

# Modification and Validation of HPLC Analytical Method for Estimation of Clotrimazole in Health Care Products

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## Abstract

**Objective:** The aim of the study was to modify the USP analytical method and validate it for the estimation of clotrimazole in health care cream by using reverse phase HPLC.

**Methods:** LC-20AD liquid chromatography equipped with SPD-20A UV-VIS detector was used for analysis. The separation was done on C18 column which was attached with loop 20  $\mu$ L. Mobile phase was consisting of methanol:  $K_2HPO_4$  (85:15) and flow rate was 1.5 mL/min. The method was validated as per the ICH guidelines.

**Results:** The method is selective and reproducible, capable to separate all possible excipients and help to quantify accurately the amount of clotrimazole in health care product. The percentage coefficient variation (% CV) of the retention times and the peak areas were 0.504% and 1.453%, respectively. The linearity was evaluated by least square method i.e.,  $r^2=0.9996$ . The limits of detection (LOD) and limits of quantitation (LOQ) for clotrimazole were 0.25  $\mu$ g/mL and 0.5  $\mu$ g/mL respectively. The results of Instrumental precision were 103.5% with %CV of 0.612% and the intermediate precision was 0.35 to 1.43%. The recoveries at three concentrations levels over a range of 80 - 120% were found within the range of 101.2 to 103.8% as per ICH guidelines. The results of robustness show no major changes occurring over changes in flow rate from 1.4 mL/min. to 1.6 mL/min. The method was also applied for the analysis of clotrimazole in formulated health care cream and marketed pharmaceutical creams that contain clotrimazole.

**Conclusion:** It was concluded that the method is so simple, easy and applicable. It could be easily used for the analysis of compound in its dosage form. The robustness and ruggedness of the method are significantly broader, reproducible and could be adjusted according the needs.

**Keywords:** Clotrimazole; Health care product; Anti-itching cream; HPLC analytical method; Method validation; Antifungal

## Introduction

Clotrimazole is an antifungal medication commonly used in the treatment of fungal infections (of both humans and other animals) such as vaginal yeast infections, oral thrush, and ringworm.

The compound (3) is produced by alkylating imidazole (1) with o-chlorotriptyl chloride (2) in acetone, with triethylamine as the base [1] (Figure 1).

Additionally, clotrimazole is used to treat the sickling of cells (related to sickle cell anemia) by blocking ion channels or clop  $\beta$  channels in the red blood cell membrane, keeping ions and water within the cell [2,3].

The primary mechanism of action of clotrimazole is against the division and growth of fungi [4]. Clotrimazole alters the permeability of the fungal cell wall and inhibits the activity of enzymes within the cell [1]. Clotrimazole causes holes to appear in the cell membranes, essential constituents of the fungal cells can leak out. This kills the fungi and treats the infection.

Before using the analytical method for the determination of any compound the adjustment and modification of method is required as per feasibility. Analytical methods must be validated to provide reliable data not only for regulatory submissions but also essential for a number of purposes, including testing for QC release, testing of stability samples, testing of reference materials and to provide data to support the specifications.

High-performance liquid chromatography (HPLC) is a chromatographic method that is used to separate a mixture of compounds in analytical chemistry and biochemistry to identify, quantify or purify the individual components of the mixture [4,5].

The objective of this study was to prepare an anti-itching cream which contains clotrimazole as active ingredient and blend with herbal and non-herbal excipients. Pick USP standard method for the analysis of Clotrimazole and carry out the modification and validation by using various parameters like system suitability, specificity range and linearity, accuracy, precision and robustness in accordance with ICH guidelines. After suitable modification, the method was used for the estimation of Clotrimazole in anti-itching cream.

