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Stability indicating RP-HPLC method for simultaneous estimation of Betamethasone dipropionate and Calcipotriene in bulk and pharmaceutical dosage form

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ABSTRACT

The aim of the present study was to develop a reverse phase high performance liquid chromatographic (RP-HPLC) method for the simultaneous estimation of Betamethasone Dipropionate (BDP) and Calcipotriene (CPT). By using phosphate buffer (pH 3.0) and mobile phase consists of methanol: phosphate buffer mixed in the ratio of 70:30 % v/ v and Inertsil C18 column (4.6 x 250mm, 5□m). The retention times (R_t) were found to be 2.669 min. and 3.855 min. for BDP and CPT respectively. For standard preparation Rt were found to be 2.569 min. and 3.842 min. BDP and CPT respectively. Both drugs produced linear responses with correlation coefficient (r2) of 0.999. The percentage relative standard deviation (% RSD) are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-101%. The limit of detection (LOD) and limit of quantification (LOQ) of BDP and CPT was found to 2.9, 10.03 and 3.0, 10.1 respectively and the results obtained was within the limit. The developed method was validated according to International Conference on Harmonization (ICH) guidelines for various parameters specified in ICH, Q2 (R1) guidelines and statistical analysis were found to be in good accordance with the prescribed values.This method was successfully validated as per ICH guidelines and proved to be suitable for routine quality control use.

Keywords: *Mobile phase, International Conference on Harmonisation, limit of detection, limit of quantification, robustness, precision.*

1. INTRODUCTION

It is obvious that, 1-3 % world's population is affected by a common skin disease psoriasis [1, 2]. Several new biological therapies have been developed, which target specific steps in the pathogenesis of psoriasis [3-5]. Betamethasone dipropionate (BDP) is belongs to the class of adrenocortical steroid, showing immunosuppressive and anti-inflammatory activities [6]. It is used in colon crohn's disease [7], prednisone sparing therapy [8], and asthma [9-13] etc. Chemically it is 9-fluoro 11β, 17, 21-trihydroxy-16β-methylpregna-1, 4-diene-3, 20-dione 17, 21-dipropionate [14].

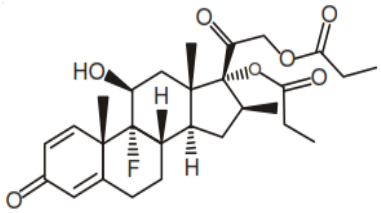

Figure 1. Structure of BDP.

Calcipotriol also called as Calcipotriene, synthesized from calcitriol. Calcipotriol (CPT) is a low-calcemic vitamin D receptor (VDR) agonist. CPT is about 200 times less potent in its effects on calcium metabolism than vitamin D (1, 25 [OH] D_3) [15]. CPT also induces expression of thymic stromal lymphopoietin, which triggers T-cell differentiation in keratinocytes [16]. Calcipotriol is chemically it is (1R,3S,5Z)-5-{2-[(1R,3aS,4E,7aR)-1-[(2R,3E,5S)-5-cyclopropyl-5-hydroxypent-3-en-2-yl]-7a-methyl-octahydro-1H -inden-4-ylidene]ethylidene}-4-methylidene-cyclo-hexane-1,3-diol. Various methods for the simultaneous estimation of BDP and CPT were reported in literature [17-19].

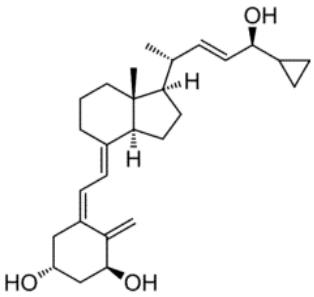

Figure 2. Structure of CPT.

To the best of our knowledge, there is no method was available for simultaneous determination of CPT and BDP by RP-HPLC. But, attempts were made in this study to reveal that determination of individual compound or combination with other drugs have been reported [20-21]. The present work has aimed was to develop and validate a simple, rapid and sensitive method for the simultaneous estimation of BDP and CPT. Validation of the method was done in accordance with ICH, Q_2 (R_1) guidelines for the assay of active ingredients [22].

2. MATERIALS AND METHODS

Reagents and Chemicals. BDP and CPT were procured from Mylon and Cipla, India respectively. Potassium di hydrogen phosphate (KH_2PO_4) from Finer chemical Ltd., Mumbai, India. Water and methanol for HPLC from Lichrosolv, Mumbai, India.

Acetonitrile for HPLC from Molychem and Ortho phosphoric acid from Merck, Mumbai, India. Membrane filters purchased from pall life sciences, Mumbai, India.

