Development of Validated Analytical Methods for Some Drugs and their Formulations

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SURYAKANT D BHOSALE

Under the Guidance of

PROF. (MRS.) SADHANA J RAJPUT

Pharmacy Department
Faculty of Technology and Engineering
The Maharaja Sayajirao University of Baroda
Vadodara – 390 001

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Pharmacy Department
Faculty of Technology and Engineering,
The Maharaja Sayajirao University of Baroda,
Post Box No. 51, Kalabhavan, Vadodara – 390 001, INDIA
Phone: (+91-0265)2434187 Fax: (0265)2423898/2418927.
E-mail: head-pharm@msubaroda.ac.in

Date:

CERTIFICATE

This is to certify that the thesis entitled, **"Development of validated Analytical Methods for Some Drugs and their Formulations"** submitted for the Ph.D. degree in Pharmacy by **Mr. Suryakant D Bhosale** (Registration Certificate No. 001681) incorporates original research work carried by him under my supervision and no part of the thesis has been submitted for any other degree.

Prof. (Mrs.) Sadhana J. Rajput
Research Guide

Head
Pharmacy Department

Dean
Faculty of Technology and Engineering,
The M S University of Baroda

DECLARATION

In accordance with the University Ordinance No. FTE/1809 Ph.D., I State that the work presented in this thesis titled "**Development of validated analytical methods for Some Drugs and their Formulations"** comprises of independent investigations carried out by me at the Pharmacy Department, Faculty of Technology & Engineering, The M. S. University of Baroda, Vadodara. Wherever references have been made to the work of others, it has been clearly indicated with the sources of information under the References in the individual chapters. The results of this work have not been previously submitted for any degree or fellowship.

Mr. Suryakant Bhosale

Certified and forwarded through Research Guide

Prof. (Mrs.) Sadhana J. Rajput

Pharmacy Department,
Faculty of Technology & Engineering,
The M. S. University of Baroda,
Kalabhavan, Vadodara – 390 001.

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Dedicated

to

The Almighty

&

My Loving Family

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1. Introduction

1.1 Importance of Analytical Methods:

Quality is important in every product or service, but it is vital in medicine as it involves life. Unlike other consumer goods, there can be and there is no second quality.

Therefore analytical methods which are a measure of quality of the drugs play a very comprehensive role in drug development and follow up activities, to assure that a drug product meets the established standard, is a stable and will continue to meet purported quality throughout its shelflife[1].

These methods should be selective and sensitive to monitor the known and unknown impurities, have to be written in a format such that they can be produced over a period of time and from laboratory to laboratory, i.e. these methods should be validated.

1.2 Introduction to Analytical Methods:

Analytical methods are required to characterize drug substance and drug product composition during all phases of pharmaceutical development[2] .Early phase methods must support changes in synthetic routes and dosage form and elucidate the structures and levels of impurities. In later phases, goals change to the development of rapid and robust methods for release and stability evaluation.

Analysis includes a wide range of simple and instrumental analytical methods, but the most widely most used analytical methods for quality assurance are spectroscopy and chromatography based. Most quantitative analysis require, measuring specified components in the presence of sample matrix and /or related substances, therefore isolation or separation of the components are required preceding quantitative analysis. In such cases chromatographic techniques are used for quantitative analysis. In cases where matrix interference is not observed quantitative measurements are made using spectroscopic or titration methods directly[3].

For the present studies analytical methods based on Reversed Phase High Performance Liquid Chromatography (RP-HPLC), Reserved Phase and Normal Phase High Performance Thin Layer Chromatography HPTLC and Infra Red Spectrophotometry have been developed

1.2.1 Chromatography Techniques:

In chromatographic methods, separation is based on variation in the distribution of different compounds between two dissimilar phases -a stationary phase and a mobile phase. Further differentiation can be made between chromatographic procedures in which the individual

components are monitored on line (HPLC) and procedures in which the components are measured in situ on the chromatographic stationary phase(TLC)[4].

A. High Performance Liquid Chromatography (HPLC):

High-performance liquid chromatography (HPLC) is an advanced form of liquid chromatography used in separating the complex mixture of molecules encountered in chemical and biological systems, in order to understand better the role of individual molecules. In liquid chromatography, a mixture of molecules dissolved in a solution (mobile phase) is separated into its constituent parts by passing through a column of tightly packed solid particles (stationary phase). The separation occurs because each component in the mixture interacts differently with the stationary phase. Molecules that interact strongly with the stationary phase will move slowly through the column, while the molecules that interact less strongly will move rapidly through the column. This differential rate of migration facilitates the separation of the molecules.

HPLC utilizes different types of stationary phases, a pump that moves the mobile phase(s) and analyte through the column, and a detector to signal the characteristic retention time for the analyte.Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase[5].

A block diagram of HPLC System is as given below.

