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Development and validation of HPLC Method for Estimation of Clotrimazole from Microemulsion Formulation

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Abstract: *Background : Clotrimazole is a broad-spectrum antifungal agent commonly used for topical treatment of fungal infections . Microemulsion based drug delivery have an advanced approach for enhancing solubility , stability , and bioavailability. Thus , microemulsion formulation were prepared, and the present study aim to develop an HPLC method for estimation of clotriazole in microemulsion formulation .*
Result : A C18 column (Phenomenex Hypersilgold)(5 µm, 250 × 4.6 mm) was used. A mixture of ACN and water (100 ml water was added with 1 drop of 1 M OPA) (70:30 v/v) was selected as a mobile phase. The flow rate was controlled at 1.2 mL/min. The injection volume was 20 µL. The wavelength detector was operated at 210 nm. The data were integrated with the PDA detector with EMPOWER software. This method was found to give a sharp peak of CTZ at a retention time of 4.6 min. No interfering peaks of other components in the formulations were seen, percent recoveries were within 100 ± 2%, and %RSD was not higher than 2, indicating for a high degree of specificity, accuracy, and precision, respectively. The linear regression analysis data for the calibration curve also exhibited a good linear relationship. CTZ was extracted from a ME .
Conclusion : From the reported experiments, the present HPLC technique was successfully used for estimation of the CLT from the microemulsion formulation .

Keywords: HPLC, Microemulsion , Clotrimazole

I. INTRODUCTION

Fungal infection , particularly superficial mycoses, continue to be a significant public health concern , especially in tropical and subtropical region .Clotrimazole, an imidazole derivative , is a broad spectrum antifungal. It inhibits the biosynthesis of ergosterol, a componentof the fungal cell membrane, and this affects the permeability of the cell membrane, which results in leakage and loss of essential intracellular compounds and finally causes cell lysis. [1]

Microemulsion are one of the best candidates as a novel drug delivery system because of their long shelf life, improved drug solubilization with ease of preparation and administration .Few methods have been reported for the determining clotrimazole individually or in combination in different formulations.These includes HPTLC, Spectroscopy etc. According to survey of analytical litreture, none of the reported methods describes a simple HPLC method for estimation of clotrimazole in microemulsion formulation .

Thus, their was a need for a validated simple HPLC method to determine clotrimazole in microemulsion formulation [2]

Fig No :1:Strcture of clotrimazole

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Methods

Chemicals : Clotrimazole from Yarrow Pharma Mumbai (99% purity)**,** HPLC - grade methanol obtained from Thermosil fine chem industries. HPLC – grade acetonitrile from Swastic Chemicals Nagpur , Distllied water , Ortho phosphoric Acid obtained from Prayojina Labotories India , Isopropyl palmitate ,Tween 80, Polyethylene glycol 400 ,Methyl cellulose , Methyl parabean obtained from Loba chemical pvt ltd .

Instruments :. Water 600 HPLC system consisting of C_{18} (Phenomenex Hypersil gold)/ 4.6x250 mm) column and 996 PDA detector with EMPOWER Software. Shimadzu UV – Vis Spectrophotometer 1900 , Magnetic stirrer of REMI, pH meter of Hanna , Ultrasonification and Homogenizer of IKA T -25 Ultra Turrex were used .[3,4]

Preformulation study :

Physical appearance : The drug clotrimazole powder was examined for its organoleptic properties like color taste and odour **.**

Solubility study : The sample was qualitatively tested for its solubility in various solvents. It was determined by taking certain amount of drug sample in 1 ml of solvent e.g. water, methanol, ethanol, pH buffer 6.8 in small test tubes and well solubilized by shaking upt o saturation .

Melting point : The Melting point was determined by the capillary method using Digital Melting point apparatus. It can be performed by filling of the drug packed by tapping the bottom of the capillary on a hard surface so that the drugs pack down in to the bottom of the tube. The apparatus was started and dip the thermometer in it & tempreture at which the drug melts was noted. Average of triplicate reading was taken and compared with litreture [5].

U.V. Spectroscopy of Drug :

(a) Determination of Wavelength of Maximum Absorbance (λ max) :

10mg of drug was weighed accurately and transferred to 10ml of volumetric flask. Then Methanol (suitable solvent) was added to dissolve the drug completely. The volume was made up to 10 ml with solvent. The prepared sample was 1000µg/ml.

