Original Article

Development and validation of a reversed-phase HPLC method for simultaneous estimation of clotrimazole and beclomethasone dipropionate in lotion and cream dosage form

Abstract

Background: The combination of Clotrimazole and Beclomethasone dipropionate is used as anti-fungal and anti-inflammatory for external use in the form of cream and lotion. **Aim:** A simple, specific, economic, precise, and accurate reversed-phase high performance liquid chromatographic method development for the simultaneous estimation of clotrimazole (CT) and beclomethasone dipropionate (BD) in lotion and cream formulations. **Materials and Methods:** The chromatographic separation was achieved on a Kromasil C18 (150 mm × 4.6 mm, 5 μm) analytical column. A mixture of acetonitrile–water (70:30, v/v) was used as the mobile phase, at a flow rate of 1 ml/min and detector wavelength at 254 nm. The validation of the proposed method was carried out for specificity, linearity, accuracy, precision, limit of detection, limit of quantitation, and system suitability test as per ICH guideline. **Results:** The retention time of CT and BD was found to be 5.4 and 4 min, respectively. The linear dynamic ranges were from 2-16 μg/ml and 80-640 μg/ml for BD and CT, respectively. Limit of detection and quantification for BD were 0.039 and 0.12 μg/ml, for CT 1.24 and 3.77 μg/ml, respectively. **Conclusions:** The developed method was validated and found to be simple, specific, accurate and precise and can be used for routine quality control analysis of titled drugs in combination in lotion and cream formulation.

Key words:
Beclomethasone dipropionate, clotrimazole, reversed-phase high performance liquid chromatographic

Introduction

Clotrimazole is 1-[(2-chlorophenyl)(diphenyl) methyl]-1*h*-imidazole.[1] It is a prescription drug indicated for the treatment and prophylaxis of fungal infections. Clotrimazole interacts with yeast 14-α demethylase, a cytochrome p-450 enzyme that converts lanosterol to ergosterol, an essential component of the membrane. In this way, clotrimazole inhibits ergosterol synthesis, resulting in increased cellular permeability.[2-4] Beclomethasone dipropionate is 9a-chloro-11b–hydroxy-16b–methyl-3,20-dioxopregna 1,4-diene-17,21-diyldipropionate.[5] It is a synthetic halogenated glucocorticoid with anti-inflammatory and vasoconstrictive effects, is used for treating steroid-dependent asthma, allergic or non-allergic rhinitis. The anti-inflammatory actions of corticosteroids are thought to involve phospholipase A2 inhibitory proteins, lipocortins, which control the biosynthesis of potent mediators of inflammation such as prostaglandins and leukotrienes.[2-4]

The chemical structures of CT and BD are shown in Figure 1a and b.

A detailed survey of analytical literature for CT revealed several methods based on varied techniques, *viz*, HPLC,[6-9] Spectrophotometry,[10-12] Spectrofluorimetry,[13] High-Performance Thin-Layer Chromatography (HPTLC),[14,15] and stability-indicating HPLC method.[16] Similarly, a survey of the analytical literature for BD revealed methods based on HPLC for determination in pharmaceuticals.[6-8,17] According

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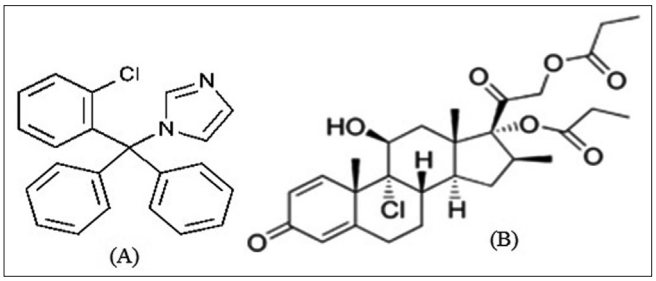

Figure 1: Chemical structure of (A) Clotrimazole and (B) Beclomethasone dipropionate[4,5]

to detailed survey of analytical literature, none of the reported analytical methods available for simultaneous estimation of CT and BD in their combined dosage form. None of the reported analytical procedures describes a simple and satisfactory RP-HPLC method for simultaneous determination of CT and BD in their combined dosage forms. So, the objective of this work was to develop simple, precise, and rapid RP-HPLC methods for combination drug products containing CT and BD.

