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DEVELOPMENT OF ANALYTICAL METHOD AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF CLOBETASOL PROPIONATE AND KETOCONAZOLE IN PHARMACEUTICAL CREAM FORMULATION BY RP-HPLC METHOD

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Abstract:

A simple, accurate rapid and precise has been developed and validated for simultaneous estimation of Clobetasol Propionate and Ketoconazole in Pharmaceutical Cream Formulation by RP-HPLC Method. The successful estimation was carried out of the drug product is developed on the ODS Qualisil *Column C_{18} (250 mm x 4.6 mm, 5µm or equivalent) at ambient temperature using Methanol: Acetonitrile: Phosphate buffer (50:20:30 v/v/v) as mobile phase composition. The flow rate was adjusted to 1.5mL/minute and the absorption maxima were observed on UV detector at 254 nm. Retention Time for Clobetasole Propionate 11.1±0.1min and Ketoconazole 12.2±0.1min. The linearity was obtained in the concentration range of 6-14µg/mL and 120-280µg/mL for Clobetasole Propionate and Ketoconazole respectively. Mean percentage recoveries were 99.16% for Clobetasole Propionate and 99.17% for Ketoconazole. The LOD of Clobetasole Propionate and Ketoconazole were found to be 0.7µg/mL and 2.0µg/mL whereas the LOQ was 3.0µg/mL and 10.0µg/mL respectively. Percentage relative standard deviation of percent assay values for replicate sample preparation was 0.28% for Clobetasole Propionate and 0.47% for Ketoconazole. The method was robust with respect to change in flow rate, temperature and composition of mobile phase. The method was validated statistically and applied successfully for the determination of Clobetasole Propionate and Ketoconazole. Validation studies revealed that method is specific, rapid, reliable, and reproducible. The high recovery and low relative standard deviation confirm the suitability of the method for the routine determination of Clobetasole Propionate and Ketoconazole in pharmaceutical cream formulation.*

Keywords: *Clobetasol Propionate, Ketoconazole, RP – HPLC, Method Validation*

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INTRODUCTION:

Clobetasol Propionate is a potent topical glucocorticoid for topical use [1]. It is a white or almost white crystalline powder that is practically insoluble in water, freely soluble in acetone and in dichloromethane, and sparingly soluble in ethanol [2]. Clobetasol Propionate is used for short-term relief of anti-inflammatory, Pruritic manifestations of moderate to severe corticosteroid responsive dermatoses and in Psoriasis [3-5]. It is also highly effective for contact dermatitis caused by exposure to poison ivy/oak. It has very high potency and typically should not be used with occlusive dressings. Different dosage forms for topical use are currently approved by United States Food and Drug administration (USFDA). It is available in dosage forms such as cream, gel, ointment, shampoo etc [6-9].

The molecular structure of the drug is given in Fig.2

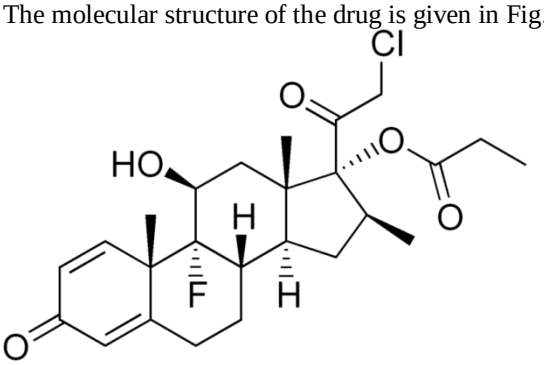


Fig 1: Molecular structure of Clobetasol Propionate

Chemical Name:- Clobetasol Propionate is Chemically 21-Chloro-9-fluoro-11β-hydroxy-16β- methyl-3, 20-dioxopregna-1,4-dien-17-yl Propanoate. Its molecular formula is $C_{25}H_{32}ClFO5_4$ and molecular weight is 466.97g/mol.

Clobetasol Propionate is also used to treat several autoimmune diseases including alopecia areata, vitiligo, lichen sclerosus, and lichen planus [10].

