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Development and Validation of Stability Indicating RP-HPLC Method for Simultaneous Estimation of Beclomethasone Dipropionate and Clotrimazole

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ABSTRACT
The aim of the present study was to develop a validated stability indicating reverse phase high performance liquid chromatography (RP-HPLC) method for simultaneous estimation of Beclomethasone dipropionate (BEC) and Clotrimazole (CL) in bulk and combined dosage form (CLOCIP-B cream). An isocratic, RP-HPLC method was developed using Hi Q Sil C_{18} (250 x 4.6 mm, 5 µm) column using 1 mM ammonium acetate buffer and acetonitrile (10:90 v/v) as mobile phase at flow rate of 1 ml/min at detection wavelength of 223 nm.
The chromatographic conditions yield good separation between drugs with retention time (RT) of 4.227 ± 0.013 min and 6.440 ± 0.027 min, for BEC and CL, respectively. The method was validated with respect to linearity, precision, accuracy and robustness. The data of linear regression analysis indicated a good linear relationship over the range of 10-60 µg/ml for BEC and 5-30 µg/ml for CL concentrations with a correlation coefficient (r^2) of 0.996 for BEC and 0.995 for CL. The drugs were subjected to forced degradation under different conditions. The developed method was found to be simple, sensitive, selective, accurate, and repeatable for simultaneous analysis of BEC and CL and can be adopted for routine analysis of these drugs in bulk and pharmaceutical dosage form.
Keywords: high performance liquid chromatography (HPLC), beclomethasone dipropionate, clotrimazole, stability indicating, validation

INTRODUCTION

Beclomethasone dipropionate (BEC) chemically is 9 α-chloro-11β-hydroxy-16 β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropionate and Clotrimazole (CL) chemically is 1-((2-chlorophenyl)(diphenyl)methyl)-1H-imidazole. Beclomethasone dipropionate is a steroidal drug used in asthma; while Clotrimazole is antifungal agent [1]. Clocip B cream (Beclomethasone Dipropionate/Clotrimazole) is a topical medication used to treat conditions that are fungus based and it is most commonly used to treat athletes' foot, jock itch, oral thrush, and ringworm or yeast infection.

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Literature survey reveals few methods viz UV spectrophotometric [2], simple HPLC [3] and stability indicating HPLC method [4] for estimation of beclomethasone dipropionate alone and in combinations with other drugs. Also simple UV spectrophotometric method [5], RP-HPLC method [6-7], stability indicating RP-HPLC [8-9] methods for estimation of Clotrimazole alone and in combinations with other drugs are reported.

Only one Simple RP-HPLC method for estimation of Beclomethasone dipropionate and clotrimazole in combination is reported [1]. To the best of our knowledge no stability indicating RP-HPLC method has been reported for simultaneous estimation of Beclomethasone dipropionate and Clotrimazole. The present work describes a simple stability indicating HPLC method for the simultaneous determination of Beclomethasone dipropionate and Clotrimazole in bulk and pharmaceutical dosage form (CLOCIP –B cream). The validation parameters performed according to the International conference on harmonization (ICH) guidelines [10-11].

MATERIALS AND METHODS

Reagents and chemicals

Authentic sample of Beclomethasone dipropionate and Clotrimazole was obtained from Cipla Pharmaceuticals Ltd, Mumbai and NuLife Pharmaceuticals Ltd., Pune, respectively. The formulation CLOCIP-B cream (Cipla Pharmaceuticals Ltd) labeled to contain Beclomethasone dipropionate (IP) 0.25 mg and Clotrimazole (IP) 10 mg was procured form local market. Acetonitritle (HPLC grade) was obtained from S. D. Fine Chem. Limited (Mumbai, India), HPLC grade water is collected at college using ELGA water purification system. Ammonium acetate, glacial acetic acid, hydrochloric acid (HCl), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2) (all are AR grade) were purchased from S. D. Fine Chem. Limited (Mumbai, India).