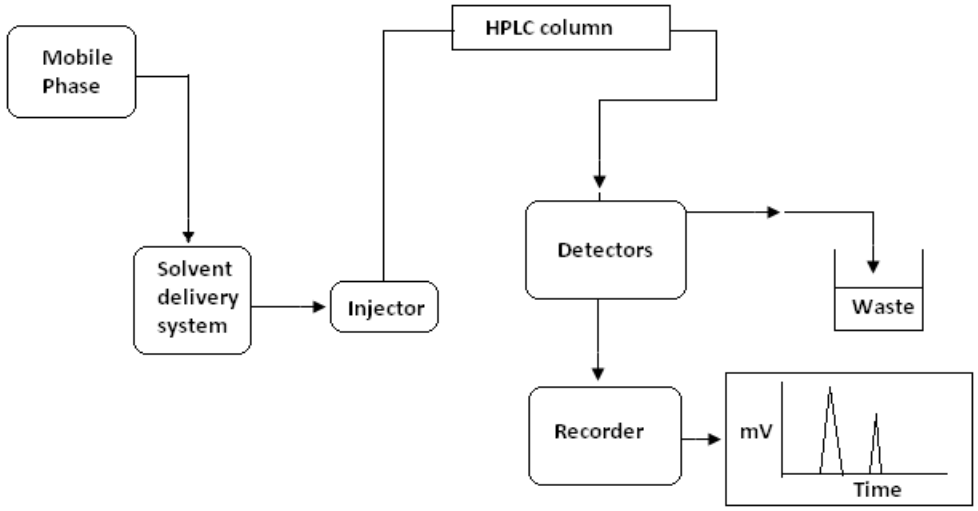


Fig: 1.1 Block diagram of HPLC System

In HPLC, a pump provides the higher pressure required to move the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes.

This allows for a better separation on columns of shorter length when compared to ordinary column chromatography.

The sample to be analyzed is introduced, in small volumes, into the stream of mobile phase. The solution moved through the column is slowed by specific chemical or physical interactions with the stationary phase present within the column. The velocity of the solution depends on the nature of the sample and on the compositions of the stationary (column) phase. The time at which a specific sample elutes (comes out of the end of the column) is called the retention time; the retention time under specified conditions is considered an identifying characteristic of a given sample.

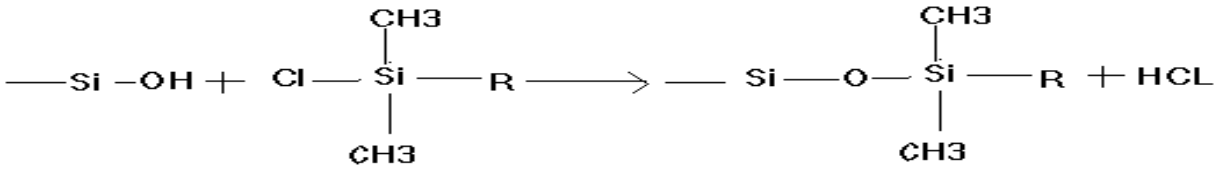
There are two types of stationary phases mainly reverse phase and normal phase. The nature of the stationary phase will determine whether a column can be used for normal phase or reverse phase chromatography.

a. Normal phase HPLC:

Normal phase chromatography utilizes a polar stationary phase and a non-polar mobile phase. Generally, more polar compounds elute later than non-polar compounds

b. Reversed phase HPLC:

About 75% of current HPLC analysis is performed using the reverse phase[6]**.** In reversed phase chromatography the Stationary phase is mainly silica chemically bonded through a siloxane (Si-o-Si-C) linkage to a low polar function group. These phases are prepared by treating the surface silanol groups of silica with an organochorosilane reagent[7]**.**The polarity of the column can be changed by varying the alkyl chain length in R.



Where R= C_6H1_3 (Hexyl), C_8H_{17} (Octyl) or $C_{18}H_{17}$ (Octadecyl).

For our studies mainly we have used C-18 columns

Thus Reversed phase HPLC has a non-polar stationary phase and an aqueous, moderately polar mobile phase. With these stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily.

Retention time can be increased by adding more water to the mobile phase; thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. Similarly, an investigator can decrease retention time by adding more organic solvent to the eluent.

HPLC Detectors[8]

Based on the method or principle used in detection the detectors available are RI detectors, UV detectors, Fluorescent detectors, Electrochemical detectors and Photo diode-array detectors (PDA).For the present study UV and PDA detectors are mainly used.

Refractive index detector is known as Universal detector, but it is not a very sensitive detector

UV Detectors

In these systems detection depends on absorption of UV ray energy by the sample.The equipment comprises of accessories in order as UV source, grating (for light defraction),sample passing through a tubing exposed to rays, photo cell, charge conductor etc.

When the UV rays emitted by lamp pass through gratings, rays split into different wavelengths. One specific wavelength rays are passed through sample. Some amount of light is absorbed by sample and the unabsorbed rays which fall on photo cell.

These rays on collision on photo cell produce electrons whose current is recorded. This is indicative of nature and quantity of sample. This UV wavelength range of absorption is specific for sample . These are the HPLC detectors used in general, unless there is requirement for analysis of special compounds. They are capable to detect very wide range of compounds. The sensitivity ranges till microgram quantity of estimation.

