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DEVELOPMENT OF NEW RP-HPLC METHOD FOR THE ESTIMATION OF BETAMETHASONE AND CALCIPOTRIENE IN THE OINTMENT DOSAGE FORM AND VALIDATION OF THE DEVELOPED METHOD.

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Abstract:
Combination therapy of calcipotriol monohydrate (CPM) and betamethasone dipropionate (BMD) is the option of choice for treatment of psoriasis. Psoriatic patients suffer from chronic dermatitis characterized by scaling, infiltration and erythema. The presant work is aimed at developing a validated RP-HPLC method for the simultaneous estimation of calcipotriol monohydrate (CPM) and betamethasone dipropionate (BMD) by using most affordable materials and techniques. Initially the mobile phase tried was methanol: Ortho phosphoric acid buffer and Methanol: phosphate buffer, Acetonitrile: methanol with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to Phosphate buffer (pH 3.0), Ethanol in proportion 45 : 55 v/v respectively. The method was performed with various columns like C18 column Phenomenex column, YMC, and Inertsil ODS column. Dikma Endeversil C18 ODS (2.1x 150mm, 3 µm) was found to be ideal as it gave good peak shape and resolution at 0.3 ml/min flow and the retention times were found to be 3.724, 5.148min.. UV spectrum of 10 µg/ml Betamethasone and 10 µg/ml Calcipotriene in diluents (mobile phase composition) was recorded by scanning in the range of 1000nm to 400nm. From the UV spectrum wavelength selected as 254 nm. At this wavelength both the drugs show good absorbance. The method developed was subjected to system suitability, assay (betamethasone dipropionate-99.97%, calcipotriol monohydrate100.64%). Precision, accuracy, robustness LOD, LOQ and degradation studies and found the results are within limits.

Keywords: *betamethasone dipropionate, calcipotriol monohydrate, Acetonitrile, psoriasis etc.*

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INTRODUCTION:

Psoriasis is a chronic inflammatory skin disease with increased epidermal proliferation. Psoriasis vulgaris, the most common form of psoriasis, is usually characterized by well-circumscribed, red, raised, scaly plagues. Lesions usually occur symmetrically, affecting the knees, elbows, buttocks, scalp, extremities and areas subjected to trauma. The prevalence of psoriasis is generally estimated at between 0.5%–4.6% of the global population with rates varying among countries[1].

Topical therapy is the mainstay of treatment for mild to moderate psoriasis and often the initial treatment for severe psoriasis. About 80% of patients with psoriasis are treated topically. Patients treated with phototherapy or systemic agents, including biological agents, can also be managed with topical agents as adjunctive therapy[8].

Topical corticosteroids and vitamin D3 analogue are the treatment of choice. Combination therapy is known to be superior to monotherapy and is commonly used. By combining medications with a different mechanism of action and safety profile, efficacy can be enhanced and/or safety improved. A calcipotriol/betamethasone dipropionate two-compound ointment (Dovobet®, Daivobet®, Taclonex®, LEO Pharma A/S Ballerup, Denmark) has been shown to be safe and effective in the treatment of psoriasis vulgaris[2].

Pharmaceutical analysis plays a vital role in the Quality Assurance and Quality control of bulk drugs. Analytical chemistry involves separating, identifying, and determining the relative amounts of components in a sample matrix. Pharmaceutical analysis is a specialized branch of analytical chemistry. Pharmaceutical analysis derives its principles from various branches of sciences like physics, microbiology, nuclear science, and electronics etc[9].

Chromatography is a separation of mixture into individual components using a stationary phase and a mobile phase. This may be regarded as an analytical technique employed for the purification and separation of organic and inorganic substances[3]. There are various advanced chromatographic techniques, widely used for the estimation of multicomponent drugs in their formulations. The various chromatographic techniques are

- High Performance Liquid Chromatography
- High Performance Thin Layer Chromatography
- Gas Chromatography

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is a type of liquid chromatography that employs a liquid mobile phase and a very finely divided stationary phase. The technique of high performance liquid chromatography is so called because of its improved performance when compared to column chromatography[4]. Advances in column technology, high-pressure pumping system and sensitive detectors have transformed liquid column chromatography into high speed, efficient, accurate and highly resolved method of separation.

The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages[10].