Chromatographic condition

HPLC system used was JASCO system equipped with model PU 2080 Plus pump, Rheodyne sample injection port (20 µl), MD 2010 PDA detector and Borwin-PDA software (version 1.5). A chromatographic column Hi Q Sil C_{18} (250 x 4.6 mm, 5 µm) was used. Separation was carried out at a flow rate of 1 ml/min using 1 mM ammonium acetate buffer: acetonitritle (10:90 v/v) as mobile phase and detection at 223 nm. The representative Chromatogram is shown in **Figure 1**.

Preparation of 1 mM ammonium acetate buffer and Mobile phase

Ammonium acetate buffer (1mM) was prepared by dissolving 7.71 mg of ammonium acetate in 80 ml of HPLC grade water, add 0.05 ml glacial acetic acid and make the volume up to 100 ml with HPLC grade water. Mobile phase was prepared by mixing 1 mM Ammonium acetate buffer: acetonitritle (10:90 v/v) and then filtered through 0.45 µm membrane filter; sonicated for 15 min.

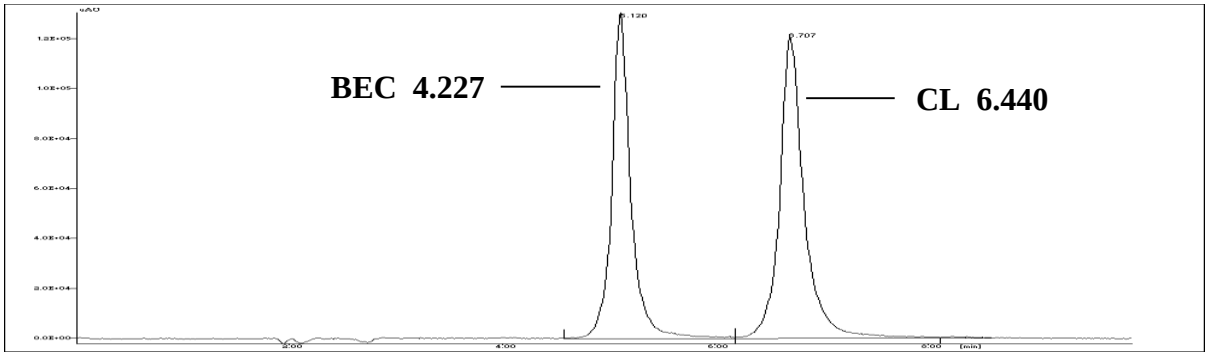

Figure 1. Chromatogram of mixture containing BEC (10 µg/ml) and CL (10 µg/ml)

Preparation of standard stock solution

Standard stock solution of each drug was prepared separately by dissolving 10 mg of the drug in 10 ml of acetonitritle to get concentration of 1000 µg/ml. From the respective standard stock solution, working standard solution was prepared containing 100 µg/ml of BEC and CL in acetonitritle separately. From this further dilutions were made in the acetonitritle to get a final concentration in the range 10-60 µg/ml for BEC and 5-30 µg/ml for CL, separately.

Selection of detection wavelength

From the standard stock solution (1000 µg/ml) further dilutions were made using methanol and scanned over the range of 200-400 nm and the spectra was obtained. It was observed that both the drug showed considerable absorbance at 223 nm.

Preparation of sample solution

For preparation of sample solution 1 gm of cream (10 mg of CL: 0.25 mg of BEC) was taken in beaker containing 10 ml of methanol, mixed well and filtered. The solution was warmed for 5 min at 50^0C, then cooled in ice-bath for 15 min and promptly centrifuged. The supernatant layer was taken and extracted with n-hexane using separating funnel. n-hexane layer was discarded and methanol layer was collected. 4 ml was pipetted out from methanol layer into 10 ml volumetric flask and diluted up to mark with mobile phase to get the concentration of 10 µg/ml of BEC (400 µg/ml of CL) for BEC determination. Further 1 ml was diluted to 10 ml with acetonitritle to get concentration of 10 µg/ml of CL (0.25 µg/ml of BEC) for CL determination.