PDA detectors: These are detectors which follow principle similar to UV detectors but the only advantages are higher sensitivity and measure the entire absorption range i.e. it gives scan of entire spectrum.

HPLC method development[9]

The wide variety of equipment, columns, eluent and operational parameters involved makes high performance liquid chromatography (HPLC) method development seem complex. The process is influenced by the nature of the analytes and generally follows the following steps:

- Step 1 – Initial studies
- Step 2 - Selection of initial conditions
- Step 3 - Selectivity optimization

- step 4 - System optimization

Step-1 Initial studies

When developing an HPLC method, the first step is always to consult the literature to know the physico chemical properties of analyte..T he properties like solubility,polarity,molecular weight and partition coefficient are key parameters in selecting the right column and mobile phase for a successful chromatographic separation.

Sample preparation. The sample matrix determines whether the sample requires dissolution, filtration, extraction, preconcentration or clean up. Chemical derivatization may be required to assist detection sensitivity or selectivity.

Types of chromatography. Reverse phase is the choice for the majority of samples, but if acidic or basic analytes are present then reverse phase ion suppression (for weak acids or bases) or reverse phase ion pairing (for strong acids or bases) should be used. The stationary phase should be C_{18} bonded. For low/medium polarity analytes, normal phase HPLC is a potential candidate, particularly if the separation of isomers is required.

Gradient HPLC.

This is only a requirement for complex samples with a large number of components (.20–30) because the maximum number of peaks that can be resolved with a given resolution is much higher than in isocratic HPLC.

Gradient HPLC will also give greater sensitivity, particularly for analytes with longer retention times, because of the more constant peak width (for a given peak area, peak height is inversely proportional to peak width).

Detectors UV detectors ,single channli,multi channel or Photodiode array are the most common detectors used in HPLC .

Fluorescence or electrochemical detectors are used for trace analysis. For preparative HPLC, refractive index is preferred because it can handle high concentrations without overloading the detector.

UV wavelength.he analytical sensitivity is maximum at λ_{max} ,but the wavelengths showing high absorbance can be used as analytical wavelengths. The UV wavelengths below 200 nm are avoided because mobile phase interferences and detector noise increase in this region. Higher wavelengths give greater selectivity.

Step 2 - Selection of chromatographic conditions.

This step determines the optimum conditions to adequately retain all analytes; that is, ensures no analyte has a capacity factor of less than 0.5 (poor retention could result in peak overlapping) and no analyte has a capacity factor greater than 10–15 (excessive retention leads to long analysis time and broad peaks with poor detectability). Selection of the following is then required.

Mobile phase solvent strength. The solvent strength is a measure of its ability to sweep analytes from the column. It is generally controlled by the concentration of the solvent with the highest strength; for example, in reverse phase HPLC with aqueous mobile phases, the strong solvent would be the organic modifier; in normal phase HPLC, it would be the most polar one. The aim is to find the correct concentration of the strong solvent.

Gradient HPLC. With samples containing a large number of analytes (20–30) or with a wide range of analyte retentivities, gradient elution becomes necessary to avoid excessive retention.

Step 3 - Selectivity optimization.

The aim of this step is to achieve adequate selectivity (peak spacing). The mobile phase and stationary phase compositions need to be taken into account. To select these, the nature of the analytes must be considered.

Once the analyte types are identified, the relevant optimization parameters may be selected The optimization of mobile phase parameters is always considered first as this is much easier and convenient than stationary phase optimization.

Step 4 - system parameter optimization.

This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved include column dimensions, column-packing particle size and flow rate. These parameters may be changed without affecting capacity factors or selectivity.

Step 5 - method validation. Proper validation of analytical methods is important for pharmaceutical analysis when ensurance of the continuing efficacy and safety of each batch manufactured relies solely on the determination of quality. The ability to control this quality is dependent upon the ability of the analytical methods, as applied under well-defined conditions and at an established level of sensitivity, to give a reliable demonstration of all deviation from target criteria.

B. High Performance Thin Layer chromatography (HPTLC) [10]:

Thin Layer chromatography utilizes a flat (Planar) stationary phase for separation therefore is also called as Planar Chromatography. In Thin Layer Chromatography (TLC) this stationary phase is supported by a glass plate or foil (plastic or aluminium).Again unlike column separations, the TLC plate constitutes an open system, which passes through the individual steps of the TLC analysis in an off-line mode.

The most advanced form of instrumental TLC is called **High Performance Thin Layer chromatography (HPTLC)**. HPTLC includes sophisticated instruments, controlled by integrated software**.**

It uses HPTLC plates featuring small particles with narrow size distribution. HPTLC plates provide improved resolution, higher detection sensitivity, and improved in situ quantification and are used for pharmaceutical densitometric analysis. Normal phase adsorption TLC on silica gel with a less polar mobile phase, such as chloroform methanol has been used for pharmaceutical analysis.Lipophilic C-18, C-8, C-2; phenyl chemically-modified silica gel plates developed with a more polar aqueous mobile phase, such as methanol-water or dioxane are used for reverse phase.

