Chapter 5

Experimental, Results & Discussion

5. EXPERIMENTAL

This chapter follows a description of the methods developed which can offer new analytical possibilities for the determination of content and identity of drug in their combination dosage form. Different HPLC, HPTLC and IR methods were developed for analyzing the proposed drugs in formulations.

Different HPLC, HPTLC and IR methods were developed for analyzing the proposed drugs in formulations. Apparatus and materials requirement for all proposed methods are described in the following section.

5.1 HPLC METHODS

5. I.a. Apparatus and software for HPLC measurement

For ***butenafine hydrochloride and betamethasone dipropionate*** chromatography was performed on an Ultimate 3000 HPLC chromatographic system equipped with an autosampler, photodiode array detector PDA detector a Rheodyne syringe-loading sample fixed loop (20 µL) injector (7725). The chromatographic separation was performed at ambient temperature on a Inertsil C18 (250 mm × 4.6 mm, 5µm) column. Data was acquired and processed by Chromalieone software, software. Degassing of the mobile phase was done by sonication in Ultrasonic bath (Ultrasonics Selec, Vetra, Italy).

For the reverse phase methods of ***etizolam, esitalopram oxalate, fluocinolone acetonide ,miconazole nitrate and hydrocortisone acetate*** chromatography was performed on Jasco chromatographic system equipped with Rhynodyne manual sampler with 20µL loading sample loop and UV detector. Data was acquired and processed by Borwin software and the chromatographic separation was performed at ambient temperature on a HiQ-sil C18HS, 5 µm column having dimensions 4.6 mm x 250 mm .

Peak purity analysis for ***stability indicating method of esctalopram and etizolam*** was performed on the HPLC system (Waters, Milford, USA), equipped with a 2996 photo-diode array (PDA) detector. Photostability studies were carried out in photostability chamber (Mack Pharmatech Pvt.Ltd. Serial No. 10/05-06) which was set at 25±1°C.The photostability chamber was equipped with an illumination bank on the inside top, as defined under Option 2 in ICH guideline Q1B. The light bank consisted of a combination of one black light UV lamp set at UV 200 W/h/m2 and 4 white fluorescent lamps set at 1.2 million lux hrs. The samples were placed at a distance of

9 in. from the light bank. Both fluorescent and UV lamps were turned on simultaneously. Thermal stability study was carried out in hot air oven at 80°C for 48 hrs.

The chromatographic study of Racecadotril was performed on Shimazdu chromatographic equipped with equipped with Shimazdu pump LC-10 ATVP,Detector: SPD –10 AVP and Rhynodyne manual sampler with 20µL loading sample loop. Data was acquired and processed by Winchrome software, The chromatographic separation was performed at ambient temperature on a Prochrome (India) C-18, 5µm column having dimensions 250 x 4.6 mm was used.

5. I.b *Apparatus and software for HPTLC measurement*

(a) *Sample Application*: Camag Linomat V (Switzerland) sample applicator was used to apply samples in the form of bands with the help of Camag, Hamilton, 100 microlitre syringe on HPTLC plates i.e silica gel precoated aluminum plate 60F 254, [20 cm × 10 cm with 250 µm thickness; E.Merck, Germany)] for normal phase and on RP-18F 254 S silica gel precoated plates for reverse phase chromatography.

HPTLC plates were prewashed with methanol and activated at 110^0C for 5 min.

(b)Development of Plates: After sample application plates were developed in a Camag (Switzerland) twin trough glass chamber saturated with the mobile phase. Development of plates was carried out in Linear ascending manner 20 cm × 10 cm twin trough glass chamber (Camag,The Muttenz, Switzerland) saturated with the mobile phase (25°C ± 2) at relative humidity of 60 % ± 5. Sample application position was kept at 10 mm and solvent front position was at 8 cm thus the plates were developed over a position of 8 cm. Developed plates were dried in a stream of air with the help of an air dryer.

(c)Scanning of plates: Developed plates were scanned densitometrically with the help of Camag TLC scanner 3 at 254 nm operated by WinCATS software. The source of radiation used was deuterium lamp. Slit dimension was 6.00x0.45 mm Concentrations of the compound chromatographed were determined from the intensity of the diffused light. Evaluation was performed by linear regression of peak areas determined by UV absorption as a function of sample amounts.

All the experiments with standards and with samples were repeated 3 or 6 times and average was accepted.

Procurement of APIs

Gift sample of butenafine hydrochloride standard drug(100.17% pure) was provided by Glenmark Pharmaceuticals Ltd (Mumbai, India). Betamethasone dipropionate standard(98.96% pure) was obtained from Halcyon Labs Pt. Ltd (Mumbai, India). Escitalopram oxalate was provided by Synergene Active Ingredients Pvt.Ltd, Quthbullapur Mandal, Andra Pradesh (India). Etizolam standard (99.3% pure) was provided by Macleods Pharmaceuticals Limited, R&D Center, Mumbai. Albendazole standard (99.82%) was obtained from Medibios Laboratories Pvt Ltd, Tarapur. Ivermectin(99.5%pure) standard was obtained from Hexa analytical Laboratories, Mumbai. Miconazole nitrate standard (99.82% pure) was obtained from Pranami Drugs Pvt.LTD, Bharuch, Gujarat. Fluocinolone acetonide (99.9% pure) was obtained from Hexa Laboratories, Mumbai.Hydrocortisone acetate(100.1% pure) was obtained from Kemwell Pvt.Lt.,Bangalore. Racecadotril standard (98.76 % pure) was obtained from Symed Labs Ltd (India).

5.1.1 RP-HPLC Method for Simultaneous Determination of Butenafine Hydrochloride and Betamethasone Dipropionate in a Cream Formulation

5.1.1.1 Experimental

***Reagents*:**

HPLC grade methanol and water were purchased from S.D. Fine Chemicals (Mumbai, India).

Chromatographic condition:

Chromatographic sepration was carried out on a Inertsil, C-18 column at 25 °C temperature. A gradient programme with mobile phase consisting of methanol and water was pumped at a flow rate of 1 mL/min. The proportion of methanol was increased from 90% to 95% and held at 95% for a period of 10 min. A gradient programme was followed:

1-8 min methanol: water (90:10)

8.1-18 min methanol: water (95:05)

18.1-21 min methanol: water (90:10)

The elution was monitored at 254 nm and the injection volume was 20 µL.

Marketed Formulation:

Each 15 g cream tube (Butenaskin-BM of Elder Health Care Ltd, Mumbai, India) containing butenafine hydrochloride (1%, w/w) and betamethasone dipropionate USP equivalent to betamethasone (0.05%, w/w) was procured from the local market.

Preparation of Mobile Phase:

Methanol and water used for the mobile phase were filtered through a 0.45 μm membrane filter (Ultipore N –66R Nylon 66; Pall Corp.,) and degassed by ultrasonication for 15 min.

Preparation of Standard Stock Solution

Standard stock solution of butenafine hydrochloride was prepared by dissolving 100 mg of butenafine hydrochloride in methanol -water(90+10) to get a solution containing 1mg/ml of butenafine hydrochloride.Standard stock solution of betamethasone dipropionate was prepared by dissolving 10 mg of betamethasone dipropionate in methanol-water (90+10,v/v/) to get a solution containing 100 μg/ml for betamethasone dipropionate. The working standard solution of butenafine hydrochloride was prepared by diluting appropriate volume of butenafine hydrochloride stock solution with methanol:water (90:10) to get a solution containing 200μg/ml of butenafine hydrochloride.Working standard solution of betamethasone dipropionate was prepared by diluting appropriate volume of standard stock solution with methanol - water(90+10) to get a solution containing 10μg/ml of betamethasone dipropionate.Binary mixture of butenfine hydrochloride and betamethasone dipropionate was prepared by transfering appropriate volume of standard stock solutions to 100 ml volumetric flask and diluting it with methanol:water(90+10,v/v) to get a solution containing 10 μg/ml of betamethasone dipropionate and 200μg/ml of butenafine hydrochloride.

Preparation of Sample Solution

In a 50 mL screw-cap centrifuge tube, 2 g cream sample was weighed and extracted with three portions of methanol–water (90 + 10, v/v). For each extraction, 25 mL methanol–water (90 + 10, v/v) was added to the centrifuge tube and heated in a water bath (60°C) with shaking to disperse the cream, vortexed for 1 min, and centrifuged for 10 min at 3000 rpm. Supernatants were collected in a 100 mL volumetric flask, and the final volume was diluted to 100 mL with methanol–water (90 + 10, v/v).

Analysis of a Marketed Formulation:

Assay of marketed cream formulation (Butenaskin-BM of Elder Health Care Ltd) containing butenafine hydrochloride (1%, w/w) and betamethasone dipropionate USP equivalent to betamethasone (0.05%, w/w) was performed by preparing the sample solutions as described in the previous section. Six of the prepared sample and standard solutions were injected. The assay of the commercial sample was calculated by comparing the areas of standard and sample peaks.

Method Validation

Calibration curve (linearity of the HPLC method)

Linearity of butenafine hydrochloride was established by triplicate injections of standard solutions prepared by diluting the aliquots of standard stock solution with methanol:water to get the solutions of butenafine hydrochlirode in the range of 50-500µg/ml.Linearity of betamethasone dipropionate was established by triplicate injections of standard solutions prepared by diluting the aliquots of standard stock solution with methanol:water to get the solutions of betamethasone dipropionate in the range of 2.5-25µg/mL

Accuracy

Accuracy of the method was studied by recovery experiments using the standard addition method at three different levels (80, 100, and 120%). Known amounts of standard solutions containing betamethasone dipropionate (8, 10, and 12 µg) and butenafine hydrochloride (160, 200, and 240 µg) were added to prequantified sample solutions to reach the 80, 100, and 120% levels. These samples were analyzed and recovery was calculated. The difference between the spiked and unspiked sample was determined for different recovery levels.

Precision (repeatability)

Precision of the assay method was demonstrated by analysing six different sample solutions containing betamethasone dipropionate equivalent to 10 µg/mL and butenafine hydrochloride equivalent to 200 µg/mL, and from the area obtained concentration was calculated, and the results were expressed as %RSD.

Intermediate precision (ruggedness)

Intermediate precision of the method was demonstrated by carrying out the experiment on different days, by different analysts, and on different instruments using different C18 column(Prochrom).

LOD and LOQ

The LOD and LOQ values were calculated from the calibration curves as k SD/b where k= 3 for LOD and 10 for LOQ. SD is the standard deviation of the response of the minimum detectable drug concentration and b is the slope of the calibration curve.

Specificity

Specificity of the method was demonstrated by injecting the blank solution, standard solution, sample solution, and solvent extracted placebo and the responses were determined.

Robustness

Robustness of the method was demonstrated by deliberately varying the chromatographic conditions. The flow rate of the mobile phase was changed from 1.0 to 0.9 mL/min and from 1.0 to 1.1 mL/min. The temperature of the column was changed from 25 to 22°C and from 25 to 27°C. The sample solutions for the robustness study were applied onto the column in triplicate, and the responses were determined.

System suitability study

System suitability was established by injecting six replicate injections of standard solution of betamethasone dipropionate and butenafine hydrochloride and the %relative standard deviation (% RSD) of peak areas, resolution factor, tailing factor and theoretical plates were determined.

5.1.1.2 Results and Discussion

Optimization of the method

To optimize the chromatographic conditions, the effect of chromatographic variables such as composition of mobile phase, pH of mobile phase and flow rate were studied. The resulting chromatograms were recorded and the chromatographic parameters such as asymmetric factor, resolution and theoretical plates were calculated. The conditions that gave the best resolution, symmetry and theoretical plate were selected for estimation. The results of these trials are reported in **Table5.1.1.1**

Table 5.1.1.1 Optimization of HPLC method

Mobile phase	Flow rate (ml/min)	Betamrthasone dipropionate		Butenafine hydrochloride	
		RT (min)	Peak Shape	RT (min)	PeakShape
Methanol:Water (90:10v/v)	1 ml	4.5	Sharp	35(late elution)	Sharp
Methanol:Water (95:05v/v)	1 ml	4.2	Sharp	28(late elution	sharp
Methanol:0.1M ammonium acetate (95:05v/v)	1 mL	4.07	Sharp	14.05	Sharp

A satisfactory separation and good peak symmetry were obtained by using the described methanol–water gradient program. Although the mobile phase composition of methanol:0.1M ammonium acetate buffer was giving good isocratic separation and sharp peak for standards, when applied the same to formulation the betamethasone dipropionate peak showed tailing, due to interference by exicipient .With methanol:water (90:10) isocratic elution peak shapes were sharp but butenafine hydrochloride elution was very late. So finally gradient elution was selected for the development of method. Quantification was achieved with UV detection at 254 nm based on peak area.

A representative chromatogram is shown in **Fig.5.1.1.1**

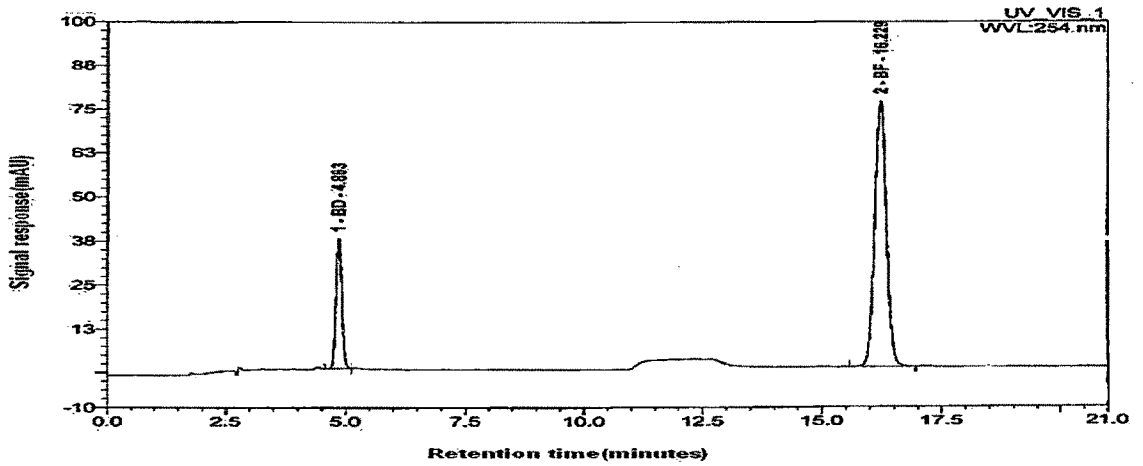


Fig.5.1.1.1: Chromatogram of Betamethasone dipropionate and Butenafine hydrochloride at 254 nm ((Betamethasone dipropionate 10µg/mL and Butenafine hydrochloride 200µg/mL)

Linearity calibration plot **(Fig.5.1.1.2).**

A good linear relationship between concentration and peak areas over a concentration range of 2.5–25µg/ml for betamethasone dipropionate **(Fig.5.1.1.2)** and 50–500 µg/ml for butenafine hydrochloride The correlation coefficient (R2) was found to be 0.9997 for betamethasone dipropionate and 0.9997 for butenafine hydrochloride which are greater than 0.999, ensure that a good correlation existed between the peak area and analyte concentration.

Table 5.1.1.2 Regression analysis of the calibration curves for betamethasone dipropionate and butenafine hydrochloride.

Parameter	Betamethasone dipropionate	Butenafine hydrochloride
Linearity range(µg/mL)	2.5-25	50-500
Regression equation	y=23022x+5239.1	y=7807.5x+38628
Correlation coefficient (r^2)	0.9997	0.9997
Slope	23022	7807.5
X-intercept	-0.2276	-4.947
Y-intercept	5239.1	38628

Accuracy

Percent recoveries were obtained from the difference between the areas of spiked and unspiked samples. The mean recovery of the added standard drug was 100.44 and 99.49% for betamethasone dipropionate and butenafine hydrochloride, respectively. These mean recovery values are well within the 98-100% indicating the method is accurate

Table 5.1.1.3 Recovery studies of betamethasone dipropionate and butenafine hydrochloride

Drug	Recovery Level(%)(n=3)	%Recovery±%RSD	Mean Recovery
Betamethasone Dipropionate	80	100.58±1.08	**100.44%**
	100	99.73±1.36	
	120	101.03±1.20	
Butenfine Hydrochloride	80	99.34±0.73	**99.49%**
	100	99.73±0.79	
	120	99.22±1.30	

Precision

RSD of mean assay values was found to be 0.21% for betamethasone dipropionate and 0.92% for butenafine hydrochloride. These %RSD values which are well below 2% indicate that the repeatability of this method is satisfactory.

Table 5.1.1.4 Precision of betamethasone dipropionate and butenafine hydrochloride

Drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean ±%RSD
Betamethasone dipropionate (%)	99.76	97.62	97.98	97.56	97.41	97.88	**97.70±0.21**
Butenafine Hydrochloride(%)	95.69	93.34	94.49	93.57	93.65	93.85	**94.09±0.92**

The intermediate precision study revealed that the method is rugged with RSD values of 0.52% for betamethasone dipropionate and 1.05% for butenafine hydrochloride. As evident the RSD values of the data obtained are well below 2% indicating that method is precise and rugged.

Table 5.1.1.5 The intermediate precision of betamethasone dipropionate and butenafine hydrochloride

Drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean ±%RSD
Betamethasone dipropionate(%)	96.61	96.45	95.47	95.76	95.53	96.41	**96.07±0.52**
Butenafine Hydrochloride(%)	94.78	93.35	92.27	92.33	92.53	93.73	**93.18±1.05**

LOD and LOQ

The LOD was found to be 0.46 µg/mL for betamethasone dipropionate and 9.32 µg/mL for butenafine hydrochloride. LOQ was found to be 1.39 µg/mL for betamethasone dipropionate and 28.25 µg/mL for butenafine hydrochloride. These values indicate that the method is sensitive.

Specificity

Good resolution was obtained between the drugs and excipients showing complete sepration of butenafine hydrochloride and betamethasone dipropionate. No interference from excipients, impurities; or degradation products ensured that the peak response was due to betamethasone dipropionate and butenafine hydrochloride only.

Robustness

The method remained unaffected by small, deliberate changes in the flow rate and column temperature. The RSD of mean assay values was found to be 0.81% for betamethasone dipropionate and 0.32% for butenafine hydrochloride with a flow rate of 0.9 mL/min. The RSD of mean assay values was found to be 0.27% for betamethasone dipropionate and 0.98% for butenafine hydrochloride with a flow rate of 1.1 mL/min. Also, RSD of mean assay values was found to be 0.81 and 0.27% for betamethasone dipropionate and butenafine hydrochloride, respectively, at 22°C and 0.50 and 1.13% for betamethasone dipropionate and butenafine hydrochloride, respectively, at 27°C.The RSD values of the data obtained are well below 2% indicating that method is robust i.e it is reliable and can be used for routine analysis of the drugs.

5.1.1.6 Robustness studies HPLC method of betamethasone dipropionate and butenafine hydrochloride

Parameter		Betamethasone Dipropionate Assay values (n=3)				Butenafine Hydrochloride Assay values (n=3)			
	Sample	1	2	3	Mean ±%RSD	1	2	3	Mean ±%RSD
Flow Rate (ml/min)	0.9	97.74	96.87	96.18	**96.93**	94.61	94.21	94.02	**94.28**
	1.1	95.76	95.83	96.24	**95.94±0.27**	97.11	97.70	95.84	**96.88±0.98**
Temp. (°C)	22	96.96	95.99	96.50	**96.48±0.50**	96.04	96.48	96.51	**96.34±0.27**
	27	96.66	96.66	95.80	**96.37±0.50**	98.60	97.07	96.40	**97.39±1.13**

System suitability tests were carried out on freshly prepared standard solutions ($n = 6$) containing butenafine hydrochloride and betamethasone dipropionate. System suitability parameters obtained with 20 µL injection volumes are summarized in **Table 5.1.1.7**

Table 5.1.1.7 System suitability test parameters for betamethasone dipropionate and butenafine hydrochloride

Parameter	Betamethasone dipropionate (±%RSD)	Butenafine hydrochloride(±%RSD)
Retention Time(min)	4.82 ± 0.80	16.18 ± 0.17
Theoretical plates	9322.98 ± 01.58	11640.99 ± 0.34
Tailing factor	1.25	1.29
Resolution	-	28.37 ± 0.19

All validation data are summarized in **Table 5.1.1.8**

Applicability of the method to Marketed formulation

The developed method was successfully applied to analyze betamethasone dipropionate and butenafine hydrochloride in marketed cream formulation. A clear separation of the drugs was achieved in tablet with no interference from excipients.

The ***assay*** results obtained by using the proposed method for the analysis of a marketed cream formulation containing butenafine hydrochloride (1%, w/w) and betamethasone dipropionate USP equivalent to betamethasone (0.05%, w/w) were in good agreement with the labeled amounts of betamethasone dipropionate and butenafine hydrochloride. The average contents of butenafine hydrochloride and betamethasone dipropionate were 19.54 mg/2 g cream (0.977%, w/w) and 0.9409 mg/2 g cream (0.047045%, w/w), respectively.