Materials and Methods

Apparatus and instruments

- A Shimadzu (Kyoto, Japan) HPLC system (LC-2010CHT) equipped with autosampler, UV and photodiode array (PDA) detector, Rheodyne injector with 20 µl loop volume
- Balance, Model ALC 210.4 (Acculab)
- Ultra Sonicator (Fast Clean Ultrasonic Cleaner)
- pH analyzer (Chemiline CL 180 µc based pH meter)
- Volumetric flasks – 10 ml, 100ml (Borosil)
- Pipettes – 1 ml, 2 ml, 5 ml, 10 ml (Durasil)
- Separating funnel
- Beaker – 250 ml, 100 ml (Borosil).

Reagents and materials

- Clotrimazole (Glenmark pharmaceuticals Ltd.)
- Beclomethasone dipropionate (Glenmark pharmaceuticals Ltd.)
- HPLC grade acetonitrile, water (Finar Chemicals Pvt. Ltd, Ahmedabad, India)
- Cyclohexane (SD Fine Chemicals Pvt. Ltd., Ahmedabad, India)
- Whatman filter paper no. 41.

Formulation

- CANDID-B lotion (Glenmark pharmaceuticals Ltd. Label claim: Clotrimazole IP (1% w/v) and beclomethasone dipropionate IP (0.025% w/v) in 15 ml.
- CANESTEN-S cream (Bayer pharmaceuticals Ltd.) Label claim: Clotrimazole IP (10 mg) and beclomethasone dipropionate IP (0.25 mg)

Chromatographic condition

- Column: Kromasil C18 (150 mm × 4.6 mm, 5 µm)
- Mobile phase: Acetonitrile:Water (70:30)
- Detection wavelength: 254 nm
- Flow rate: 1 ml/min
- Injection volume: 10 µl
- Temperature: 40°C

Preparation of combined standard stock solution of CT and BD

Accurately weighed CT (40 mg) and BD (1 mg) were transferred into 10 ml volumetric flask and diluted up to the mark with ACN (Acetonitril) to give a stock solution having strength of 4000 µg/ml:100 µg/ml of CT:BD.

Preparation of test solution

Lotion

1 ml of lotion (10 mg:0.25 mg of CT:BD) was taken in 10 ml volumetric flask, and volume was made up with methanol. Then, extraction of prepared solution was done with cyclohexane using separating funnel. cyclohexane layer was discarded, and methanol layer was collected. 3 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 300 µg/ml:7.5 µg/ml of CT:BD.

Cream

1 gm of cream (10 mg:0.25 mg of CT:BD) was taken in beaker containing 10 ml of methanol, mixed well, and filtered. The solution was warmed for 5 min at 50°C, then cooled in ice-bath for 15 min and promptly centrifuged. The supernatant layer was taken and extracted with cyclohexane using separating funnel. cyclohexane layer was discarded, and methanol layer was collected. 3 ml was pipetted out from methanol layer into 10ml volumetric flask and diluted up to mark with mobile phase to get the concentration of 300 µg/ml:7.5 µg/ml of CT:BD.

Method development and optimization

The wavelength for the analysis of was selected from the UV spectrum of CT and BD by scanning in the range of 200-400 nm. From this, the wavelength of 254 nm was selected for the final method as these drugs has shown good absorbances. For HPLC analysis, initially various mobile phases and stationary phase were tried in attempts to obtain the best separation and resolution between CT and BD. The mobile phase consisting a combination of acetonitrile and water in the ratio of 70:30 v/v was found to be an appropriate mobile phase allowing adequate separation of two drugs using a Kromasil C18 (150 mm × 4.6 mm, 5 µm) with flow rate of 1 ml/min at 254 nm and 40°C temperature. 40°C Temperature was used for good resolution and faster elution of compounds. A HPLC chromatogram of separation of CT and BD were shown in Figure 2.

Method Validation

As per the ICH guidelines Q2R1, the method validation

parameters studied were specificity, linearity, accuracy, precision, limit of detection, limit of quantitation, and system suitability test.[18]

Specificity

Specificity of an analytical method is its ability to measure the analyte accurately and specifically in the presence of component that may be expected to be present in the sample matrix. Chromatograms of standard and sample solutions of CT and BD were compared, and peak purity spectra obtained from using photo diode array detector (PDA) were recorded in order to provide an indication of specificity of the method.

Linearity

Different volume (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 ml) were withdrawn from combined standard stock solution containing 4000:100 µg/ml of CT:BD and diluted upto 10 ml with mobile phase to get the linear concentrations of CT (80-640 µg/ml) and BD (2-16 µg/ml). Each solution was injected under the operating chromatographic conditions. Calibration curves were constructed by plotting peak areas versus concentrations, and the regression equation was calculated. Each response was average of three determinations.