C. Infra Red Spectroscopy[11]

Infrared (IR) spectroscopy is one of the most common spectroscopic techniques used for qualitative analysis by organic and inorganic chemist. Simply, it is the absorption measurement of different IR frequencies by a sample positioned in the path of an IR beam. The main goal of IR spectroscopic analysis is to determine the chemical functional groups in the sample. Different functional groups absorb characteristic frequencies of IR radiation. Using various sampling accessories, IR spectrometers can accept a wide range of sample types such as gases, liquids, and solids. Thus, IR spectroscopy is an important and popular tool for structural elucidation and compound identification.

Quantitative[12,] IR spectroscopy was generally considered to be able to provide only qualitative and semi quantitative analyses of common samples, especially when the data were acquired using the conventional dispersive instruments. However, the developments of reliable FTIR instrumentation and strong computerized data-processing capabilities have greatly improved the

performance of quantitative IR work. Thus, modern infrared spectroscopy has gained acceptance as a reliable tool for quantitative analysis.

The basis for quantitative analysis of absorption spectrometry is the Bouguer–Beer–Lambert law, commonly called Beer's law. For a single compound in a homogeneous medium, the absorbance at any frequency is expressed as

$A = abc$

Where, A is the measured sample absorbance at the given frequency, a is the molecular absorptivity at the frequency, b is the path length of source beam in the sample, and c is the concentration of the sample.

In the present work,it has been tried to quantitate albendazole and ivermectin in the combined dosage form(Tablet) by IR spectroscopy,because solubility of albendazole is the major problem for its analysis in the solution form. However taking advantage of DRS or ATS ,IR technology the IR spectra of samples can be directly taken with less sample preparation and without using mineral acids for solublizing albendazole and also avoiding degradation of ivermectin if any in acidic medium.

1.3 Validation of analytical methods

The objective of any analytical measurement is to obtain consistent, reliable and accurate data. Validated analytical methods play a major role in achieving this goal. The results from method validation can be used to judge the quality, reliability and consistency of analytical results, which is an integral part of any good analytical practice. Validation of analytical methods is also required by most regulations and quality standards that impact laboratories[13].

"Validation is the process of collecting documented evidence that the method performs according to its intended purpose" .This is based on analytical experiments performed according to the validation protocols that comply with the international guidelines i.e. ICH guidelines on method validation. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) is a unique project that brings together the regulatory authorities of Europe, Japan and the United States and experts from the pharmaceutical industry in the three regions to discuss scientific and technical aspects of product registration.

Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the

International Conference on Harmonization (ICH) guidelines (Q2A and Q2B)[14,15] The US Food and Drug Administration (FDA)[16,17]and US Pharmacopoeia (USP) both refer to ICH guidelines, i.e. these methods should be validated.

All analytical procedures require some form of validation, regardless of whether the method is used for stability, in –process analysis ,release, or acceptance testing.

The extent of guidelines for validation requirements provided by different organizations varies widely, but the objective of validation is always to achieve valid analytical test results. This is important to ensure the quality and safety of products that are measured using the analytical method.

Method validation has received considerable attention in literature from various industrial committees and regulatory agencies. There is a wide variety of information and guidance available, as mentioned below;

Literature from industrial committees and regulatory agencies

Committees and regulatory agencies	**Guidelines available**
ICH	a) $Q2R_1$Guidelines are guidelines for new method development and its validation. b) Q_1R_1 Guidelines are for development and validation of stability indicating analytical methods includes methodology
The United States Food and Drug Administration	Two industry guidelines: a)for the validation of analytical methods2 b)forthe validation of bioanalytical methods 3.
IUPAC	"Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis".
EURACHEM	detailed guide for method validation primarily developed for ISO/IEC accredited laboratories but because of its completeness it is also a good source for (bio)pharmaceutical laboratories
AOAC	technical document for the verification of analytical methods for the ISO 17025 accreditation.

Huber	Has published a technical document for the verification of analytical methods for the ISO 17025 accreditation.
Viswanathan and co-authors	An overview for validationof bioanalytical methods.

1.3.1 Validation Parameters[18]

As per ICH guidelines following types of analytical procedures need to be validated:

- Identification test
- Quantitation tests for impurities content
- Limit test for the control of impurities
- Quantitative tests of the active ingredient or other main components of the drug

These various parameters for validation of any analytical method are:

1. Accuracy

The accuracy of an analytical procedure ***expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found***.

Accuracy can also be described as ***the extent to which test results generated by the method and the true value agree.***

The ICH document on validation methodology recommends accuracy to be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range (for example, three concentrations with three replicates each). Accuracy should be reported as percent recovery by the assay of known added amount of standard in the sample or as the difference between the mean and the accepted true value, together with the confidence intervals.