Table: 5.1.1.8 Analysis Cream Formulation (n=6)

Cream	Drug (mg / 2 gm of cream)	% drug obtained ± %R.S.D
Butenaskin BM	Betamthasone dipropionate-20mg Butenafine hydrochloride-1mg	**97.70 ±0.22** **94.09 ±0.93**

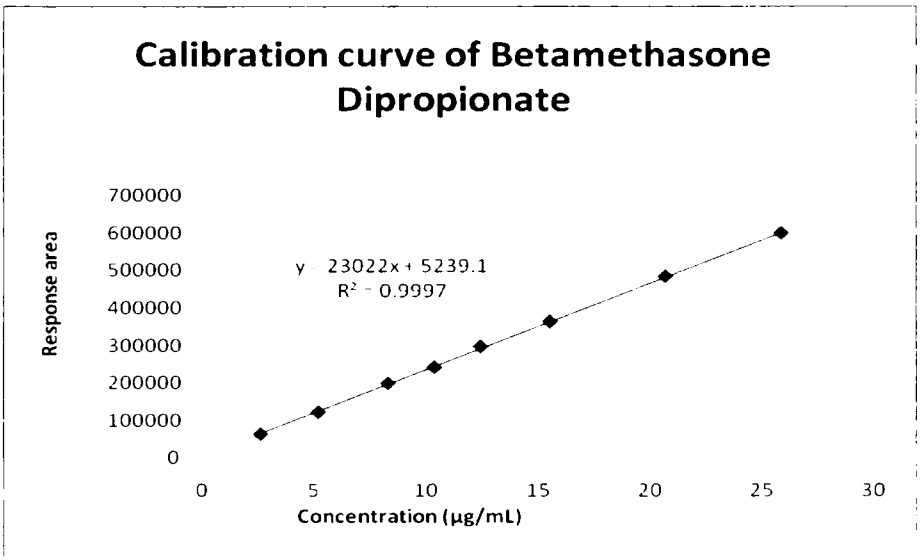


Fig. 5.1.1.2: Calibration Curve for betamethasone dipropionate.(2.5-25µg/ml)

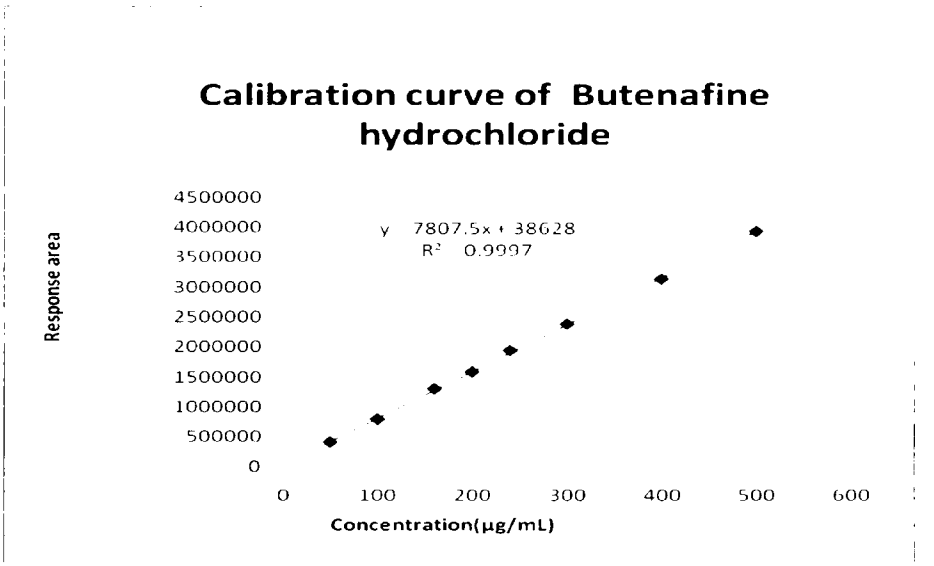


Fig.5.1.1.3 Calibration Curve for butenafine hydrochloride (50-500µg/ml).

Table 5.1.1.9 Summary of validation parameters for the proposed HPLC method for betamethasone dipropionate and butenafine

Parameter	Betamethasone Dipropionate (±RSD)	Butenafine Hydrochloride (±RSD)
LOD (µg/ml)	0.46	9.32
LOQ (µg/ml)	1.39	28.25
Accuracy (% recovery)	100.44	99.49
Precision[a]	97.70 ±0.22	94.09 ±0.93
Ruggedness[a]	96.07 ±0.53	93.19 ±1.05
Robustness (22° C Column oven Temp.)[b]	96.48 ± 0.81	96.34 ± 0.27
Robustness (27° C Column oven Temp)[b]	96.37 ± 0.50	97.26 ± 1.13
Robustness (0.9ml/min Flow rate)[b]	96.93 ± 0.81	94.61 ± 0.32
Robustness (1.1ml/min Flow rate)[b]	95.94 ± 0.27	96.88 ± 0.98

[a]mean asay values of 6 replicates

[b] mean assay values of 3 replicates

5.1.2.RP-HPLC method for simultaneous estimation of Escitalopram Oxalate and Etizolam in bulk and in Tablet Formulation

5.1.2.1 Experimental

Reagents

HPLC grade methanol, water, potassium dihydrogen ortho phosphate, trietyalmine used was purchased from S.D. Fine Chemicals (Mumbai, India).

Chromatographic condition:

Isocratic chromatography was performed on a HiQ-sil C18HS, 5 µm column having dimensions 4.6 mm x 250 mm. A mobile phase consisted of methanol: phosphate buffer pH -5 (70:30 v/v) was pumped at a flow rate of 1 mL/min.The elution was monitored at 254 nm and the injection volume was 20 µL.

Marketed Formulation :

Etizola 5 plus (Macleods pharma) Tablets containing 0.5 mg etizolam and escitalopram oxalate equivalent to 5 mg of escitalopram were procured from local market.

Preparation of Buffer (pH-5)

6.8 gm of Potassium dihydrogen phosphate was dissolved in 2000 mL of distilled water and adjusted to pH 5 with triethlamine.

Preparation of Mobile Phase

Methanol and phosphate buffer pH-5 used for mobile phase were filtered through 0.22 µm membrane filter (Durapore Membrane, Miilipore GV 0.22µm) and degassed by ultrasonication for 15 min.

Preparation of Standard Stock Solution:

The standard stock solution of etizolam was prepared by dissolving10 mg etizolam in100 ml of methanol to get a solution containing 100µg/ml of etizolam.The standard stock solution of escitalopram oxalate was prepared by dissolving 100 mg escitalopram in 100 ml of methanol to get a solution containing 1 mg/ml of escitalopram oxalate.Working standard solution of etizolam was prepared by diluting appropriate aliquot of stock solution with methanol to get a solution containing 20 µg/ml etizolam. Working standard solution of escitalopram oxalate was prepared by diluting appropriate aliquot of standard stock solution of escitalopram oxalate with methanol to get a solution containing 200µg/ml of esctalopram oxalate.Binary mixture of etizolam and

escitalopram was prepared by tranfering appropriate aliquots of etizolam and escitalopram standard stock solutions to a 100 ml flask and diluted with methanol to get a solution containing 20µg/mL of of etizolam and 200µg/mL of escitalopram oxalate.

Preparation of Sample Solution:

Twenty tablets (each tablet containing escitalopram oxalate equivalent to escitalopram 5 mg and etizolam 0.5 mg were accurately weighed, their mean weight was determined and the tablets were powdered in a glass mortar. An amount of powder equivalent to two tablets was dissolved in 50 mL of methanol and was sonicated for 20 min.The resulting mixture was filtered through 0.45µ membrane filter(SY25TG,mdi Membrane Technologies,California USA).The filtrate ,thus obtained containing escitalopram oxalate equivalent to 200 µg/mL and etizolam equivalent to 20 µg/mL was used for analysis.

Analysis of a Marketed Formulation:

Assay of marketed tablet formulation containing etizolam 0.5 mg and escitalopram oxalate equivalent to escitalopram 5 mg was performed by preparing the sample solutions as described earlier in the *preparation of the sample.* Six injections of above prepared sample and standard solutions were injected and the peak areas were determined .The assay of the commercial sample was calculated by comparing the areas of standard and sample peaks.

Validation of the Method:

Calibration curve (linearity of the HPLC method)

Linearity of the etizolam was established by injecting in triplicate the standard solutions of etizolam prepared by diluting different aliquots of standard stock solution with methanol to get the solution in the concentration range of 5- 550 µg/mL for etizolam. Linearity of the escitalopram oxalate was established by injecting in triplicate the standard solutions of escitalopram oxalate prepared by diluting different aliquots of standard stock solution with methanol to get the solution in the concentration range of 55-550 µg/mL for escitalopram oxalate.

Accuracy (recovery)

Accuracy of the method was studied by recovery experiments using standard addition method at three different levels (80%, 100% and 120%). The known amounts of standard solutions containing escitalopram oxalate (160, 200 and 240 µg) and etizolam (16, 20, 24µg) were added

to prequantified sample solutions to reach the 80,100 and 120 % levels. These samples were analyzed and from the difference between peak areas of etizolam and escitalopram oxalate present in the spiked and unspiked samples the % recovery of added drugs was determined.

Precision (repeatability)

Precision of the assay method was demonstrated by determining the responses for six repeatedly injected sample solutions and from the peak areas RSD of mean assay value was calculated.

Intraday Precision

Intraday precision was demonstrated by injecting six different sample solutions containing escitalopram oxalate equivalent to 200 µg/mL and etizolam equivalent to 20 µg/mL at different time intervals within the same day and % RSD of mean assay value was calculated.

Intermediate precision (ruggedness)

Intermediate Precision of the method was demonstrated by carrying out the experiment on different day, by different analyst and on different instrument using different C-18 column.

LOD and LOQ

LOD and LOQ of escitalopram oxalate and etizolam were calculated using the following equations as per International Conference on Harmonization (ICH) guidelines.

LOD = 3.3 x σ/S LOQ = 10 x σ/S Where, σ = Standard deviation of response, S = Slope of regression equation.

Specificity

Specificity of the method was studied by injecting blank, standard, placebo and sample solutions.

Robustness

Robustness of the method was demonstrated by deliberately varying certain chromatographic parameters, only one at a time. The flow rate of the mobile phase was changed from 1.0 mL/min to 0.9 mL/min and also from 1.0 mL/min to 1.1 mL/min. The composition of mobile phase was changed from methanol: phosphate buffer pH-5 (70:30, v/v) to methanol: phosphate buffer pH-5 (66.5:30 v/v) and also from methanol: phosphate buffer pH-5 (70:30, v/v) to methanol: phosphate buffer pH-5 (73.5:30 v/v).The solutions for robustness study were applied on the column in triplicate and the responses were determined.

System suitability study

For this study first upon a 20 µL of blank solution (methanol) was injected and ran for 12 minutes. After this 6 replicates of freshly prepared standard solution were injected and the % relative standard deviation (% RSD) of the response peak areas, theoretical plates, tailing factor and resolution factors were calculated.

5.1.2.2 Results and Discussion

Optimization of the method

Method was optimized by varying chromatographic parameters like mobile phase compositions, pH of mobile phase, flow rate to satisfy system suitability testing. Various mobile phase compositions and buffers were tried (Table5.1.2.1) A satisfactory separation and good peak symmetry was obtained by using the mobile phase containing methanol: phosphate buffer pH-5 (70:30, v/v) and reverse phase chromatography with isocratic technique. Quantification was achieved with UV detection at 254 nm based on peak area. A representative chromatogram is shown in **fig.5.1.2.1**

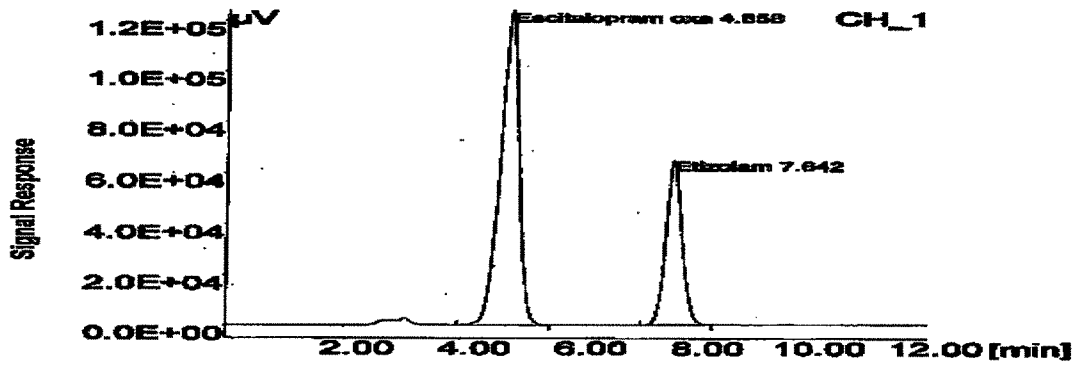


Fig.5.1.2.1Chromatogram of Escitalopram oxalate and Etizolam at 254nm (Escitalopram oxalate 200µg/ml and Etizolam 20µg/ml)

Table 5.1.2.1 Optimization of the method

Mobile phase	Flow rate (ml/min)	Escitalopram oxalate		Etizolam	
		RT (min)	Peak Shape	RT (min)	Peak Shape
Methanol:Water (90:10v/v)	1 ml	No peak for 1 hr	-	No peak for 1 hr	-
Methanol:Phosphate buffer pH-3.6(50:50v/v)	1 mL	5.83	Split	28.55(late elution)	Sharp
Methanol:Phosphate buffer pH-6.8(50:50v/v)	1 mL	17.87(late elution)	Not sharp	26.62(late elution)	Sharp
Methanol:Aetate buffer pH-5 (50:50v/v)	1 mL	21.291	Not Sharp	48.33(late elution)	Sharp
Methanol:Phosphate buffer pH-5 (50:50v/v)	1 mL	8.533	Sharp(late elution)	28.33(late elution)	Not Sharp
Methanol:Phosphate buffer pH-5 (50:50v/v)	1 mL	8.533	Sharp(late elution)	28.33(late elution)	Not Sharp
Methanol:Phosphate buffer pH-5 (70:30v/v)	1 mL	4.933	Sharp	7.65	Sharp

Linearity regression data is summarized in **Table 5.1.2.2** which shows a good linear relationship between concentration and peak areas over a concentration range of 55-550µg/ml for escitalopram oxalate (**Fig 5.1.2. 2**) and 5 - 50µg/ml for etizolam (**Fig.5.1.2.3**). The correlation coefficient (R^2) was found to be 0.9993 for escitalopram oxalate and 0.9996 for etizolam.The R^2 values for both the drugs were greater than 0.999 which shows that there exist a good correlation between analyte concentration and response area.

Table 5.1.2.2. Regression analysis of the calibration curves for escitalopram oxalate and etizolam

Parameter	Escitalopram Oxalate	Etizolam
Linearity range(µg/mL)	55-550	5 – 50
Regression equation	y=10903x+165905	y=57853x+34312
Correlation coefficient(R^2)	0.9993	0.9996
Slope	10903	57853
X-intercept	-15.216	-0.5931
Y-intercept	165905	34312

Accuracy studies indicate that the mean percent recovery of the added standard drug to be 98.35% and 98.03% for escitalopram oxalate and etizolam respectively. As the % recovery of the added drugs is well within the limits of 98-102%, it indicates the accuracy of this method.

Table 5.1.2.3 Recovery study of escitalopram oxalate and etizolam.

Drug	Recovery Level (%) (n=3)	%Recovery	Mean Recovery %
Escitalopram oxalate	80	98.37	**98.35**
	100	98.34	
	120	98.35	
Etizolam	80	98.59	**98.03**
	100	97.49	
	120	98.02	

In the ***precision*** studies, RSD of mean assay values were found to be 0.51 for escitalopram oxalate and 0.59 for etizolam. These RSD values which are well below 2% indicate that the repeatability of this method is satisfactory. This indicates a closeness of aggrement in repeated measurements of peak response.

Table 5.1.2.4 Precision study of escitalopram oxalate and etizolam HPLC method.

Drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean ±%RSD
Escitalopram oxalate	95.81	98.80	96.37	95.35	95.78	96.20	**95.76±0.51**
Etizolam	96.36	95.74	97.27	95.84	96.57	96.70	**96.41±0.59**

In the ***intraday precision*** studies RSD of mean assay values were found to be 0.54(95.15%) for escitalopram oxalate and 0.72(94.97) for etizolam which shows closeness of agreement in measurement of peak responses over a period of time. It also indicates that drug is stable sufficiently for the time of analysis.

Table 5.1.2.5 Intraday precision of escitalopram oxalate and etizolam HPLC method.

Drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean ±%RSD
Escitalopram oxalate	94.24	95.15	95.13	95.10	96.18	95.12	**95.15±0.51**
Etizolam	96.70	96.50	95.90	96.79	95.50	97.45	**96.41±0.59**

Intermediate precision (Ruggedness) study reveals that the method is rugged with %RSD values of 0.55 for escitalopram oxalate and 0.96 for etizolam. As evident the RSD values of the data obtained for precision studies are well below 2% indicating that method can be repeated successfully on different days,on different column and by different analyst.

Table 5.1.2.6 Intermediate precision of escitalopram oxalate and etizolam HPLC method.

Drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean ±%RSD
Escitalopram oxalate	97.71	96.66	95.37	96.24	96.40	95.10	**96.24±0.55**
Etizolam	95.54	93.90	95.66	94.15	94.29	95.33	**94.81±0.96**

LOD and LOQ

The limit of detection was found to be 15.27µg/mL for escitalopram oxalate and 1.15µg/mL for etizolam. The limit of quantification was found to be 46.27µg/mL for escitalopram oxalate and 3.48 µg/mL for etizolam. These values indicate that the method is sensitive.

Specificity

Both the drugs were resolved properly and there was no any interference from the exipients, impurities and degradation products. The peak response was due to escitalopram oxalate and etizolam only,making this method a very specific for these drugs.

Robustness

Robustness study signified that the results of the method remained unaffected by small, deliberate changes in the flow rate and mobile phase composition. The RSD of mean assay values was found to be 0.81 for escitalopram oxalate and 0.71 for etizolam with a flow rate of 0.9 mL/min .The RSD of mean assay values was found to be 0.63 for escitalopram oxalate and 0.49 for etizolam with a flow rate of 1.1 mL/min. Also RSD of mean assay values was found to be 0.77 and 1.14 for escitalopram oxalate and etizolam respectively with mobile phase composition of methanol: phosphate buffer pH-5(66.5:30, v/v) and 0.90 and 0.35 for escitalopram oxalate and etizolam respectively with mobile phase composition methanol: phosphate buffer pH-5 (73.5:30, v/v). As evident, the RSD values of the data obtained are well below 2% indicating that method is reliable for normal usage.

Table 5.1.2.7 Robustness of escitalopram oxalate and etizolam HPLC method.

Parameter		Escitalopram Oxalate Assay values (n=3)				Etizolam Assay values (n=3)			
	Sample	1	2	3	Mean± %RSD	1	2	3	Mean± %RSD
Flow Rate (ml)	0.9	101.44	101.83	100.25	**101.18 ±0.81**	94.27	93.04	93.18	**93.49 ±07**
	1.1	101.92	100.99	102.23	**101.72 ±06**	96.34	95.42	95.68	**95.81 ±049**
Mobile phase	66.5:30	94.17	95.12	95.62	**94.97 ±0.77**	96.76	95.13	94.67	**95.53 ±114**
	73.5:30	94.46	95.11	96.17	**95.25 ±0.90**	94.24	94.89	94.44	**94.52 ±0.35**

System suitability tests were carried out on freshly prepared standard solutions (n = 6) containing escitalopram oxalate and etizolam. System suitability parameters obtained with 20 µL injection volume are summarized in **Table 5.1.2.2** As it is evident from the system suitability paremeters, escitalopram oxalate and etizolam get eluted well before ten minutes and their resolution is very satIsfactory,thus making it a very economical method for routine analysis of both the drugs .

Table 5.1.2.8 System suitability test parameters for escitalopram oxalate and etizolam

Parameter	Escitalopram Oxalate	Etizolam
Retention Time(min)	4.85 (± 0.31)	7.65 (± 0.56)
Theoretical plates	2408.43(±1.31)	3979.09(±0.83)
Tailing factor	0.852±1.9	1.088±1.265
Resolution	-	5.249±1.029

Applicability of the developed method for the analysis of marketed tablets.

The assay results obtained by using the proposed method for the analysis of marketed tablet formulation containing etizolam 0.5 mg and escitalopram oxalate equivalent to esctalopram 5 mg per tablet were in good agreement with the labeled amounts of escitalopram oxalate and etizolam. The average contents of etizolam and escitalopram oxalate were 0.48205 mg per tablet (96.41%) and 4.788 mg (95.76) per tablet respectively.This indicates that present method can be successfully used for the estimation of escitalopram oxalate and etizolam in a combined tablet dosage form without interference of any impurity or excipient.

Table 5.1.2.9:Analysis of Marketed Tablet Sample

Tablet	Drug Mg/Tablet	% Drug obtained ± %R.S.D
EtizolaPlus 5	Etizolam-0.5 mg Escitalopram Oxalate-5 mg	96.41±0.59 95.76±0.51

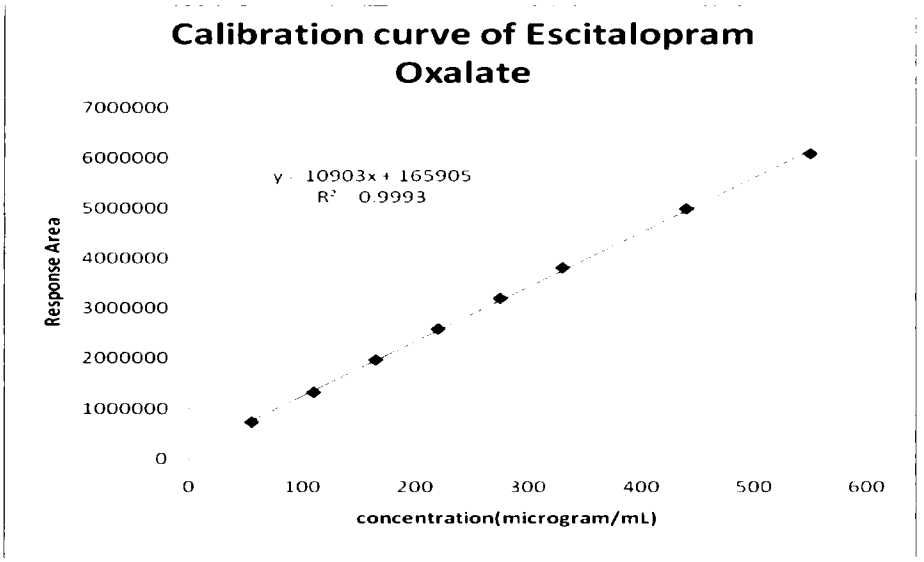


Fig.5.1.2.1 Calibration curve diagram for Escitalopram oxalate(55-500µg/ml).

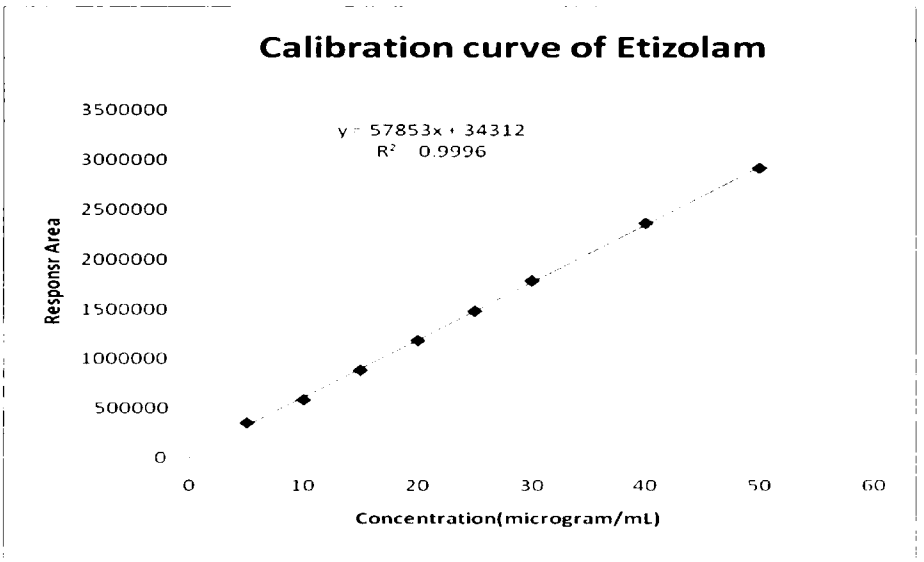


Fig.5.1.2.3 Calibration curve diagram for Etizolam(5-50µg/ml).