- Greater sensitivity (various detectors can be employed)
- Improved resolution (wide variety of stationary phases)
- Reusable columns (expensive columns but can be used for many analysis)
- Ideal for the substances of low viscosity
- Easy sample recovery, handling and maintenance.
- Instrumentation leads itself to automation and quantification (less time and less labour)
- Precise and reproducible
- Integrator itself does calculations.

Betamethasone is a systemic corticosteroid used to relieve inflammation in various conditions, including but not limited to allergic states, dermatologic disorders, gastrointestinal diseases, and hematological disorders[6]. Calcipotriol (INN) or calcipotriene (USAN) is a sythetic derivative of calcitriol or Vitamin D. In humans, the natural supply of vitamin D depends mainly on exposure to the ultraviolet rays of the sun for conversion of 7-dehydrocholesterol to vitamin D3 (cholecalciferol) in the skin[4].

Materials: following are the materials used and their suppliers Betamethasone Supplied by KP LBAS, Calcipotriene Supplied by KP LBAS, Ortho phosphoric acid by FINAR chemical LTD, Water and Methanol for HPLC by Standard solutions Ltd, Acetonitrile for HPLC by Standard solutions Ltd, HCl, NaOH from MERCK.

Apparatus: following are the instruments used in this work. HPLC machine make by WATERS, software: Empower, 2695 separation module.2487 UV detector. UV/VIS spectrophotometer-LABINDIA UV 3000+. pH meter-Adwa – AD 1020, Weighing machine-Accost ER-200A, Pipettes and Burettes, Beakers etc.

HPLC METHOD DEVELOPMENT:

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Ortho phosphoric acid buffer and Methanol: phosphate buffer, Acetonitrile : methanol with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to Phosphate buffer (pH 3.0), Ethanol in proportion 45 : 55 v/v respectively[4,5,6,7].

Wave length selection:

UV spectrum of 10 µg/ml Betamethasone and 10 µg/ml Calcipotriene in diluents (mobile phase composition) was recorded by scanning in the range of 1000nm to 400nm. From the UV spectrum wavelength selected as 254 nm. At this wavelength both the drugs show good absorbance[8].

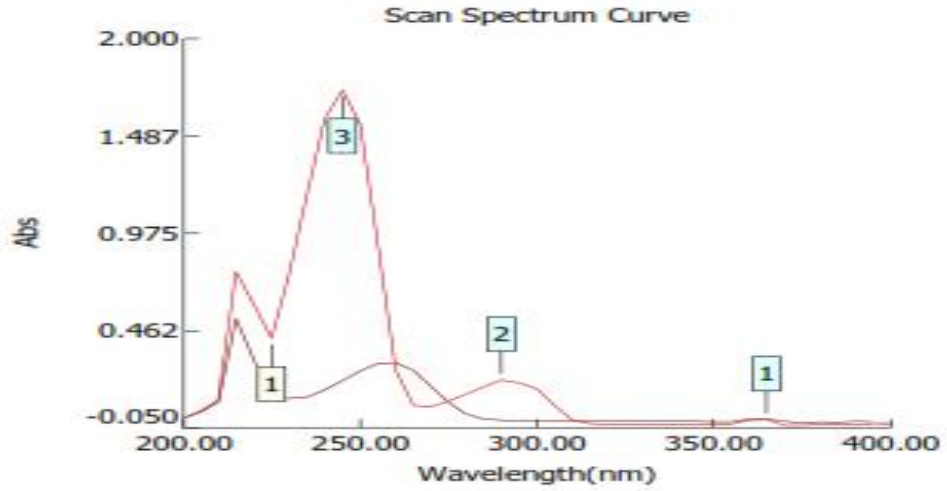

Figure 1: Isobestic point of Betamethasone and Calcipotriene

Optimization of Column:

The method was performed with various columns like C18 column Phenomenex column, YMC, and Inertsil ODS column. Dikma Endeversil C_{18} ODS (2.1x 150mm, 3 µm) was found to be ideal as it gave good peak shape and resolution at 0.3 ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Instrument used	:	Waters HPLC 2695 Model with auto sampler and 2996 PDA detector.
Temperature	:	Ambient
Column	:	Dikma Endeversil C_{18} ODS (2.1 x 150mm, 3.0 µm)
Buffer	:	3.4g of KH_2PO_4 in 1000 ml of HPLC water Ph was adjusted with OPA up to 3.0.
pH	:	3.0
Mobile phase	:	45% buffer 55% Ethanol
Flow rate	:	0.3 ml per min
Wavelength	:	254 nm
Injection volume	:	4 µl
Run time	:	3 min.