Precision

The repeatability was checked by repeatedly (n = 6) injecting CT (400 µg/ml) and BD (10 µg/ml) standard solutions and recording the responses. The intra-day and inter-day precisions of the proposed method was determined by measuring the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for test mixture of 3 different concentration of CT (320,400,480 µg/ml) and BD (8,10,12 µg/ml). The results were reported in terms of relative standard deviation.

Accuracy (% Recovery)

The accuracy of the method was determined by calculating recoveries of CT and BD by the standard addition method. Known amount of standard solutions of CT (0,192,240,288 µg/ml) and BD (0,4.8,6.0,7.2 µg/ml) were added to a pre-quantified sample solution of CT (240 µg/ml) and BD (6 µg/ml). Each solution was injected in triplicate, and the percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equation of the respective calibration curves.

Limit of detection and limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation of y-intercept of calibration curve (N) and slope (S) of the calibration curve.

$$LOD = 3.3 \times N/S, LOQ = 10 \times N/S$$

System-suitability test

System suitability tests are used to verify that the resolution and repeatability of the system are adequate for the analysis intended. The parameters used in this test were retention time, capacity factor, theoretical plate, tailing factor, resolution.

Result and Discussion

Method validation

Specificity

The analytical method was found to be specific as no interference of excipients was found in separation. Study has shown that the CT and BD peaks were free from excipients and co-eluting impurities, as the peak purity index was >0.99 [Table 1].

(The peak mentioned as excipient in chromatogram was not observed in case of standard and blank, and it was only observed in test sample so we can confirm it as excipient. As we had used marketed sample, a detail about placebo preparation was not available; so, we had not prepared it and also not injected it.) Comparative HPLC chromatogram of std, test and blank of CT and BD were shown in Figure 3.

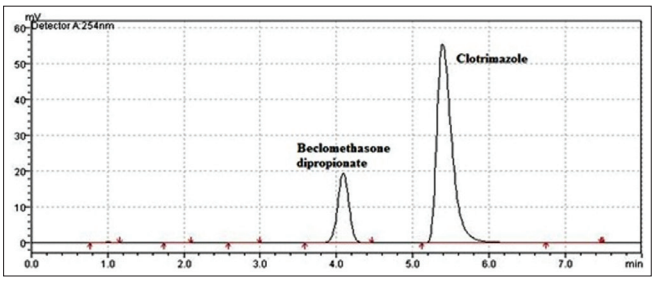

Figure 2: HPLC chromatogram of CT (400 µg/ml) and BD (10 µg/ml)

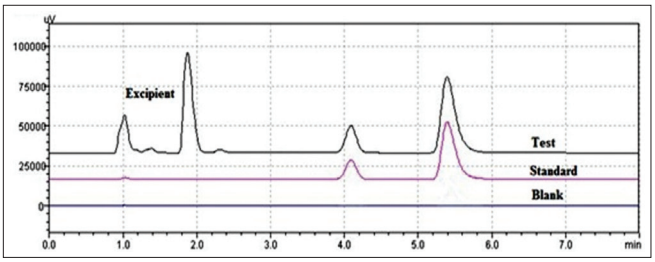

Figure 3: Comparative HPLC chromatogram of std and test of CT and BD

Table 1: Peak purity table of CT and BD

	Peak purity index	
	CT	BD
Standard	0.9999	1.0
Sample (lotion)	0.9999	0.9999
Sample (cream)	0.9999	0.9999

CT – Clotrimazole; BD – Beclomethasone dipropionate

Linearity

Linear correlation was obtained between peak area and concentration of CT and BD in the range of 80-640 µg/ml and 2-16 µg/ml, respectively. The linearity of the calibration curves was validated by the value of correlation coefficients of the regression [Tables 2 and 3].

Table 2: Linearity of CT and BD

CT (µg/ml)	Mean peak area±S.D. (*n*=3)*	%RSD	BD (µg/ml)	Mean peak area±S.D. (*n*=3)*	%RSD
80	101608±403	0.39	2	26732±89	0.33
160	197886±278	0.14	4	52407±105	0.20
240	298694±338	0.11	6	76682±124	0.16
320	386473±657	0.16	8	99877±119	0.11
400	485023±865	0.17	10	126453±225	0.17
480	577781±1034	0.17	12	151279±368	0.24
560	662979±989	0.14	14	174322±289	0.16
640	766392±831	0.10	16	201784±305	0.15

**n* – Number of repetition; CT – Clotrimazole; BD – Beclomethasone dipropionate; RSD – Relative standard deviation; S.D. – Standard deviation

Table 3: Linearity data of developed method

Parameter	CT	BD
Linearity range, µg/mL	80-640	2-16
Correlation coefficient, R[2]	0.9997	0.9998
Slope±SD (*n*=3)*	1177.8±92	12413±125
Intercept±SD (*n*=3)*	10599±445	1979.2±150