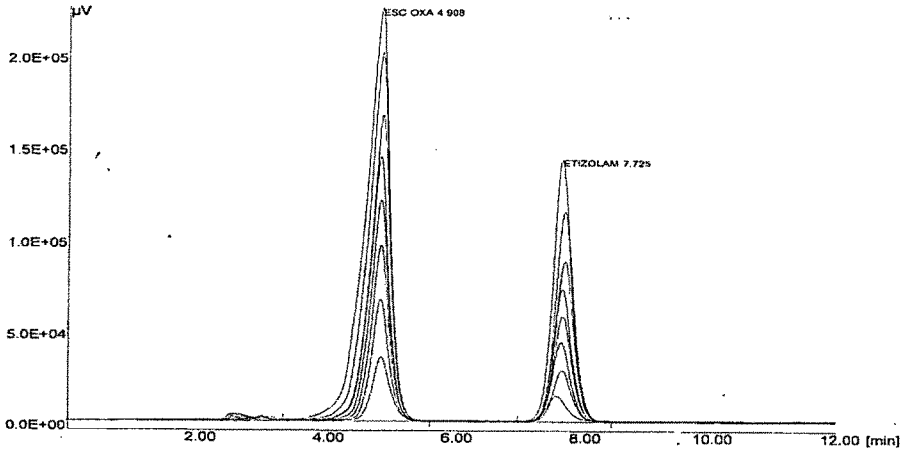


Fig.5.1.2.4 Overlay View of Linearity Chromatograms of Escitalogram Oxalate and Etizolam

Table 5.1.2.10 Summary of validation parameters for the proposed HPLC method for escitalopram oxalate and etizolam

Parameter	Escitalopram Oxalate	Etizolam
LOD (µg/ml)	15.27	1.15
LOQ (µg/ml)	46.27	3.48
Accuracy(% recovery)	98.35	98.03
Precision[a]	95.76±0.51	96.41±0.59
Intraday Precision	95.15±0.54	94.97± 0.72
Ruggedness[a]	96.24±0.55	94.81±0.96
Robustness [b] (methanol: phosphate buffer pH-5 (66.5:30, v/v))	94.97±0.77	95.53±1.14
Robustness [b] (methanol: phosphate buffer pH-5 (73.5:30, v/v))	95.25±0.90	94.52±0.35
Robustness [b] (0.9 ml/min Flow rate)	101.18±0.81	93.49±0.71
Robustness [b] (1.1 ml/min Flow rate)	101.72±0.63	95.81±0.49

[a] mean assay values of 6 replicates ,

[b] mean assay values of 3 replicates

5.1.3. Stability indicating RP-HPLC method for simultaneous estimation of Escitalopram Oxalate and Etizolam in a Tablet Formulation

5.1.3.1 Experimental

Reagents , Marketed Formulation , Preparation of Mobile Phase, Preparation of Standard Stock Solution, Preparation of Sample Solution, Analysis of a Marketed Formulation all these requirements are as per 5.1.2. The mobile phase was modified to get retention times which were suitable for stability indicating method.

Photo stability studies were carried out in a photo stability chamber. Peak purity analysis was carried out with another HPLC system (Waters Corp., Milford, MA), equipped with a 2996 photodiode array (PDA) detector. The thermal stability study was carried out in a dry-air oven.

Chromatographic condition:

A mobile phase consisted of methanol: phosphate buffer pH- 5 (62:38 v/v) was pumped at a flow rate of 1 mL/min.The elution was monitored at 254 nm and the injection volume was 20 µL.

Forced Degradation Studies

For forced degradation studies of escitalopram oxalate and etizolam standards were forced to degrade under acid hydrolysis, alkaline hydrolysis, oxidation, photolytic and thermal stress.Standard stock solution of ecitalopram oxalate(10mg/mL) and etizolam(1 mg/mL) were prepared by dissolving 100 mg of escitalopram oxalate and 10 mg of etizolam in 10 mL of methanol separately. Binary mixture of these drugs was prepared by dissolving 10 mg of etizolam and 100 mg of escitalopram in 10ml of methanol to get a solution containing 10mg/ml of escitalopram oxcalate and 1 mg/ ml of etizolam.

For *acid* degradation 1ml each of escitalopram oxalate standard, etizolam standard and binary mixture solutions were transferred to 50 ml round bottom flasks separately and 1 ml of 1N HCL wad added to the flasks and about 35 ml of methanol was added and refluxed for 6 hrs .After six hrs contents of the flasks were cooled and neutralized with 1 N NaOH and transferred to 50 ml volumetric flask and the final volume was made to 50 ml with methanol to get a solution containing 20µg/ml of etizolam and 200µg/ml of escitalopram oxalate

For *alkali* degradation 1ml each of escitalopram oxalate standard, etizolam standard and binary mixture solutions were transferred to 50ml round bottom flasks separately and 1 ml of 1N NaOH was added to the flasks and about 35 ml of methanol was added and refluxed for 6 hrs .After

six hrs contents of the flasks were cooled and neutralized with 1 N HCL and transferred to 50 ml volumetric flask and the final volume was made to 50 ml with methanol to get a solution containing 20µg/ml of etizolam and 200µg/ml of escitalopram oxalate.

For *oxidative* degradation 1ml solution each of escitalopram oxalate standard solution,etizolam standard and binary mixture solutions was transferred to 50ml round bottom flasks separately and 5 ml of 3%H_2O_2 was added to the these flasks and about 35 ml of methanol was added and refluxed for 6 hrs .After six hrs contents of the flasks were transferred to 50 ml volumetric flask and the final volume was made to 50 ml with methanol to get a solution containing 20µg/ml of etizolam and 200µg/ml of escitalopram oxalate. In accordance with the ICH guidelines the drug samples were placed at a distance of 9 in. from the light bank. Both fluorescent and UV lamps were turned on simultaneously. The samples were exposed for a total of 15 days.

For thermal degradation standard drugs were kept in an oven at 80°C for 48 hrs.

Forced Degradation Studies of Tablets

For degradation induced by acid hydrolysis, base hydrolysis, and oxidation, 1mL of1N HCl, 5 mL of 1N NaOH, and 1mL of 3.0% H_2O_2 were separately added to 100 ml round bottom flasks each containing tablet powder equivalent to weight of 2 tablets. Acid, base, and H_2O_2 mixtures were refluxed for 6 hrs with 35 ml of methanol on water and then cooled to room temperature. Acid mixture was neutralized with 1 ml 1N NaOH, basic mixture was neutralized with 5 ml of 1N HCl .The contents of flasks were transferred to 50 ml volumetric flasks and the final volume was made to 50 ml with methanol. Suitable dilutions of degraded samples were made with methanol to obtain the concentrations of etizolam (20 µg/mL) and escitalopram oxalate (200 µg/mL) for chromatographic analysis.

The tablets were also subjected to thermal stress at 80°C for 48 h. An amount of the thermally stressed tablet powder equivalent to 2 tablets was transferred to a 50 mL volumetric flask and diluted to volume with methanol to obtain concentrations of Etizolam (20 µg/mL) and escitalopram oxalate(200 µg/mL) for chromatographic analysis.

The photostability of the drug was also studied by exposing the tablets in a photostability chamber for 15 days and then continuing as indicated for dry heat degradation. The resulting solutions were analyzed as degraded samples by using the same chromatographic conditions.

Validation of the Method

Calibration curve (linearity of the HPLC method)

Calibration curve was constructed by plotting concentrations of escitalopram oxalate and etizolam vs. peak areas, and the regression equations were calculated. The linearity of this method was investigated by injecting the escitalopram oxalate and etizolam solutions in the range of 55-550 and 10-50 µg/mL respectively in duplicate.

Accuracy (recovery)

Accuracy of the method was studied by recovery experiments using standard addition method at three different levels (80%, 100% and 120%). The known amounts of standard solutions containing escitalopram oxalate (160, 200 and 240 µg) and etizolam (16, 20, 24µg) were added to prequantified sample solutions to reach the 80,100 and 120 % levels. These samples were analyzed by injecting the sample solution and recovery was calculated from the difference between the areas of spiked and unspiked samples.

Precision (repeatability)

Precision of the assay method was demonstrated by injecting six different sample solutions containing escitalopram oxalate equivalent to 200 µg/mL and etizolam equivalent to 20 µg/mL and RSD of mean assay value was calculated.

Intraday Precision

Intraday precision was demonstrated by injecting six different sample solutions containing escitalopram oxalate equivalent to 200 µg/mL and etizolam equivalent to 20 µg/mL at different time intervals within the same day and RSD of mean assay value was calculated.

Intermediate precision (ruggedness)

Intermediate Precision of the method was demonstrated by carrying out the experiment on different day, by different analyst and on different instrument using different C-18 column.

LOD and LOQ

LOD and LOQ of escitalopram oxalate and etizolam were calculated using the following equations as per International Conference on Harmonization (ICH) guidelines.

$LOD = 3.3 \times \sigma/S$ $LOQ = 10 \times \sigma/S$

Where, σ = Standard deviation of response, S = Slope of regression equation

Specificity

Specificity of the method was studied by injecting blank, standard, placebo and sample solutions. Specificity of the method was also demonstrated by finding out the peak purity of the drugs under various stress conditions.

Robustness

Robustness of the method was demonstrated by deliberately changing the chromatographic conditions. The flow rate of the mobile phase was changed from 1.0 mL/min to 0.9 mL/min and also from 1.0 mL/min to 1.1 mL/min. The composition of mobile phase was changed from methanol: phosphate buffer pH-5 (62:38, v/v) to methanol: phosphate buffer pH-5 (60:38 v/v) and also from methanol: phosphate buffer pH-5 (62:38, v/v) to methanol: phosphate buffer pH-5 (65:38 v/v).The solutions for robustness study were applied on the column in triplicate and the responses were determined.

System suitability study

For this study 20 µL of blank solution (methanol) was injected and ran for 20 minutes. After this 20 µL of standard solutions in 6 replications were injected and the % relative standard deviation (% RSD) of the response peak areas, theoretical plates, resolution factor and tailing factor was calculated.

5.1.3.2 Results and Discussion

Optimization of Method

Method developed in 5.1.2 was further modified for its application as a stability indicating method for etizolam and escitalopram oxalate in tablet formulation. The mobile phase combination of methanol:phosphate pH -5 buffer and flow rate were further optimized to get proper resolution of drug peaks from degradation products mainly. A satisfactory separation and good peak symmetry was obtained by using the mobile phase containing methanol: phosphate buffer pH-5 (62:38, v/v) and reverse phase chromatography with isocratic technique.

Table: 5.1.3.1 Optimization of Method

Mobile phase	Flow rate (ml/min)	Escitalopram oxalate		Etizolam	
		RT (min)	Peak Shape	RT (min)	Peak Shape
Methanol: Phosphate buffer pH-5 (70:30v/v)	1 mL	4.933	Sharp	7.65	Sharp
Although above method was an optimized method,it was further optimized to lengthen the retention time of escitalopram oxalate for stability indicating method for proper sepration of degradation products, escitalopram oxalate and etizolam. Mainly in alkaline degradation of escitalopram oxalate it was required to optimize the method for the proper resolution of degradation product,					
Methanol: Phosphate buffer pH-5 (67:33v/v)	1 mL	5.233	Sharp but under alkaline degradation not resolved properly	9.550	Sharp
Methanol: Phosphate buffer pH-5 (67:33v/v)	1 mL	6.167	Sharp but under alkaline degradation not resolved properly	10.6	Sharp
Methanol: Phosphate buffer pH-5 (65:35v/v)	1 mL	6.317	Sharp but under alkaline degradation not resolved properly	11.033	Sharp
Methanol: Phosphate buffer pH-5 (62:38v/v)	1 mL	6.642	Sharp and under alkaline degradation ,resolved properly	13.842	Sharp

After changing the mobile phase, the method was validated again and these validation parameters are reported in **Table 5.1.3.3** Quantification was achieved with UV detection at 254 nm based on peak area. A representative chromatogram is shown in **Fig.5.1.3.1**

Fig.5.1.3.1Chromatogram of Escitalopram oxalate and Etizolam at 254nm (Esitalopram oxalate 200 µg/ml and Etizolam 20µg/ml)

Linearity regression data is summarized in **Table 5.1.3.2** which shows a good linear relationship between concentration and peak areas over a concentration range of 100-500µg/ml for escitalopram oxalate (**fig5.1.3. 2**) and 10- 50µg/ml for etizolam (**fig.5.1.3.3**). The correlation coefficient ($R^{2)}$ was found to be 0.9999 for escitalopram oxalate and 0.9998 for etizolam. R^2 greater than 0.999 indicates a good correlation between concentration of the drugs and peak responses within the range of linearity.

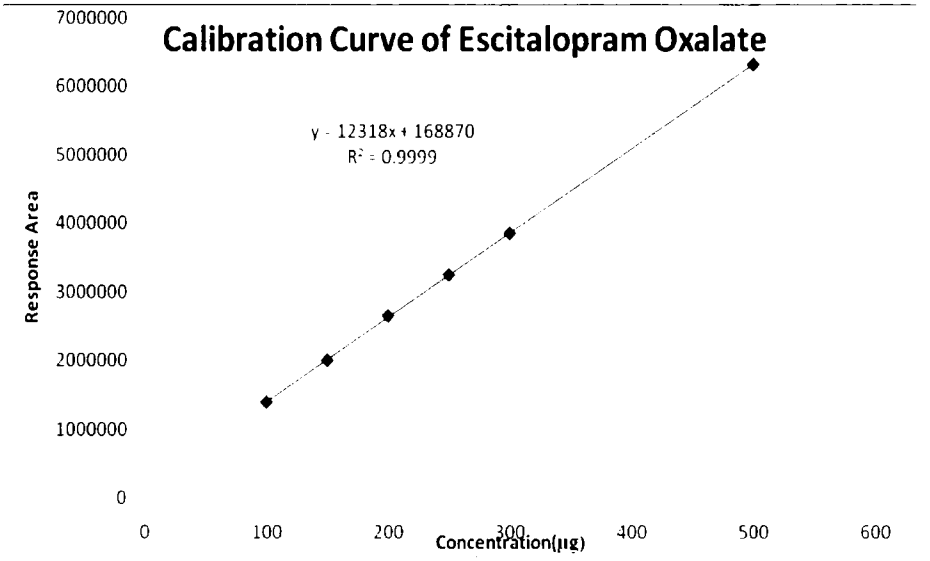


Fig.5.1.3.2 Calibration curve diagram for Escitalopram oxalate(55-550µg/ml)

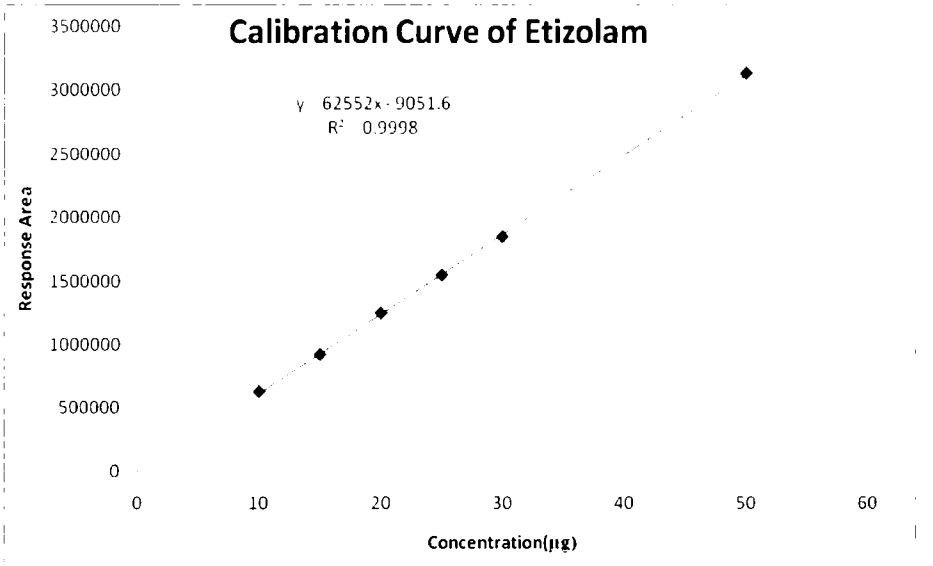


Fig.5.1.3.3 Calibration curve diagram for Etizolam(10-50µg/ml).

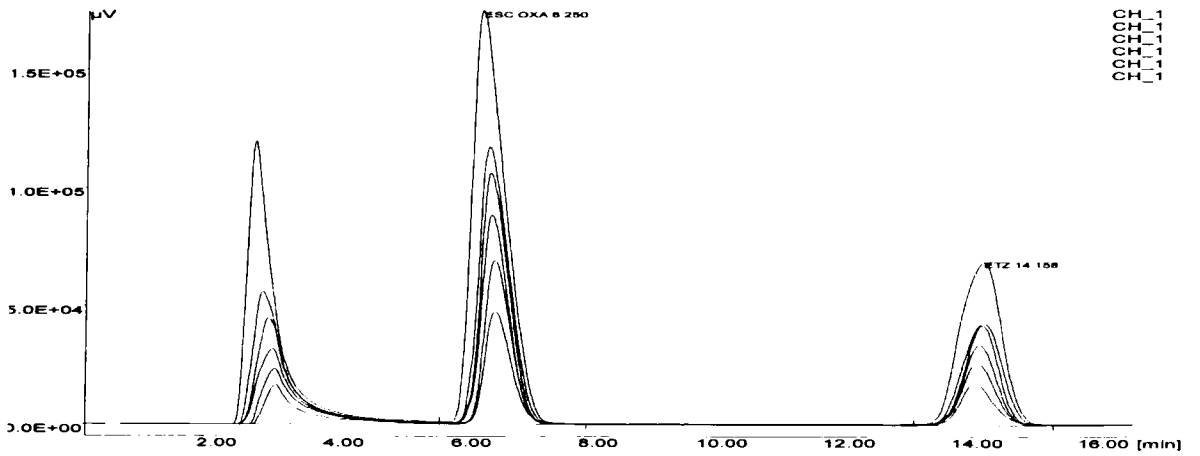


Fig.5.1.3.4 Overlay chromatograms of Escitalopram Oxalate and Etizolam

Table 5.1.3.2. Regression analysis of the calibration curves for escitalopram oxalate and etizolam

Parameter	Escitalopram Oxalate	Etizolam
Linearity range(µg/mL)	100-500	10- 50
Regression equation	y=12318x+168870	y=62552x-9051.6
Correlation coefficient(R^2)	0.9999	0.9998
Slope	12318	62552
X-intercept	-13.710	0.1447
Y-intercept	168870	

Accuracy

These studies indicate that the mean percent recovery of the added standard drug was 98.47% and 98.64 % for escitalopram oxalate and etizolam respectively.

Table 5.1.3.3 Recovery study of escitalopram oxalate and etizolam

Drug	Recovery Level % (n=3)	%Recovery	Mean Recovery %
Escitalopram oxalate	80	98.37	98.35
	100	98.34	
	120	98.35	
Etizolam	80	98.59	98.03
	100	97.49	
	120	98.02	

Precision

RSD of mean assay values was found to be 0.54 for escitalopram oxalate and 0.72 for etizolam. These % RSD values which are well below 2% indicate that the repeatability of this method is satisfactory.

Table 5.1.3.4 Precision study of escitalopram oxalate and etizolam HPLC method.

Drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean ±%RSD
Escitalopram oxalate	95.80	94.14	94.69	95.17	95.03	94.59	**94.90±0.54**
Etizolam	96.00	94.68	95.37	94.79	94.13	94.38	**94.89±0.72**

Intraday precision

% RSD of mean assay values were found to be 1.20 for escitalopram oxalate and 1.06 for etizolam.

5.1.3.5 Intraday precision study of escitalopram oxalate and etizolam HPLC method.

Drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean ±%RSD
Escitalopram oxalate	93.28	96.64	94.97	96.66	95.08	95.11	**95.29±1.20**
Etizolam	96.95	96.40	96.95	96.79	97.084	94.38	**96.42±1.06**

Intermediate precision **(Ruggedness)** study reveals that the method is rugged with %RSD values of 0.55 for escitalopram oxalate and 0.96 for etizolam. As evident the RSD values of the data obtained are well below 2% indicating that method is precise and rugged. It indicates the method can be used for routine analysis of both the drugs.

5.1.3.6 Intermediate precision study of escitalopram oxalate and etizolam HPLC method

Drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean ±%RSD
Escitalopram oxalate	94.54	95.0939 7	94.251 55	94.116 22	94.036 55	93.503 48	**94.24±0.55**
Etizolam	95.10	95.05	95.30	94.10	94.20	95.11	**94.81±0.96**

LOD and LOQ

The limit of detection was found to be 5.32µg/mL for escitalopram oxalate and 0.64µg/mL for etizolam. The limit of quantification was found to be 16.14 µg/mL for escitalopram oxalate and 1.95µg/mL for etizolam. These values indicate that the method is sensitive.

Specificity

Specificity of the method was established through resolution of drug peaks from the peaks of degradation product or excipient peaks. Studies indicated that there is no interference from exipients, impurities and degradation products and assured that the peak response was due to escitalopram oxalate and etizolam only.

Robustness

Robustness study signified that the results of the method remained unaffected by small, deliberate changes in the flow rate and mobile phase composition. The RSD of mean assay values was found to be 0.18 for escitalopram oxalate and 0.65 for etizolam with a flow rate of 0.9 mL/min .The RSD of mean assay values was found to be 0.83 for escitalopram oxalate and 0.55 for etizolam with a flow rate of 1.1 mL/min. Also RSD of mean assay values was found to be 0.53 and 0.97 for escitalopram oxalate and etizolam respectively with mobile phase composition of methanol: phosphate buffer pH-5 (60:38, v/v) and 0.67 and 0.77 for escitalopram oxalate and etizolam respectively with mobile phase composition methanol: phosphate buffer pH-5 (65:35, v/v). As evident the RSD values of the data obtained are well below 2% indicating that method is robust.

System suitability tests were carried out on freshly prepared standard solutions (n = 6) containing escitalopram oxalate and etizolam. System suitability parameters obtained with 20 µL injection volume are summarized in **Table 5.1.2.1**.

Table 5.1.3.7 System suitability test parameters for Escitalopram Oxalate and Etizolam

Parameter	Escitalopram Oxalate	Etizolam
Retention Time(min)	6.178 (±) 0.116	13.71± 0.10
Theoretical plates	2349.82(±0.64)	3971.56(±)0.54
Tailing factor	0.98(±1.006)	1.067(±0.65)
Resolution	5.12(±0.65)	8.87±0.086

Application of the method to marketed tablets.

Assay results obtained by using the proposed method for the analysis of marketed tablet formulation containing etizolam 0.5 mg and escitalopram oxalate equivalent to esctalopram 5 mg per tablet were in good agreement with the labeled amounts of escitalopram oxalate and etizolam. The average contents of etizolam and escitalopram oxalate were 0.474 mg per tablet (94.90%)94 and 4.745 mg (94.89%) per tablet respectively.