The aliquot portion of stock solution were diluted appropriately with HPLC grade methanol to obtain concentration 10 µg/ml. The solutions were scanned in the range of 400-200 nm in 1 cm cell against blank .

(b) Preparation of Calibration Curve of Clotrimazole :

The calibration curve was plotted between the concentration and absorbance. The different concentrations between 5-30µg/ml were scanned at 264nm and absorbances were recorded.

Fourier-Transform Infra Red spectroscopy (FT-IR) : The IR spectrum of drug substance was authenticated using IR spectroscopy. The presence of characteristic peaks associated with specific structural characteristics of the drug molecule was noted.

Method of Preparation of Microemulsions :

Preparation of aquous phase: (Dissolved, PEG -400 by heating)

Preparing oil phase : Dissolved isopropyl palmitate , tween 80 , drug and preservative by heating

Emulsifying aquous & oil phase : Mixed in suitable vessel and given time to equilibrium ,using emulsification techniquies – using high shear homogenizer by applying 600 rpm for 15 min.

Evaluation :

1 Organoleptic Characteristics Organoleptic characters were monitored to detect any visible signs of instability such as creaming, cracking phase separation or color changes.

2 pH Determination: The pH of fresh sample and samples kept in different storage conditions was determined by a digital pH meter. The pH measurements were also taken for the samples at 24 h on days 7, 15, and 30.

3 .Viscosity: The viscosities of the samples were determined at 25°C spindle speeds ranging from 100 to 200 rpm while using a spindle CP 41 in a Brookfield programmable Rheometer. Rotate the spindle in the microemulsion till we get a constant dial reading on the display of the viscometer .

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4 . Particle size : Microscopic analysis was performed on optical microscope (400X) in order to measure globule size and multiple natures of all formulations. The oil droplet size was measured by dynamic laser light scattering technique using particle size analyzer (Malvern Mastersizer 2000**).**

5 . Drug content analysis : 1ml of clotrimazole Microemulsion was taken in 10 ml volumetric flask containing 1 ml methanol and Volume was made up to 10 ml with phosphate butter 6.4 pH. From the above solution. 1 ml was further diluted with 10 ml phosphate buffer to get 10 ml. The resultant solution was filtered through Whatman filter paper and absorbance of the solution was measured at 210 nm using UV spectrophotometer.

6 . Freeze Thaw :

Freeze-thaw cycle testing is a part of stability testing that allows to determine the microemulsion formulation will remain stable under various conditions. It consists of quick freezing and thawing were to kept in test tube for 24 hrs at freezing temperature and 24 hrs at room temperature and then measured the temperature by using thermometer heating up to 50 ^{0}C to observed the formulation was stable at under conditions.

7 .Centrifugation: Those formulations that passed the heating cooling cycle then subjected to were centrifuged test. The microemulsion were centrifuged at 3500 rpm for 5 min. Those formulations that did not show any phase separation some formulation show phase separation test.

8. **In-Vitro Diffusion study**: The in-vitro drug release study was carried out on a simple dissolution cell using cellophane membrane.Prior to release studies, the cellophane membrane was soaked in distilled water for 6 hours, washed frequently 4 times by changing distilled water, then immersed in 5% v/v glycerol solution for at least 60 min and washed finally with 5 portions of distilled water.

15 ml freshly prepared micro emulsion was added to donor chamber, made up of a hollow glass tube (2.5 cm in diameter and 10 cm in length) and membrane was tied on bottom end of the tube with a nylon string. This tube was dipped into 250 ml vessel containing 100 ml of PBS pH 6.8 and was stirred at 100 rpm on a magnetic stirrer and maintained at 37 °C which acted as receiving chamber. Once move sample as from the receptor compartment at 1,2,3,4,5,6,7,8 hours and replace same volume of medium .

Aliquots of 1ml were collected from receiving chamber at predetermined time intervals and the drug content was determined on UV spectrophotometer at 210nm after suitable dilution .

II. DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR ESTIMATION CLOTRIMAZOLE

Method development strategy :

Selection of common solvent (Diluent) :-

Methanol of HPLC grade was selected as common solvent for preparation of stock solution and developing spectral characteristics of drugs, further dilutions from stock solutions were made in mobile phase.The selection was made after assessing the solubility of clotrimazole in different solvents i.e Methanol, Acetonitrile and water.

Preparation of standard stock solution

Accurately weigh 1mg of clotrimazole was dissolved in 10 ml methanol. this solution used as a standard solution.

Preparation of diluents :-

Methanol of HPLC grade was selected as a common solvent for preparation of stock solution and developing spectral characteristics of drug , further dilution from stock solutions were made in the mobile phase.

Procedure

The mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. the standard solution containing clotrimazole was injected in different combinations of solvents, to get a stable peak with good peak characters. Each solution was filtered through membrane filter (size0.15 micron).To achieve acceptable separation using selected chromatographic conditions .the following chromatographic condition were established by trial and error were kept constant through out the method **.**

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Optimized Chromatographic Parameters:

Column:C_{18} (Phenomenex Hypersilgold)/4.6x250 mm, **Flow Rate :**1.2ml/min **,Wavelength :**210 nm , **Mode :**Isocratic **Injection volume :**20µl **, Column oven Temperature:** Ambient (25^0C), **Run Time :**10 minutes , **Mobile Phase :**100 ml water was added with 1 drop of 1M OPA solution and ACN (30 :70) %v/v .

Method validation :- The method has been evaluated for system suitability, specificity, linearity, precision, accuracy, robustness, and stability of solution as per ICH guideline Q2(R1) .

System Suitability : System suitability parameters (capacity factor, tailing factor, number of theoretical plates, and resolution of peak) were assessed by filtering mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. A 20 mL std. drug solution was injected which was made in five replicates and the system suitability parameters were recorded.

Specificity : The specificity of the method has been established by comparing the chromatograms obtained by injecting blank (mobile phase), solution of clotrimazole (20 µg/ ml), and sample solution of clotrimazole microemulsion. Prepared the placebo solution by weighing equivalent amount of placebo present in the sample to be taken for assay preparation in triplicate, diluted it as per the test method and injected into the HPLC system. Evaluate the % interference from placebo and recorded the observation.

Precision was assessed by injecting three samples of clotrimazole solution (20 µg/ml) on the same day (repeatability) and a different day (intermediate precision). The precision of the method was investigated as %RSD of the response .

Linearity: 20,25,30,35 µg/ml of clotrimazole solutions were prepared by diluting the solution from mobile phase. Solutions were filtered through 0.45 µm syringe filter and injected in an HPLC system to measure the peak area. The calibration curve (peak area v/s concentration) was plotted. The correlation coefficient of the calibration curve was determined to ensure the linearity of the analytical method.

Accuracy : The accuracy samples were prepared by spiking the standard into the pre-analyzed formulation sample at different concentrations (80%, 100% and 120%) and injected each in triplicate. The resultant mixture was injected and recovery of standard spiked was calculated.

Robustness Deliberate minute variations in the chromatographic conditions such as flow rate (±0.1 ml/min), acetonitrile ratio in the mobile phase (± 2%) have been made. After each change, assay results were checked by injecting the clotrimazole solution (20 µg/ml) into the chromatographic system and the results were compared with those under the original chromatographic conditions.

Results and Discussion :-Preformulation studies it is observed that clotrimazole is a white fine powder having no odor . Solubility was determined in various solvents found that freely soluble in ethanol ,methanol, slightly soluble in Distilled water . Melting point was observed in range 147-148 degree celcius .Maximum wavelength was determined at 210 nm by scanning sample from 200-400 nm and calibration curve was obtained by absorbance of aliquots from 5–30 (µg/ml) with following linear equation y=0.024x-0.01 . R^2 = 0.999 . Stability confirmed by FT-IR studies .All six clotrimazole formulation were odorless , washable , homogeneous and stable was evaluated under various parameters , pH of all formulations were observed at 6.8 and viscosity 26.6 to 40.23 centipoise .All of six, formulation F6 was found to be excellent on the basis of stability , agains colour change , creaming and phase separation.