**n* – Number of repetition; CT – Clotrimazole; BD – Beclomethasone dipropionate; SD – Standard deviation

Table 4: Results of repeatability (*n*=6)*

Sr.no.	Assay (µg/ml) of CT (400 µg/ml)	Assay (µg/ml) of BD (10 µg/ml)
1	402.80	10.02
2	399.36	10.04
3	403.65	10.01
4	400.24	10.05
5	400.95	10.00
6	403.64	10.04
Mean	401.77	10.03
S.D.	1.83	0.018
%RSD	0.45	0.18

**n* – Number of repetition; CT – Clotrimazole; BD – Beclomethasone dipropionate; RSD – Relative standard deviation; S.D. – Standard deviation

Overlay chromatogram of CT (80-640 µg/ml) and BD (2-16 µg/ml) were shown in Figure 4.

Precision

The % RSD for repeatability of CT and BD were found to be 0.45 and 0.18, respectively. For lotion, the % RSD for intra-day precision was found to be in the range of 0.22-0.56 and 0.11-0.71; while inter-day precision was found to be in the range of 0.99-1.12 and 0.98-1.22 for CT and BD, respectively. While for cream, the % RSD for intra-day precision was found to be in the range of 0.16-0.62 and 0.28-0.73; while inter-day precision was found to be in the range of 1.07-1.25 and 1.05-1.19 for CT and BD, respectively, which indicates the method is precise [Tables 4 and 5].

Accuracy (% Recovery)

The accuracy study was carried out by the standard addition method. For lotion, the percent recovery was found in the range of 99.97-100.46% and 100-100.62% for CT and BD, respectively. For cream, the percent recovery was found in the range of 98.95-99.62% and 99.37-99.58% for CT and BD, respectively, which indicates accuracy of the method [Tables 6 and 7].

Limit of detection and limit of quantification

LOD and LOQ value of CT and BD were listed in following table [Table 8].

System suitability test

The % RSD of system-suitability test parameters was found satisfactory [Table 9].

Analysis of formulation

The proposed RP-HPLC method was successfully applied

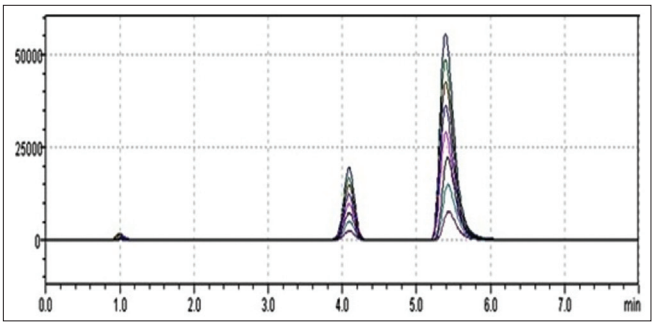

Figure 4: Overlay chromatogram of CT (80-640 µg/ml) and BD (2-16 µg/ml)

Table 5: Results of intra-day and inter-day precision (*n*=3)*

Concentration of test (µg/ml)		Lotion mean assay (µg/ml)±% RSD				Cream mean assay (µg/ml)±% RSD			
		Intra-day precision		Inter-day precision		Intra-day precision		Inter-day precision	
CT	BD	CT	BD	CT	BD	CT	BD	CT	BD
320	8	319±0.56	7.9±0.11	318±0.99	7.9±1.01	315±0.62	7.5±0.28	316±1.07	7.6±1.19
400	10	398±0.42	9.8±0.49	399±1.04	9.9±1.22	395±0.31	9.6±0.73	394±1.16	9.7±1.13
480	12	479±0.22	11.9±0.71	478±1.12	11.8±0.98	474±0.16	11.4±0.35	475±1.25	11.5±1.05

**n* – Number of repetition; CT – Clotrimazole; BD – Beclomethasone dipropionate; RSD – Relative standard deviation

Table 6: Results of recovery study (lotion) (*n*=3)*

Amt of sample (µg/ml)		Amt. of Std added (µg/ml)		Amt. found (µg/ml)		Amt. recovered (µg/ml)		% Recovery±S.D. (*n*=3)	
CT	BD	CT	BD	CT	BD	CT	BD	CT	BD
240	6			239.8	5.99				
240	6	192	4.8	431	10.76	191.1	4.77	100.46±0.55	100.62±0.10
240	6	240	6	479.9	11.98	240.0	5.99	99.97±0.40	100.16±0.80
240	6	288	7.2	527	13.19	287.1	7.2	100.31±0.22	100±0.47