Instrumentation: The chromatography analysis was performed using HPLC **(**WATERS, software: Empower, 2695 separation module, PDA detector) and UV visible spectrophotometer (LABINDIA UV 3000^{+}). pH meter and Weighing machine from Adwa – AD 1020 and Afcoset ER-200A respectively.

Wave length selection: UV spectrum of 10 µg / ml BDP and CPT in diluents (mobile phase composition) was recorded by scanning in the range of 200 nm to 400 nm. From the UV spectrum wavelength selected as 260 nm.

Optimization of Column: Inertsil C_{18} ODS (4.6 x 250mm, 5µm) was used for this method and found to be ideal as it gave good peak shape and resolution at flow rate 0.8 ml/min.

Optimized Chromatographic Conditions: The HPLC system (WATERS, software: Empower, 2695 separation module, PDA detector) with dual λ Absorbance UV detector. The wavelength of detection as set at 260 nm. Separation was carried out in gradient mode on inertsil C_{18} column (4.6 x 250mm, 5µm) and the retention time of BDP and CPT were found to be 2.952 min. and 6.832 min. respectively (shown in Figure 3 and Figure 4), using 70:30 v/v methanol: phosphate buffer as mobile phase at a flow rate of 0.8 ml/min. The mobile phase filtered through nylon Millipore (0.45µm) membrane filter, and degassed with ultrasonicator prior to use. Chromatography was carried out at 25^0C room temperature and the column temperature should be 32^0C.

Preparation of phosphate buffer: Exactly weighed 6.8 gm of KH_2PO_4 was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3.0 with orthophosphoric acid.

Preparation of mobile phase: Accurately measured 300 ml (30%) of phosphate buffer and 700 ml of methanol HPLC (70%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45µ filter under vacuum filtration.

Diluent preparation: Mobile phase was used as diluent.

Standard solution preparation: Accurately weigh10 mg of BDP and CPT, into a 10ml and dry volumetric flask to this add 7ml of diluent and sonicate to dissolve it completely and make up to the mark with diluent.

Sample solution preparation: Accurately weigh 10 tablets crush in mortar and pestle and transfer equivalent to 10 mg of BDP and CPT (marketed formulation) sample into a 10ml clean dry volumetric flask add diluent (7ml) and sonicate to dissolve it completely and make up to the mark with the diluent. Additional pipette 3 ml of BDP and CPT of the above stock solution into a volumetric flask (10ml) and dilute up to the mark with diluent.

Procedure: Inject 20 µl of the standard, sample into the chromatographic system and measure the areas for BDP and CPT peaks and calculate the percentage assay by using the formulae. 3.

VALIDATION OF THE METHOD.

The analytical method was validated for linearity, accuracy, and precision, limit of detection (LOD), limit of quantification (LOQ), specificity, and robustness, in accordance with ICH guidelines. Further statistical evaluations were performed.

System suitability: System suitability experiment was assessed by injecting five consecutive injections of both drugs at a concentration of 10.0 µg/ml. Resolution between two drugs must be not less than 2. Theoretical plates should not less than 2000. Tailing factor must be not less than 0.9 and not more than 2. The percentage relative standard deviation (%RSD) of retention time and tailing factor were calculated. Values with RSD of ≤ 2% for peak areas and retention time (R_t) were accepted.

Linearity: Linearity of BDP and CPT were determined at five different concentrations in triplicate and plotted using linear regression of the mean peak area versus concentration. A straight line fit was made through the data points by least square regression analysis in order to obtain linear regression equation (y= mx+c).

Accuracy: It was performed in triplicate for various concentrations of sample solutions, prepared by spiking at about 50%, 100% and 150% of specification limit to placebo and analyzed by the proposed HPLC method. The % recovery for each level should be between 98.0 to 102.0%. The accuracy of the analytical method was established in triplicate across its range according to the assay procedure. Accuracy values within the range of 85–115% and % RSD of ≤ 2 was the acceptance criteria.

Precision: Precision was measured in terms of repeatability of application and measurement and this study was carried out by injecting five replicates of the same standard (system precision).

Robustness: Robustness of the method was assessed by deliberately modifying the experimental conditions in terms of two factors such as change in mobile phase composition (1.0 ± 0.1 mL) and flow rate (0.8 ± 0.2 ml/min). By introducing two factors, robustness of the described method was studied. The chromatographic variations were evaluated by analysing the effect on peak areas and R_t of the three QC samples of BDP and CPT in triplicate. The results were expressed in terms of % mean difference. Values within a range of ±5% were accepted.

Sensitivity: LOD and LOQ were estimated by measuring the signal to noise ratio (S/N). Stock solution of both drugs were serially diluted with methanol to prepare the series of samples with low concentrations and injected into the HPLC system.