Ketoconazole is an imidazole derivative with a wide antifungal spectrum and possesses some antibacterial activity. It is widely used in the treatment of dermal and systemic mycoses. Ketoconazole presents advantage over other imidazole derivatives in sustaining adequate blood levels following oral administration [11-13]. Due to its advantages of oral administration, lower toxicity than most azole antimycotics, and effective against many fungal and gram positive microorganisms. Ketoconazole has been widely used as an antifungal drug [14-15]. It inhibits cytochrome P450 dependent lanosterol C14 demethylase, which is responsible for production of ergosterol, a necessary component in fungal cell wall synthesis. Ketoconazole is a weak base with pKa values of 2.94 and 6.51 [16-17]. The pharmaceutical dosage formulations of Ketoconazole: cream, shampoo and tablet are applied in the treatment of human systemic fungal infections [18-19].

The molecular structure of the drug is given in Fig.1

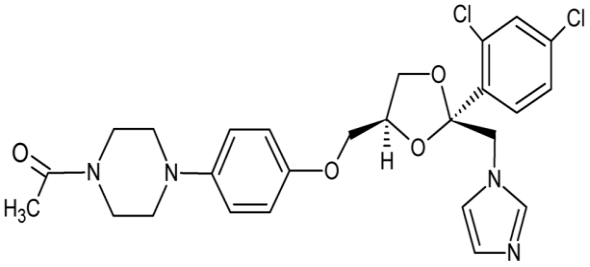


Fig 2: Molecular structure of Ketoconazole

Chemical Name:- Ketoconazole is Chemically Cis-1-acetyl-4-[4-[2-(2,4-dichlorophenyl)-2H-imidazolyl methyl)-1,3-dioxolan-4-yl] methoxy] phenyl]-piperazine. Its molecular formula is $C_{26}H_{28}Cl_2N_4O_4$ and molecular weight is 531.431 g/mol.

It interferes with the fungal synthesis of ergosterol, a constituent of cell membrane specific for fungi [20]. Ketoconazole also inhibits biosynthesis of triglycerides, phospholipids and oxidative or peroxidative enzyme activity, resulting in intracellular buildup of toxic concentrations of hydrogen peroxide [21]. Additionally, in the treatment of Candida albicans infections, it inhibits the transformation of blastospores to invasive mycelial forms [22]. However, Ketoconazole is lipophilic and practically insoluble in water [23], therefore its clinical use has some practical disadvantages. Ketoconazole is an antifungal drug approved by the US FDA in 1981.

From Literature survey it has been concluded that many methods have been done for Clobetasol Propionate and Ketoconazole in combination with other drugs like RP-HPLC [24-26], UV Spectrophotometry [27-30], HPTLC [31-32] etc. However, no method is reported for simultaneous estimation of Clobetasol Propionate and Ketoconazole by RP-HPLC in any literature. In the present investigation, a specific RP-HPLC method is described for the simultaneous estimation of Clobetasol Propionate and Ketoconazole in pharmaceutical cream formulation.

MATERIALS AND METHODS:

Materials and Reagents: Acetonitrile (HPLC grade), Methanol (HPLC grade), Ketoconazole RS, Clobetasol Propionate RS, Distilled Water and Potassium Dihydrogen Phosphate.

Instrumentation and Chromatographic condition: Chromatographic separation was performed on a Analytical technology HPLC instrument (Software: HPLC Work Station) equipped with Deuterium lamp as detector, HPLC pump and manual Rheodyne sample injecting facility programmed at 20 µL capacity per injection was used.

Experimental Design:

Selection of detection wavelength: Sensitivity of HPLC method with UV detection depends on proper selection of detection wavelength. An ideal wavelength is the one, at which both drugs gives good response. In the present study, standard solution of Clobetasol Propionate and Ketoconazole were scanned over the range of 200-400 nm. Wavelength of Clobetasol Propionate and Ketoconazole, 254nm was selected for analysis because of Clobetasol Propionate is low dose concentration in cream formulation.

Selection and Optimization of chromatographic conditions: Selection of proper HPLC method depends upon nature of drugs (ionic or neutral molecule), its molecular weight and its solubility. RP-HPLC method was selected initial separation because of its simplicity, efficiency, reproducibility and its recommended use for ionic and moderate to non-polar compounds. To optimize the chromatographic conditions the effect of chromatographic variables such as mobile phase, pH, flow rate, column temperature and solvent ratio were studied. The condition that gave best resolution, symmetry, theoretical plates and capacity factor was selected for estimation.