Thus, accuracy of the method was studied by recovery experiments using standard addition method at three different levels (80%, 100% and 120. Known amounts of Standard solutions containing analyte were added to prequantified sample solutions to get 80,100 and 120 %. These samples were analyzed by injecting the sample solution and % recovery was calculated.

In the present study %recovery was calculated by the following formula.

$$\frac{\text{AREA OF SPIKED SAMPLE-AREA OF UNSPIKED SAMPLE}}{\text{AREA OF STANDARD}} \times \frac{\text{CONC.OF STANDARD}}{\text{CONC. OF ADDED DRUG}} \times 100$$

Acceptance limit for % recovery is 98-100%

2. Precision

The precision of an analytical procedure expresses ***the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions***. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility

Precision is a measure of the reproducibility of the whole analytical method (including sampling, sample preparation and analysis) under normal operating circumstances. Precision is determined by using the method to assay a sample for a sufficient number of times to obtain statistically valid results (i.e. between 6 - 1 0). Therefore for present work sample were assayed six times and the %RSD for obtained assay values was obtained. Precision is then expressed as the relative standard deviation.

$$\%RSD = \frac{\text{STD DEV}}{\text{MEAN}} \times 100$$

Assay values were calculated with the following formula

$$\%\ \text{Assay} = \frac{\text{Area of Sample}}{\text{Area of Standard}} \times \frac{\text{Concentration of Standard}}{\text{Concentration of Sample}} \times \frac{\text{Purity of Standard}}{100} \times 100$$

Repeatability

Repeatability ***expresses the precision under the same operating conditions over a short interval of time***. Repeatability is also termed intra-assay precision.

Intermediate precision

Intermediate precision ***expresses within-laboratories variations: different days, different analysts, different equipment, etc.***

Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

3. Specificity

ICH defines specificity as "the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically this might include impurities, degradants, matrix, etc."USP<1225> refers to the same definition but also comments that other reputable authorities such as IUPAC and AOAC use the term "selectivity" for the same meaning.

This reserves the use of "specific" for those procedures that produce a response for a single analyte only. ISO/IEC most likely has the same understanding because it requires a method to be "selective" rather than specific. Our goal is to distinguish and quantify the response of the target compounds from the responses of all other compounds Selectivity is the ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix.

Specificity for an assay ensures that the signal measured comes from the substance of interest, and that there is no interference from excipient and/or degradation products and/or impurities.

Determination of this can be carried out by assessing the peak identity and purity.

4. Detection Limit

The detection limit of an individual analytical procedure ***is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value*.**

Limit of detection (LOD) was calculated by using the formula

LOD = 3.3 x σ/S Where, σ = Standard deviation of response

S = Slope of regression equation.

5. Quantitation Limit

The quantitation limit of an individual analytical procedure ***is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.*** The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

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

6. Linearity

The linearity of an analytical procedure ***is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.*** This is the method's ability (within a given range) to obtain results which are either directly, or after mathematical transformation proportional to the concentration of the analyte within a given range.

Linearity is determined by calculating the regression line using a mathematical treatment of the results (i.e. least mean squares) vs. analyte concentration.. Calibration curve was constructed by plotting peak area vs concentrations of analyte.

7. Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The range is normally expressed in the same units as the test results. For assay tests, ICH requires the minimum specified range to be 80 to 120 percent of the test concentration.

8. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Thus Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e. different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc. (i.e. from laboratory to laboratory, from analyst to analyst.)

Table:1.1 Important validation parameters suggested by regulatory agencies

Parameter	**ICH**	**USP**	**ISO 17025**
Specificity	**X**	**X**	-
Selectivity	-	-	**X**
Precision		**X**	-
Repeatability	**X**	-	**X**
Intermediate precision	**X**	-	-
Reproducibility	**X**	**X (RUGGEDNESS)**	**X**
Accuracy	**X**	**X**	**X**
Linearity	**X**	**X**	**X**
Range	**X**	**X**	-
Limit of detection	**X**	**X**	**X**
Limit of quantitation	**X**	**X**	**X**
Robustness	**X**	**X**	**X**
Ruggedness	**X**	**X**	-

1.3.2 System Suitability Tests (SST)[19,20]

System suitability testing is an integral part of HPLC procedures..The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

Once a method or system has been validated the task becomes one of routinely checking the suitability of the system to perform within the validated limits.

System suitability test parameters are:

- Plate number or number of theoretical plates (n)
- Capacity factor (capacity ratio) k
- The selectivity or Separation Factor (relative retention) α
- Peak Resolution R
- Peak asymmetry factor (As).

These are measured on a peak or peaks of known retention time and peak width.

1. Plate number or number of theoretical plates (n)

This is a measure of the sharpness of the peaks and therefore the efficiency of the column. This can be calculated in various ways, for example the USP uses the peak width at the base and the BP at half the height.