3. RESULTS AND DISCUSSION

HPLC and HPTLC are the reported analytical methods for compounds such as BDP and CPT, either individually or in combination with other dosage form. The optimization of the chromatographic conditions have to done to obtain symmetric peaks with better resolution and system suitability. Finally, the mobile phase was optimized as potassium dihydrogen phosphate with buffer (pH 3.0), Methanol in proportion 30: 70 v/v respectively.

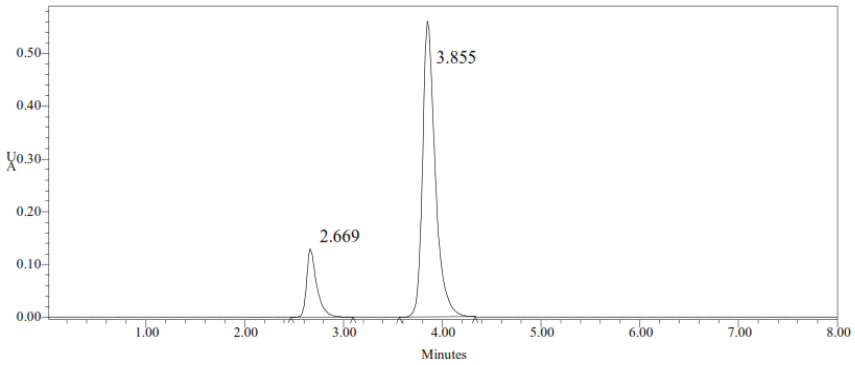

Figure 3. Optimized method for BDP and CPT sample solutions.

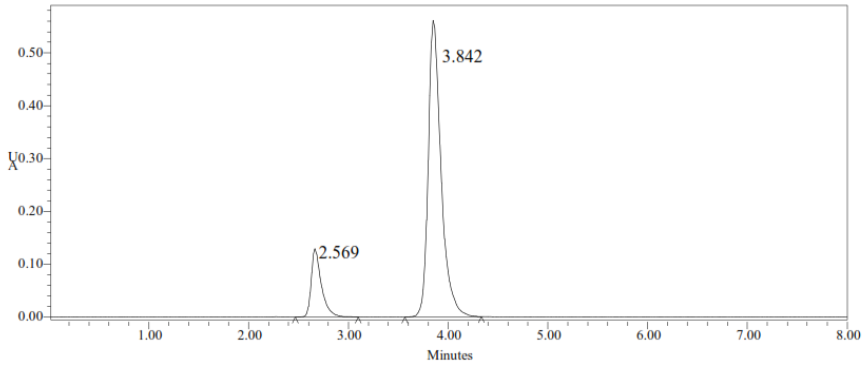

Figure 4. Retention times for BDP and CPT standard solutions.

Linearity: The linearity responses were determined by preparing and injecting standard solutions. The linearity range was found to be 100-500µg/ml of BDP and 5-25µg/ml of CPT. The linearity was established in the range of 10-50% of BDP and 5-25% of CPT. For both drugs correlation coefficient (R^2) was found to be 0.999 and shown in below Table 1.

Table 1. Linearity results of BDP and CPT.

Sl. No.	BDP		CPT	
	Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area
1	100ppm	668934	5ppm	66510
2	200ppm	956781	10ppm	94701
3	300ppm	1313873	15ppm	124802
4	400ppm	1563458	20ppm	152731
5	500ppm	1867084	25ppm	179732

Accuracy: Spiked samples were prepared by spiking pre-analyzed sample solutions with pure drug at three different concentration levels each in triplicate and mean percentage recovery values were calculated. The % recovery of BDP (98.02-100.45%) and CPT (99.79-100.20%) at each level was within the limits of 98% and 102% (shown in following Table 2 and Table 3). Hence, accuracy was established for the present work and the method was said to be accurate.

Table 2. Accuracy (recovery) data for BDP.

% Concentration (at specification level)	Area	Amount added (mg)	Amount found (mg)	% Recovery	Mean recovery
50%	656659.5	5.0	5.036	100.7%	99.84%
100%	1304258	10.0	10.003	100.0%	
150%	1854608	14.4	14.224	98.780%	

Table 3. Accuracy (recovery) data for CPT.

% Concentration (at specification level)	Area	Amount added (mg)	Amount found (mg)	% Recovery	Mean recovery
50%	65800	5.3	5.34	100.8%	100.51%
100%	124353	10	10.10	100.01%	
150%	177940	14.2	14.45	99.68%	

Precision: The intraday precision was demonstrated by injecting six test solutions at 25 µg/ml concentration as per the test procedure (shown in Table 4) and recording the chromatograms of six test solutions. The % RSD of BDP and CPT was found to be 0.207 and 0.324 respectively.

Table 4. Results of method precession for BDP and CPT.

