	1.5	1.4

CONCLUSION

HPLC method was developed and validated as per ICH guidelines. The method is specific for simultaneous estimation of MF, SA, MP and PP in pharmaceutical dosage form. The method has linear response in stated range of 1-10 µg/ml for MF, 35-350 µg/ml for SA, 2-20 µg/ml for MP and 0.2-2 µg/ml for PP and is accurate and precise. The %RSD during precision were always less than 2 and recovery studies at various levels i.e. 80, 100, 120% were in the range of 98-102%. The assay value was in the range of 98-102%. Robustness studies did not show any significant change in the various system suitability parameters and assay values were within limit. LOD and LOQ values show that the method is sensitive. Statistical analysis proves that the method is suitable for the analysis of MF, SA, MP and PP as bulk drugs and in pharmaceutical formulations without any interference from the excipients.

All the above discussions and statistics prove that the method is simple, precise, and accurate and has a wide industrial application and thus can be used for inhouse estimation of MF, SA, MP and PP simultaneously forms IPQC samples and finished formulations.

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