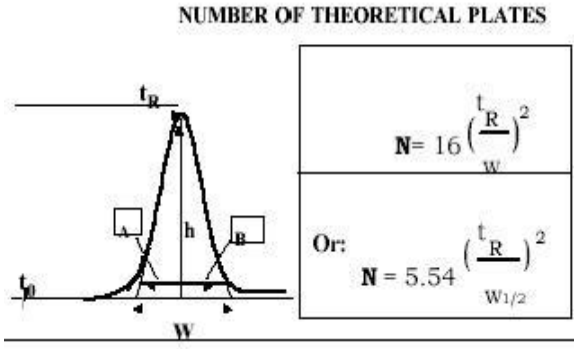


Fig: 1.2 Plate number or number of theoretical plates

WhereWhere, tR is the retention time and W is the peak width.

$W_{1/2}$= peak width at peak height

Wb = peak width at base

t = retention time of peak

Therefore the higher the plate number the more efficient the column. The plate number depends on column length - i.e. the longer the column the larger the plate number.

2. Capacity factor (capacity ratio) k

Capacity factor is the ratio of the reduced retention volume to the dead volume. Capacity factor, k", is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. Capacity factor is a measure of how well the sample molecule is retained by a column during an isocratic separation. The ideal value of k" ranges from 2-10. Capacity factor can be determined by using the formula,

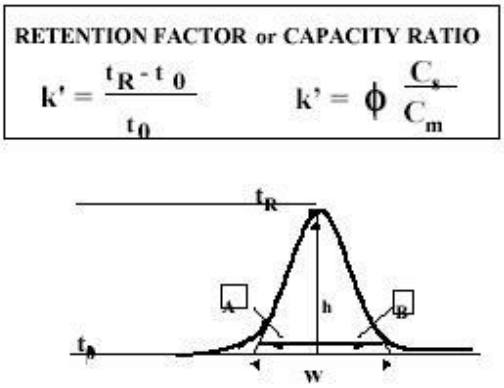


Fig: 1.3 Capacity factor (capacity ratio) k

Where, tR = retention volume at the apex of the peak (solute) and t0 = void volume of the system.

3. The selectivity or Separation Factor (relative retention) α

The selectivity (or separation factor), **α**, is a measure of relative retention of two components in a mixture. Selectivity is the ratio of the capacity factors of both peaks, and the ratio of its adjusted retention times. Selectivity represents the separation power of particular adsorbent to the mixture of these particular components.

This parameter is independent of the column efficiency; it only depends on the nature of the components, eluent type, and eluent composition, and adsorbent surface chemistry. In general, if the selectivity of two components is equal to 1, then there is no way to separate them by improving the column efficiency.

The ideal value of **α** is 2. It can be calculated by using formula,

α = V2 – V1 / V1 – V0 = k1"/ k2"

Where, V0 = the void volume of the column,

V1 and V2 =the retention volumes of the second and the first peak respectively

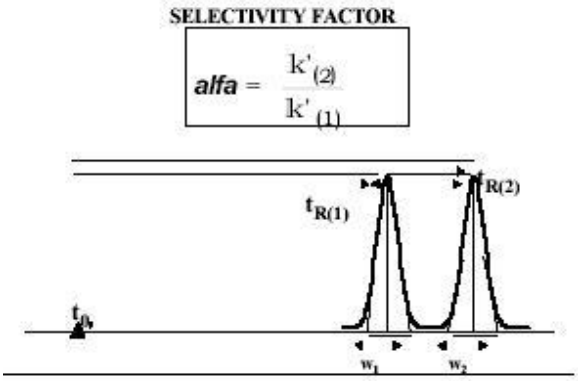


Fig: 1.4 The selectivity (or separation factor), **α**

4. Peak Resolution R

Resolution (Rs): Resolution is the parameter describing the separation power of the complete chromatographic system relative to the particular components of the mixture.

The resolution, Rs, of two neighboring peaks is defined as the ratio of the distance between two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of Rs is 1.5. It is calculated by using the formula

This is not only a measure of the separation between two peaks, but also the efficiency of the column. It is expressed as the ratio of the distance between the two peak maxima. (At) to the mean value of the peak width at base (Wb).

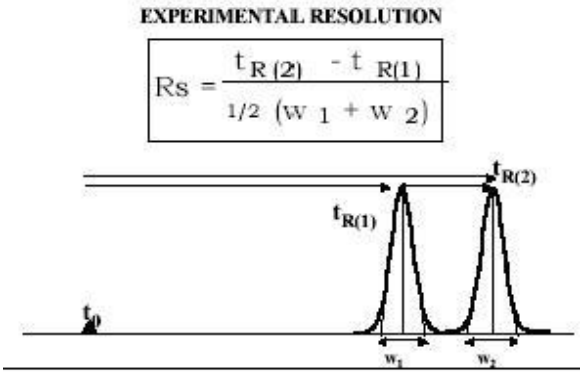


Fig: 1.5 Peak Resolution R

Where, Rt1 and Rt2 are the retention times of components 1 and 2 and W1 and W2 are peak width of components 1 and 2

5. Peak asymmetry factor (Tf): Peak asymmetry factor, Tf, can be used as a criterion of column performance. The peak half width, b, of a peak at 10% of the peak height, divided by the corresponding front half width, gives the asymmetry factor.