Stress degradation studies of bulk drug

Stability studies were carried out to provide evidence on how the quality of drug varies under the influence of a variety of environmental conditions like hydrolysis, oxidation, temperature, etc. to get insight on specific storage conditions. Dry heat and photolytic degradation were carried out in solid state.

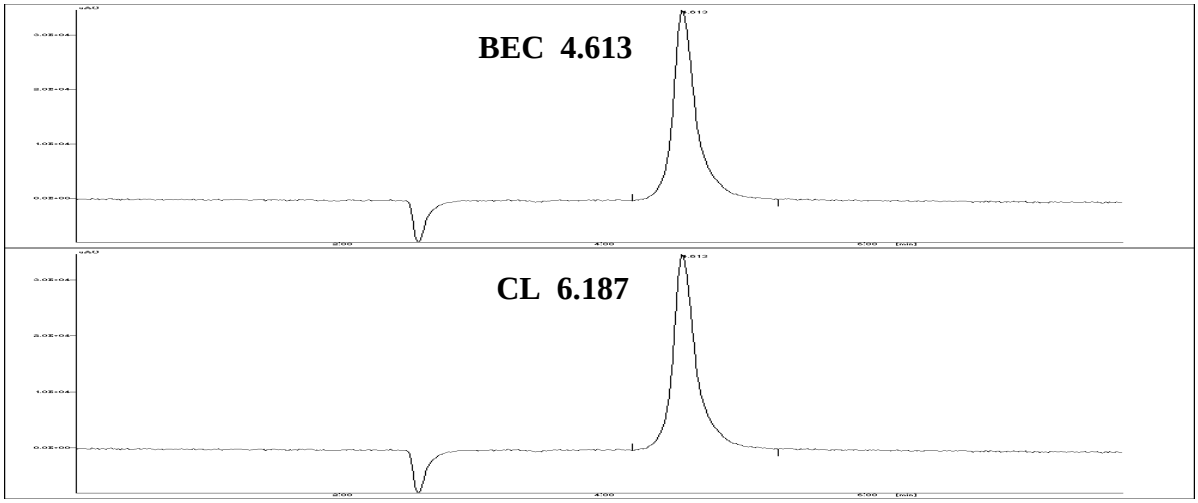

Figure 2. Chromatogram of: BEC and CL after alkaline hydrolysis

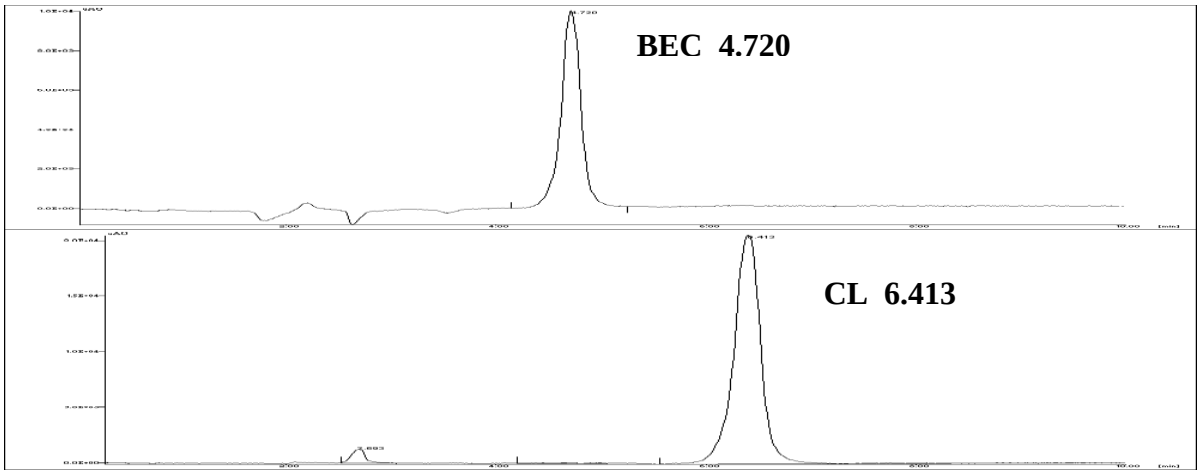

Figure 3. Chromatogram of: BEC and CL after acid hydrolysis

Alkaline hydrolysis

For alkaline hydrolysis, 1 ml working standard solution of BEC (100 µg/ml) was mixed with 1 ml of 0.01 N NaOH and kept aside for 4 hours at room temperature, after exposure the volume was made up to 10 ml with the acetonitritle and injected; CL was treated in a similar manner to BEC. After alkaline treatment, BEC & CL showed no peak of degradation product. The percent recovery of BEC and CL were 71.89 % and 86.37%, respectively. The representative chromatogram is shown in **Figure 2**.

Acid hydrolysis

For acid hydrolysis , 1 ml working standard solution of BEC (100 µg/ml) was mixed with 1 ml of 0.01 N HCl and kept aside for 6 hours at room temperature, after exposure the volume was made up to 10 ml with the acetonitritle and injected; CL was treated in a similar