Fig No :2: Prepared Microemulsion formulation of clotrimazole drug

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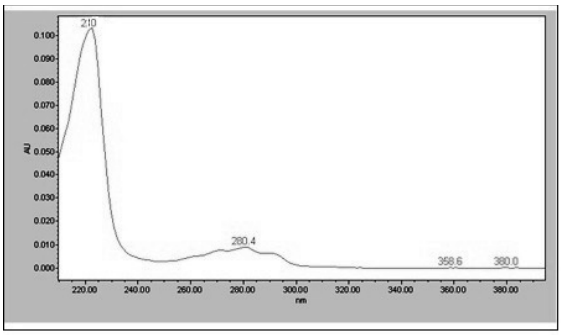

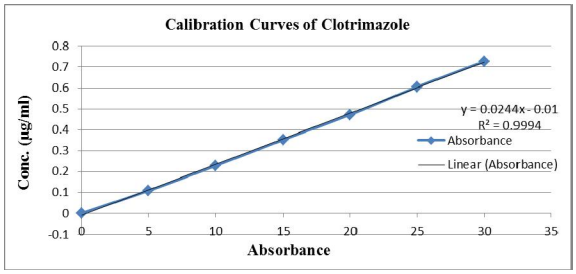

Fig No :2: Scanning wavelength maxima of clotrimazole . Fig No:3: Calibration Curve

Sr. no.	Clotrimazole (mg)	Isopropyl palmitate (ml)	Tween 80 (ml)	PEG 400 (ml)	Methyl cellulose (mg)	Methyl paraben (mg)	Distilled water (ml)
F1	100	10	2.5	0.5	120	20	qs
F2	100	10	3 .0	0.7	120	20	-
F3	100	7	2.0	0.6	120	20	-
F4	100	13	3.0	0.7	120	20	-
F5	100	13	3.5	0.5	120	20	-
F6	100	13	3.5	0.9	120	20	-

Table No1: Composition of micro –emulsion formulation Evaluation of prepared Multiple Emulsion formulation

Batches	Particle size µm	pH	Density (g/cc)	Viscosity (centipoises)	Drug content (%)	Centrifugation (3500rpm)	Freeze thaw study
F1	0.04	6.65	0.96	26.6	65.90	Phase Separation	Phase Separation
F2	0.02	6.97	0.95	34.4	71.04	Stable	Stable
F3	0.03	5.97	0.98	17.6	60.62	Phaseseparation	PhaseSeparation
F4	0.02	6.86	0.97	40.23	81.04	Stable	Stable
F5	0.02	6.67	0.98	45.56	70.60	Stable	Stable
F6	0.02	6.86	0.98	56.38	85.23	Stable	Stable

Table No :2: Evaluation of prepared Multiple Emulsion formulation

In Vitro Drug Release Profile Of Clotrimazole Microemulsion %CDR increases with increase in the ratio of oil :water as well as the ratio of surfactant : co surfactant , as compared to F1 –F6 % cumulative drug release data and graph indicate that F6 shows maximum pearmeation of clotrimazole after 8 hours with compared to other formulation

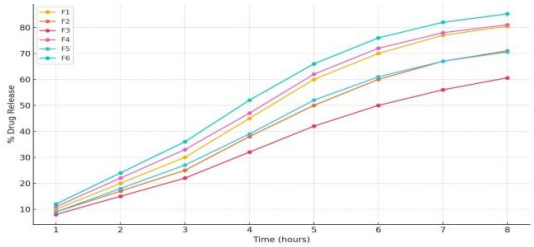

Fig No : 4:In vitro drug release study

Selection of Optimized formulation There were total 6 batches of different concentration are prepared. Out of these F6 formulation containing drug and excipient were found to be an optimized batch they have good stability and have no phase separation . These batch shows better result of other evaluation parameter.

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Method Validation

1 .System suitability After setting the optimum conditions, system suitability parameters for the method are determined and compared with recommended limits. The clotrimazole was repeatedly eluted at 5.6 min, tailing factor forpeak less than 2 indicating good peak symmetry, and a number of theoretical plates always greater than 2000 ensure good column efficacy.