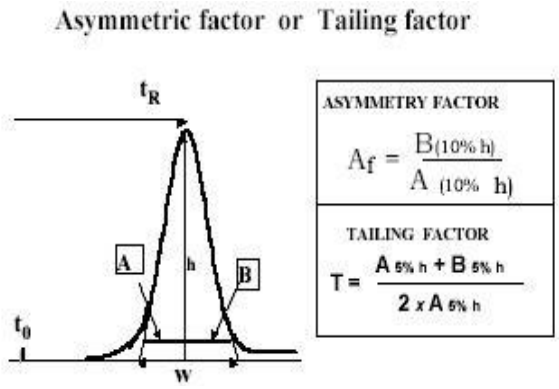


Fig: 1.6 Peak Asymmetry facor(Tf)

Table 1.2 SYSTEM SUITABILITY TEST PARAMETERS AND RECOMMENDATIONS

Parameters	**Recommendation**
Theoretical Plates (N)	should be > 2000
Capacity Factor ($k^{\prime}$)	The peak should be well-resolved from other peaks and the void Volume ,generally $k^{\prime}$>2.0
Relative retention	Not essential as long as the resolution is stated.
Resolution (Rs)	R_S of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard) etc.
Tailing Factor (T)	T of </= 2

1.3.3 Stability indicating assay method

Patients taking a pharmaceutical product for a particular malady expect the product to be safe and efficacious. Pharmaceutical regulatory agencies worldwide demand that the product retains its identity, quality, purity, and potency for the time the product is commercially available. Consequently the agencies expect to see stability data supporting the proposed expiration date of the product in the marketing submission. Therefore it is necessary to conduct stability studies to predict, evaluate, and ensure drug product safety[21].

Stability studies of drug substances via acid hydrolysis, base hydrolysis, oxidation and thermal and photolytic stress testing are a part of development strategy under the ICH requirements[22].

These studies provide information on a drug's inherent stability and help to validate analytical methods to be used for evaluation stability. Stability assays are currently being developed by using the stress testing approach of the ICH guidelines, Q1 A[R2].The approach has been further extended to stress test of drug combinations. These tests allow accurate and precise quantification of drugs, their degradation products, and their interaction products.

Stability-indicating methods according to 1987 guideline were defined as the **'*Quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured*.'**

The revised drug stability guideline Q1AR from the International Conference on Harmonization (ICH) requires that stability samples be tested using validated stability-indicating assay methods. The guideline suggests that stress testing be performed to establish the intrinsic stability of the drug substance and to validate the stability-indicating nature of the analytical method. It also recommends that stress testing include the evaluation of the effect of temperature, humidity (when appropriate), oxidation, and photolysis on the drug substance plus its susceptibility to hydrolysis across a wide range of pH values when in solution or suspension. Although applicable to new drugs, the ICH guidelines have recently been sought to be extended to generic drugs.

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OBJECTIVES OF THE STUDY

A patient taking a pharmaceutical product expects the product to be safe and efficacious.

Due to abundance of pharmaceutical agents available in the pharmaceutical market in various various dosage forms either as a single drug component or in combination with other drugs and also due to potency of the most of the drugs, it becomes necessary to quantitate these agents in their formulations in a precise manner.

Pharmaceutical regulatory agencies worldwide demand that the product retains its quality, purity, and potency for the time the product is commercially available. Consequently the agencies expect to see stability data supporting the proposed expiration date of the product in the marketing submission. In the broader sense the stability studies that are conducted should provide evidence of how the quality of the drug substance and drug product changes over time when subjected to various environmental conditions, such as temperature, humidity, and light.

It has also been observed that mainly in case of the cream formulations ,interference by cream components by cream base components hamper the analysis of active constituents ,which makes it a challenging task.

Therefore there is always a need to develop validated analytical methods which are precise, accurate, selective, and sensitive and can be used for routine analysis and stability studies of the drug products.

The objective of the present work was to develop validated analytical methods with the help of which we can separate and simultaneously quantitate drug components from the pharmaceutical formulations.

The specific aim of the work undertaken was:

- To develop validated analytical method based on HPLC for Racecadotril as a single component in bulk and in a commercial powder formulation.Racecadotril was selected because it was recently introduced in the Indian market and no method was available in the literature for estimation of the drug in the formulation at the time of undertaking the study.
- To study applicability of developed HPLC method for the determination of racecadotril in presence of its degradation products.
- To develop validated HPLC method for simultaneous analysis of Butenafine hydrochloride and Betamethasone dipropionate in bulk and in cream formulation. This combination was selected for study as the cream formulations are always difficult to analyze and butenafine

was recently introduced in the market at the time of undertaking the project.. Also not a single analytical method was reported in the literature for simultaneous estimation of the butenafine hydrochloride and betamethasone dipropionate.