**n* – Number of repetition; CT – Clotrimazole; BD – Beclomethasone dipropionate; S.D. – Standard deviation

Table 7: Results of recovery study (cream) (*n*=3)*

Amt of sample (µg/ml)		Amt. of Std added (µg/ml)		Amt. found (µg/ml)		Amt. recovered (µg/ml)		% Recovery±S.D. (*n*=3)	
CT	BD	CT	BD	CT	BD	CT	BD	CT	BD
240	6			235	5.93				
240	6	192	4.8	425	10.7	190	4.77	98.95±0.67	99.37±0.18
240	6	240	6	473	11.9	238	5.97	99.16±0.41	99.5±0.59
240	6	288	7.2	522	13.1	287	7.17	99.62±0.52	99.58±0.71

**n* – Number of repetition; CT – Clotrimazole; BD – Beclomethasone dipropionate; S.D. – Standard deviation

Table 8: LOD and LOQ of CT and BD

	CT	BD
LOD (µg/ml)	1.24	0.039
LOQ (µg/ml)	3.77	0.120

CT – Clotrimazole; BD – Beclomethasone dipropionate; LOD – Limit of detection; LOQ – Limit of quantification

Table 9: System suitability test parameter (*n* =3)*

Parameter	CT	%RSD	BD	%RSD
Retentiontime (min)±SD	5.402±0.0017	0.031	4.087±0.0016	0.039
Theoritical plates±SD	3920.447±20.45	0.52	3463.633±25.56	0.73
Capacity factor±SD	4.352±0.045	1.03	3.049±0.022	0.72
Tailing factor±SD	1.307±0.011	0.84	1.029±0.002	0.19
Resolution±SD	4.22±0.01	0.23	6.88±0.03	0.43

**n* – Number of repetition; CT – Clotrimazole; BD – Beclomethasone dipropionate; SD – Standard deviation; RSD – Relative standard deviation

Table 10: Result of analysis of formulation (*n*=3)*

Formulation	Label claim		Assay% of label claim±%RSD	
	CT	BD	CT	BD
Lotion	10 mg	0.25 mg	99.34%+0.44	99.20%+0.28
Cream	10 mg	0.25 mg	96.46%+0.55	97.61%+0.39

**n* – Number of repetition; CT – Clotrimazole; BD – Beclomethasone dipropionate; RSD – Relative standard deviation

for determination of CT and BD from lotion and cream. The percentage of CT and BD was found to be satisfactory, which is comparable with the corresponding claim amount [Table 10].

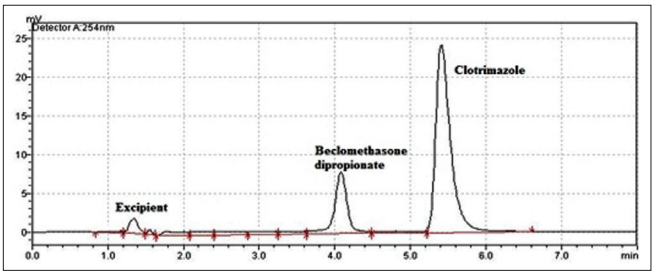

Figure 5: HPLC chromatogram of test (lotion) containing CT (400 µg/ml) and BD (10 µg/ml)

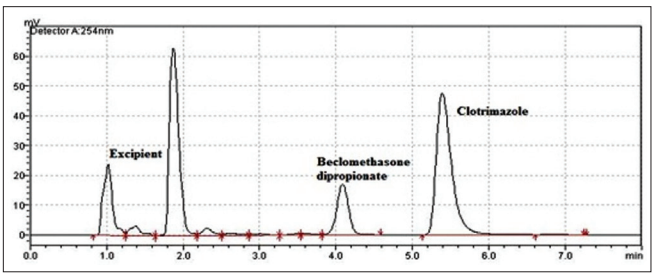

Figure 6: HPLC chromatogram of test (cream) containing CT (400 µg/ml) and BD (10 µg/ml)

HPLC chromatogram of test (lotion) containing CT (400 µg/ml) and BD (10 µg/ml) were shown in Figure 5.

HPLC chromatogram of test (cream) containing CT (400 µg/ml) and BD (10 µg/ml) were shown in Figure 6.

Conclusion

A simple, specific, economic, linear, precise, and accurate RP-HPLC method has been developed and validated

for quantitative determination of clotrimazole and beclomethasone dipropionate in lotion and cream formulation using simple mobile phase. The method is very simple and specific as both peaks are well separated from excipient peaks, which makes it especially suitable for routine quality control analysis work.

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