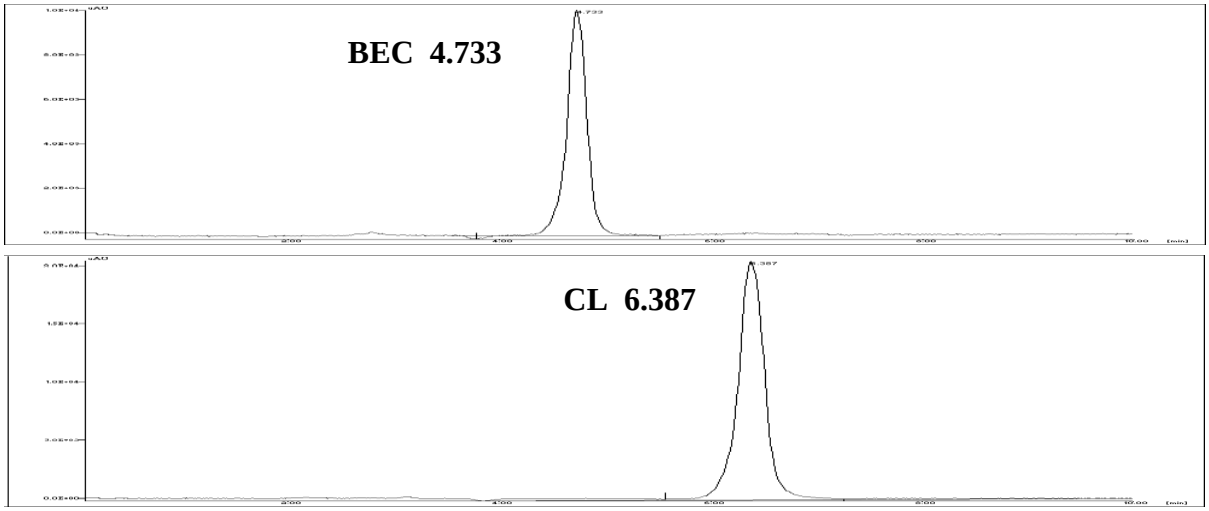

Figure 4. Chromatogram of: BEC and CL after neutral hydrolysis

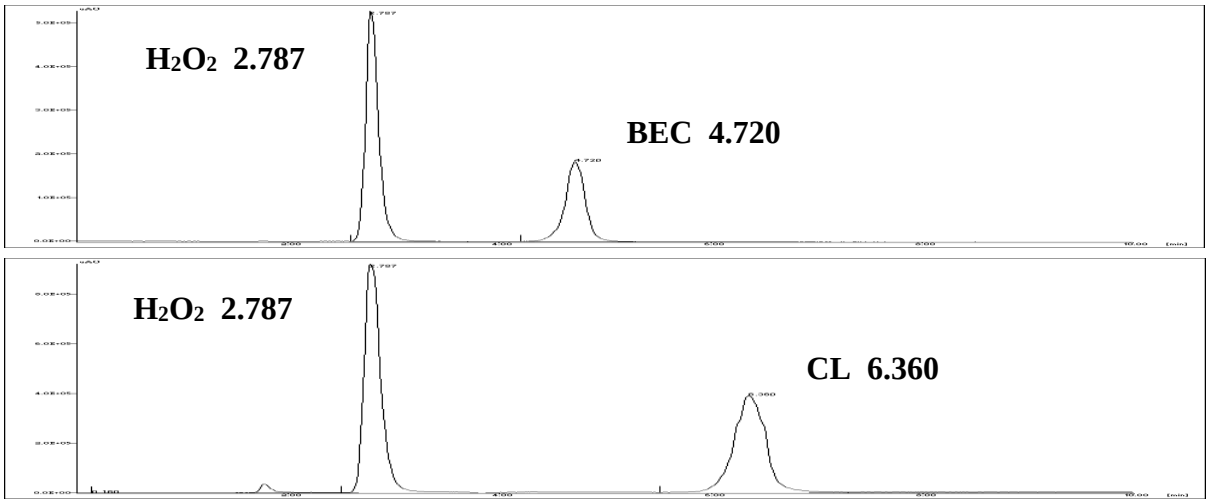

Figure 5. Chromatogram of: BEC and CL after oxidation with 30 % v/v H_2O_2

manner to BEC. After acid hydrolysis, BEC & CL showed no peak of degradation product. The percent recovery of BEC and CL were 85.74 % and 67.40 %, respectively. The representative chromatogram is shown in **Figure 3**.

Neutral hydrolysis

For neutral hydrolysis, 1 ml working standard solution of BEC (100 µg/ml) was mixed with 1 ml water , and was kept aside for 24 hours at room temperature, after exposure the volume was made up to 10 ml with the acetonitritle and injected; CL is treated in a similar manner to BEC. On neutral hydrolysis, BEC 68.66% and CL 66.78 % were recovered with no peak of degradant. The representative chromatogram is shown in **Figure 4**.