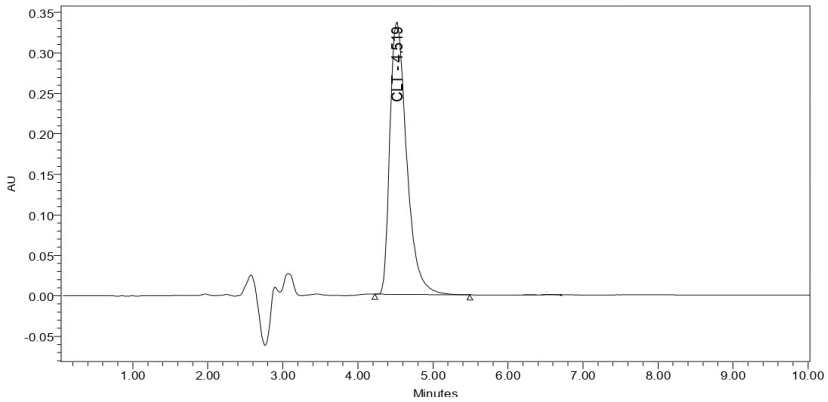

Fig No :4: Chromatogram for system suitability showing retenstion time 4.5

Sr. No	Peak area	Retention Time	Symmetry	No. of theoretical Plates
	CLT	CLT	CLT	CLT
1	460866	4.602	1.89	8475
2	439073	4.683	1.92	8214
3	437334	4.632	1.81	8654
4	432571	4.6541	1.89	8723
5	433709	4.6067	1.92	8462
Mean	435671.8	4.5059	1.886	8505.6
S.D	3044.044	0.011597	0.045056	198.2128
%R.S.D	0.698701	0.45017	2.388946	2.33038

Table No 3: Summary of system suitability of Test results

	CLT		
	Levels		
	80%	100%	120%
Amt added (µg/ml)	16	20	24
	16	20	24
	16	20	24
Amt taken (µg/ml)	16	20	24
	16	20	24

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	16	20	24
Amt recovered (µg/ml)	15.97	19.92	23.97
	15.96	19.95	23.94
	15.94	15.96	15.99
% Recovery	98.80	98.50	98.87
	98.70	98.69	98.72
	98.50	98.76	98.96
Mean % recovery	98.61	98.65	98.85
% RSD	0.15	0.13	0.12

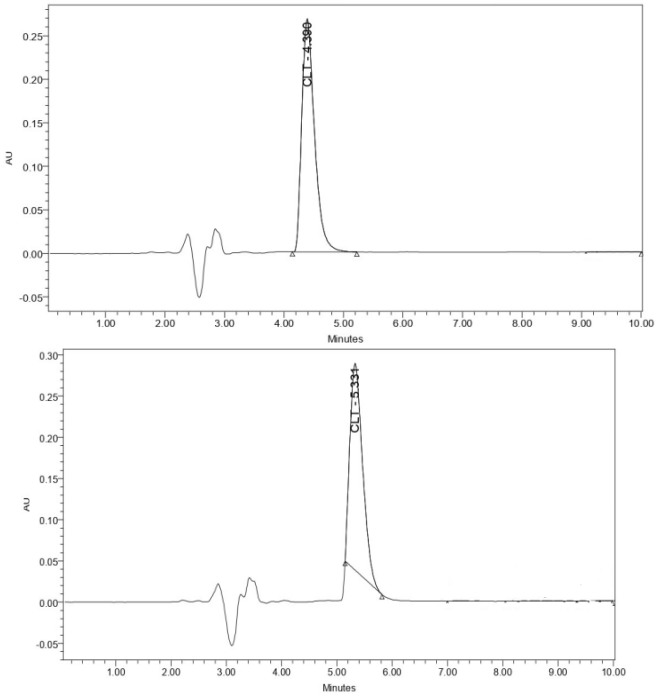
Table No :4: Data showing accuracy results

III. RESULTS FOR ROBUSTNESS

3.1 Effect of Variation in flow rate of mobile phase by ± 10%:

Prepared the system suitability solution (Standard Preparation) and inject into the HPLC system at –10% flow rate (1.1mL/min) and +10% flow rate (1.3mL/min) when compared with the test method flow rate.

Procedure: Injected standard solution into the HPLC System in normal conditions and followed by the robust conditions. Measured the peak response for the major peaks .