PREPARATION OF BUFFER AND MOBILE PHASE:

Preparation of Phosphate buffer:

3.4g of KH_2PO_4 in 1000 ml of HPLC water Ph was adjusted with OPA up to 3.0.final solution was filtered through 0.44 µm Membrane filter and sonicate it for 10 mins.

Preparation of mobile phase:

Accurately measured 450 ml (45%) of above buffer and 550 ml of Ethanol HPLC (100%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

PREPARATION OF THE BETAMETHASONE & CALCIPOTRIENE STANDARD & SAMPLE SOLUTION:

Standard Solution Preparation (*500 ppm of Betamethasone and 50 ppm of Calcipotriene)*

Accurately weigh and transfer 100 mg of Betamethasone and 10 mg of Calcipotriene working

standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 0.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation: (*500 ppm of Betamethasone and 50 ppm of Calcipotriene)*

Weigh accurately about 1000 mg of sample from top, middle and bottom position of the tube into a 10 ml clean dry volumetric flask add about 7 mL diluents sonicate for 20 minutes with intermediate shaking. Cool to room temperature. Make up to the volume with diluents and mix well. Filter through 0.45µ PVDF filter and Inject.

Procedure:

Inject 4 µl of the standard, sample into the chromatographic system and measure the areas for Betamethasone and Calcipotriene peaks and calculate the %Assay by using the formulae.

Validation parameters:

1. SYSTEM SUITABILITY
2. LINEARITY
3. PRECISION
4. INTERMEDIATE PRECISION/RUGGEDNESS
5. ACCURACY
6. LIMIT OF DETECTION
7. LIMIT OF QUANTIFICATION
8. ROBUSTNESS
9. DEGRADATION STUDIES

- Hydrolytic degradation under acidic condition
- Hydrolytic degradation under alkaline condition
- Thermal induced degradation
- Oxidative degradation
- Photo degradation

RESULTS AND DISCUSSION:

SYSTEM SUITABILITY:

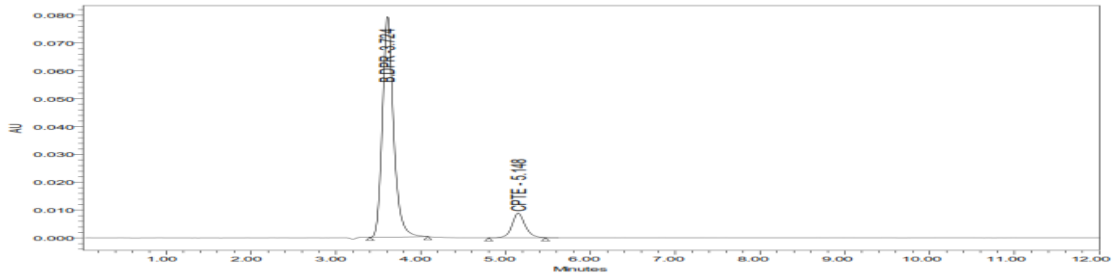

Figure 2: Chromatogram for system suitability

Table 1: Results of system suitability parameters

S.No	Name	RT(min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Betamethasone	3.724	854796	71179		1.14	4230.25
2	Calcipotriene	5.148	7536	25154	5.75	1.08	9959.10

Acceptance criteria:

- Resolution between two drugs must be not less than 2.
- Theoretical plates must be not less than 2000.
- Tailing factor must be not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

VALIDATION PARAMETERS:

1. ASSAY:

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown below.