## Materials and Methods

### Instruments

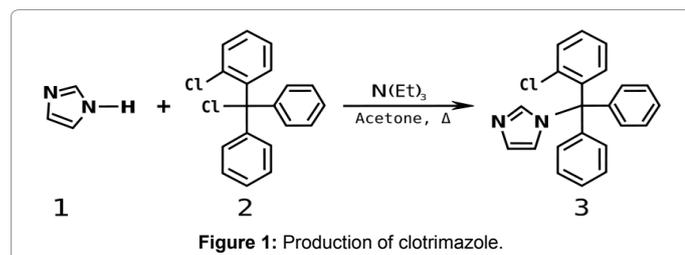
The HPLC system utilized consists of a LC-20AD liquid chromatography equipped with SPD-20A UV-VIS detector. Chromatographic separations were performed on C18 (4.6  $\times$  250 mm, L1; 5  $\mu$ m packing) which was attached with loop 20  $\mu$ L and connected with HPLC-Dell system. In addition, electronic balance, microliter syringe, micropipette and micro-pore filtration assembly were used in this study.

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## Materials

Clotrimazole (Reference standard) powder, methanol, acetonitrile, dibasic potassium phosphate and double distilled water, all solvents were of HPLC grade and reagents of analytical grade. Clotrimazole anti-itching cream was provided by Dabur international Ras Al Khaimah. While the marketed brands were collected from local market of Ras Al Khaimah.

## Preparation of anti-itching cream

It consisted of two phases, oil and aqueous. In aqueous phase added oleth. Oil phase contained cetostearyl alcohol, arlacel, cetareth 20, petroleum jelly and linseed oil. Temperature was maintained at 75°C for both phases separately and oil phase was transferred in to water phase. The mixture was homogenized for 15 minutes. When temperature reached at 45-40°C optiphen mint plus and pre blended mixture of clotrimazole, boric acid and zinc oxide (dispersed) in propylene glycol was added. Lastly when temperature reached at 35°C the pre-blend having menthol and perfume sweet floral was added and mixed for 15-20 minutes to get a clear formulation.

## Preparation of mobile phase

Mobile phase consisted on methanol:  $K_2HPO_4$  (85:15).  $K_2HPO_4$  solutions were filtered through filtration unit (Millipore, 0.45  $\mu$ m pore size) and degassed before use. The flow rate was maintained at 1.5 mL/min and volume of injection was 20  $\mu$ L. Detection was performed at a wavelength of 254 nm and analysis was carried out at ambient temperature.

## Preparation of stock solution

50 mg of clotrimazole RS was weighed accurately and transferred to 100 mL volumetric flask. Diluted with methanol, the concentration of stock solution was 500  $\mu$ g/mL (on dried basis).

## Preparation of sample solution

Accurately weighed 2.5 g of clotrimazole herbal health care product (1%) into a 100 mL volumetric flask dissolved and diluted to volume with methanol (250  $\mu$ g/mL).

## Method optimization and validation

The meaning of analytical validation, to the pharmaceutical world, is the process to confirm that an analytical procedure does what it purports to do: that is, to document through laboratory studies that the measurement procedure can reliably assess the identity, strength and quality of a bulk drug substance, excipient or finished pharmaceutical product.

The present experiment was carried-out according to the official specifications of United State Pharmacopeias (USP-36), International Conference on Harmonization (ICH-1996) and Centre of Drug Evaluation and Research (CDER-1994) [6-8]. In the present study analytical method for the estimation of clotrimazole was validated with respect to system suitability, specificity, linearity, limit of detection,

limit of quantification, precision, accuracy and ruggedness. Than the modified method was used to estimate the percentage content of clotrimazole (1%) in the finish product of Dabur international and verified this method by analyzing different pharmaceutical creams containing clotrimazole (1%) as mentioned in Table 1.

## System suitability

It is dependent on, the objective of the analytical procedure, its robustness and the availability of reference substances. The assessment for the suitability of the system was done using six (6) drug replicas at concentration of 500  $\mu$ g/mL. It was used to confirm that the resolution and reproducibility of the chromatographic system is adequate for the analysis to be done. The method was evaluated by analyzing the repeatability, retention time, peak area, tailing factor, theoretical plates (Tangent) of the column with relative standard deviation and base line correction (ICH Q2B, 1996) [7].