Table 5.1.3.8: Analysis of Marketed Tablet Sample

Tablet	Drug (mg/Tablet)	% Drug obtained ± %R.S.D
EtizolaPlus 5	Etizolam-0.5 mg Escitalopram Oxalate-5 mg	96.41±0.59 95.76±0.51

Table 5.1.3.9 Summary of validation parameters for the proposed HPLC method for escitalopram oxalate and etizolam

Parameter	Escitalopram Oxalate	Etizolam
LOD (µg/ml)	3.77	0.75
LOQ (µg/ml)	11.44	2.28
Accuracy(% recovery)	98.47	98.64
Precision[a]	94.90±0.54	94.89±0.72[a]
Intraday Precision	95.29±1.20	96.42±1.06
Ruggedness[a]	94.24±0.55	94.81±0.96[a]
Robustness (methanol: phosphate buffer	96.15±0..53[b]	95.62±0.97[b]

pH-5 (60:38, v/v))[b]		
Robustness (methanol: phosphate buffer pH-5 (65:38, v/v))[b]	95.01±0.67[b]	94.85±0.77[b]
Robustness (0.9 ml/min Flow rate)[b]	94.23±0.18[b]	96.81±0.65[b]
Robustness (1.1 ml/min Flow rate)[b]	94.89±0.83[b]	95.95±0.55[b]

[a] mean assay values of 6 replicates,

[b] mean assay values of 3 replicates

Forced Degradation Studies Results

The following degradation behavior of the drugs was observed during the HPLC studies:

Acidic conditions

The individual drugs and their combination were heated in 1N HCl for 6 hrs. No significant degradation was observed for etizolam as well as escitalopram oxalate.

Basic condition

Escitalopram oxalate degraded significantly in basic condition i.e.about 51.81% while etizolam was not degraded significantly under same condition i.e. up to 4.55%.

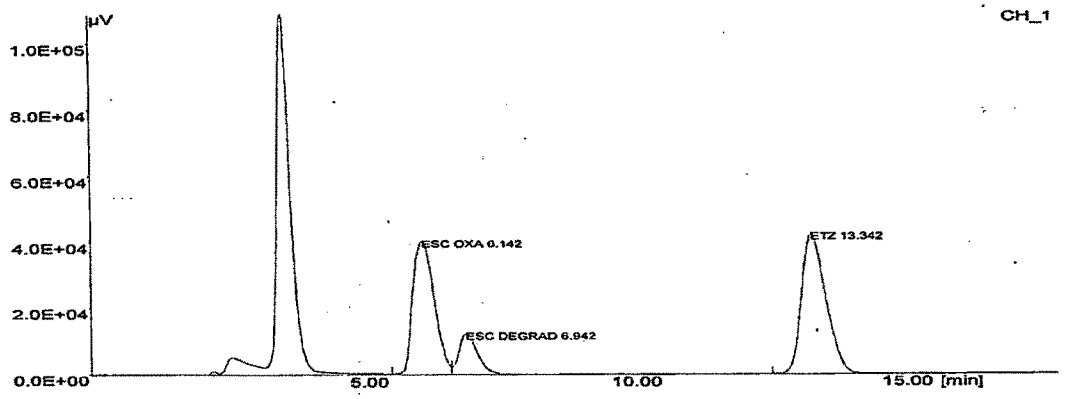


Fig 5.1.3.5 Degradation of Escitalopram under basic condition (1N NaOH)

Oxidative condition

Escitalopram oxalate degraded significantly in peroxide degradation i.e. about 31.42% while etizolam was not degraded under same condition.

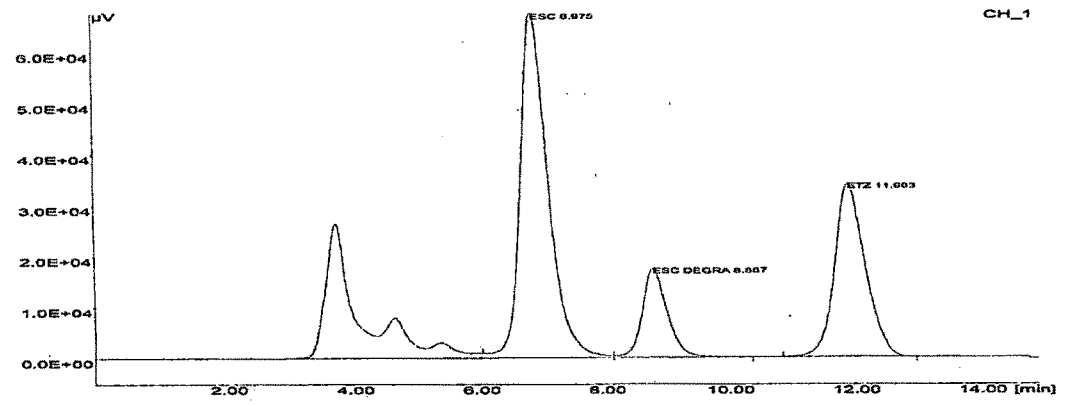


Fig 5.1.3.6 Degradation of Escitalopram under oxidative condition

PDA detection to determine the purity of escitalopram oxalate and etizolam peaks showed purity angle (PA) values and Thrshold values as given in **Table.5.1.3.4**

The Purity Angle (PA)Value was less than the Threshold v (TH) values (as evident from the purity plots. The PA value was less than the TH values, thereby indicating that the escitalopram oxalate and etizolam peaks were free from any co eluting peaks.

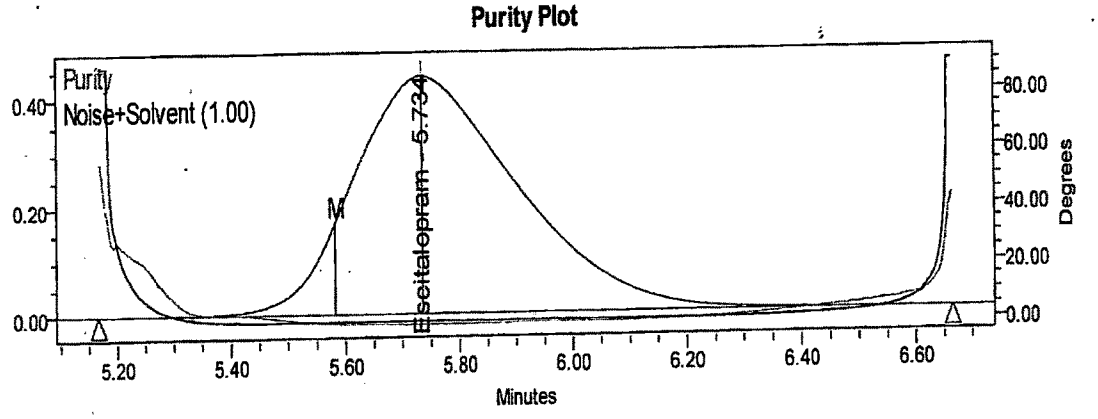
Thermal, photo and humidity conditions

No significant degradation was observed in case of thermal, photo and humidity conditions of both etizolam as well as escitalopram oxalate. From this it is clear that escitalopram oxalate is susceptible to basic and oxidative degradation.

Table 5.1.3.10: Peak purity and peak threshold for various` stress conditions

Stress condition	Etizolam PA	Etizolam TH	Escitalopram oxalate PA	Escitalopram oxalate TH
Standard	0.738	1.151	0.239	1.020
Control Sample	0.124	1.06	0.199	1.006
Basic 1N NaOH	0.52	19.27	1.07	2.976
Oxidation H_2O_2	0.119	1.074	0.281	1.012
Acid	0.139	1.126	0.318	1.013
Photo	0.639	1.180	0.190	1.022
Thermal	0.122	1.090	0.166	1.009
Humidity	0.207	1.066	0.172	1.008

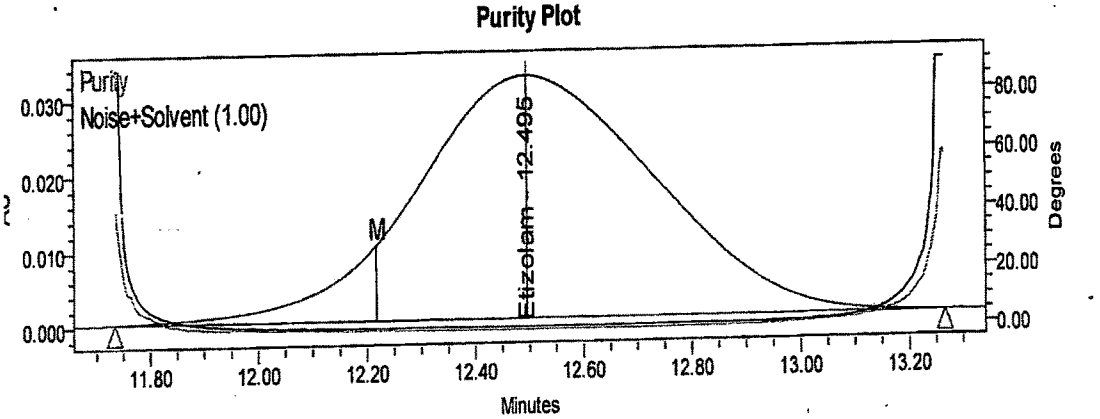
The Purity Angle (PA)Value was less than the Threshold v (TH) values (as evident from the purity plots in The PA value was less than the TH value, thereby indicating that the escitalopram oxalate and etizolam peaks were free from any co eluting peaks, and the method can be used as stability indicating method.



PA: 0.199 TH: 1.006

SampleName B.NoA090624Control 6Hrs Vial 11 Injection 1 Date Acquired 9/20/2011 1:29:16 AM Purity1 Angle 0.199 Purity1 Threshold 1.006

Fig.5.1.3.7 Peak Purity of Esitalopram Oxalate in Control Sample.



PA: 0.124 TH: 1.061

SampleName B.NoA090624Control 6Hrs Vial 11 Injection 1 Date Acquired 9/20/2011 1:29:16 AM Purity1 Angle 0.124 Purity1 Threshold 1.061

Fig.5.1.3.8 Peak Purity of Etizolam in Control Sample

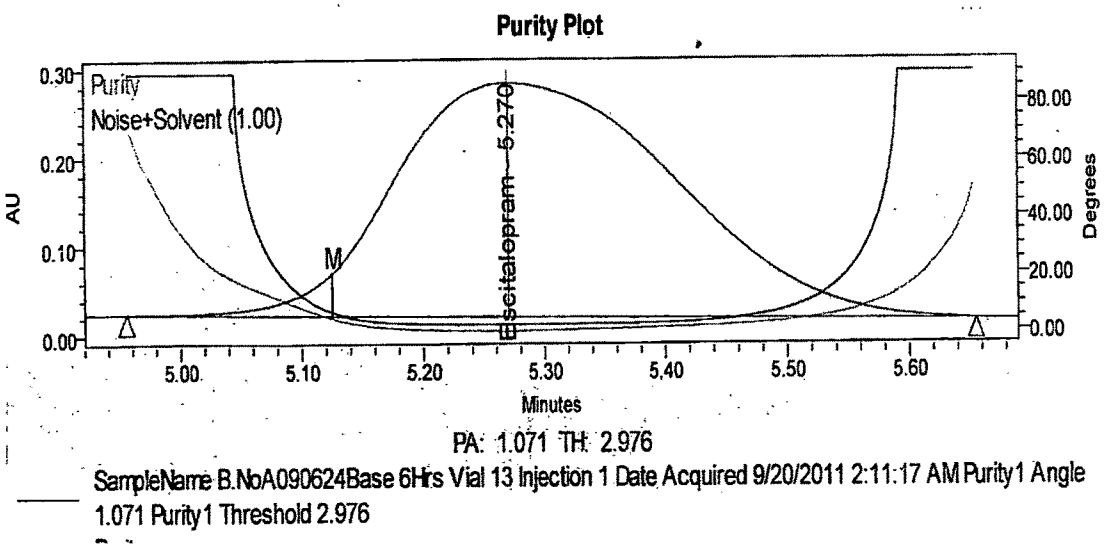


Fig.5.1.3.9 Peak Purity of Escitalopram Oxalate in alkaline condition

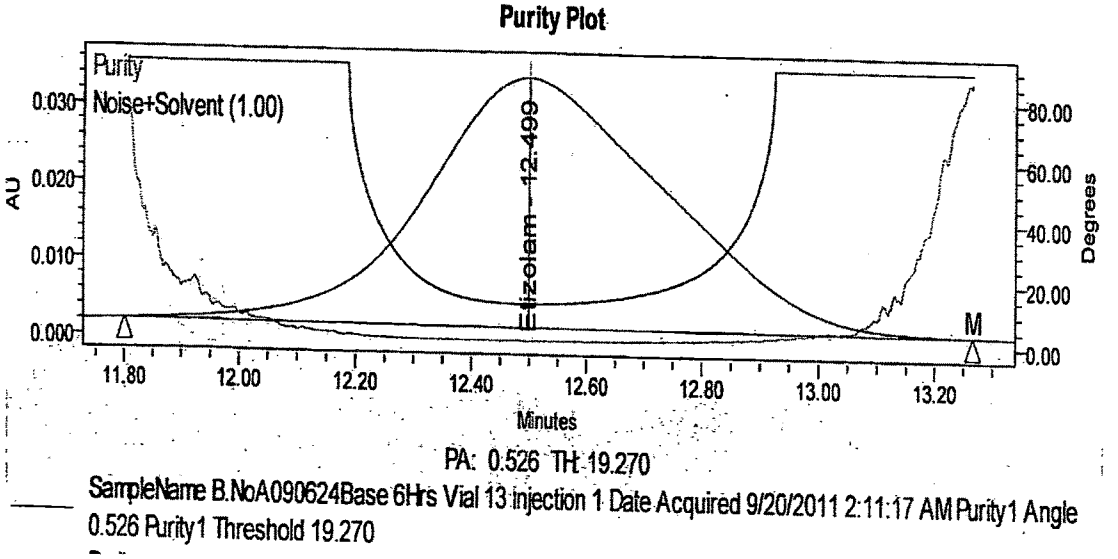


Fig.5.1.3.10 Peak Purity of Etizolam in alkaline condition

5.1.4 Application of developed RP HPLC method for the dissolution studies of etizolam and escitalopram oxalate

RP-HPLC method developed as per 4.1.2 was applied for the dissolution studies of ETIZOLA 5 tablet containing etizolam 0.5 mg and escitalopram oxalate equivalent to 5 mg of escitalopram.For this study the for dissolution studies of etizolam tablets described in Supplement I, Japan Pharmacopoiea -XV was referred.

5.1.4.1 Experimental

This study was a single point dissolution study, therefore sample was withdrawn after 45 minutes as per JP specifications.

Instrumentation

For this study HPLC Instrumentation was used as mentioned in 5.1.2.Dissolution study was carried out using Veego Dissolution Test Apparatus.

Dissolution study conditions

Dissolution medium: 900 ml Distilled Water was used as dissolution medium.

Temperature-37°C, Speed-75 rpm

Study was carried out for 45 minutes.

Standard solution was prepared

For the preparation of standard solution 14 mg of etizolam standard and 140 mg of escitalopram oxalate was dissolved in 25 ml of methanol and diluted to 50 ml with water. 2.5 ml of this solution was diluted to 50ml of water.4 ml of this solution was further diluted to 100 ml to get 0.56 µg/ml of etizolam and 5.6µg/ml of escitalopram oxalate. This solution was injected for study.

Dissolution

For this study, ETIZOLA PLUS 5 containing escitalopram oxalate and etizolam, Etizola 5 tablet containing only etizolm and Nexito containing only esitalopram oxalate tablet were put in different baskets in 900 mL of dist.water and study was carried out as per dissolution study conditions, After 45 minutes 20 ml of sample was pipetted out. First 10 ml of this sample was discarded and remaining 10 ml was filtered through 0.45 µm filter. This filtered solution was used for study.

Dissolution rate calculation

HPLC chromatogram of standard solution and sample solutions were obtained by dissolution study was carried by using the chromatographic conditions used in 5.1.2, and with the help of response areas, assay values for dissolved etizolam and escitalopram oxalate were calculated. With the help of these values % dissolution rate was calculated. This study was carried out three times.

5.1.4.2 Results and Discussion

Dissolution study reveals that after 45 minutes 95.20%(±1.003) of Etizolam and 86.32%(±0.50) of Ecitalopram oxalate was released in water dissolution medium. As per the Supplement I, Japan Pharmacopoiea.XV, dissolution of about 70% of etizolam should take place in 30 minutes. Thus the developed method described in 5.1.2 can be successfully applied for dissolution studies of etzolam and escitalopram in combined tablet dosage form.

5.1.5 RP-HPLC Method for Simultaneous Determination Fluocinolone acetonide and Miconazole Nitrate in bulk and in Ointment Formulation

5.1.5.1 Experimental

Chemicals and Reagents

HPLC grade methanol and water were purchased from S.D. Fine Chemicals (Mumbai, India). Placebo for the formulation was obtained from Hexa labs, Mumbai.

Chromatographic Condition

The gradient program used was as follows:

0–2 min, methanol–water (95 + 5, v/v); 2–4 min, methanol–water (95 + 05, v/v) to methanol–wate(100+0 v/v) r; and 4–6 min, methanol–water (100+0, v/v), 6–8min, methanol–water (100+0, v/v) to methanol–water (95 + 5, v/v)

Marketed Formulation

Each 15 g ointment tube (ZOLE-F of REXEL (RANBAXY) containing miconazole nitrate (2%, w/w) and fluocinolone acetonide (0.01%, w/w) was procured from the local market.

Preparation of Mobile Phase

Methanol and water used for the mobile phase were filtered through a 0.45μm membrane filter (Ultipore N –66R Nylon 66; Pall Corp.,) and degassed by ultrasonication for 15 min.

Preparation of Standard Stock Solution

The standard stock solution of fluocinolone acetonide was prepared by dissolving 10 mg of fluocinolone acetonide in methanol to get a solution containing 10μg/ml of fluocinolone acetonide.Standard stock solution of miconazole nitrate was prepared by dissolving 200 g of miconazole nitrate in methanol to obtain a solution containing 2 mg/mLof miconazole nitrate . Working standard solution of fluocinolone acetonide was prepared by diluting appropriate

aliquot of standard stock solution with methanol were prepared by placing 2 mL fluocinolone acetonide stock solution and 20 mL miconazole nitrate stock solution in a 100 mL volumetric flask. The solutions were diluted to 100mL with methanol to obtain a solution containing 2 µg /mL of fluocinolone acetonide and 400 µg /mL miconazole nitrate

Preparation of Sample Solution

In a 100 mL conical flask, 0.5 g ointment sample was weighed and dissolved in about 30 ml of methanol by sonification for about 5 minutes with intermittent shaking. The ointment gets completely dissolved in methanol. Then it was transferred to 50 ml volumetric flask .The conical flask was rinsed with 3 portions of 5 mL methanol and transferred to volumetric flask, and the final volume was made to 50 mL with methanol.

Analysis of a Marketed Formulation:

Assay of marketed ointment formulation (ZOLE-F of REXEL (RANBAXY) containing miconazole nitrate (2%, w/w) and fluocinolone acetonide (0.01%, w/w) was performed by preparing the sample solutions as described in the previous section. Six of the prepared sample and standard solutions were injected. The assay of the commercial sample was calculated by comparing the areas of standard and sample peaks.

Validation of the Method

The validation of the method was done following the ICH guidelines.

Calibration curve (linearity of the HPLC method)

The calibration curve was constructed by plotting concentrations of fluocinolone acetonide and miconazole nitrate and versus peak areas, and the regression equations were calculated. The linearity of the method was investigated by using concentrations in the range 0.5-4.5 µg/mL for fluocinolone acetonide and 200- 900 µg/mL for miconazole nitrate. These concentrations were prepared by diluting appropriate volumes of working standard with methanol.

Accuracy (recovery)

Accuracy of the method was studied by recovery experiments using the standard addition method at three different levels (80, 100, and 120%). Known amounts of standard solutions containing fluocinolone acetonide (1.6, 2, and 2.4 µg) and miconazole nitrate (320, 400, and 480 µg) were added to prequantified sample solutions to reach the 80, 100, and 120% levels. These

samples were analyzed and the recovery of recovery was calculated from the difference of peak areas of spiked and unspiked sample.

Precision (repeatability)

Precision of the assay method was demonstrated by injecting six different sample solutions containing fluocinolone acetonide equivalent to 2 µg/mL and miconazole nitrate equivalent to 400µg/mL, and RSD of the mean assay value was calculated.

Intraday Precision

Intraday precision was demonstrated by injecting six different sample solutions containing fluocinolone acetonide equivalent to 2 µg/mL and miconazole nitrate equivalent to 400 µg/mL at different time intervals within the same day and RSD of mean assay value was calculated.

Intermediate precision (ruggedness)

Intermediate precision of the method was demonstrated by carrying out the experiment on different days, by different analysts, and on different instruments using different C18 column.

LOD and LOQ

LOD and LOQ of fluocinolone acetonide and miconazole nitrate were calculated using the following equations according to ICH guidelines:

$LOD = 3.3 \times \sigma/S \quad LOQ = 10 \times \sigma/S$

Where, σ = Standard deviation of response

S = Slope of regression equation.

Specificity

Specificity of the method was demonstrated by injecting the blank solution, standard solution, sample solution, and solvent extracted placebo and the responses were determined. The retention times of fluocinolone acetonide and miconazole nitrate were 3.52 (±0.32) and 6.55 (±1.10) min, respectively.

Robustness

Robustness of the method was demonstrated by deliberately changing the chromatographic conditions. The flow rate of the mobile phase was changed from 1.0 to 0.9 mL/min and from 1.0 to 1.1 mL/min.In another study methanol used was changed from S.D Chemicals to Merck Chemicals. The sample solutions described for the robustness study were applied onto the column in triplicate, and the responses were determined.

System suitability study

For this study, 20 µL blank solution [methanol–water (95 + 5, v/v)] was injected and run for 20 min with the gradient program. After this, 20 µL standard solutions in six replicates were injected, and the RSD of the resultant peak areas, theoretical plates, tailing factor and resolution factor of the peaks was calculated.

5.1.5.2 Results and Discussion

Optimization of method

Several mobile phase compositions were tried. A satisfactory separation and good peak symmetry were obtained by using the described methanol–water gradient program. Quantification was achieved with UV detection at 238 nm based on peak area. A representative chromatogram is shown in **Fig.5.1.5.1**

A gradient elution technique was used because, it was found that some of the excipients in the cream formulation was getting eluted lately after about half an hour with isocratic elution with methanol and water. This late eluting excipient was increasing the total time of analysis.