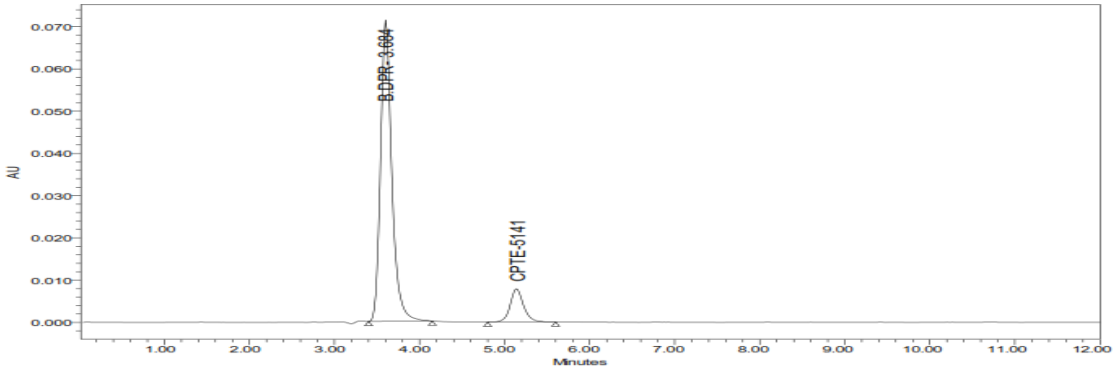

Figure 3: Chromatogram for Standard

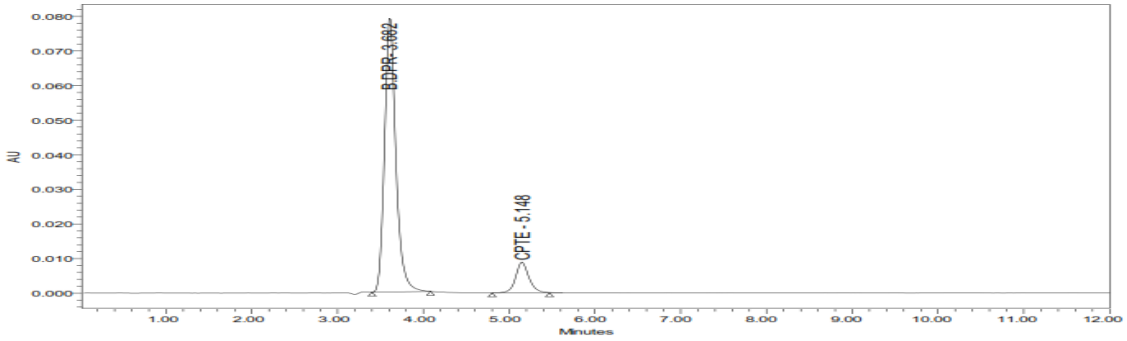

Figure 4: Chromatogram for Sample

Assay Results: (Betamethasone)

$$\frac{855999}{854516.7} * \frac{100}{10} * \frac{0.5}{10} * \frac{10}{1000} * \frac{1}{1} * \frac{100}{0.5} * \frac{99.8}{100} * 100 = 99.97\%$$

Assay Results: (For Calcipotriene)

$$\frac{115671}{114706} * \frac{10}{10} * \frac{0.5}{10} * \frac{10}{1000} * \frac{1}{1} * \frac{100}{0.05} * \frac{99.8}{100} * 100 = 100.64\%$$

Table 2: Results of Assay for Betamethasone and Calcipotriene

	Label Claim (mg)	**% Assay**
Betamethasone	0.5mg	99.97
Calcipotriene	50 mcg	100.64

2. LINEARITY:

Preparation of stock solution:

Accurately weigh and transfer 100 mg of Betamethasone and 10 mg of Calcipotriene working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I: (*250 ppm of Betamethasone and 25 ppm of Calcipotriene)*

0.25 ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent.

Preparation of Level – II: (*375 ppm of Betamethasone and 37.5 ppm of Calcipotriene)*

0.375 ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent.

Preparation of Level – III: (*500 ppm of Betamethasone and 50 ppm of Calcipotriene)*

0.5 ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent.

Preparation of Level – IV : (*625 ppm of Betamethasone and 62.5 ppm of Calcipotriene)*

0.625 ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent

Preparation of Level – V: (*750 ppm of Betamethasone and 75 ppm of Calcipotriene)*

0.75 ml of above stock solutions has taken in 10ml of volumetric flask, dilute up to the mark with diluent

Procedure:

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient

The linearity range was found to lie from 250µg/ml to 750µg/ml of Betamethasone, 25µg/ml to 75µg/ml of Calcipotriene and chromatograms are shown below.