**Specificity:** of the method demonstrates that there is no interference between the pharmacologically active ingredients from excipients that incorporated in their dosage forms (ICH Q2B, 1996).

**Linearity:** refers to a proportional relationship between the drug concentration and the instrument response (or signal) used to measure the drug (ICH Q2B, 1996). The linearity for the present method was determined by analyzing standard solution of clotrimazole in the concentration range of 0.01-0.07% (100-700  $\mu$ g/mL). To evaluate the LOD and LOQ of the method, serial dilutions were made from the stock solution in the range of 0.25-10  $\mu$ g/mL.

**Accuracy:** refers to the difference between the average assay values and the true (known) drug concentrations (ICH Q2B, 1996). To validate the accuracy and recovery of the method from herbal health care cream, samples were prepared by known amount of drug at three different concentration levels (in duplicate) i.e., 80%, 100% and 120% of the label claim and were analyzed according to the procedures.

**Precision:** relates to the variation or reproducibility of the data. Precision measurements should be obtained by replication of various drug concentrations and by the replication of standard concentration curves prepared separately on different days (ICH Q2B, 1996). The method precision was analyzed under the quality of repeatability that had been done by six (6) determinations at 100% of the test concentration, intermediate precision (intra-day and inter-day variation), and reproducibility (an inter-laboratory trial).

**Robustness:** studies were done on method precision using a sample concentration of 250  $\mu$ g/mL by making slight variations in flow rate and concentration of methanol: $K_2HPO_4$  solution in the mobile phase.

**For content assay of cream:** 2.5 g were accurately weighed and transferred carefully to a 100 mL volumetric flask. The cream was dissolved in methanol and shaken for about 15 minutes. The solution was filtered through 0.22  $\mu$ m Millipore filter paper. The filtered sample solution was then injected. The final concentration of working solution was equivalent to 0.25 mg/mL.

## Results and Discussion

Clotrimazole prevents the growth of fungi by interfering with the production of the membrane that surrounds fungal cells. It is commonly available without a prescription in various dosage forms, such as a cream, vaginal tablet, or as a prescription troche or throat lozenge. In the present study a health care cream was prepared and a HPLC analytical method was modified for the estimation of clotrimazole in health care products. It is a simple, sensitive, and an easy HPLC method

Brand Name	Dabur Anti-itch Cream	Cream -A	Cream -B	Cream -C
Origin	UAE	USA	Oman	Spain
Strength of Clotrimazole	1.0%	1.0%	1.0%	1.0%
Batch No	-	5NNA25003	5ES8015A	BXPJLLH
Mfg Date	-	03-2015	12-2015	06-2015
Exp Date	-	02-2018	12-2018	06-2018

Table 1: Label information of different pharmaceutical products containing clotrimazole.

to operate, using UV detection for the determination of clotrimazole not only as raw material as well in the herbal health care products. The method is selective and reproducible, capable to separate all possible excipients and help to quantify accurately the amount of clotrimazole in health care product (Figure 3).

One of the most important advantages of analytical method validation is reduction of pharmaceutical cost and increase the confidence of developer as well as the user. Similarly it plays a supreme importance during the process of drug discovery, release to market and development, culminating in a marketing approval.

System suitability was conducted by injecting six (6) replicated injection of freshly prepared clotrimazole RS solution at concentration of 500 µg/mL. The percentage coefficient variation (% CV) of the retention times and the peak areas of clotrimazole were 0.504% and 1.453%, respectively. The theoretical plate count, based on USP tangent calculations [9] for clotrimazole was  $3656.63 \pm 29.66$  (Table 2). The average tailing factor for six consecutive peaks was 1.036, factor equal to or near to 1.0 is signified as complete symmetrical (Table 2).

The linearity of the peak area response was determined by making a seven concentration points in the range of 0.01 – 0.07%. Calibration curve was plotted against peak area versus concentration. The linearity was evaluated by linear regression analysis using least square method. The linear regression coefficient was  $r^2=0.9996$  (Figure 4).