Table 5.1.5.1 Optimization of method

Mobile phase	**Flow rate (ml/min)**	**Fluocinolone Acetonide**			**Miconazole Nitrate**		
		RT (min)	**Peak Shape**	**Theoretical plates**	**RT (min)**	**Peak Shape**	**Theoretical plates**
CH_3OH:Water (90:10)	1	3.96	Sharp	1618	10.16	Sharp	Above 2000
CH_3OH:0.1M Ammonium acetate (90:10)	1	3.97	Sharp	1231	31.94	Not Sharp	Above 2000
CH_3OH:Water (90:10)	1	3.96	Sharp	1618	10.16	Sharp	Above 2000
CH_3OH:Water (87.5:12.5)	1	4.12	Sharp	2017	12.80	Sharp	Above 2000
Although the CH_3OH:Water (87.5:12.5) mobile phase gave sharp peaks for both the drugs, the mobile phase could not be used for the development of method as one cream excipient was found to be eluting at 35 min, giving a long run time for the chromatographic procedure. Therefore gradient elution was tried for early elution of exicipient to avoid its interference in succeeding injection.							
CH_3OH:Water 0-8 Min 88.5-98, 8-25 98 25-30 98-88.5	1	4.08	Sharp	Above 2000	12.53	Sharp	Peak on raised baseline,Excipient peak at

							26.417 min
CH_3OH:Water 5.5-7.5 Min 88.5-98, 7.5-20 98 25-25 98-88.5	1	4.07	Sharp	Above 2000	12.40	Sharp	Peak on raised baseline,Excipient peak at 25.95min
CH_3OH:Water 5.5-7.5 Min 88.5-98, 7.5-20 98 25-25 98-88.5	1	4.07	Sharp	Above 2000	12.40	Sharp	Peak on raised baseline,Excipient peak at 25.95min
CH_3OH:Water 0-2 Min 88.5 2-4 88.5-100 4-12 100 12-15 100-85.5	1	4.08	Sharp	Above 2000	11.04	Sharp	Peak on raised baseline,Excipient peak at 20.30min
CH_3OH:Water 0-2 Min 88.5 2-4 88.5-100 4-10 100 10-12 100-85.5	1	4.02	Sharp	Above 2000	10.86	Sharp	Peak on raised baseline,Excipient peak at 15.38min
CH_3OH:Water 0-2 Min 90 2-4 90-95 4-8 100 8-10 100-85.5	1	3.85	Sharp	Above 2000	9.425	Sharp	Excipient peak at 13.917min
CH_3OH:Water 0-2 Min 90 2-4 90-95 4-6 100 6-8 100-85.5	1	3.89	Sharp	Above 2000	9.533	Sharp	Excipient peak at 14 min
CH_3OH:Water 0-2 Min 95 2-4 95-100 4-6 95-100 6-8 100-95	**1**	**3.52**	**Sharp**	**Above 2000**	**6.55**	**Sharp**	**Excipient peak at 13 min. min**

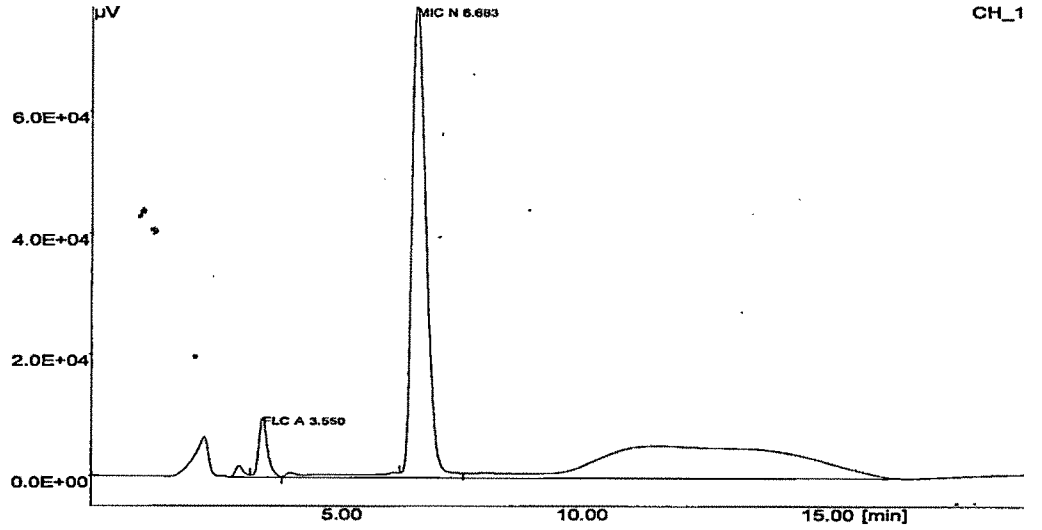


Fig.5.1.5.1: Chromatogram of Fluocinolone Acetonide and Miconazole Nitrate 238 nm (Fluocinolone acetonide 2µg/ml andMiconazole nitrate 400µg/ml)

Linearity regression data are summarized in Table 4.1.5.2, which shows a good linear relationship between concentration and peak areas over a concentration range of 0.5 -4.5µg for fluocinolone acetonide (**Fig.5.1.5.2**) and 300-900µg for miconazole nitrate (**Fig. 5.1.5.3**). The correlation coefficient (R2) was found to be 0.9996 for fluocinolone acetonide and 0.9994 for miconazole nitrate.

Table 5.1.5.2 Regression analysis of the calibration curves for fluocinolone acetonide and miconazole nitrate

Parameter	**Fluocinolone acetonide**	**Miconazole nitrate**
Linearity range(µg/mL)	0.5 -4.5µg	300-900
Regression equation	y=51092x +12916	Y=2656.2+410675
Correlation coefficient (R^2)	0.9996	0.9994
Slope	51092	2656.2
Y-intercept	12916	410675

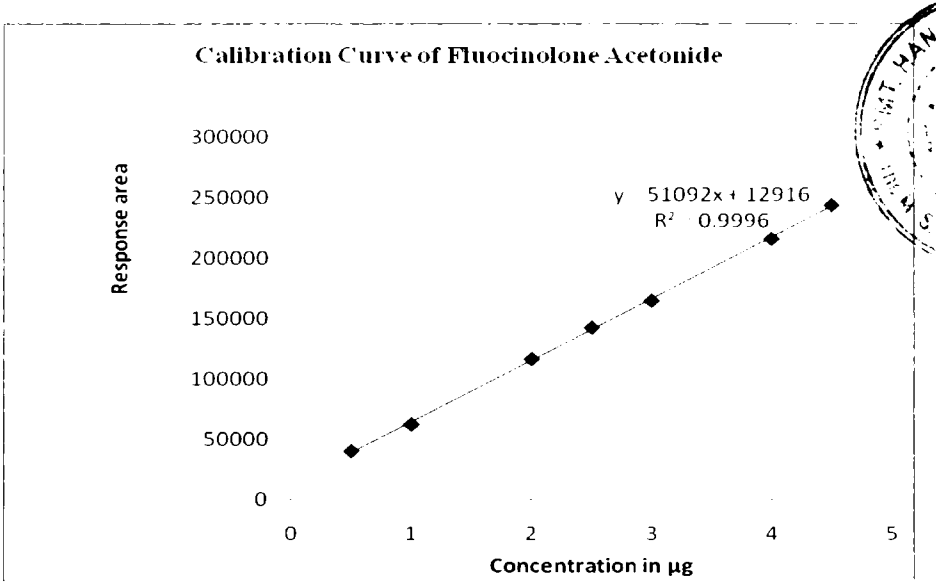


Fig.5.1.5.2:Calibration Curve for fluocinolone acetonide (0.5-4.5µg/ml))

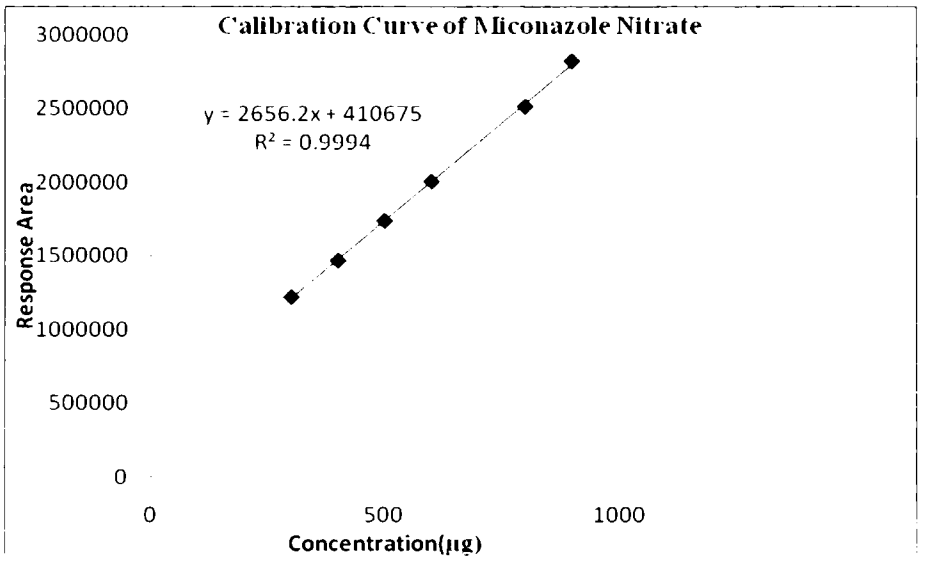


Fig. 5.1.5.3: Calibration Curve for Miconazole Nitrate (300-900µg/ml)

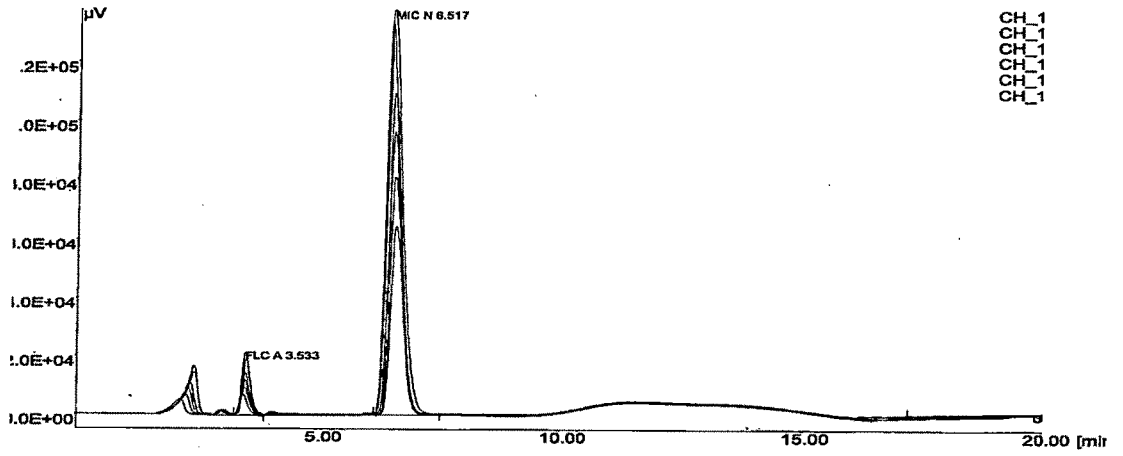


Fig:5.1.5.4 Overlay chromatogram of fluocinolone acetonide and miconazole nitrate

Accuracy studies indicated that the mean recovery of the added standard drug was 98.43%and 99.88% for fluocinolone acetonide and miconazole nitrate, respectively. Specificity studies indicated no interference from excipients, impurities, or degradation products, and ensured that the peak response was due to fluocinolone acetonide and miconazole nitrate only.

Table 5.1.5.3 recovery study of fluocinolone acetonide and miconazole nitrate

Drug	Recovery Level (%) (n=3)	%Recovery±%RSD	Mean Recovery (%)
Fluocinolone acetonide	80	98.54±0.34	**98.43±0.13**
	100	98.29038±0.21	
	120	98.45667±0.17	
Miconazole Nitrate	80	101.2168±0.37	**99.88±1.32**
	100	99.88397±0.007	
	120	98.56255±0.47	

In the ***precision*** studies, RSD of mean assay values was found to be 0.51 for fluocinolone acetonide and 0.46 for Miconazole Nitrate. These values indicate that the repeatability of this method is satisfactory.

Table 5.1.5.4 Precision study of fluocinolone acetonide and miconazole nitrate

Drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean ±%RSD
Fluocinolone acetonide	98.47	98.58	99.11	98.36	99.46	99.51	**98.92±0.51**
Miconazole nitrate	100.41	99.07	99.99	99.90	100.26	99.81	**99.91±0.46**

In the ***intraday precision*** studies RSD of mean assay values were found to be 0.67 for fluocinolone acetonide and 0.45 for miconazole nitrate

Table 5.1.5.5 Intraday precision study of fluocinolone acetonide and miconazole nitrate

Drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean ±%RSD
Fluocinolone acetonide	99.76	99.20	100.98	100.64	100.27	99.58	**100.07±0.67**
Miconazole Nitrate	97.47	98.11	97.46	97.25	97.18	98.25	**97.62±0.45**

The intermediate precision study revealed that the method is rugged with RSD values of for fluocinolone acetonide and for Miconazole Nitrate.

Table 5.1.5.6 Intermediate precision study of fluocinolone acetonide and miconazole nitrate

Drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean ±%RSD
Fluocinolone Acetonide	97.46	98.42	98.72	97.40	96.46	97.55	**97.67±0.82**
Miconazole Nitrate	96.18	96.38	96.13	96.73	95.97	95.74	**96.19±0.35**

LOD and LOQ

The LOD was found to be 0.13µg/mL for fluocinolone acetonide and 20.40 µg /mL for miconazole nitrate. The LOQ was found to be 0.39 µg /mL for fluocinolone acetonide and 61.82 µg/ mL for miconazole nitrate. These values indicate that the method is sensitive.

Specificity studies indicated that there is no interference from exipients, impurities and degradation products and assured that the peak response was due to escitalopram oxalate and etizolam only.

Robustness studies signified that the results of the method remained unaffected by small, deliberate changes in the flow rate and by using the methanol solvent of a different manufacturer The RSD of mean assay values was found to be 0.125593 for fluocinolone acetonide and 1.408821 for miconazole nitrate with a flow rate of 0.9 mL/min. The RSD of mean assay values was found to be 0.962301 for fluocinolone acetonide and 1.091665 for miconazole nitrate with a flow rate of 1.1 mL/min. Also, RSD of mean assay values was found to be 0.542503 and 0.452552 for fluocinolone acetonide and miconazole nitrate, respectively, after using the methanol solvent of a different manufacturer. All validation data are summarized in **Table 5.1.5.9**

Table 5.1.5.7 Robustness study of fluocinolone acetonide and miconazole nitrate

Parameter		Fluocinolone acetonide Assay values (n=3)				Miconazole Nitrate Assay values (n=3)			
	Sample	1	2	3	Mean± %RSD	1	2	3	Mean± %RSD
Flow Rate(ml)	0.9	101.50	101.69	101.74	101.64 ±0.12	98.5	101.28	100.63	100.16 ±1.40
	1.1	98.84	99.98	100.75	99.86	99.42	101.22	101.41	100.6 ±1.09
Change of Reagent		95.19	94.33	95.25	94.92 ±0.54	101.18	100.31	100.49	100.66 ±0.45

System suitability tests were carried out on freshly prepared standard solutions (n = 6) containing Miconazole Nitrate and fluocinolone acetonide. System suitability parameters obtained with 20 mL injection volumes are summarized in **Table 5.1.5.1.**

Table 5.1.5.8 System suitability test parameters for fluocinolone acetonide and miconazole nitrate

Parameter	Fluocinolone acetonide	Miconazole nitrate
Retention Time (min)	3.527833(±0.320872)	6.557(±1.103)
Theoretical plates	2345.236(±1.909277)	3004(±1.383157)
Tailing factor	1.46675(±1.008749)	1.51825(±1.980619)
Resolution	-	7.8±(0.481)

Analysis of a Marketed Formulation

The ***assay*** results obtained by using the proposed method for the analysis of a marketed cream formulation containing fluocinolone acetonide (0.01%, w/w) and miconazole nitrate (2%, w/w) were in good agreement with the labeled amounts of fluocinolone acetonide and miconazole nitrate. The average contents of fluocinolone acetonide and miconazole nitrate were 0.009892g/100g cream (0.0098% w/w) and 1.9784g/ 100g cream (1.9784 %, w/w), respectively.

Cream	Drug (mg / 100 gm of cream)	% of Drug obtained ±RSD
Zole-F	Fluocinolone acetonide (0.1mg/100gm) Miconazole nitrate (2mg/100gm)	**98.92±0.51** **99.91±0.46**

Table 5.1.5.9 Summary of validation parameters for the proposed HPLC method for fluocinolone acetonide and miconazole nitrate

Parameter	Fluocinolone acetonide	Miconazole nitrate
LOD (µg/ml)	0.13	20.40
LOQ (µg/ml)	0.39	61.82
Accuracy(% recovery)	98.4333	99.88776
Precision[a]	98.92056(±0.51563)	99.91119±(0.46917)
Intraday precision[a]	100.07±0.67	97.62±0.45
Ruggedness[a]	97.67476(±0.824081)	96.19428±(0.354551)
Robustness (Reagent)[b]	94.92631±0.542503	100.665±0.452552
Robustness (0.9 ml/min Flow rate)[b]	101.6474(±0.12559)3	100.1645±(1.40882)

Robustness (1.1 ml/min Flow rate)[b]	99.86476±(0.962301)	100.6917±(1.091665)

[a] mean assay values of 6 replicates, [b] mean assay values of 3 replicates

5.1.6 RP-HPLC method for simultaneous estimation of Hydrocortisone acetate and Miconazole nitrate in a Cream Formulation

5.1.6.1 Experimental

Reagents

HPLC grade acetonitrile, water, ammonium acetate(pure) used was purchased from S.D. Fine Chemicals (Mumbai, India).

Marketed Formulation

Each 10 g Cream tube (FUMIC) containing 2% miconazole nitrate and 1% hydrocortisone acetate was provided by Apoorva Biopharmaceuticals Pvt.Ltd,Thane, India

Preparation of Mobile Phase

Acetonitrile and 0.1 M ammonium acetate used for mobile phase were filtered through 0.22 µm membrane filter (Durapore Membrane, Miilipore GV 0.22µm) and degassed by ultrasonication for 15 min.

Preparation of ammonium acetate buffer

For the preparation of 1 liter of 0.1M ammonium acetate buffer, 7.71 gm of ammonium acetate was dissolved in 200 ml of distilled water in a beaker and to it 8 ml of glacial acetic acid was added and final volume was made to 1000 ml with distilled water in a volumetric flask.

Preparation of Standard Stock Solution

The standard stock solution was prepared by dissolving miconazole nitrate and hydrocortisone acetate in 100 ml of acetonitrile to get a solution containing 200 µg /mL of miconazole nitrate and 100µg /mL of hydrocortisone acetate. Working standard solution was prepared by diluting 1 mL of the stock solution with mobile phase to 10 mL to get a solution containing 20µg/mL of miconazole nitrate and 10µg/mL of hydrocortisone acetate

Preparation of Sample Solution

In a 50 mL screw-cap centrifuge tube, 1 g cream sample was weighed and extracted with three portions of acetonitrile:0.1 M ammonium acetate (80:20,v/v). For each extraction, 25 mL acetonitrile :0.1 M ammonium acetate (80:20,v/v) was added to the centrifuge tube and heated

in a water bath (60°C) with shaking to disperse the cream, vortexed for 1 min, and centrifuged for 10 min at 3000 rpm. Supernatants were collected in a 100 mL volumetric flask, and the final volume was diluted to 100 mL with acetonitrile :0.1 M ammonium acetate (80:20,v/v).1mL of this solution was diluted with mobile phase to get the solution containing hydrocortisone acetate equivalent to 10 µg/mL and miconazole nitrate equivalent to 20µg/mL

Analysis of a Marketed Formulation

Assay of marketed cream formulation containing miconazole nitrate2% and hydrocortisone acetate 1% was performed by preparing the sample solutions as described earlier in the *preparation of the sample.* Six injections of above prepared sample and standard solutions were injected. The assay of the commercial sample was calculated by comparing the areas of standard and sample peaks.

Validation of the Method

The validation of the method was done following the ICH guidelines.

Calibration curve (linearity of the HPLC method)

Calibration curve was constructed by plotting concentrations of hydrocortisone acetate and miconazole nitrate vs. peak areas, and the regression equations were calculated. The linearity of this method was investigated by using the concentrations 5,10,15,20,30,25 and 30µg/mL for hydrocortisone acetate and,10,20,30,40,50 and 60 µg/mL for miconazole nitrate. These concentrations were prepared by diluting appropriate volume of working standard with mobile phase. The retention times of hydrocortisone acetate and miconazole nitrate were 4.055(±0.16) min and 13.14(±0.31) min respectively (Fig 2.1 and 2.2).

System suitability study

For this study first upon a 20 µL of blank solution (mobile phase)was injected and ran for 20 minutes. After this 20 µL of standard solutions in 6 replicates were injected and the % relative standard deviation (% RSD) of the response peak areas was calculated.

Accuracy (recovery)

Accuracy of the method was studied by recovery experiments using standard addition method at three different levels (80%, 100% and 120%). The known amounts of standard solutions containing hydrocortisone acetate (160, 200 and 240 µg) and miconazole nitrate (18, 20, 22µg)

were added to prequantified sample solutions to reach the 80,100 and 120 % levels. These samples were analyzed by injecting the sample solution and recovery was calculated.

Precision (repeatability)

Precision of the assay method was demonstrated by injecting six different sample solutions containing hydrocortisone acetate equivalent to 10µg/mL and miconazole nitrate equivalent to 20µg/mL and RSD of mean assay value was calculated.

Intraday Precision

Intraday precision was demonstrated by injecting six different sample solutions containing hydrocortisone acetate equivalent to 10 µg/mL and miconazole nitrate equivalent to 20 µg/mL at different time intervals within the same day and RSD of mean assay value was calculated.

Intermediate precision (ruggedness)

Intermediate Precision of the method was demonstrated by carrying out the experiment on different day, by different analyst and on different instrument using different C-18 column.

Specificity

Specificity of the method was studied by injecting blank, standard, placebo and sample solutions.

Robustness

Robustness of the method was demonstrated by deliberately changing the chromatographic conditions. The flow rate of the mobile phase was changed from 1.0 mL/min to 0.9 mL/min and also from 1.0 mL/min to 1.1 mL/min. The composition of mobile phase was changed fromacetonitrile: 0.1 M ammonium acetate (80:20, v/v) to acetonitrile: 0.1 M ammonium acetate (76:20 v/v) and also from acetonitrile: 0.1M ammonium acetate (80:20, v/v) to acetonitrile: 0.1M ammonium acetate (84:20 v/v).The solutions for robustness study were applied on the column in triplicate and the responses were determined.

LOD and LOQ

LOD and LOQ of hydrocortisone acetate and miconazole nitrate were calculated using the following equations as per International Conference on Harmonization (ICH) guidelines.

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

Where, σ = Standard deviation of response S = Slope of regression equation.