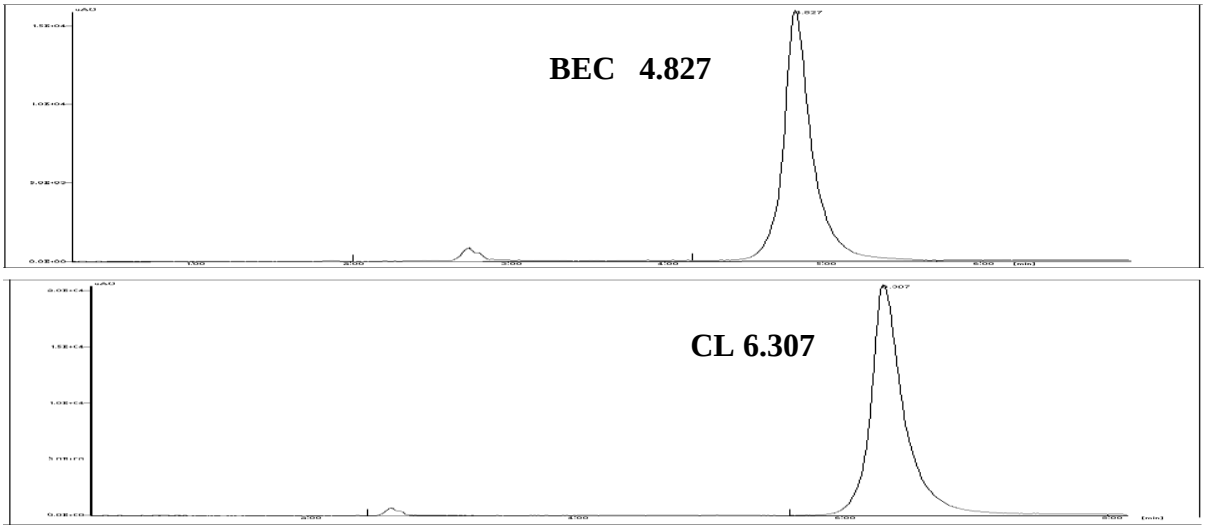

Figure 6. Chromatogram of: BEC and CL after dry heat degradation

Degradation under oxidative condition

For oxidation study, 1 ml working standard solution of BEC (100 µg/ml) was mixed with 1 ml of 30 % solution of H_2O_2 and was kept aside for 2 hours at room temperature, after exposure the volume was made up to 10 ml with the acetonitrile and injected; CL was treated in a similar manner to BEC. In the oxidative condition, percent recovery obtained for BEC was 75.37 % and for CL was 79.91% with no peak of degradant. The representative chromatogram is shown in **Figure 5**.

Degradation under dry heat

Dry heat studies were performed by keeping drug sample separately in oven (80 °C) for a period of 24 hours. A sample were withdrawn after 24 hours, dissolved in acetonitrile to get solution of 1000 µg/ml and further diluted with acetonitrile to get 10 µg/ml as final concentration and was injected. In the dry heat degradation condition, percent recovery obtained for BEC was 89 % and CL was 70.08 % with no peak of degradant. The representative chromatogram is shown in **Figure 6**.

Photo-degradation studies

Photolytic studies were carried out by exposure of drug to UV light up to 200 watt hours/square meter and subsequently to cool fluorescent light to achieve an illumination of 1.2 million Lux Hrs. Sample was weighed, dissolved in and diluted with acetonitrile to get 10 µg/ml.

After the photo degradation study for UV light and fluorescence light, BEC was 93.74% and CL was 98.16 % recovered, with no peak of degradant. The representative chromatogram is shown in **Figure 7**.