Table 3: Area of different concentration of Betamethasone and Calcipotriene

S. No	Betamethasone		Calcipotriene	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	250	244841	25	29672
2	375	525756	37.5	68336
3	500	856654	50	113345
4	625	1150925	62.5	159680
5	750	1435608	75	204473

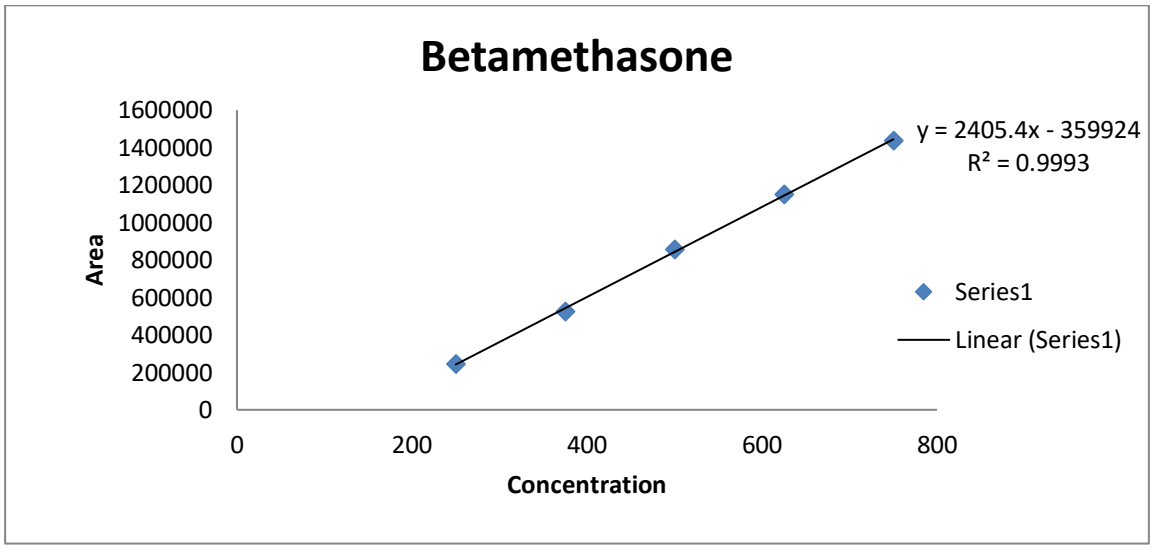

Figure 5: Calibration graph for Betamethasone

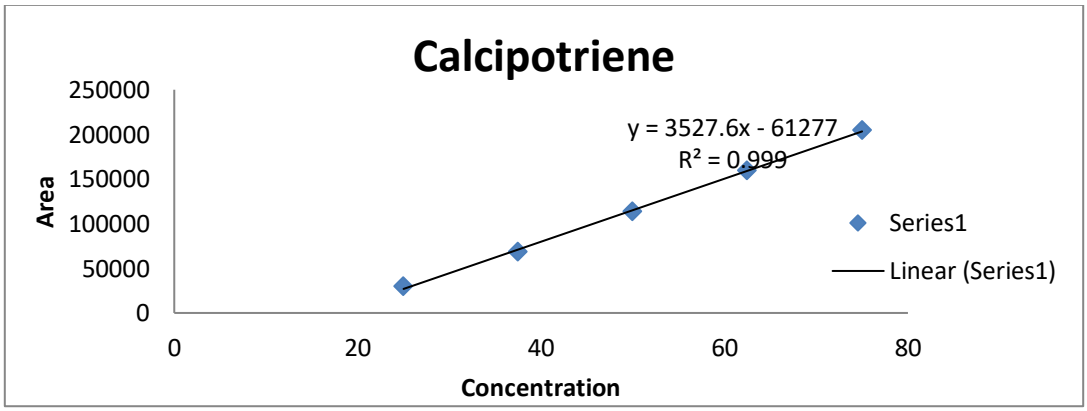

Figure 6: Calibration graph for Calcipotriene

Table 4: Analytical performance parameters of Betamethasone and Calcipotriene

Parameters	**Betamethasone**	**Calcipotriene**
Slope (m)	2405	3527
Intercept (c)	35992	61277
Correlation coefficient (R^2)	0.999	0.999

Acceptance criteria:

Correlation coefficient (R^2) should not be less than 0.999

- The correlation coefficient obtained was 0.999 which is in the acceptance limit.