5.1.6.2 Results and Discussion

Optimization of the method

For the optimization of method various mobile phase combinations were tried as mentioned in the table.Main problem with different mobile phase composition was that the theoretical plates were not increasing beyond 2000.This problem was solved by replacing methanol with acetonitrile.

Table 5.1.6.1 Otimization of the method

Mobile phase	Flow rate (ml/min)	Hydrcortisone acetate			Miconazole Nitrate		
		RT (min)	Peak Shape /Resolution	Theoretical Plates	RT (min)	Peak Shape	Theoretical Plates
Methanol :water (85:15)	1 mL	3.750	No proper resolution from dead volume peak	Less than 2000	Not detected at 20µg/ml conc.		
Methanol :0.1M Ammonium acetate (95:05)	1 mL	3.792	Fronting	1875	6.475	sharp	Above 2000
Methanol:0.1M Ammonium acetate (90:05)	1 ml	3.981	Not Sharp	1647	9.508	Tailing	Above 2000
Methanol : 0.1% Formic acid (90:10)	1mL	3.25	Sharp but not getting resolved properly from the excipient peak	622.57	9.350	Sharp	3002.82
Methanol : 0.1% Formic acid (90:10)	0.8mL	3.60	Sharp but didnot getting resolved properly from the excipient peak	335.45	9.862	Sharp	1485
ACN:Water (90:10)	1 ml	3.750		Above 2000	Did not elute upto 30 min.		
ACN:0.1M Ammonium acetate (90:10)	1 mL	3.558	Sharp but did not getting resolved properly	Above 2000	9.100	Sharp	Above 2000

			from the excipient peak(Resolution 1.20)				
ACN:0.1M Ammonium acetate (87.5:12.5)	1 mL	3.717	Sharp but did not getting resolved properly from the excipient peak(Resolution 1.35)	Above 2000	10.575	Sharp	Above 2000
ACN:0.1M Ammonium acetate(85.:15)	1 mL	3.825	Sharp but did not getting resolved properly from the excipient peak(Resolution 1.75)	Above 2000	12.417	Sharp	Above 2000
ACN:0.1M Ammonium acetate(85.:15)	1 mL	4.05	Sharp , resolved properly from the excipient peak(Resolution 2.50)	Above 2000	13.14	Sharp	Above 2000

A satisfactory separation and good peak symmetry was obtained by using the mobile phase containing acetonitrile: 0.1 M ammonium acetate (80:20, v/v). Quantification was achieved with UV detection at 225 nm based on peak area. A representative chromatogram is shown in **fig.5.1.6.1**

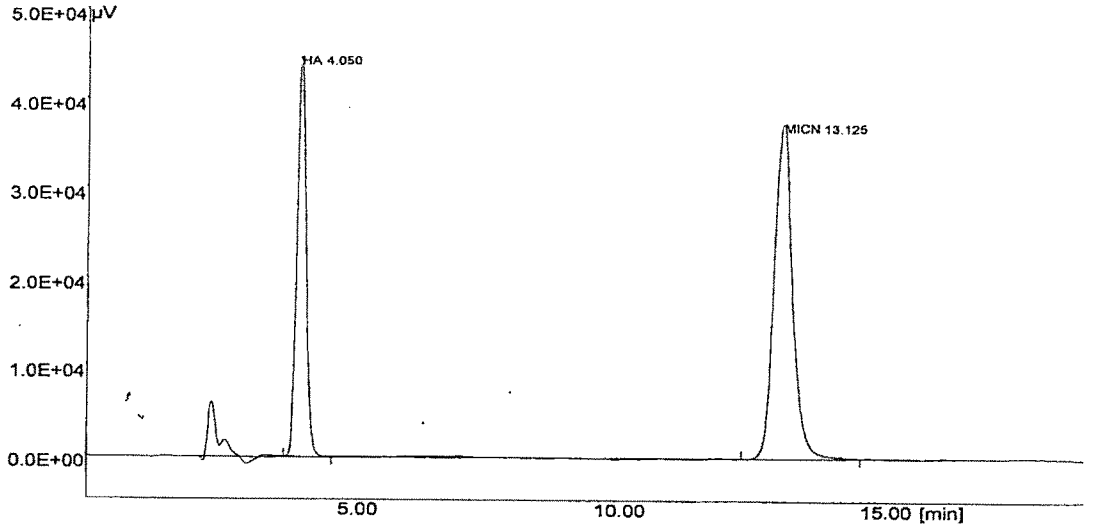


Fig.5.1.6.1 Chromatogram of Hydrocortisone acetate and and Miconazole nitrate at 225nm (Hydrocortisone acetate 10µg/ml,miconazole nitrate 20µg/ml)

Accuracy studies indicate that the mean percent recovery of the added standard drug to be 98.69% and 100.37% for hydrocortisone acetate and miconazole nitrate respectively.

Linearity regression data is summarized in **Table 5.1.6.2** which shows a good linear relationship between concentration and peak areas over a concentration range of 5-30µg/ml for hydrocortisone acetate (**Fig. 5.1.6.2**) and 10 - 60µg/ml for miconazole nitrate (**Fig. 5.1.6.3**). The correlation coefficient (R^2) was found to be 0.9994 for hydrocortisone acetate and 0.9991 for miconazole nitrate.

Table 5.1.6.2 Regression analysis of the calibration curves for hydrocortisone acetate and iconazole nitrate

Parameter	Hydrocortisone acetate	Miconazole nitrate
Linearity range(µg/mL)	5-30	10 – 60
Regression equation	y=49909x+22990	y=41107x+41044
Correlation coefficient(R^2)	0.9994	0.9991
Slope	49909	41107
X-intercept	-0.46	-0.99
Y-intercept	22990	41044

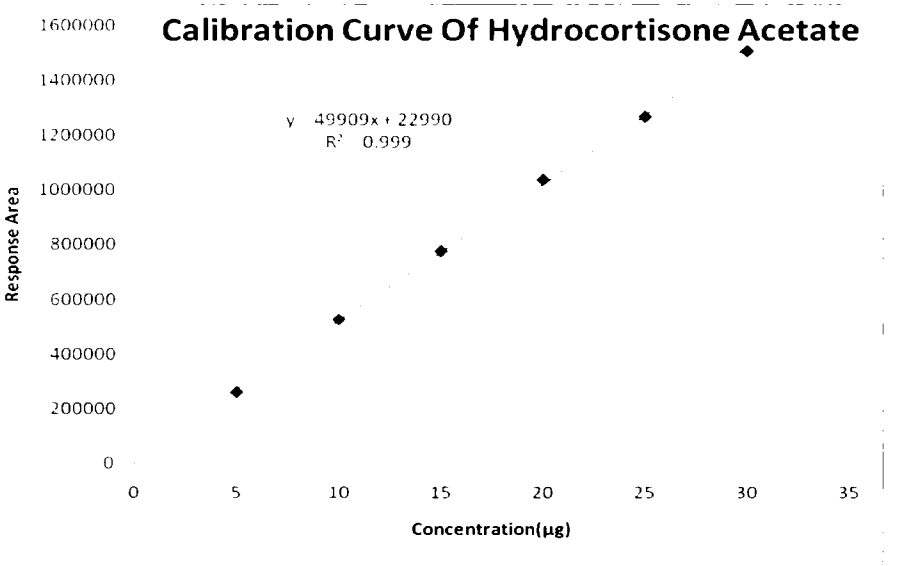


Fig. 5.1.6.2 Calibration curve diagram for Hydrocortisone acetate(5-30µg/ml)

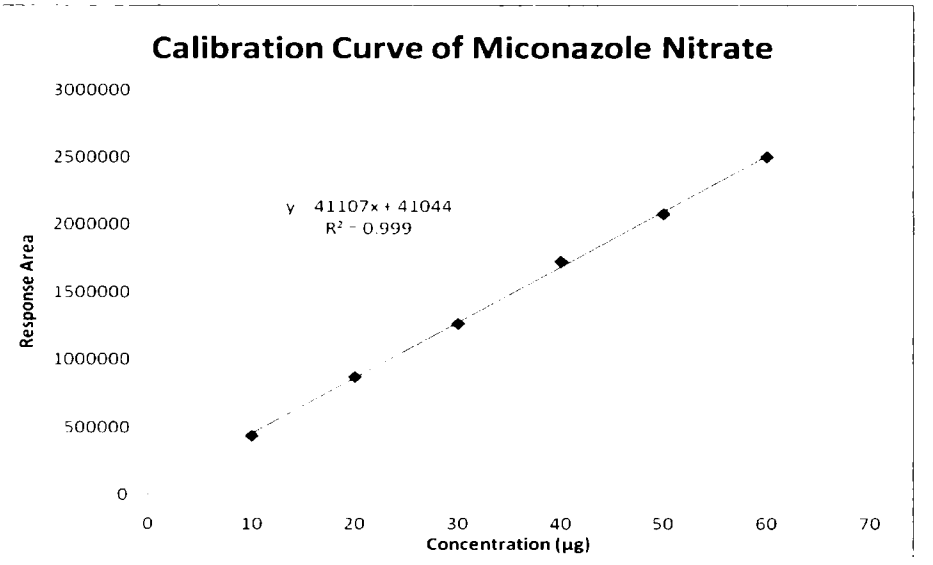


Fig. 5.1.6.3 Calibration curve diagram for Miconazole nitrate (10-60µg/ml)

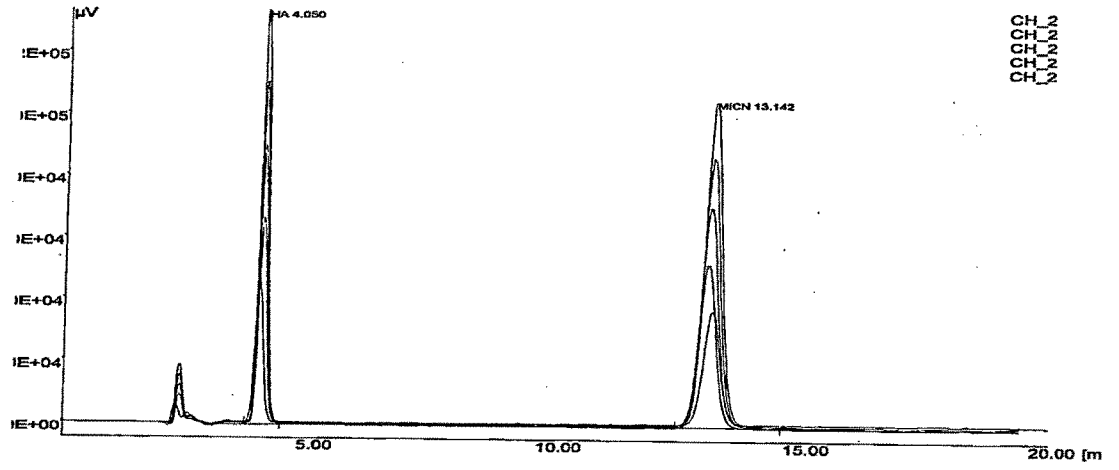


Fig. 4.1.6.3 Overlay calibration Chromatogram for Miconazole nitrate and hydrocortisone acetate

In the ***precision*** studies, RSD of mean assay values was found to be 1.13for hydrocortisone acetate and 0.85 for miconazole nitrate. These values indicate that the repeatability of this method is satisfactory. In the ***intraday precision*** studies RSD of mean assay values were found to be 1.06 for hydrocortisone acetate and 1.36 for miconazole nitrate.

Intermediate precision (Ruggedness) study reveals that the method is rugged with RSD values of 1.045 for hydrocortisone acetate and 1.34 for miconazole nitrate.

LOD and LOQ

The limit of detection was found to be 0.866639 µg/mL for hydrocortisone acetate and 2.03577 µg/mL for miconazole nitrate. The limit of quantification was found to be 2.62618µg/mL for hydrocortisone acetate and 6.169022 µg/mL for miconazole nitrate. These values indicate that the method is sensitive.

Specificity studies indicated that there is no interference from exipients, impurities and degradation products and assured that the peak response was due to hydrocortisone acetate and miconazole nitrate only.

Robustness study signified that the results of the method remained unaffected by small, deliberate changes in the flow rate and mobile phase composition. The RSD of mean assay values was found to be 0.46 for hydrocortisone acetate and 0.64 for miconazole nitrate with a flow rate of 0.9 mL/min .The RSD of mean assay values was found to be 0.32 for hydrocortisone acetate and 0.47 for miconazole nitrate with a flow rate of 1.1mL/min. Also RSD of mean assay

values was found to be 0.82 and 1.21for hydrocortisone acetate and miconazole nitrate respectively with mobile phase composition of acetonitrile: 0.1 M ammonium acetate(76:20,v/v) and 0.62 and 1.31for hydrocortisone acetate and miconazole nitrate respectively with mobile phase composition acetonitrile: 0.1 M ammonium acetate (84:20, v/v). System suitability tests were carried out on freshly prepared standard solutions (n = 6) containing hydrocortisone acetate and miconazole nitrate. System suitability parameters obtained with 20 µL injection volume are summarized in **Table 5.1.6.1**

Table 5.1.6.3 System suitability test parameters for hydrocortisone acetate and miconazole nitrate

Parameter	Hydrocortisone acetate	Miconazole nitrate
Retention Time(min)	4.05(± 0.141)	13.14 (± 0.31)
Theoretical plates	2865.048(±0.978221)	8665.925(±1.18129)
Tailing factor	1.19±1.70	1.18±1.62
Resolution	-	20.64±0.49

Analysis of a Marketed Formulation

The ***assay*** results obtained by using the proposed method for the analysis of marketed cream formulation containing miconazole nitrate equivalent to 20 mg /gm and hydrocortisone acetate equivalent to 10mg/gm cream ,were in good agreement with the labeled amounts of hydrocortisone acetate and miconazole nitrate. The average contents of miconazole nitrate and hydrocortisone acetate were 19.328mg per cream (96.64%) and 10.019mg (100.19%) per gram of cream respectively.

Table 5.1.6.4 Summary of validation parameters for the proposed HPLC method for hydrocortisone acetate and miconazole nitrate

Parameter	Hydrocortisone acetate	Miconazole nitrate
LOD (µg/ml)	0.866639	2.03577
LOQ (µg/ml)	2.62618	6.169022
Accuracy(% recovery)	98.69	100.37
Precision	100.19±1.13[a]	96.64±0.85
Intraday precision	99.48±1.06	97.65±1.36
Ruggedness	98.85±1.04[a]	96.50±1.34[a]
Robustness (acetonitrile: 0.1 M ammonium acetate (76:20, v/v))	98.67±0.82[b]	95.53±1.21[b]
Robustness (acetonitrile: 0.1 M ammonium acetate (84:30, v/v))	98.25±0.62[b]	95.23±1.31[b]
Robustness (0.9 ml/min Flow rate)	98.35±0.46[b]	95.83±1.27[b]
Robustness (1.1 ml/min Flow rate)	99.83±0.32[b]	95.75±0.47[b]

[a] mean assay values of 6 replicates , [b] mean assay values of 3 replicates

5.1.7 A stability indicating validated RP-HPLC Assay Method for Racecadotril in bulk and in formulation (Oral Powder)

5.1.7.1 Experimental

HPLC Instrumentation

High performance liquid chromatography including a Shimazdu pump LC-10 ATVP equipped with Detector: SPD –10 AVP Winchrome software, Prochrome (India) C-18, 5µm column having dimensions 250 x 4.6 mm was used.

Reagents

HPLC grade acetonitrile, water and glacial acetic acid used were purchased from S.D. Fine Chemicals (India).

Chromatographic condition

Prochrome (India), C-18 column was used at ambient temprature.The mobile Phase consisted of water: acetonitrile: glacial acetic acid (49:50:1) was pumped at a flow rate of 1.2 mL/min.The elution was monitored at254 nm and the injection volume was 20 µL.

Marketed Formulation

Each Sachet containing 30 mg of racecadotril was procured from local market (Redotil-30, Dr.Reddys Laboratory).

Preparation of Mobile Phase

Mobile phase was prepared by mixing water, acetonitrile and glacial acetic Acid (49:50:1) v/v/v. The mobile phase was filtered through 0.45 micron membrane filter (Ultipore N –66R Nylon 6) and degassed by ultrasonication (Mansi ultrasonicator) for 15 min.

Preparation of Standard Solution

Working standard solutions were prepared by dissolving 25 mg of Racecadotril in 50 ml of volumetric flasks and made up the volume with mobile phase.

Preparation of Sample solution

The powder equivalent to 25 mg of Racecadotril of Racecadotril commercial sample **REDOTIL-30** was dissolved in 50 ml of methanol, ultratonicated for 15 minutes and filtered through 0.45 micron membrane with mobile phase and final volume was made up with mobile phase to obtain the concentration of 500 µg

***Analysis of a Marketed Formulation*:**

Assay of Racecadotril commercial sample (oral powder of racecadotril-**REDOTIL-30**) was performed. The powder equivalent to 25 mg of Racecadotril was dissolved in 50 ml of methanol, ultratonicated for 15 minutes and filtered through 0.45 micron membrane with mobile phase and final volume was made up with mobile phase to obtain the concentration of 500 µg/ml

Validation of the Method

Accuracy (recovery)

Accuracy of the method was studied by recovery experiments using standard addition method at three different levels (80%, 100% and 120%). The known amounts of standard solutions containing racecadotril(400, 500 and 600 µg) were added to prequantified sample solutions to reach the 80,100 and 120 % levels. These samples were analyzed by injecting the sample solution and recovery was calculated.

Calibration curve (linearity of the HPLC method)

Calibration curve was constructed by plotting peak area vs concentrations of racecadotril, and the regression equations were calculated Linearity of the method was investigated by using the concentrations 250, 375, 500, 625 and 750 µg/mL. The retention time was 15.49 min.

Precision (repeatability)

Precision of the assay method was demonstrated by injecting six different sample solutions containing Racecadotril equivalent to 500µg/mL and RSD of mean assay value was calculated.

Intermediate precision (ruggedness)

Intermediate Precision of the method was demonstrated by carrying out the experiment by different analyst and on different day.

LOD and LOQ

LOD and LOQ of racecadotril was calculated using the following equations as per International Conference on Harmonization (ICH) guidelines.

$LOD = 3.3 \times \sigma/S$ $LOQ = 10 \times \sigma/S$

Where, σ = Standard deviation of response, S = Slope of regression equation.

Specificity

Specificity of the method was studied by injecting blank, standard and sample solutions.

Robustness

Robustness of the method was demonstrated by variation in composition of mobile phase i.e. from water acetonitrile: glacial acetic acid (49:50:1) to water: acetonitrile: glacial acetic acid (52.5:50:1)

System suitability study

For this study first upon a 20 µL of blank solution (mobile phase) was injected and ran for 12 minutes. After this 20 µL of standard solutions in 6 replicates were injected and the % relative standard deviation (% RSD) of the response peak areas was calculated.

Forced Degradation Study

For this study standard Racecadotril drug was forced to degrade under various conditions like acid, alkali, oxidation, uv light, thermal and high humidity to study the percentage degradation and interference of degraded products in analytical method.

Effect of acid was studied by weighing accurately 25 mg of Racecadotril in 50ml volumetric flasks.1 ml of 1N HCL was added and allowed to rest for 48 hrs. Excess of acid was then neutralized with 1 ml of 1NNaOH and volume was made to 50mL with mobile phase to get 500µg/ml of Racecadotril solution.

Effect of base was studied by weighing accurately 25 mg of Racecadotril in 50 /ml volumetric flasks.1 ml of 1NNaOH was added and allowed to rest for 48 hrs. Then neutralized with 1 ml of 1N HCL and volume was made to 50mL with mobile phase to get 500µg/Ml of Racecadotril solution.

Effect of oxidation was studied by weighing accurately 25 mg of Racecadotril in 50 ml volumetric flasks.1 ml of 6%H_2O_2 was added and allowed to rest for 48 hrs. Then volume was made to 50mL with mobile phase to get 500µg/ml of Racecadotril solution.

Effect of UV light was studied by weighing accurately 25 mg of Racecadotril in a petri dish and exposing to short and long U.V.light for 48 hrs. and then dissolving the exposed drug in mobile phase and diluting it to 50 ml with mobile phase to get 500µg/ml of Racecadotril solution.

Effect of heat was studied by weighing accurately 25 mg of Racecadotril in a 100 ml beaker and keeping it in oven having constant 105°C temperature dissolving the heated drug in mobile phase and diluting it to 50 ml with mobile phase to get 500µg/ml of Racecadotril solution.

5.1.7.2 Results and Discussion

Optimization of the method

For the optimation of the method several mobile phase compositions were tried. A satisfactory isocratic separation and good peak symmetry was obtained by using the mobile phase containing water:acetonitrile: glacial acetic acid (49:50:1) with reverse phase chromatography. Quantification was achieved with UV detection at 254 nm based on peak area

Table 5.1.7.1 Otimization of the method.

Mobile phase	Flow rate (ml/min)	Racecadotril	
		RT (min)	Peak Shape
Acetonitrile:D/W:G.A.A::(80:20:05) final pH3.7	1.2	11.62	Tailing
Acetonitrile:D/W:G.A.A::(80:20:05) final pH3.6	1.2	20.18	Tailing
Acetonitrile:D/W:G.A.A::(49:50:1) final pH 3.6	1.2	15.75	Sharp

A representative chromatogram is shown in **Fig.5.1.7.1**

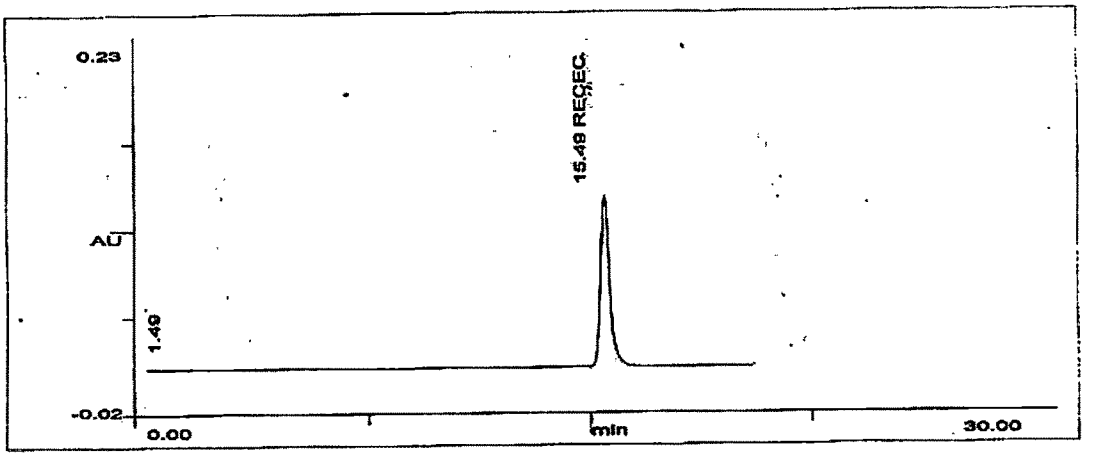


Fig:5.1.7.1 Representative chromatogram of racecadotril.