Flow rate 1.3 (µg/ml) Flow rate 1.2 (µg/ml)

Sr. No.	System Suitability parameter	Observations for flow rate	Limits

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			Unchanged	1.1 ml	1.3 ml	
1	The % RSD of peak area response for five replicate Injections	CLT	0.698701	1.33	0.89	NMT 2.0
2	Theoretical plates	CLT	7497.53	7438.7	7457.9	NLT 2000
3	Tailing factor	CLT	1.49	1.46	1.54	NMT 2.0
4	Retention Time (Min)	CLT	4.632	5.193	4.390	

Table No :5: Result for variation in flow rate

Change in organic composition ± 10% (ACN : WATER (1M OPA)

System suitability dilution was prepared and injected into the HPLC system at –10% and + 10 % ACN (Organic phase) compared with the optimized method mobile phase concentration.

Procedure: Injected standard solution into the HPLC system in normal conditions and followed by the robust conditions. Measure the peak response for the major peaks. Check the system suitabilityand record the results in the table .

Sr. No.	System Suitability parameter		Observations			Limits
			Unchanged	- 10%	+ 10%	
1	The % RSD of peak area response for five replicate injections	CLT	0.698701	0.76	0.51	NMT 2.0
2	Theoretical plates	CLT	7497.53	7496	7347.6	NLT 2000
3	Tailing factor	CLT	1.49	1.40	1.42	NMT 2.0
4	Retention Time (Min)	CLT	4.632	5.331	4.600	

Table No :6:Result for variation in mobile phase composition

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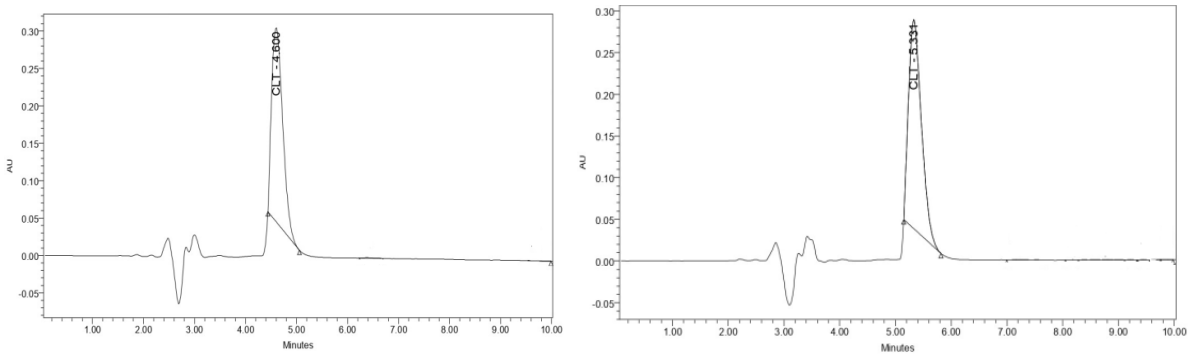

10% ACN: (Water:ACN 30:80) -10% ACN: (Water:ACN 40:60)

Linearity & Range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. Linearity was carried out for five levels in the range of 80% to 150%. A graph was plotted with concentration on X axis and mean peak areas on Y-axis. The R2value was found to be 0.999 for CLT. The result show that an excellent correlation exists between concentration and mean peak areas within the concentration range. Thus the method developed is accurate, precise, specific, & linear. Hence it can be said that, RP-HPLC is the most accurate, precise and reproducible among all methods.

Specificity

It Is the ability to assess unequivocally the analyte in the presence of impurities, degradants, matrix etc. It is evaluated by injecting the blank, placebo and the control sample solution prepared as per the proposed method to check for the interference if any peak at the retention time of CLT.

Thus, no interference was found at the Retention time of CLT .

IV. CONCLUSION

The prepared emulsion of clotrimazole had shown excellent promising results for all the parameters . Formulation F6 was found to be excellent on the basis of stability , against colour change , creaming and phase separation . F6 formulation can be further study for preclinical and clinical evaluations .

From the results of the study it can be concluded that the present HPLC technique was successfully used for the estimation of the CLT in the Microemulsion formulation.

The method showed good reproducibility, it was accurate, precise, specific, reproducible and sensitive.The analysis of Microemulsion formulation of CLT was done by the developed and validated HPLC Method

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