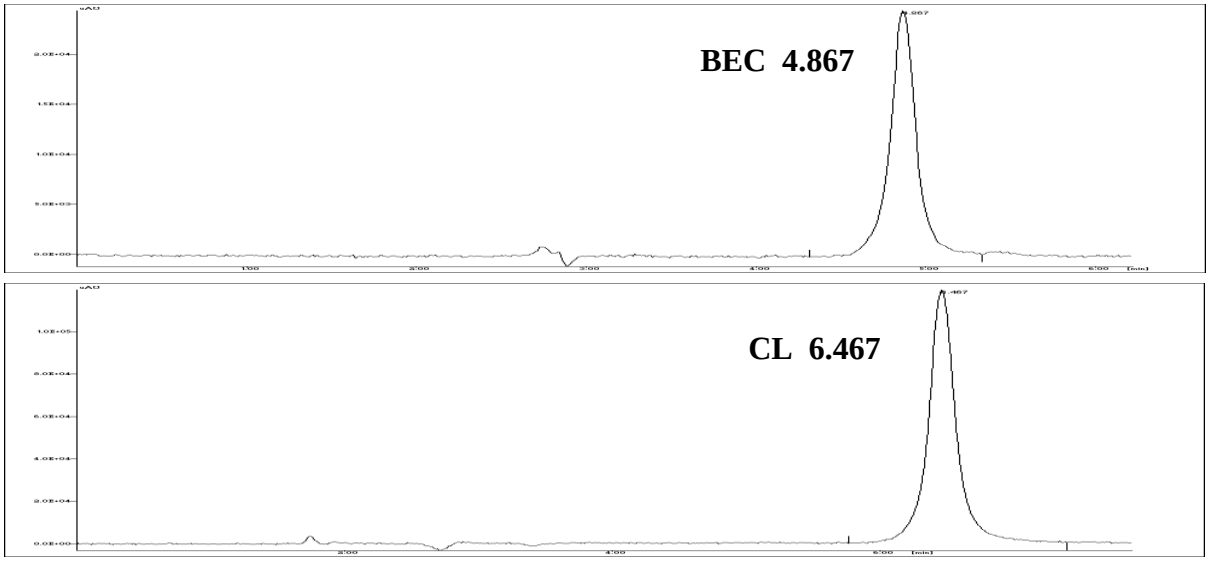

Figure 7. Chromatogram of: BEC and CL after photolytic degradation

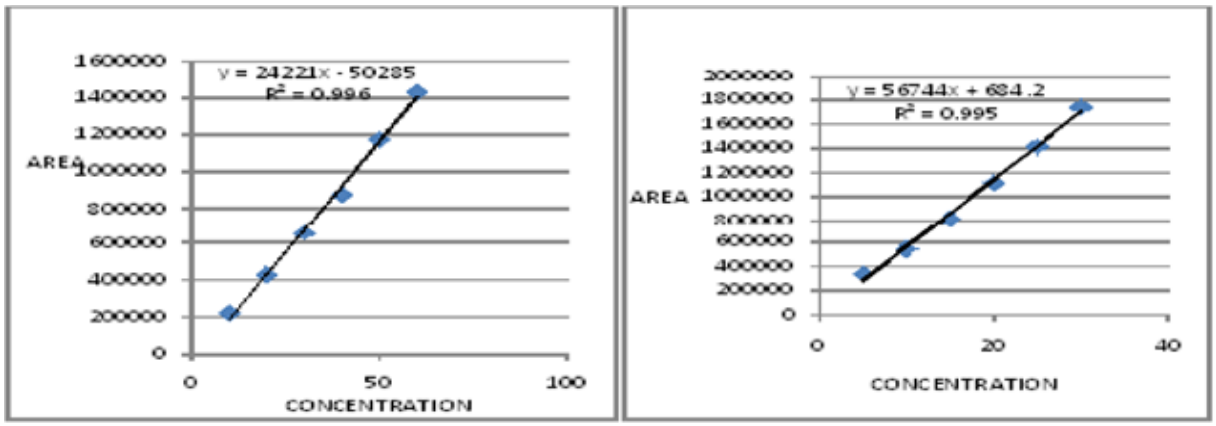

Figure 8. (a) Calibration curve of BEC, **(b)** Calibration curve of CL

Validation of analytical method

Specificity

The specificity of the method was ascertained by peak purity profile studies. The peak purity values were found to be more than 995, indicating the no interference of any other peak of degradation product, impurity or matrix.

Linearity

The linearity (relationship between peak area and concentration) was determined by analyzing six solutions over the concentration range of 10-60 µg/ml for BEC and 5-30 µg/ml for CL, six replicates per concentrations were analyzed, the equation of calibration curve was found to be y = 24221x - 50285 for BEC with r^2 = 0.996 and y = 56744x + 684.2 for CL with r^2=

Table 1. Intraday and Interday variation studies data for BEC

Concentration (µg/ml)	Intra-day Precision		Inter-day Precision	
	Average area	% R.S.D	Average area	% R.S.D
10	220608.60	0.277	222684.36	0.593
30	663433.99	0.585	666750.34	0.702
60	1423963.2	0.971	1419443.07	0.248

Table 2. Intraday and Interday variation studies data for CL

Concentration (µg/ml)	Intra-day Precision		Inter-day Precision	
	Average area	% R.S.D	Average area	% R.S.D
5	336049.92	1.5	333368.95	1.358
20	1116284.6	0.576	1118702.78	0.218
30	1735671.67	0.342	1736310.14	0.270

0.995. The peak area of drug was plotted against the corresponding concentrations to obtain the calibration curve as shown in **Figure 8**.