Accuracy

Recovery studies that mean recovery of added Racecadotril to prequqntified sample lies within the limit of 98-102%. This indicates that method is accurate.

Table 5.1.7.2 Accuracy of racecadotril HPLC method

Recovery level	% of added drug recovered	Mean Recovery%
80%	99.75	98.27
100%	96.04	
120%	99.24	

Linearity regression data is summarized in **Table 5.1.7.3** which shows a good linear relationship between concentration and peak areas over a concentration range of 250 µg – 750µg/ml for . racecadotril **Fig.5.1.7.2** .The correlation coefficient (R^2) was found to be 0.9998.The limit of detection was found to be 5.6µg/mL. The limit of quantification was found to be17.50 µg/mL for racecadotril . These values indicate that the method is sensitive.

Table 5.1.7.3 Regression analysis of the calibration curves for Racecadotril

Parameter	Racecadotril
Linearity range (µg/mL)	250-750
Regression equation	Y=28006x-29444
Correlation coefficient (R^2)	0.9999
Slope	28006
X-intercept	1.051
Y-intercept	-29444

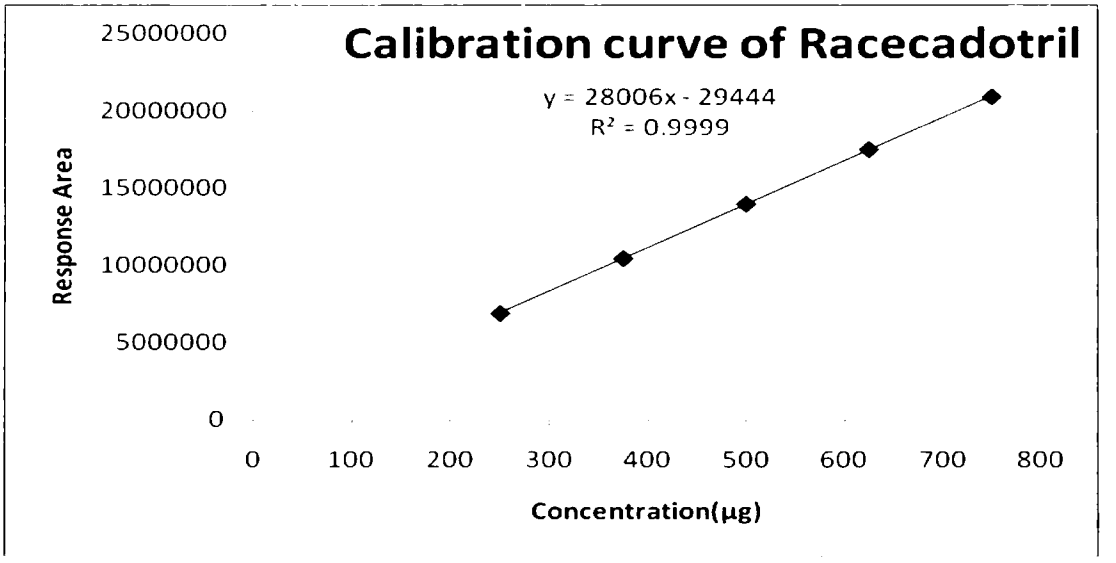


Fig:5.1.7.2 Calibration curve of Racecadotril (250-750µg/ml)

In the **precision** studies, RSD of mean assay values was found to be 1.67 which is well below 2 and indicates that the repeatability of this method is satisfactory.

Table 5.1.7.4 Precision of the Racecadotril HPLC method

Drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean ±%RSD
Racecadotril	96.59	97.17	98.58	100.43	100.70	100.52	**99.00±1.81**

Intermediate precision (Ruggedness) study reveals that the method is rugged with RSD values of 0.23.Accuracy studies indicate that the mean percent recovery of the added standard drug to be 98.27%.

Table 5.1.7.5 Intermediate Precision of the Racecadotril HPLC method

Drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean ±%RSD
Racecadotril	100.05	99.79	99.62	100.17	99.91	99.62	**99.86±3**

Specificity studies indicated that there is no interference from exipients, impurities and degradation products and assured that the peak response was due to racecadotril only.

Robustness study signified that the results of the method remained unaffected by small, deliberate changes in the mobile phase composition.

Table 5.1.7.6 Robustness study of the Racecadotril HPLC method

Drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sampl e 6	Mean ±%RSD
Racecadotril	100.05	99.79	99.62	100.17	99.91	99.62	**99.89±0.28**

Table 5.1.7.7 Summary of validation parameters for the proposed HPLC method for Racecadotril

Parameter	Racecadotril
LOD (µg/ml)	5.60
LOQ (µg/ml)	17.50
Accuracy (% recovery)	98.27
Precision	98.46(±0.67)
Ruggedness	99.86(±0.23)
Robustness (water : acetonitrile: glacial acetic acid 52.5:50:1v/v/v)	99.89(±0.28)

System suitability tests were carried out on freshly prepared standard solutions (n = 6) containing racecadotril. System suitability parameters obtained with 20 µL injection volume are summarized in **Table 5.1.7.8**

Table 5.1.7.8 System suitability test parameters for racecadotril

Parameter	**Racecadotril**
Retention Time(min)	15.42± 0.050
Theoretical plates	13774
Tailing factor	1.2

Forced Degradation study

Racecadotril was found to be susceptible mainly to the basic degradation.(Fig).Reults of forced degradation studies are summarized in the Table:

Table 5.1.7.9 Summary of Forced Degradation study

Agent	**Exposure Time (Hrs.)**	**Assay %**	**Degradation %**
0.1N HCl	48	93.97	06.03
0.1N NaOH	48	85.66	14.36
6% H_2O_2	04	99.00	01.00
(Room Temp).	48	98.75	01.25
6%H_2O_2	04	98.77	01.23
(37°C)	48	98.60	01.40
Heat (105 ^{0}C)	48	98.32	01.68
UV Light	48	98.56	01.44

The degradation data suggested racecadotril does not undergo significant degradation under specified conditions

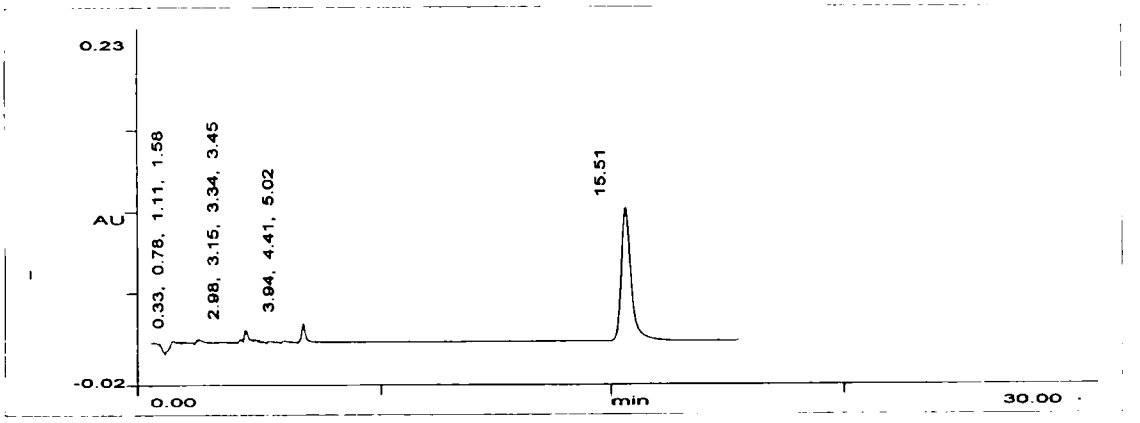


Fig: 5.1.7.3 Degradation of Racecadotril in alkali (0.1 N Na OH)

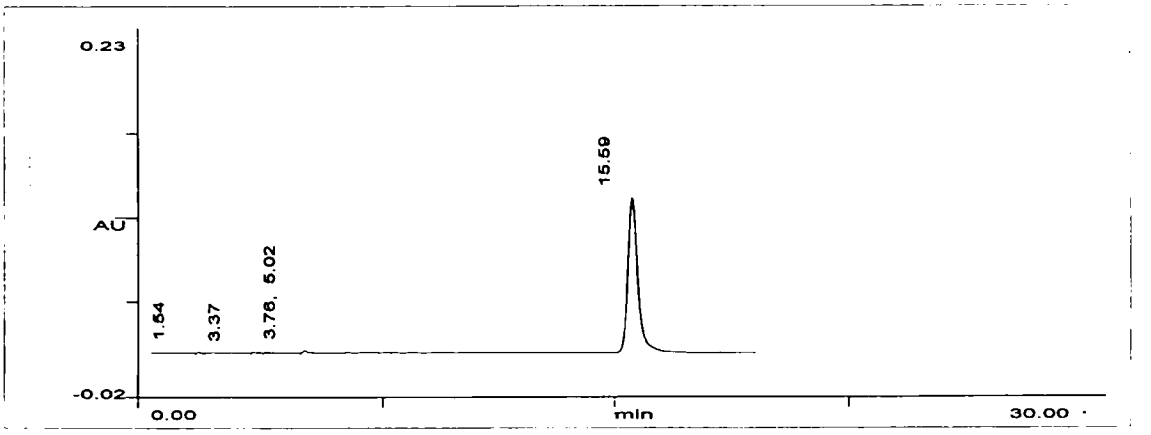


Fig:5.1.7.4 Thermal Degradation of Racecadotril

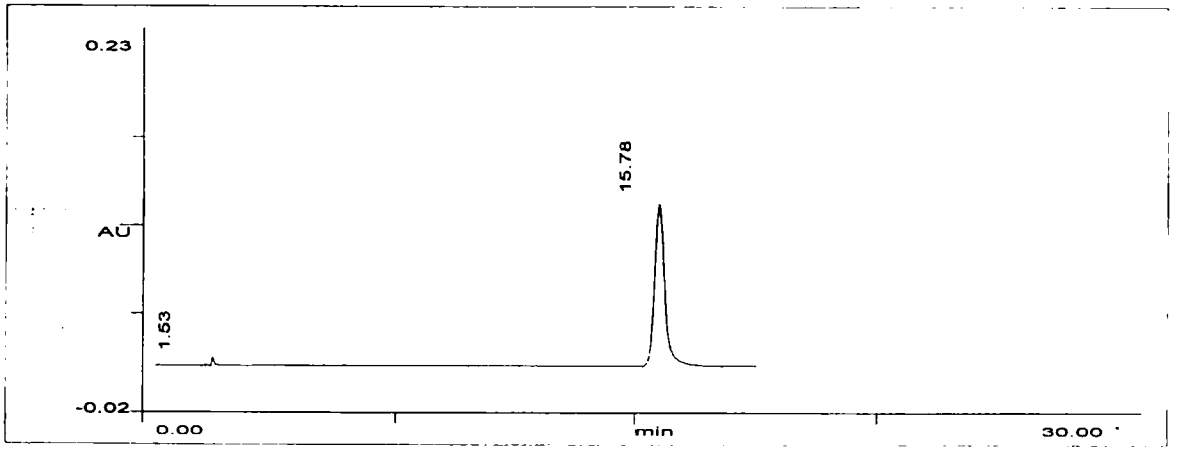


Fig:5.1.7.5 Degradation of racecadotril with hydrogen peroxide

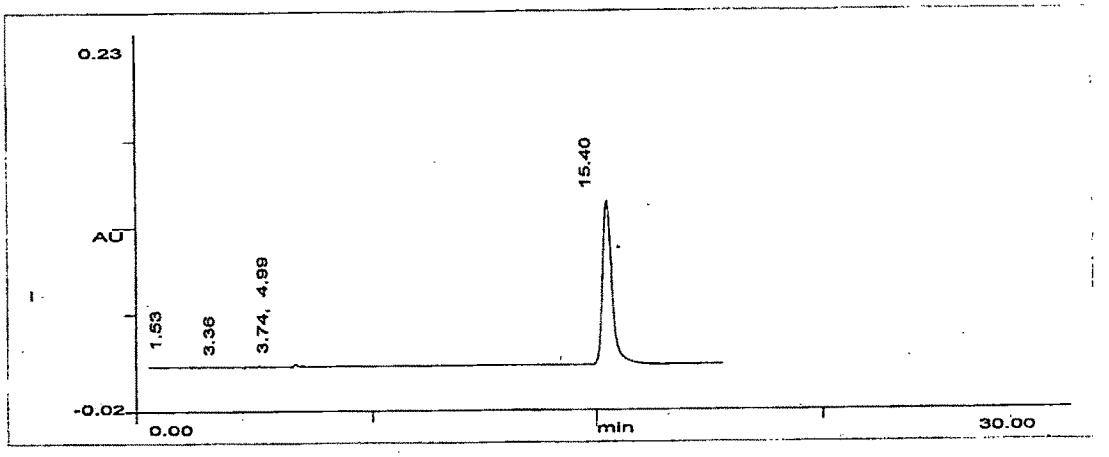


Fig:5.1.7.6 Degradation of racecadotril in UV light

5.2 HPTLC METHODS

5.2.1 Simultaneous HPTLC Determination of Albendazole and Ivermectin in bulk and in tablet dosage Form

5.2.1.1 Experimental

***Reagents*:** Analytical grade Toluene, Dietyl ether, Alcohol, Formic Acid were purchased from S.D. Fine Chemicals (Mumbai, India).

Chromatographic condition: Mobile phase composition Toluene:Diethyl ether:Ethanol:Formic acid(03:05:0.2:0.5v/v).

Marketed Formulation:

Bandy Plus(Mankind) tablets containing 400mg albendazole and 12 mg ivermectin were procured from local market.

Preparation of Standard Stock Solution:

Standard stock solution of albendazole and ivermectin were prepared by dissolving 10 mg of albendazole and 13 mg of ivermectin in 25mL of methanol:chloroform(70:30) solvent mixture separately. Working standard solution of albendazole was prepared by transferring 1mL albendazole stock solution to 10 ml volumetric flask and making the volume to get 40 ng/µL in Chloroform:Methanol(70:30) solvent mixture. Working standard solution of ivermectin was prepared by transferring 5 mL ivermectin stock solution to 10 ml volumetric flask and making the volume to get 130 ng/µL in Chloroform:Methanol(70:30) solvent mixture.

Preparation of Sample Solution:

To prepare the sample solution 20 tablets (Bandy plus) were weighed, their mean weight was determined and then were powdered.The sample solution for the determination of albendazole was prepared by transferring the weight of tablet triturate containging albendazole equivalent to 10.50 mg to a 50 ml conical flask and extracted with the 20 mL solvent mixture chloroform:methanol(70:30),by sonication for 15 minutes with intermittent shaking of the flask content.The contents of the flask then filtered through 0.45 μ syringe filter.The conical fask was again rinsed with 3 ml of with chloroform:methanol (70:30) solvent mixture and filtered through 0.45μ syringe filter,and added to volumetrif flask and the final volume was made to 25Ml to get 420ng/ml of albendazole,1 Ml of this solution was further further diluted in 10 Ml volumetric flask to get a solution containg 40ng/μL in chloroform:methanol mixture(70:30). The sample solution for the determination of ivermectin was prepared by transferring the weight of tablet triturate containging ivermectin equivalent to 3.04 mg to a 50 ml conical flask and extracted with the 20 mL solvent mixture of chloroform:methanol (70:30),by sonication for 15 minutes with intermittent shaking of the flask content.The contents of the flask then filtered through 0.45 μ syringe filter.The conical fask was again rinsed with 3 ml of with chloroform:methanol (70:30) solvent mixture and filtered through 0.45μ syringe filter,and added to volumetric flask and the final volume was made to 25Ml to get 121ng/μl of ivermectin in chloroform:methanol solvent mixture(70:30).

Analysis of a Marketed Formulation:

Assay of marketed tablet containing 400 mg albendazole and 12 mg ivermectin was performed by preparing the sample solutions as described in the previous section. Six of the prepared sample and standard solutions were injected.The assay of the commercial sample was calculated by calibration curve equation.

Validation of the Method

Accuracy (recovery).—Accuracy of the method was studied by recovery experiments using the standard addition method at three different levels (80, 100, and 120%). Known amounts of standard solutions containing albendazole (120,160 and 200 ng) and ivermectin(242, 363, and 565 ng) were spiked on prequantified sample solutions to reach the 80, 100, and 120% levels. These samples were analyzed and recovery was calculated.

Calibration curve (linearity of the HPTLC method).—

The calibration curve was constructed by plotting concentrations of albendazole and ivermectin versus peak areas, and the regression equations were calculated. The linearity of the method was investigated by applying bands of working standard solution to get the 80,160,240,320,400 and 480ng/spot for albendazole and and 260,520,780,1040,1300 anf 1560ng/Spot of ivermectin.

Precision (repeatability).—Precision of the application and measurement was demonstrated by Repeatability studies.Repetability of application was studied by applying 3 µl of standard albendazole soluition and 3 µl of ivrmectin standard solution six times on the same day and RSD of the mean area value was calculated.Repeatability of measurement was studied by scanning the same spot six times for albendazole and ivermectin

LOD and LOQ.—LOD and LOQ of ivermectinand albendazole were calculated using the following equations according to ICH guidelines:

$LOD = 3.3 \times \sigma/S$ $LOQ = 10 \times \sigma/S$

Where, σ = Standard deviation of response,S = Slope of regression equation.

5.2.1.2 Results and Discussion

Optimization of the method

Optimization of the mobile phase was carried out with the intention of obtaining satisfactory resolution of the albendazole and ivermectin without any interference by excipients of tablet.Also sample preparation was important limitation for this combination because,albendazole is mainly soluble in mineral acid and degradation of ivermectin was taking place when preparation was tried with HCl. Therfore a co solvent was used for dissolving these drugs simultaneously. Several mobile phase compositions were tried. A satisfactory separation and good Rf values were obtained by using the mobile phase composition Toluene :Diethyl ether:Ethanol:Formic acid(3:5:0.2:0.0.5v/v) were obtained .Various mobile phase combinations tried are as mentioned below.Quantification was achieved with UV detection at 254 nm based on peak area. A representative chromatogram is shown in **Fig.5.2.1.1**

Table 5.2.1.1 Optimization of the method

Mobile phase	Composition v/v
Chloroform:Diethyl ethyl ether:Glacial acetic acid	6:1:1
Toluene:Chloroform:Ethanol	4:4:1
Toluene:Chloroform:Ethanol:Formic acid	4:4:0.5:1 drop
Toluene:Chloroform:Ethanol:Formic acid	4:4:0.5:5 drops
Toluene:Diethyl ether:Ethanol:Formic acid	3:5:1:5 drops
Toluene:Diethyl ether:Ethanol:Formic acid	4:5:0.5:5 drops
Toluene:Diethyl ether:Ethanol:Formic acid	3:5:0.2:0.5

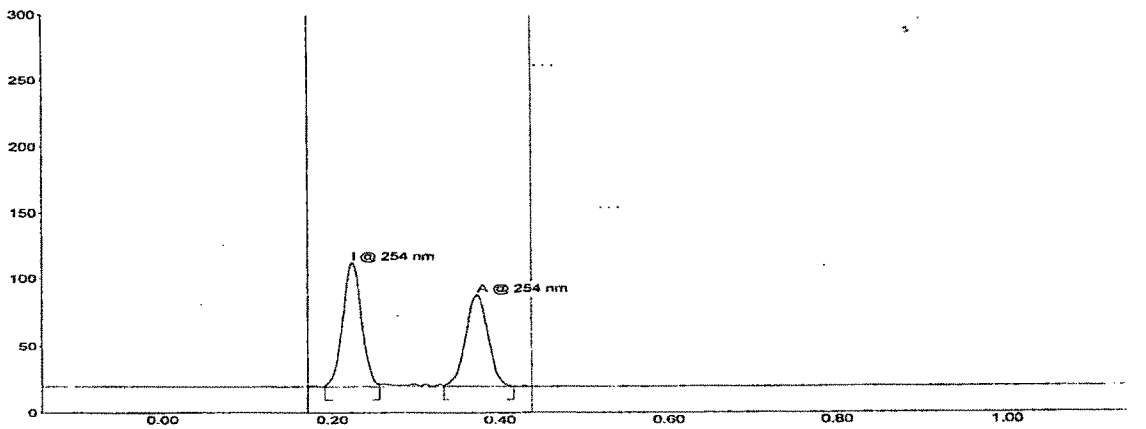


Fig.5.2.1.1 HPTLC Chromatogram of Ivermectin and Albendazole at 254 nm (Albendazole 160 ng/µl and ivermectin 260ng/µl)

Accuracy studies indicated that the mean recovery of the added standard drug was 98.84% for ivermectin and 99.16 fo ralbendazole, respectively.

Linearity regression data are summarized in **Table 5.2.1.2**,which shows a good linear relationship between concentration and peak areas over a concentration range of 80-480 ng for albendazole **(Fig.5.2.1.2)** and 260-1560ng for ivermectin**(Fig.5.2.1.3)**. The correlation coefficient (R2) was found to be 0.9988 for albendazole and 0.9983for ivermectin

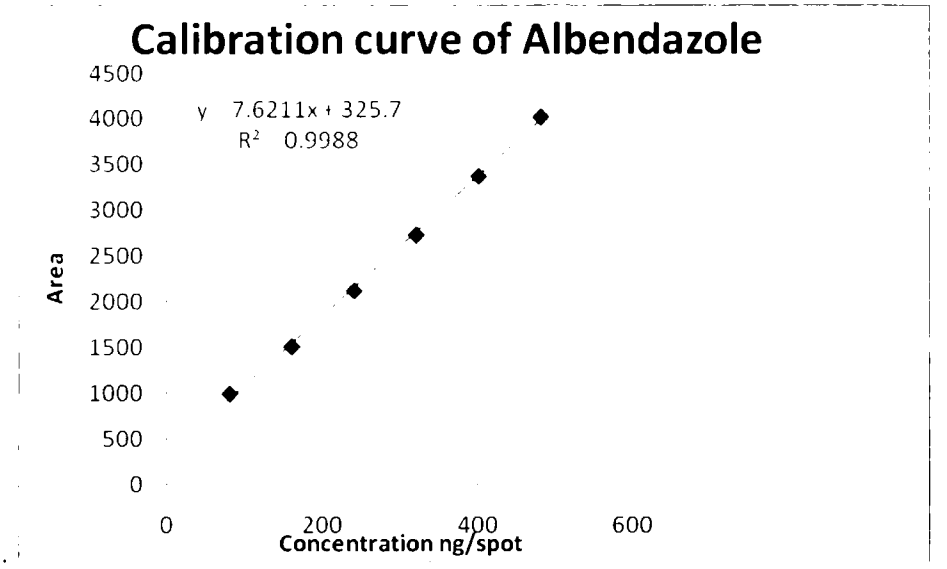


Fig.5.2.1.2 Calibration Curve for Albendazole(80-480 ng/µl)

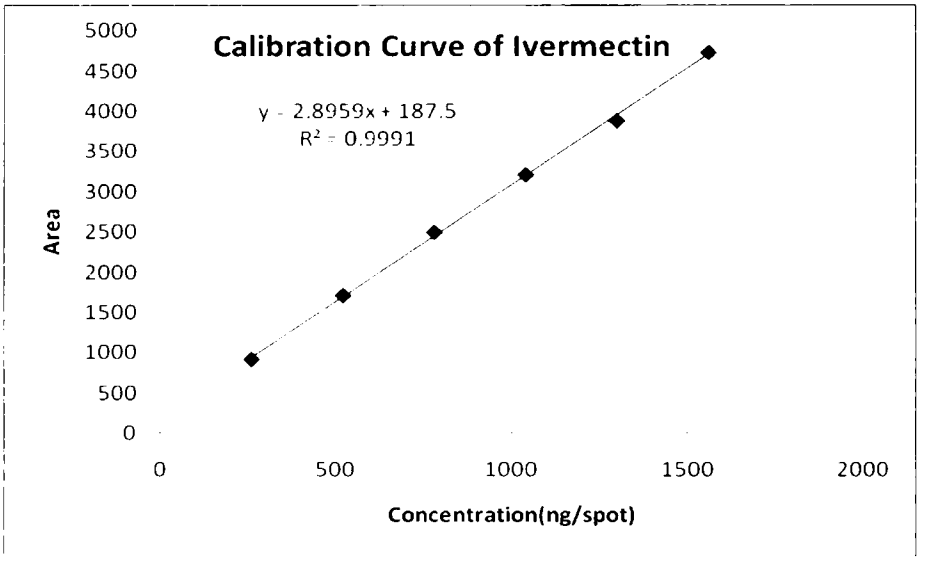


Fig.5.2.1.3 Calibration Curve for Ivermectin.(260-1560 ng/µl)

Table 5.2.1.2 Regression analysis of the calibration curves for ivermectin and albendazole.