Chromatographic conditions for developed RP-HPLC method:

Stationary Phase: ODS Qualisil C-18 column, (250 mm x 4.6 mm, 5µm or equivalent)
Flow rate: 1.5ml/min.
Column Temperature: Ambient Temperature
Detection wavelength: 254 nm

Methodology:

Preparation of Phosphate Buffer 0.05M: Weight accurately 6.8g of Potassium Dihydrogen Phosphate, Transfer in 1000mL of volumetric flask add 700mL of distilled water sonicate properly and markup to volume.

Preparation of Mobile phase: The mobile phase was prepared by adding the ratio of (50:20:30 v/v/v) Methanol: Acetonitrile: Buffer 0.05M (Potassium Dihydrogen Phosphate) and then filtered through 0.45 µm membrane filter; sonicated for 15 min.

Diluent Preparation: The Mobile phase was used as diluent.

Preparation of standard stock solution

a) Standard stock solution for Clobetasol Propionate: Weigh accurately about 10.0 mg Clobetasol Propionate RS in 20 mL volumetric flask. Dilute with mobile phase to mark. Take 2 mL from above solution in 10 mL volumetric flask and dilute to mark with mobile phase (Solution A).

b) Standard stock solution for Ketoconazole: Weigh accurately about 10.0 mg Ketoconazole RS in 25 mL volumetric flask. Dilute with mobile phase to mark (Solution B).

c) Combined standard solution: Mix 1 mL from (Solution A) and 5 mL from (Solution B) in to 10 mL volumetric flask and dilute to mark with mobile phase.

d) Sample Solution: Weigh accurately cream about 1.0g in to a 50.0 mL volumetric flask, and add 30.0 mL of mobile phase, sonicate to dissolve, dilute to volume with mobile phase (Centrifuge for better separation 5000rpm for 5min).

Procedure: Filter both Sample and Standard Solution with 0.2 micron filter paper and inject 20 µl.

HPLC Chromatogram for Clobetasol Propionate and Ketoconazole:

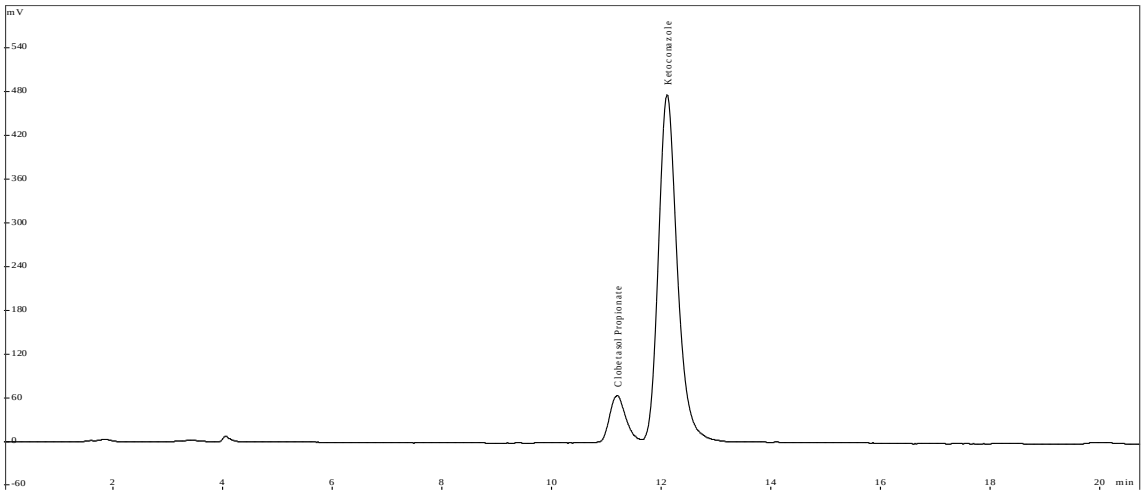


Fig. 3: Chromatogram for Clobetasol Propionate and Ketoconazole of STD

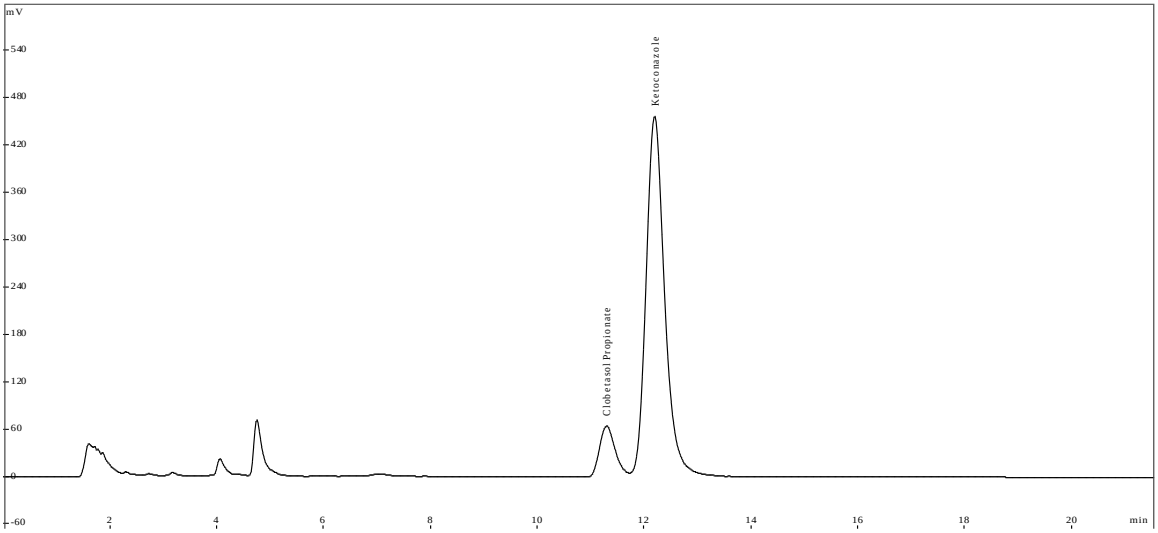


Fig. 4: Chromatogram for Clobetasol Propionate and Ketoconazole of SAMPLE

Validationof Proposed Method:

All of the analytical validation parameters for the proposed method were determined according to International Conference on Harmonization (ICH) guidelines [33].

Analysis of sample was carried out using the above method and the result are show in table 1.

System suitability:

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. In that the Theoretical Plates, Tailing factor and Peak Retention time were calculated for the standard solutions table 2. The values obtained demonstrated the suitability of the system for the analysis of this drug combination.

Linearity:

Linearity of the method was established by analysis of combined standard solution. The range of an analytical procedure is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

Mixed standard solution of Clobetasol Propionate and Ketoconazole were prepared with mobile phase in such a way that the five different concentrations of Clobetasol Propionate and Ketoconazole in the range of 4-12 µg/mL and 160-280 µg/mL respectively. The peak area was recorded for all the peaks as shown in table II for linearity of Clobetasol Propionate and Ketoconazole. The plots of peak area versus the respective concentration were found to be linear with regression coefficient (r2=0.999) for Clobetasol Propionate and (r2=0.998) for Ketoconazole as shown in Graph 1.

Table 1: Analysis of sample

Contents	Label claim	Found %w/w	Assay % of label amount
Clobetasol Propionate	0.05%	0.04978%	99.56%
Ketoconazole	1.0%	9.983%	99.83%