Precision

The precision of the method was demonstrated by intra-day and inter-day variation studies. For the intra-day studies, 3 replicates at 3 different concentrations (10, 30, 60 µg/ml for BEC and 5, 20, 30 µg/ml for CL) were analyzed in a day and percentage relative standard deviation (%RSD) was calculated.

For the inter day variation studies, 3 different concentrations were analyzed on 3 consecutive days and % RSD was calculated. The results obtained for intraday and inter day variations were found to be within limits (less than 2% RSD). The results obtained are shown in **Table 1** and **2**.

Limit of detection (LOD) and limit of quantitation (LOQ)

From the linearity data the LOD and LOQ was calculated, using the formula LOD = 3.3 σ/S and LOQ = 10 σ/S where, σ = standard deviation of the y intercept of linearity equations and S = slope of the calibration curve of the analyte. LOD of BEC and CL was found to be 0.773 µg/ml and 0.121 µg/ml, respectively. LOQ of BEC and CL was found to be 2.345 µg/ml and 0.369 µg/ml, respectively.

Assay

CLOCIP-B Cream formulation analysis was carried out as mentioned under section preparation of sample solution. Procedure was repeated for six times. The results obtained are shown in **Table 3**.

Accuracy

To check accuracy of the method, recovery studies were carried by spiking the standard drug to the CLOCIP-B cream sample solution, at three different levels around 50, 100 and

Table 3. Assay of marketed formulation

Drug	Peak Area (Avg.)	Amount Recovered (µg/ml)	%Recovery	%RSD
BEC	190895.60	9.956	99.566	1.502
CL	562903.748	9.907	99.071	0.751

Table 4. Accuracy of BEC and CL

Level%	Standard		Sample		%Recovery, ±%RSD	
	Bec(µg/ml)	CL(µg/ml)	Bec(µg/ml)	CL(µg/ml)	Bec(µg/ml)	CL(µg/ml)
50	5	5	10	10	101.10 ±1.67	98.66 ± 0.66
100	10	10	10	10	101 ± 0.50	100.33 ± 1.25
150	15	15	10	10	99.6 ± 0.40	100.13 ± 0.83

150%. Basic concentration of sample solution chosen was 10 µg/ml of BEC and 10 µg/ml of CL. % recovery was determined from linearity equation. The results obtained are shown in **Table 4.**

Robustness

Robustness of the method was checked by carrying out the analysis under conditions during which mobile phase composition (± 2% composition), detection wavelength (± 2 nm), flow rate (± 0.05 ml/min) were altered and the effect on the area were noted. Robustness of the method checked after deliberate alterations of the analytical parameters showed that areas of peaks of interest remained unaffected by small changes of the operational parameters indicating that the method is robust.

RESULTS AND DISCUSSION

Method development and optimization

For HPLC analysis, initially various mobile phases and stationary phase were tried in attempts to obtain the best separation and resolution between CL and BEC. The mobile phase consisting a combination of acetonitrile and 1 mM ammonium acetate buffer in the ratio of 90:10 v/v was found to be an appropriate mobile phase allowing adequate separation of two drugs using a Hi Q Sil C_{18} (250 x 4.6 mm, 5 µm) with flow rate of 1 ml/min at 223 nm and ambient temperature was used for good resolution and faster elution of compounds.

The results indicated the suitability of the method to study stability of Beclomethasone dipropionate and Clotrimazole under various forced degradation conditions like acidic, basic, hydrolysis, oxidation, dry heat and photolysis.

CONCLUSION

The developed method is found to be simple, sensitive, selective, accurate, and precise for analysis and can be adopted for routine analysis of drug in bulk and pharmaceutical dosage form.

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