The calculations for Limit of detection (LOD) and limit of quantification (LOQ) were done on the basis of peak responses of clotrimazole standard solution. LOD is determined by the signal to noise ratio and generally acceptable detection limit ratio is 3:1 and it was found 0.25 µg/mL. LOQ is determined by the signal to noise ratio and a typical signal to noise ratio is 10:1 which is acceptable for estimating the quantification limit. It was found to be 0.5 µg/mL for clotrimazole (Table 3).

Accuracy of the method was determined by preparing samples of anti-itching cream in duplicate at the three concentration levels over a range of 80-120% and assayed according to the procedure. The recoveries at three concentrations were found within the range of 101.2 to 103.8% of the label claim of clotrimazole and %CV values for each level ranged from 0.01 to 0.59% as per ICH guidelines (Table 4).

The precision of the method was evaluated for repeatability (Instrumental precision) and intermediate precision. Instrumental precision expresses the precision under the same operating conditions over a short interval of time. It is also termed intra-assay precision [10]. Six samples were prepared at the 100% level and assayed according to the procedure described. The results of the precision study are summarized in Table 5. The mean percent recovery of clotrimazole was 103.5% of the label claim with %CV of 0.612%. Intermediate precision of the method was demonstrated by different analyst on different day, using different HPLC column and system. For intermediate precision, the percentage relative standard deviation of clotrimazole was found to be 0.35 to 1.43%. These values were well within the acceptable limit of  $\leq 2\%$  as per USP (Table 6).

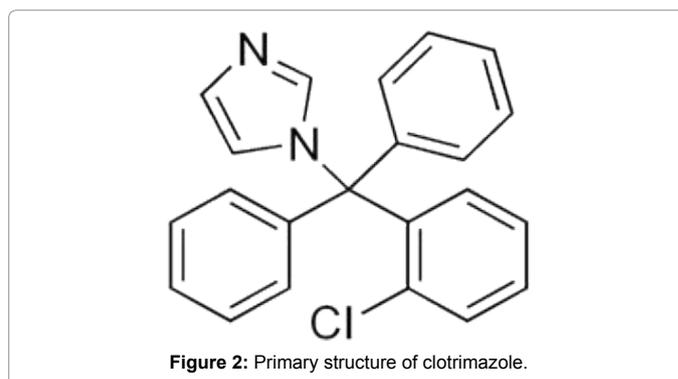


Figure 2: Primary structure of clotrimazole.

The robustness of a method is its ability to remain unaffected by small changes. Robustness study was performed on method precision sample by making slight variations in flow rate, amount of methanol and buffer and changes in pH one at a time. The results of robustness in the present method showed no major changes occurring over changes in flow rate from 1.4 mL/min. to 1.6 mL/min, showing a recovery of approximately 100%. In case of variation in the amount of methanol 80-85% and  $K_2HPO_4$  buffer 15-20%, there was no significant changes in recovery percentage.

After modification and validation, the method applied for the analysis of clotrimazole in formulated herbal cream as well it was used to analyze different pharmaceutical creams that contain clotrimazole, available in local market of Ras Al Khaimah (Table 1). The developed method is so simple, easy and applicable. It could be easily used for the analysis of compound in its dosage form. Retention time is between 6-7 minutes that could also be adjusted according the needs.

Table 7 represents the results of the assay of clotrimazole cream. The active contents recovery of the three (3) brands was between 98.43-100.16%, indicating that in spite of the difference in the excipients used by manufacturers, the recovery of active drug was possible by developed method as per BP/USP specifications indicating its good selectivity. The drug content in the health care cream (1%) was estimated, were similar to the labeled claim of marketed formulations. The standard and sample chromatograms of Dabur Antiitch, Cream-A, Cream-B and Cream-C were shown in Figure 5, indicating that the proposed method is also suitable for the separation of Betamethasone from combined dosage form (Figures 5c and 5d).