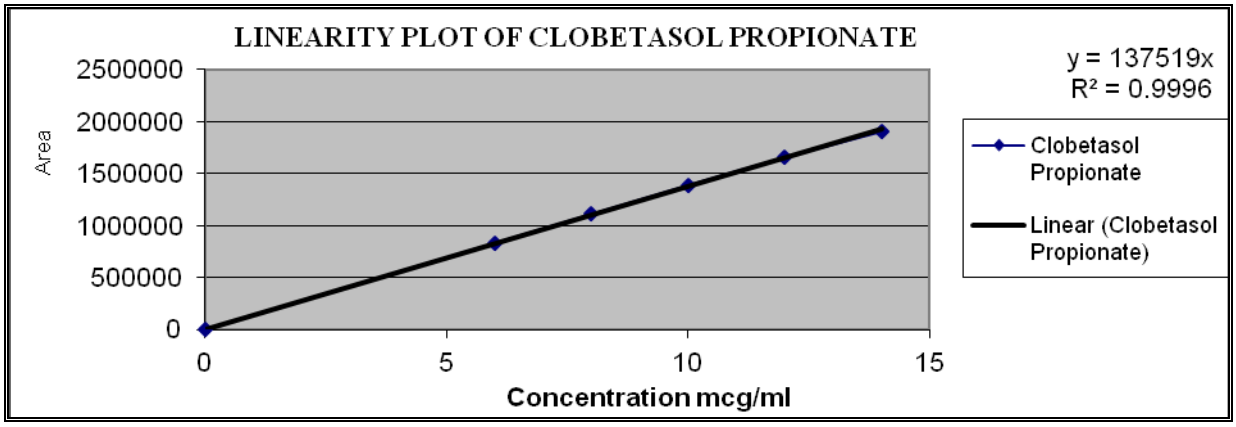
Sample in-house production batch

Table 2: System Suitability Parameter

Parameter	Clobetasol Propionate	Ketoconazole
Precision of the method (n = 5)	0.28%	0.47%
Theoretical Plates	2185	4726
Tailing factor	1.000	1.000
Retention time	11.124	12.219

Table No. 3: Linearity and Statistical analysis data for Clobetasol Propionate

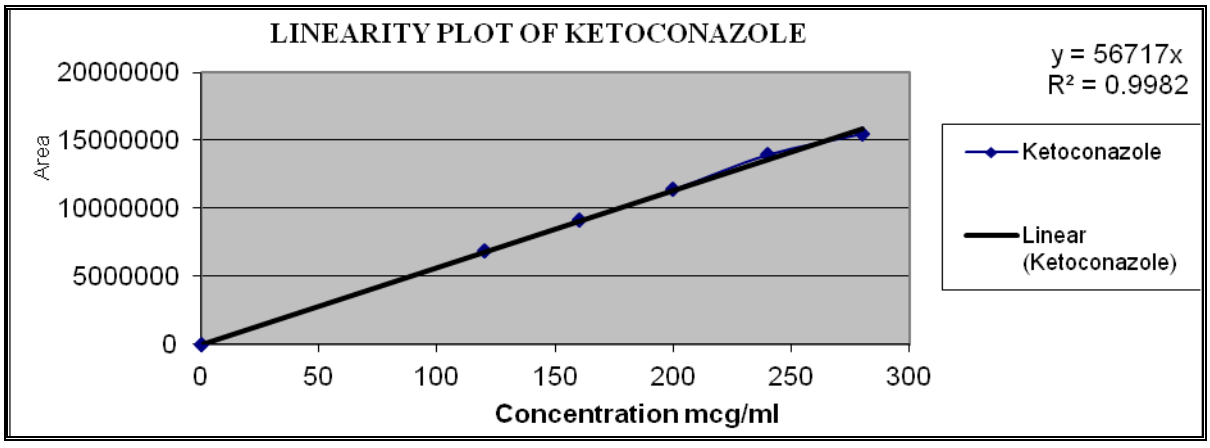
S. No.	**Concentration (µg/mL)**	**Area**
1.	6µg/mL	832564
2.	8µg/mL	1110156
3.	10µg/mL	1387583
4.	12µg/mL	1655055
5.	14µg/mL	1903358
Correlative Coefficient(r^2)		**0.999**



Graph No 1: Linearity Graph of Clobetasol Propionate

Table No. 4: Linearity and Statistical analysis data for Ketoconazole

S. No	**Concentration (µg/mL)**	**Area**
1.	120µg/mL	6836245
2.	160µg/mL	9119012
3.	200µg/mL	11398762
4.	240µg/mL	13967245
5.	280µg/mL	15698355
Correlative Coefficient(r^2)		0.998



Graph No 2: Linearity Graph of Ketoconazole

Accuracy:

For accuracy study data from nine determinations over three concentrations at 80%, 100% and 120% of expected sample concentration covering the specified range was determined & expressed as recovery values. Accuracy of the proposed method was evaluated by spiking standard stock solution containing Clobetasol Propionate and Ketoconazole into placebo equivalent to amount present in sample preparation to achieve at 80%, 100% and 120% of the target concentration. Measured recovered concentration versus added concentration for Clobetasol Propionate and Ketoconazole and calculated % recovery. % recovery for all components were found more than 97.0%, this indicate accuracy of the method. Recovery results are tabulated in Table 5 and 6.

Precision Studies:

Precision of method was studies by analysis of multiple sampling of homogeneous sample. The precision of analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous Sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogenous authenticated sample. Precision Expressed as % RSD is given in Table-7 which should be less than 2%.