Parameter	Albendazole	Ivermectin
Linearity range(ng)	80-480	260-1560
Regression equation	Y=7.6211X+325.7	Y=2.8959X+187.5
Correlation coefficient (r^2)	0.9988	0.9991
Slope	7.621	2.8959
X-intercept	-42.73	-64.746
Y-intercept	325.7	187.5

In the ***precision*** studies, RSD of mean areas values for application was found to be 1.58 for ivermectin and 1.42 for albendazole respectively. These RSD values which are well below 2%, indicate that the repeatability of this method is satisfactory. In the precision study of RSD value was found to be 1.34 for ivermectin and 0.904 for albendazole. As evident the RSD values of the data obtained are well below 2% indicating that method is precise and rugged.

LOD and LOQ

The LOD was found to be 19.43ng/µL for albendazole and 68.99ng/µL for ivermectin. The LOQ was found to be 58.90ng/µL for albendazole and 209.08ng/µL for ivermectin. These values indicate that the method is sensitive.

Specificity studies indicated no interference from excipients, impurities, or degradation products, and ensured that the peak response was due to ivermectin and albendazole only.Rf value for albendazole and ivermectin was found to be 0.38±1.06 and 0.23±1.76

All validation data are summarized in **Table 5.2.1.3**.

Analysis of Marketed formulation

The assay results obtained by using the proposed method for the analysis of a marketed tablet formulation containing albendazole 400 mg and ivermectin 12 mg were in good agreement with the labeled amounts of ivermectin andalbendazole . The average contents of albendazole and ivermectin were 400.60 mg/tablet (100.15%) and 12.31mg(102.60%,),respectively.

Table 5.2.1.3 Summary of validation parameters for the proposed HTPLC method for ivermectinand albendazole

Parameter	Albendazole(±RSD)	Ivermectin(±RSD)
LOD (µg/ml)	19.43872	54.53888
LOQ (µg/ml)	58.90521	165.2693
Accuracy(% recovery)	99.16	98.84
Precision (Application)[a]	1.42	1.58
Precision (Measurement)[a]	0.904	1.34

[a] mean asay values of 6 determinations

5.2.2 Simultaneous Reverse Phase HPTLC Determination of Betamethasone dipropionate and Butenafine hydrochloride in a cream formulation

5.2.2.1Experimental

HPTLC Instrumentation

(a) ***Sample Application*** Camag Linomat V (Switzerland) sample applicator was used to apply samples in the form of bands with the help of Camag, Hamilton , 100 microlitre syringe on HPTLC plates i.e precoated silica gel RP-18F 254 S , [20 cm × 10 cm with 250 µm thickness; E.Merck, Germany)] .

(b)Development of Plates:After sample application plates were develoed in a Camag (Switzerland)twin trough glass chamber saturated with the mobile phase for 15 min..Development of plates was carried out in Linear ascending manner in 20 cm × 10 cm itwin trough glass chamber (Camag,, Switzerland). Sample application position was kept at 10 mm and solven front t position was at 8 cm thus the plates were developed over a position of 8 mm.Developed plates were dried in a stream of air with the help of an air dryer.

(a)Scanning of plates:Developed plates were scanned d densitometrically with the help of Camag TLC scanner 3 at 254 nm operated by WinCATS software. The source of radiation used was deuterium lamp .Slit dimention was Concentrations of the compound chromatographed were determined from the intensity of the diffused light. Evaluation was performed by linear regression of peak areas determined by UV absorption as a function of sample amounts

***Chemicals and Reagents*:.**

Analytical grade Methanol,ammonia were purchased from S.D. Fine Chemicals (Mumbai, India).

Chromatographic condition:

Mobile phase composition-Methanol:ammonia (9:1 v/v).Total of 20 ml mobile phase was prepared.

***Marketed Formulation*:**

Each 15 g cream tube (Butenaskin-BM of Elder Health Care Ltd, Mumbai, India) containing buteafine hydrochloride (1%, w/w) and betamethasone dipropionate USP equivalent to betamethasone (0.05%, w/w) was procured from the local market

Preparation of Standard Stock Solution:

Standard stock solution of Betamethasone dipropionate was prepared by dissolving 10 mg of Betamethasone dipropionate and 200 mg of butenafine hydrochloride in 100 ml of methanol. To get 100ng/µl of betamethasone dipropionate and 2000ng/µl of butenafine hydrochloride.

Preparation of Sample Solution: In a 50 mL screw-cap centrifuge tube, 10g cream sample was weighed and extracted with three portions of methanol. For each extraction, 25 mL methanol was added to the centrifuge tube and heated in a water bath (60°C) with shaking to disperse the cream, vortexed for 1 min, and centrifuged for 10 min at 3000 rpm. Supernatants were collected in a 100 mL volumetric flask, and the final volume was diluted to 100 mL with methanol.

Analysis of a Marketed Formulation:

Assay of marketed cream formulation (Butenaskin-BM of Elder Health Care Ltd) containing butenafine hydrochloride (1%, w/w) and betamethasone dipropionate USP equivalent to betamethasone (0.05%, w/w) was performed by preparing the sample solutions as described in the previous section

Validation of the Method

Calibration curve (linearity of the HPTLC method)

The calibration curve was constructed by plotting concentrations of Betamethasone dipropionate and butenafine hydrochloride versus peak areas, and the regression equations were calculated. The linearity of the method was investigated by applying bands of working standard solution to

get the100,200,300, 400, 500,and 600ng/spot for Betamethasone dipropionate and and 200,4000,3000,4000,5000 and 6000ng/Spot of butenafine hydrochloride.

Accuracy (recovery)

Accuracy of the method was studied by recovery experiments using the standard addition method at three different levels (80, 100, and 120%). Known amounts of standard solutions containing Betamethasone dipropionate (160,200 and 240 ng) and butenafine hydrochloride(3200, 4000, and 4800 ng) were spiked on prequantified sample solutions to reach the 80, 100, and 120% levels. These samples were analyzed and recovery was calculated.

Precision (repeatability)

Precision of the application and measurement was demonstrated by Repeatability studies.Repetability of application was studied by applying 4µl of of sample solution six times on the same day and RSD of the mean assay value was calculated.Repeatability of measurement was studied by scanning the same spot six times for Betamethasone dipropionate and butenafine hydrochloride ans % %RSD was calculated.

(LOD and LOQ.

LOD and LOQ of butenafine hydrochlorideand Betamethasone dipropionatewere calculated using the following equations according to ICH guidelines:

$LOD = 3.3 \times \sigma/S$ $LOQ = 10 \times \sigma/S$

Where, σ = Standard deviation of response,S = Slope of regression equation.

5.2.2.2 Results and Discussion

Optimization of the mobile composition was carried to get proper resolution of the drugs and also to getthe Rf values for both drugs in between 0.3-0.7 which is considered as a good chromatography.Various solvent systems were tried on normal phase silica gel HPTLC plates but after lot of trials it was found that this combination can be seprated on reverse phase HPTLC plates..With most of the solvent systems, either tailing of the drug spots or proper resolution of the drug peaks was not taking place.

Table 5.2.2.1 Optimizationof the mobile phase

Mobile phase	Composition v/v	Phase
Tolune:Ethyl acetate	6:4	Normal
Tolune:Ethyl acetate:dichloromethane	5:2.5:2.5	Normal
Tolune:Ethyl acetate:dichloromethane	5:3:2	Normal
Tolune:Ethyl acetate:dichloromethane:diehyl ether	5:1:3:1	Normal
Tolune:dichloromethane:diehyl ether	5:4:1	Normal
Hexane: Ethyl acetate:dichloromethane:diehyl ether	5:0.5:0.5:4	Normal
Hexane: dichloromethane:diehyl ether	1.2:0.6:0.2	Normal
Diethylether:Dichloromethane:Ethyl acetate:Hexane	1:1:8:10	Normal
Methanol:Ehyl acetate	9:1	Normal
Methanol:Ammonia	5:5	Reverse
Methanol: Ammonium	7.3	Reverse
Methanol: Ammonium	9:1	Reverse

Several mobile phase compositions were tried to get proper resolution of the drug peaks. A satisfactory separation and good Rf values were obtained by using the mobile phase composition methanol:ammonia(9:1 v/v) .Quantification was achieved with UV detection at 254 nm based on peak area. A representative chromatogram is shown in **Fig.5.2.2.1**

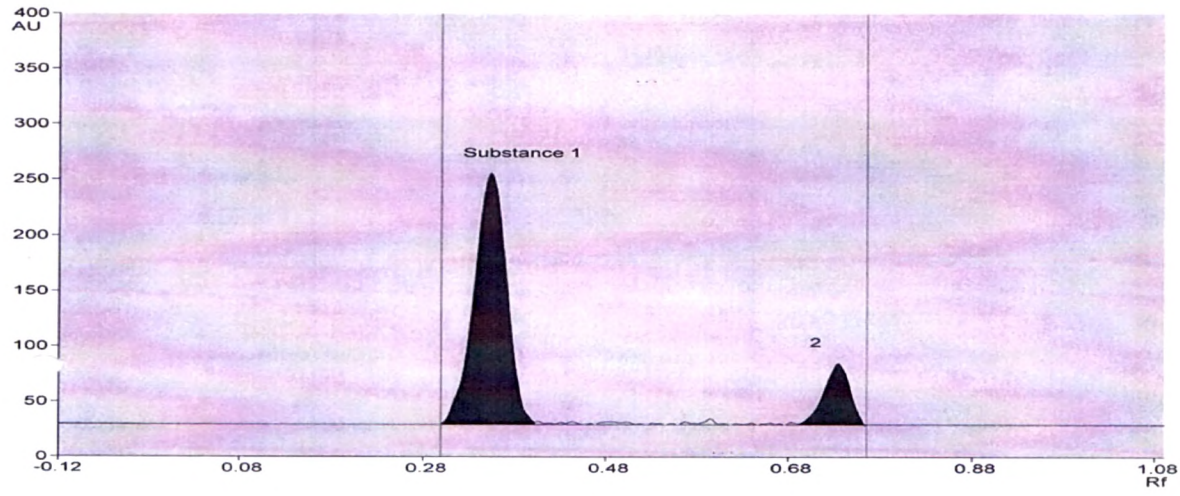


Fig. 5.2.2.1Chromatogram of Butenafine hydrochloride and Betamethasone dipropionateat 254 nm

Specificity studies indicated no interference from excipients, impurities, or degradation products, and ensured that the peak response was due to butenafine hydrochlorideand betamethasone dipropionateonly.Rf value for butenafine hydrochloride and betamethasone dipropionate was found to be 0.26± 1.93 and 0.645± 1.62 respectively.

Linearity regression data are summarized in **Table 5.2.22**,which shows a good linear relationship between concentration and peak areas over a concentration range of 100-600 ng for Betamethasone dipropionate **(Fig. 5.2.2.2)** and 2000-12000ng for butenafine hydrochloride**(Fig.5.2.2.3)**. The correlation coefficient (R2) was found to be 0.9927for Betamethasone dipropionate and 0.9918 for butenafine hydrochloride. The LOD was found to be 46.05457 ng/µL for Betamethasone dipropionate and 1258.746ng/µL for butenafine hydrochloride. The LOQ was found to be and 139.5593 ng/µL for Betamethasone dipropionate and 3814.382ng/µL for butenafine hydrochloride. These values indicate that the method is sensitive.

In the ***precision*** studies, RSD of mean assay values for application was found to be 0.01 for butenafine hydrochloride and 1.90 for Betamethasone dipropionate respectively. These RSD values which are well below 2%, indicate that the repeatability of this method is satisfactory.The rsd value of scanning measurement was found to be *Accuracy* studies indicated that the mean recovery of the added standard drug was 98.73% for butenafine hydrochloride and for 98.2%Betamethasone dipropionate, respectively. All validation data are summarized in **Table 5.2.2.3**

The assay results obtained by using the proposed method for the analysis of a marketed cream formulation containing butenafine hydrochloride (1%, w/w) and betamethasone dipropionate USP equivalent to betamethasone (0.05%, w/w) were in good agreement with the labeled amounts of betamethasone dipropionate and butenafine hydrochloride. The average contents of butenafine hydrochloride and betamethasone dipropionate were 99.30mg/10 g cream (0.99%, w/w) and 4.96 mg/10 g cream (0.0496% w/w), respectively.

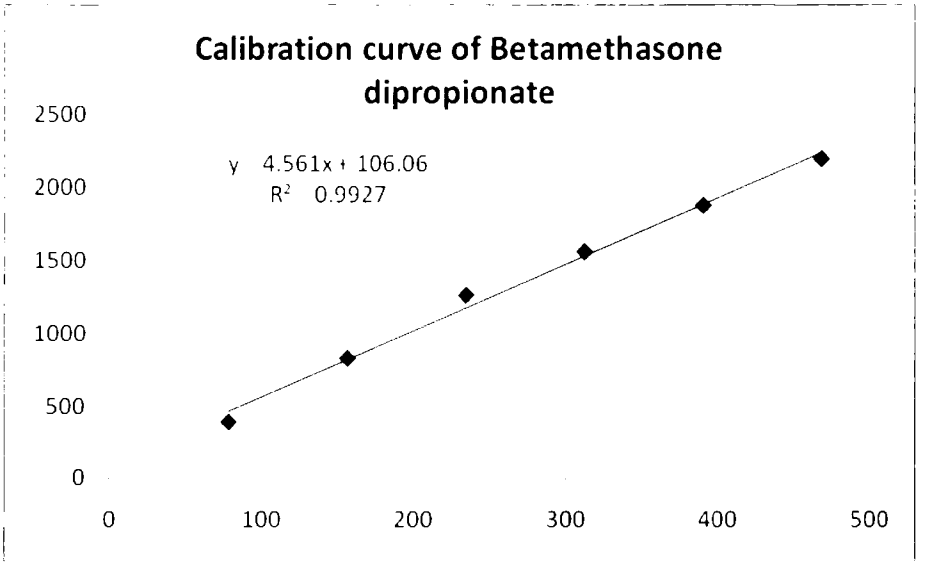


Fig. 5.2.2.2 Calibration Curve for Betamethasone dipropionate (100-600 ng/µL)

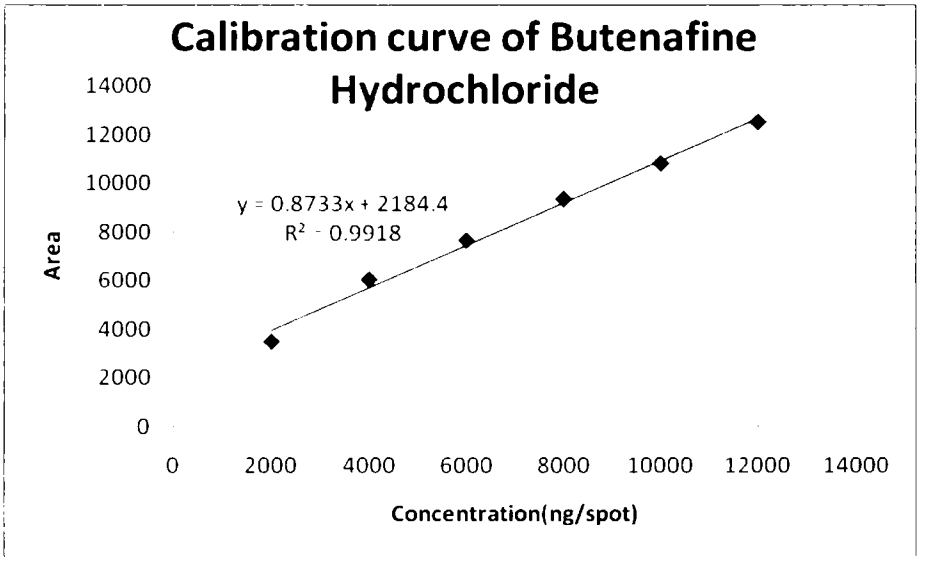


Fig. 5.2.2.3 Calibration Curve for Butenafine hydrochloride(2000-12000ng/µL)

Table 5.2.2.2 Regression analysis of the calibration curves for butenafine hydrochlorideand butenafine hydrochloride.

Parameter	Betamethasone dipropionate	Butenafine hydrochloride
Linearity range(ng)	100-600	2000-12000
Regression equation	Y=4.561X+106.06	Y=0.8733X+2184.4
Correlation coefficient (r^2)	0.9918	0.9927
Slope	4.561	0.8733
X-intercept	-23.254	2503.1
Y-intercept	106.16	2184.4

Table 5.2.2.3 Summary of validation parameters for the proposed HPTLC method for butenafine hydrochloride and Betamethasone dipropionate

Parameter	Betamethasone Dipropionate (±RSD)	Butenafine Hydrochloride (±RSD)
LOD (ng/µl)	46.0545	1258.74
LOQ (ng/ml)	139.5593	3814.38
Accuracy (% recovery)	99.16	98.84
Precision[a]	99.16±1.90	99.27±0.01

[a] mean assay values of 6 determinations

5.3. IR METHOD

5.3.1 Development of IR spectroscopic method for the estimation of Albendazole and Ivermectin in combined tablet dosage form.

It was found that albendazole needs mineral acid or a co solvent like chloroform:methanol(70:30) to prepare a solution. Therefore sample preparation of tablet containing albendazole and ivermectin for simultaneous estimation was difficult, as Ivermectin is susceptible to degradation with mineral acids.Therefore an attempt was made to take the advantage of IR spectroscopy(ATS),which requires minimum sample preparation and determinations can be carried out in the solid state of the drugs directly, to quantitate albendazole and ivermectin in a combined tablet dosage form.

Instrumentation

IR spectra were measured on JASCO FTIR -4100 using ATR 450 accessory.This FTIR comprises of a ceramic source,Michelson interfereter and TGS detector.The crystal used for measurement

Preparation of standard mixtures

Standard albendazole powder and standard ivermectin powder were mixed by trituration in the glsss mortar and pestel the following manner

Standard mixture 1-5 mg of ivermectin + 167 mg albendazole

Standard mixture 2-10 mg of ivermectin + 333.5 mg of albendazole

Standard mixture 3-20 mg of ivermectin + 667 mg albendazole

Standard mixture 4-30 mg of ivermectin +1000 mg of albendazole

Standard mixture 5-40 mg of ivermectin + 1.333 gm of albendazole

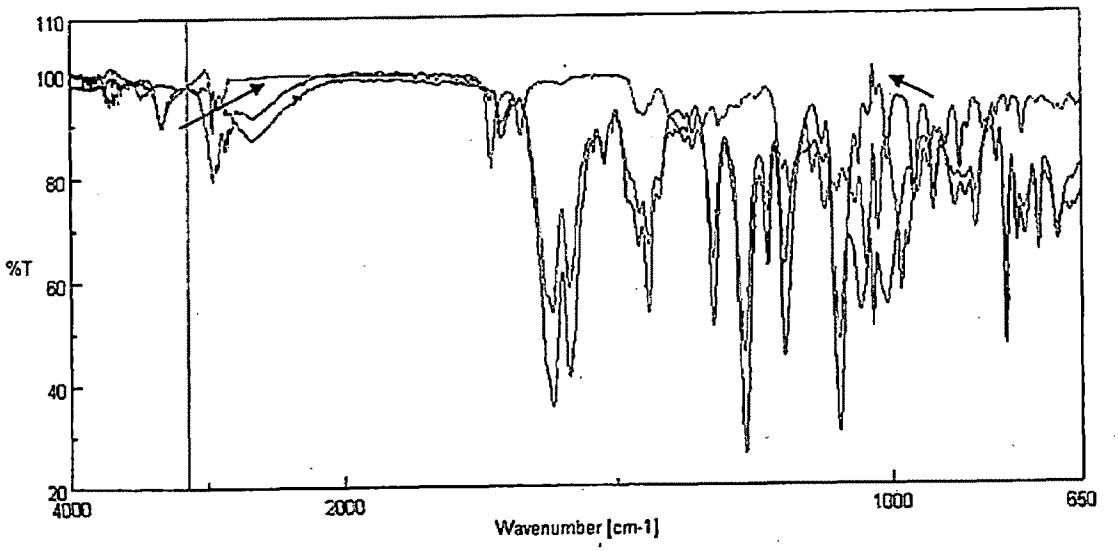
Standard mixture -50 mg of ivermectin + 1.67 gm albendazole

Preparation of marketed sample

Two tablets of Bandy plus tablet were powdered in glass mortar pestel and used for spectra measurement

Above prepared standard mixtures and sample powder were directly put in the sample cell and their IR spectra were recorded

5.3.2 Results and discussion



Blue : Albendazole Std

Green : IVR Std

Red : Formulation 12:400 (IVR:Albendazole)

Fig. 5.3.1 IR spectrum of Albendazole standard, Ivermectin Standard and Tablet Formulation

Numbers of wave numbers showing 100% transmittance for albendazole were tried for quantification of ivermectin. As the proportion of ivermectin to albendazole in tablet is 12:400, the ivermectin peaks were masked by intense albendazole peaks due to which correlation between the area of peak or height of the peak with concentration of albendazole and ivermectin in the tablet could not be established.