The good thing of this method is that the preparation of standard and sample solution is very simple and easy just in the methanol; the mobile phase is consisted on methanol and phosphate buffer, capable to separate the clotrimazole from herbal cream by using simple C18 column. Whereas different HPLC analytical method for estimation of clotrimazole from cream are available in literature but they have complicated method of sample and standard preparation [11], estimation is done for lozenges dosage form by Gracemart C18 column [12], separation is done by using Kromasil C18 with maintaining 40°C temperature [13].

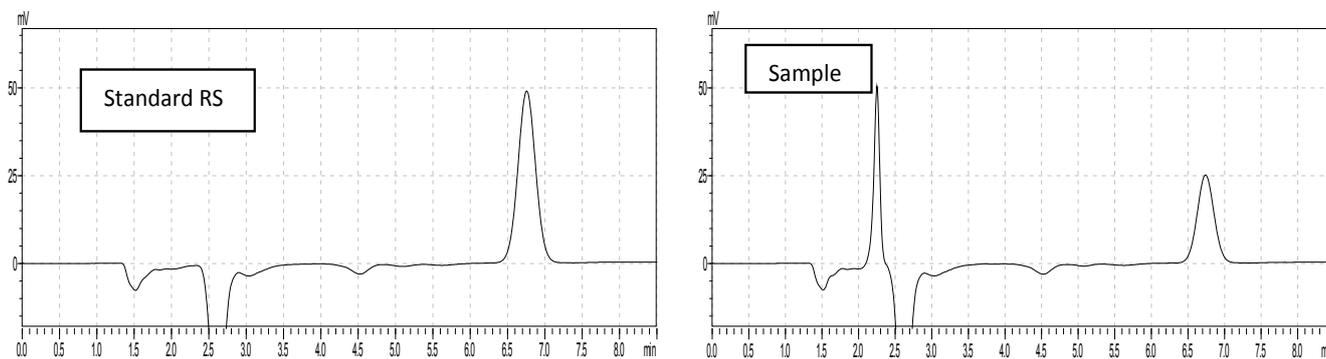


Figure 3: a) 510 µg/ml clotrimazole RS in methanol, b) 251 µg/ml concentration of clotrimazole from herbal health care product.

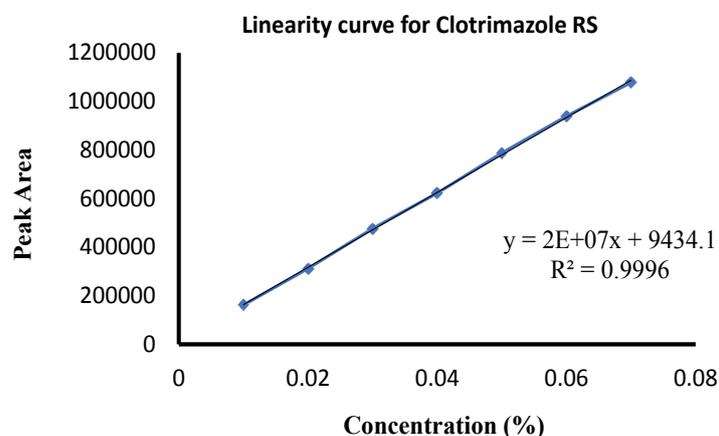


Figure 4: Calibration curve shows linearity over the concentration range.

Injection number	Retention time (min)	Peak area of Clotrimazole	Tailing factor	USP Tangent Plate count	Base line Correction As per MP
1	6.754	827606	1.035	3642.78	Complies
2	6.792	855512	1.037	3683.11	
3	6.756	820504	1.036	3703.77	
4	6.725	831857	1.034	3629.46	
5	6.702	840827	1.036	3637.73	
6	6.709	838495	1.035	3642.93	
<b>Mean</b>	<b>6.739667</b>	<b>835800.2</b>	<b>1.036</b>	<b>3656.63</b>	
<b>SD</b>	<b>0.034016</b>	<b>12147.04</b>	<b>0.00105</b>	<b>29.66</b>	
<b>%CV</b>	<b>0.504</b>	<b>1.453</b>	<b>0.101</b>	<b>0.811</b>	

Table 2: System Suitability parameters for Clotrimazole.