Table No. 5: Accuracy (by Recovery) data for the proposed RP-HPLC method for Clobetasol Propionate

Accuracy Level %		Amount Added (mg)	Amount Recovery (mg)	Area	%Recovery	Mean%
80%	1	8.3mg	8.2mg	1130586	99.05%	99.40%
	2	8.1mg	8.08mg	1111699	99.80%	
	3	8.1mg	8.04mg	1112658	99.37%	
100%	1	10.2mg	10.07mg	1387544	98.76%	98.99%
	2	10.1mg	10.03mg	1386469	99.32%	
	3	10.2mg	10.19mg	1389255	99.97%	
120%	1	12.1mg	12.03mg	1665347	99.46%	99.09%
	2	12.2mg	12.09mg	1666148	99.11%	
	3	12.2mg	12.17mg	1667755	99.77%	

Table No. 6: Accuracy (by Recovery) data for the proposed RP-HPLC method for Ketoconazole

Accuracy Level %		Amount Added (mg)	Amount Recovery (mg)	Area	%Recovery	Mean%
80%	1	8.1mg	8.02mg	9118542	99.12%	99.2%
	2	8.1mg	99.06mg	9118629	99.57%	
	3	8.2mg	8.11mg	9119591	98.91%	
100%	1	10.2mg	10.21mg	11399762	99.28%	99.40%
	2	10.3mg	10.22mg	11408739	99.32%	
	3	10.1mg	10.06mg	11398562	99.62%	
120%	1	12.0mg	11.93mg	13968689	99.42%	98.93%
	2	12.1mg	11.92mg	13965655	98.53%	
	3	12.2mg	12.05mg	13984284	98.85%	

Table No. 7 system precision result of the proposed RP-HPLC Method:

Sample	**Clobetasol Propionate**	**Ketoconazole**
Sample 1	99.55%	99.42%
Sample 2	98.94%	99.09%
Sample 3	99.52%	98.31%
Sample 4	99.23%	99.67%
Sample 5	99.60%	98.95%
%RSD	**0.28%**	**0.47%**

Robustness and Ruggedness of the Method:

Robustness of the method

Robustness is a measure of its capacity to remain unaffected by small but deliberate variations in the chromatographic method parameters and provides an indication of its reliability. This was done by small deliberate changes in the chromatographic conditions at 3 different levels and retention time of Clobetasol Propionate and Ketoconazole was noted. The factor selected were flow rate, pH and % Methanol in the mobile phase. It was observed that there were no deliberate changes in the chromatogram, which demonstrated that the RP-HPLC method developed, are robust. Results describe in Table 8.

Ruggedness of the method:

The USP guideline defines ruggedness as "the degree of reproducibility" of the test result obtained by the analysis of the same samples under a variety of normal test condition such as; different Laboratory, different analyst, different instrument etc. Here this was done by changing the instrument and analyst. Results, presented in the Table 9 that indicates the selected factors are remained unaffected by small variations of this parameter.

Table 8: Robustness of the method

Factor	**Level**	**Retention time**	
	Flow rate ml/min	Clobetasol Propionate	Ketoconazole
1.3	-0.2	11.124	12.240
1.5	0	11.121	12.249
1.7	+0.2	11.119	12.245
Column Temperature			
23°C	-2	12.845	13.952
25°C	0	11.138	12.262
27°C	+2	10.172	11.251
% Methanol in the mobile phase			
55	-0.5	11.624	12.753
60	0	11.157	12.242
65	+0.5	10.732	11.645

Table 9: Ruggedness of the method

	Clobetasol Propionate	Ketoconazole
Between instrument **I** and **II**		
Instrument	% Content	% Content
I	99.55%	99.28%
II	99.81%	98.97%
% Error	0.26%	0.31%
Between instrument **I** and **II**		
Analyst	% Content	% Content
I	100.17%	99.91%
II	99.23%	99.35%
% Error	0.94%	0.56%

CONCLUSION:

Based on the results, this study is a typical example of the development of an assay method following ICH guidelines. A new isocratic RP-HPLC method has been developed and validated for determination of Clobetasol Propionate and Ketoconazole in the pharmaceutical cream formulation. The results of the validation studies showed that the RP-HPLC method possesses significant linearity, precision, accuracy, specificity, sensitivity, high efficiency and resolution, and no interference from the excipients, as were demonstrated. The proposed method was successfully applied and is suggested for the quantitative analysis of Clobetasol Propionate and Ketoconazole in combined pharmaceutical cream formulations for QC, where economy and time are essential and to assure therapeutic efficacy.

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