Injection	Area of BDP	Area of CPT
Injection-1	1302729	123149
Injection-2	1302947	123766
Injection-3	1303236	124271
Injection-4	1303977	124691
Injection-5	1309759	124956
Average	1304529.8	124162.7
Standard Deviation	2961.1	725.6
%RSD	0.2	0.6

Intermediate Precision: Intermediate precision of the analytical method was determined by performing method precision in three successive days by different analysts under same experimental condition. Assay of all six replicate sample preparations was determined and the mean % RSD of BDP and CPT was found to be 0.259 and 0.353 respectively.

Robustness: Retention times were significantly changed with change in the flow rate and mobile phase composition but no change was found with change in wavelength. However, % assay values were within limits (%RSD < 2) and these results indicated minor changes in the flow rate and mobile phase composition didn't affected the assay results. The parameters like theoretical plate number and tailing factor were also found to be within the limits. The standard and samples of BDP and CPT 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. Percentage RSD should be below 2. The %RSD obtained for change of flow rate, variation in mobile phase was found to be below 1, which is within the acceptance criteria. Hence the method is robust.

Robustness of the proposed method was determined by varying various parameters, the %RSD reported was found to be less than 2 %.As the system suitability parameters for the standard and test chromatograms of BDP and CPT were within limits for variation in flow rate (±0.2ml) and mobile phase composition, the allowable variation in flow rate, organic solvent ratio in mobile phase composition and column temperature should be 0.8±0.2ml/min, Actual±10% and 30±5^{0}C respectively (shown in Table 5-8).

Table 5. Flow rate (ml/min) data for BDP.

Sl. No.	Flow rate (ml/min)	System suitability results	
		USP plate count	USP tailing
1	0.6	5339.9	1.4
2	0.8	4673.4	1.3
3	1.0	5216.0	1.4

Table 6. Flow rate (ml/min) data for CPT.

Sl. No.	Flow rate (ml/min)	System suitability results	
		USP plate count	USP tailing
1	0.8	7063.3	1.3
2	1.0	6090.3	1.2
3	1.2	6998.0	1.3

Table 7. Change in organic composition in the mobile phase for BDP.

Sl. No.	Change in organic composition in the mobile phase	System suitability results	
		USP plate count	USP tailing
1	10% less	4508.4	1.3
2	*Actual	4673.4	1.4
3	10% more	4318.1	1.3

Table 8. Change in organic composition in the mobile phase for CPT.

Sl. No.	Change in organic composition in the mobile phase	System suitability results	
		USP plate count	USP tailing
1	10% less	6387.7	1.2
2	*Actual	6090.3	1.2
3	10% more	6232.5	1.2

System suitability: To study system suitability 10 µl of the stock solution of 10ml was injected into the chromatograph (n=5) and determined the following parameters: the number of theoretical plates, the number of theoretical plates per meter, coefficient of asymmetry and height equivalent to theoretical plate. Measurement results are shown in Table 9. Based on results, it was found that all the system suitability parameters for developed method were within the limit.

Table 9. Results of system suitability parameters for BDP and CPT.

Sl. No.	Name	Retention time (min.)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	BDP	2.5	124505	213642	xxxx	1.2	4673.4
2	CPT	3.9	1308495	154566	6 0	1.3	6090.3

Limit of Detection and Limit of Quantification: In the present study, the LOD and LOQ were calculated according to the standard deviation of the response and the slope of the calibration curve i.e., 3.3σ/S and 10σ/S criterions, respectively; where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

Limit of Detection: The lowest concentration of the samples were prepared with respect to the base line noise and measured the signal to noise ratio. Results of LOD determined by signal to noise ratios and it was found to be 2.9 and 3 for BDP and CPT respectively. Results are shown in Table 10.

Table 10. Results of LOD.

Drug name	Baseline noise (µV)	Signal obtained (µV)	S/N ratio
BDP	52	152	2.9
CPT	52	156	3

Limit of Quantification*:* The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio. Results of LOQ determined by signal to noise ratios and it were found to be 10.03 and 10.1 for BDP and CPT respectively. Results are shown in Table 11.

Table 11. Results of LOQ.

Drug name	Baseline noise (µV)	Signal obtained (µV)	S/N ratio
BDP	52	522	10.03
CPT	52	524	10.1

4. CONCLUSIONS

This method is advantageous over reported methods and is capable of improving efficiency and output of an analytical laboratory. The results indicated the suitability of the method to study stability of both drugs. Validation parameters meet the specifications laid down in ICH guidelines. This can be applied for day to day analysis of BDP and CPT, without any interference from the excipients. The successful application of the developed method warrants that the method will be a valuable addition in the quality control department of pharmaceutical industry as it will reduce the analysis time and cost greatly. Based on all the results, it was concluded that the present method was fast, easy to perform, cost effective, sensitive, selective and repeatable.

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