Standard number	Concentration µg/mL	Peak Area
1	10	19316
2	5	8747
3	2	3349
4	1	1656
5	0.5	855
<b>Slope</b>		1944.4
<b>Intercept</b>		409.53
<b>R<sup>2</sup></b>		0.9978
<b>LOD</b>		0.25 µg/mL
<b>LOQ</b>		0.5 µg/mL

Table 3: Estimation of LOD and LOQ.

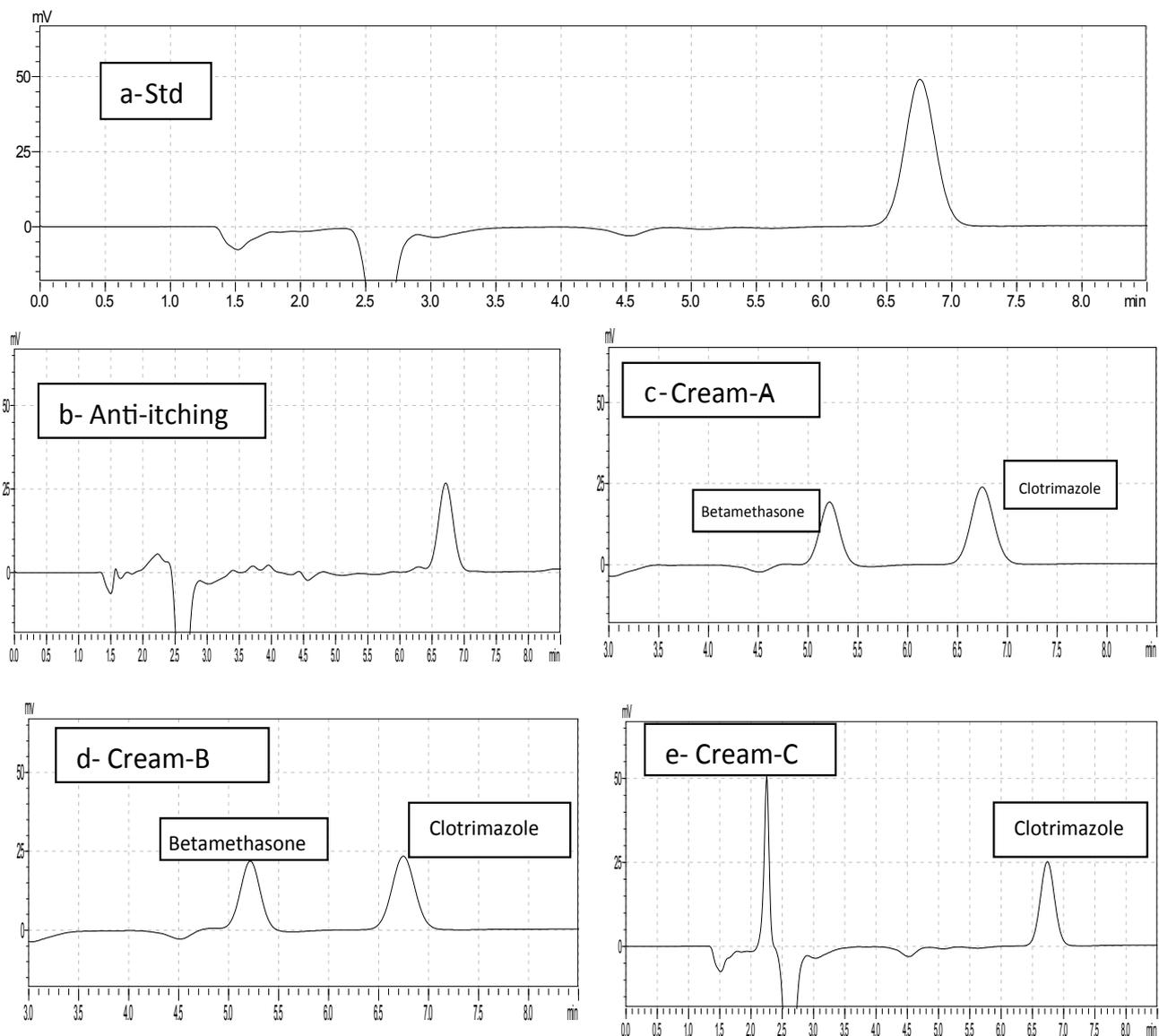


Figure 5: a) Standard clotrimazole RS in methanol; b) Separation of clotrimazole from herbal health care product; c) Separation of clotrimazole from Cream-A; d) Separation of clotrimazole from Cream-B; e) Separation of clotrimazole from Cream-C.

80% Level							
Assay	Sample Amount (gram)	Run-I	Run-II	Mean	% Recovered	% CV	% of LC
Sample-1	2.05	318449	319853	319151	79.6	0.01	102.3
Sample- 2	2.07	327063	320779	323921	80.74		103.8
100% Level							
Sample-1	2.51	395243	407836	401540	100.09	0.1128	103.0
Sample- 2	2.49	402273	402117	402195	100.25		103.1
120% Level							
Sample-1	3.01	486943	485721	486332	121.23	0.59	102.1
Sample- 2	3.09	484449	479986	482218	120.22		101.2

Table 4: Accuracy data for the assay of clotrimazole cream.

Sample Number	Sample Amount (gram)	Clotrimazole		% Recovery
		Theoretical peak area	Measured peak area	
1	2.494	389982	406239	104.2
2	2.485		404743	103.8
3	2.485		405031	103.9
4	2.483		404192	103.6
5	2.467		401772	103.0
6	2.510		399877	102.5
Mean	2.487	389982	403643	103.5
SD	0.0142		2360.53	0.633
%CV	0.57		0.585	0.612

Table 5: Precision Repeatability Data.

Conc. (%)	1 <sup>st</sup> day	2 <sup>nd</sup> day	Mean	± SD	%RSD
0.01	163830	162180	163005	1166.73	0.72
	164871	161582	163227	2325.68	1.43
Mean	164351	161881	163116	1746.2	1.07
0.02	317293	312634	314964	3294.41	1.05
	311985	310449	311217	1086.12	0.35