3. PRECISION:

Precision of the method was carried out for both sample solutions as described under experimental work. The corresponding chromatograms and results are shown below.

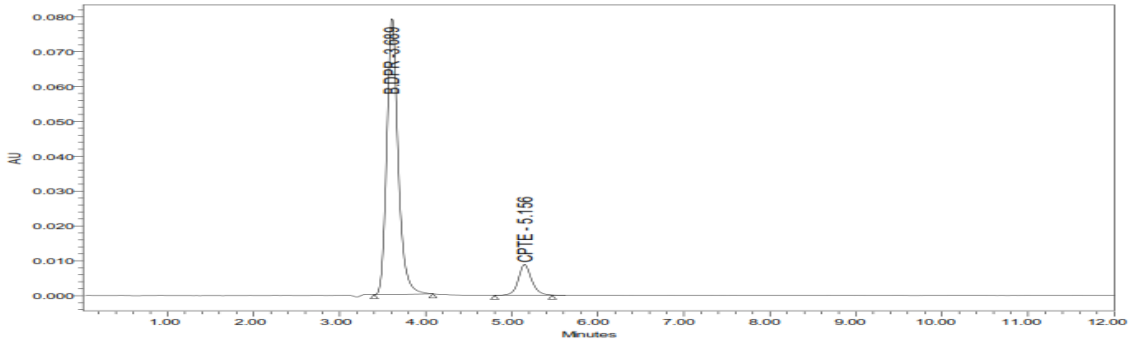

Figure 7: Chromatogram for Precision

Table 5: The results are summarized for Betamethasone and Calcipotriene

Injection	**Area for Calcipotriene**	**Area for Betamethasone**
Injection-1	111368	852828
Injection-2	112717	852337
Injection-3	112655	858355
Injection-4	113939	852839
Injection-5	111576	858513
Injection-6	112282	857582
Average	112662.3	855409.0
Standard Deviation	845.7	12.524.5
%RSD	0.8	0.4

Acceptance criteria:

- %RSD for sample should be NMT 2

- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

4. INTERMEDIATE PRECISION (ruggedness)

There was no significant change in assay content and system suitability parameters at different conditions of ruggedness like day to day and system to system variation.

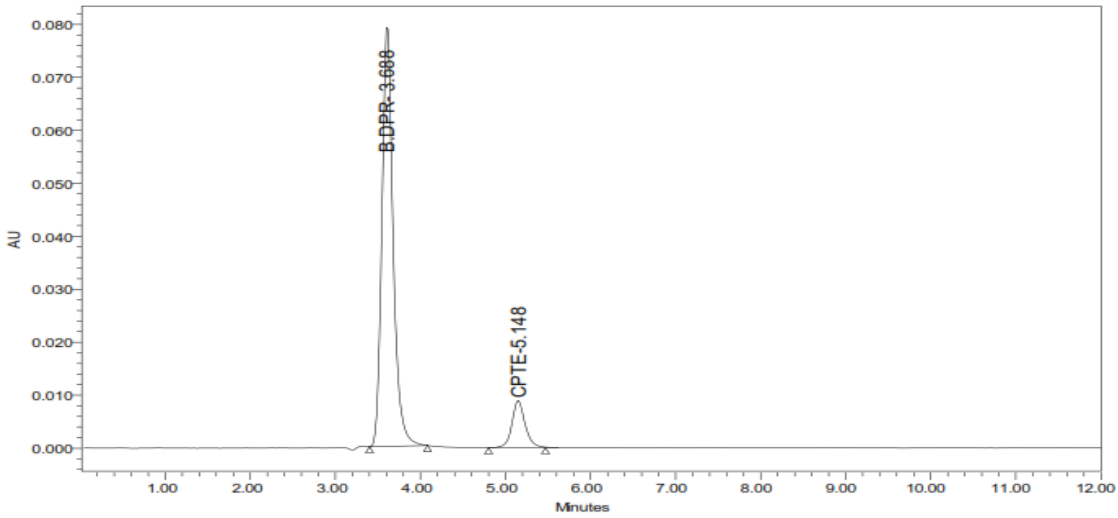

Figure 8: Chromatogram for Precision

Table 6: The results are summarized for Betamethasone and Calcipotriene

Injection	**Area for Betamethasone**	**Area for Calcipotriene**
Injection-1	859453	112535
Injection-2	857162	111224
Injection-3	859458	112915
Injection-4	858377	113391
Injection-5	858482	113108
Injection-6	859771	112959
Average	858783.8	112688.7
Standard Deviation	976.1	769.7
%RSD	0.1	0.7

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

5. ACCURACY:

Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated.