Mean	314639	311542	313091	2190.27	0.70
0.03	484746	476301	480524	5971.52	1.24
	486301	481249	483775	3572.31	0.74
Mean	485524	478775	482150	4771.91	0.99

Table 6: Intermediate Precision Data.

	Standard Peak area			Dabur Anti-itch Cream		Cream-A		Cream-B		Cream-C	
	Std 1	Std 2	Std 3	Sp 1	Sp 2	Sp1	Sp2	Sp1	Sp2	Sp1	Sp2
	840827	831857	838495	407836	407171	384837	383654	380992	380440	405254	402117
Average Area	837060			407504		384246		380716		403686	
RSD	0.56			0.12		0.22		0.1		0.55	
Sp. Wt (gm)	0.051			2.51		2.369		2.357		2.456	
%age Purity				0.9892		0.9883		0.9843		1.0016	
Tolerance Limit as per BP/USP				95.00 - 105.00% of the labeled amount of Clotrimazole							
				98.92%		98.83%		98.43%		100.16%	
Label Claim				1.00%							

Table 7: Compression of content assays of Clotrimazole cream with marketed cream.

## Conclusion

A HPLC method for determination of Clotrimazole in cream was modified and validated as per ICH and FDA guidelines. Linearity, accuracy, precision, and robustness results reveal that the proposed method is accurate, precise, rapid and simple. The sample preparation is simple, and the elution is isocratic. The method is amenable to the analysis of large number of samples with excellent precision and accuracy. Statistical analysis proves that method is repeatable, sensitive and selective for the analysis of Clotrimazole in herbal health care cream as well in pharmaceutical formulation. Based on this evidence the method can be stated as highly economical and it is recommended for routine use in quality control studies.

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