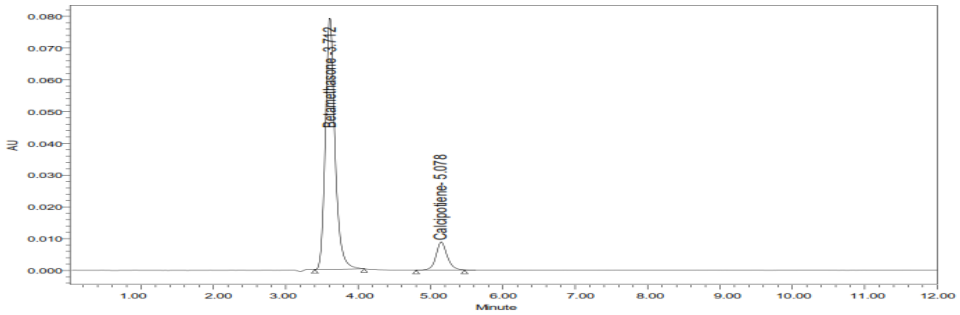

Figure 9: Chromatogram for Accuracy 100%

Table 7: The accuracy results for Betamethasone

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	427928	50	49.98	99.96	99.86
100%	854989	100	99.86	99.86	
150%	1281399	150	149.66	99.77	

Table 8: The accuracy results for Calcipotriene

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	57620	5	5.01	100.26	99.96
100%	114986	10	10.00	100.04	
150%	171648	15	14.93	99.56	

*Average of three determinations

Acceptance Criteria:

- The percentage recovery was found to be within the limit (97-103%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

6. LIMIT OF DETECTION FOR BETAMETHASONE AND CALCIPOTRIENE

The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio.

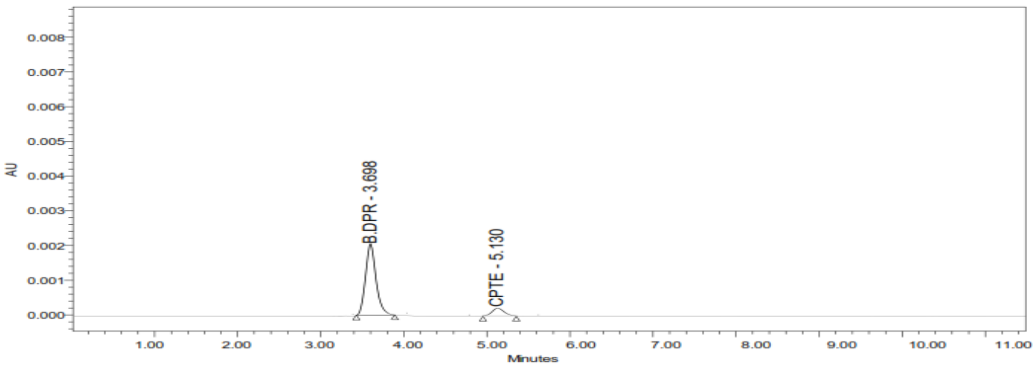

Figure 10: Chromatogram of Betamethasone, Calcipotriene showing LOD

Table 9: Results of LOD

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
Betamethasone	61	181	2.97
Calcipotriene	61	182	2.98

- Signal to noise ratio shall be 3 for LOD solution
- The result obtained is within the limit.

7. LIMIT OF QUANTIFICATION FOR BETAMETHASONE AND CALCIPOTRIENE

The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio.

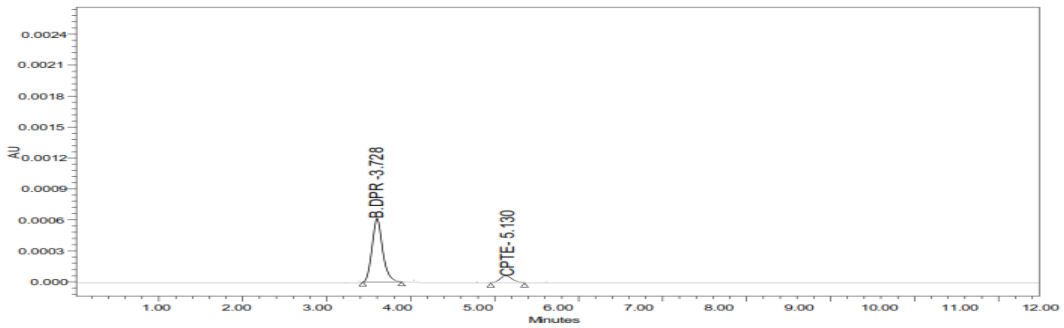

Figure 11: Chromatogram of Betamethasone, Calcipotriene showing LOQ

Table 10: Results of LOQ

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
Betamethasone	61	608	9.97
Calcipotriene	61	609	9.98

- Signal to noise ratio shall be 10 for LOQ solution
- The result obtained is within the limit.

8. ROBUSTNESS:

The standard and samples of Betamethasone and Calcipotriene were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Variation in flow

Table 11: Results for variation in flow for Betamethasone

S. No	Flow Rate (ml/min)	Results	
		USP Plate Count	USP Tailing
1	0.9	4361.01	1.24
2	1.0	4281.91	1.14
3	1.1	4137.68	1.18

Table 12: Results for variation in flow for Calcipotriene

S. No	Flow Rate (ml/min)	Results		
		USP Plate Count	USP Tailing	USP Resolution
1	0.9	9749.13	1.09	6.44
2	1.0	9959.43	1.13	5.66
3	1.1	9286.06	1.04	6.11

***** Results for actual flow (1.0 ml/min) have been considered from Assay standard.

Table 13: Results for variation in mobile phase composition for Betamethasone

S. No	Change in Organic Composition in the Mobile Phase	Results	
		USP Plate Count	USP Tailing
1	10% less	4962.22	1.35
2	*Actual	4281.91	1.14
3	10% more	4940.49`	1.44

Table 14: Results for variation in mobile phase composition for Calcipotriene

S. No	Change in Organic Composition in the Mobile Phase	Results		
		USP Plate Count	USP Tailing	USP Resolution
1	10% less	9256.47	0.91	8.69
2	*Actual	9959.43	1.13	5.66
3	10% more	92676.52	1.23	4.06

***** Results for actual Mobile phase composition (45:55) Buffer pH 3: Ethanol has been considered from Accuracy standard.

Acceptance criteria:

The Retention time, USP plate count, USP tailing factor obtained for change of flow rate, variation in mobile phase was found to be within the acceptance criteria. Hence the method is robust.

DEGRADATION STUDIES:

Table 15: Results for degradation studies of both drugs

Sample Name	Betamethasone	
	Area	% Degraded
Standard	114736	
Acid	106691	7.0
Base	109733	4.4
Peroxide	109509	4.6
Thermal	109294	4.7
Photo	107294	6.5
Sample Name	**Calcipotriene**	
	Area	**% Degraded**
Standard	854796	
Acid	797354	6.7
Base	814877	4.7
Peroxide	805816	5.7
Thermal	814022	4.8
Photo	832999	2.5

CONCLUSION:

The presant work is aimed at developing a validated RP-HPLC method for the simultaneous estimation of calcipotriol monohydrate (CPM) and betamethasone dipropionate (BMD) by using most affordable materials and techniques. The method was developed successfully and validated. The Forced degradation also done for the proposed method and it is suitable for the stability Studies of betamethasone and Calcipotriene in its pure and Ointment Dosage form. Hence the Developed method is used for the routine analysis of the drugs in the quality control of the industry.

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Conflicts of interest:

The authors express no conflicts of interest regarding the publication, all the authors worked and provided support equally and credited equally.

Abbreviations:

BMD- betamethasone dipropionate
Conc.-concentration
CPM- calcipotriol monohydrate
LOD- limit of detection
LOQ- limit of quantification
Mg-milligram
Min.-minute
Ml- millilitre
Mm-millimetre
NMT-not more than
RP-HPLC-reverse phase high pressure liquid chromatography
UV/VIS- ultraviolet/visible

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