- To develop validated HPTLC method for simultaneous analysis of Butenafine hydrochloride and Betamethasone dipropionate in bulk and in a cream formulation.
- To develop validated HPLC method for simultaneous analysis of Escitalopram oxalate and Etizolam in bulk and in a tablet formulation..Etizolam is not official in any pharmacopoeia and not a single analytical method was reported for the simultaneous estimation of these drugs in the formulation. The literature survey also revealed that no stability indicating method was available for this combination of drugs.
- To apply developed validated developed HPLC method for the determination of Escitalopram oxalate and Etizolam in presence of their degradation products.
- To study applicability of developed HPLC method for dissolution studies of Escitalopram oxalate and Etizolam in a tablet dosage form..
- To develop validated HPTLC method for the simultaneous analysis of Albendazole and Ivermectinin in bulk and in a tablet dosage form .This combination was selected for study because ,although analytical methods for indivisual drugs were available,but not a single method was reported for the simultaneous estimation of this these drugs in combined dosage form at the time of selection. Also proportion of ivermectin to albendazole (12:400) in tablet dosage form was also found to be challenging.
- To develop IR method for the simultaneous analysis of Albendazole and Ivermectin in bulk and in a tablet dosage form. Solubility of albendazole in mineral acid was the measure concern in the simultaneous method development as ivermectin is sensitive to mineral acid degradation. Therefore it was decided to take the advantage of IR for estimation of drugs in solid dosage form without much sample preparation.
- To develop validated HPLC method for simultaneous analysis of Fluocinolone acetonide and Miconazole nitrate in bulk and in an ointment. This combination was selected because, although analytical methods for individual drugs were available, not a single method was reported for the simultaneous estimation of this these drugs in combined dosage form.Also proportion of fluocinolone acetonide to miconazole nitrate (1:200) was also found to be challenging.

- To develop validated HPLC method for simultaneous analysis of Hydrocortisone acetate and Miconazole nitrate in bulk and in a cream formulation. This combination was selected because, although analytical methods for individual drugs were available, not a single method was reported for the simultaneous estimation of this these drugs in combined dosage form.
- To overcome typical challenges encountered while developing and validating methods for pharmaceutical products containing single and more than one active ingredients.

3. DRUG PROFILE [1-5]

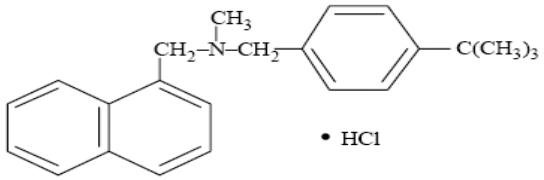
3.1 Butenafine Hydrochloride

a. Category:Antifungal

b. Molecular formula: $C_{23}H_{27}N$•HCl

c. Molecular weight: 353.93g/mole

d. Chemical Structure:



e. Chemical Name: *N*-4-*tert*-butylbenzyl-*N*-methyl-1-naphthalene methylamine hydrochloride

f. Physicochemical Properties:

- **Description:**A white, odorless, crystalline powder.
- **Solubility:** It is freely soluble in methanol, ethanol, and chloroform, and slightly soluble in water.
- **Melting point:** 210-214 ºC

g. Official Status: Not official in any pharmacopoeia

Marketed Formulations	Company	Composition
BUTENASKIN BM CREAM	**Elder Pharmaceuticals Ltd.**	**Butenafine 1% + Betamethasone dipropionate 0.05 %**

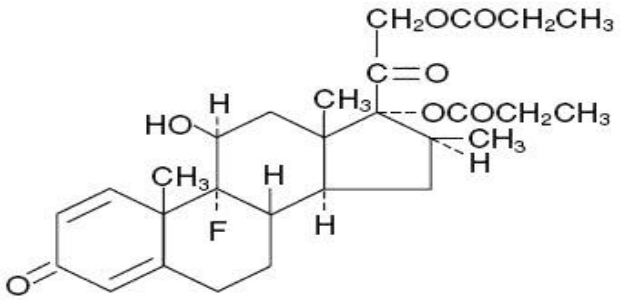
3.2 Betamethasone dipropionate [6-13]

a. Category: Glucocorticoid steroid with anti-inflammatory and immunosuppressive abilities

b. Molecular formula: $C_{28}H_{37}FO_7$

c. Molecular weight: 504.59 g/mole

d. Chemical Structure:



e. Chemical Name: 9_-fluro-11_, 17_, 21-trihydroxy-16_-methylpregna-1, 4-diene-3,20-dione

f. Physicochemical Properties:

- **Description:** a white to pale yellowish white, crystalline powder
- **Solubility:** It is freely soluble in acetone, in 1,4-dioxane and in chloroform, soluble in methanol, sparingly soluble in ethanol, slightly soluble in diethyl ether, and practically insoluble in water and in hexane.
- **Melting point:** 176 – 1809C

g. Official Status: official in U.S. Pharmacopoeia (USP), European Pharmacopoeia, and British Pharmacopoeia

Marketed Formulations	Company	Composition
BUTENASKIN BM CREAM	**Elder Pharmaceuticals Ltd.**	**Butenafine 1% + Betamethasone dipropionate 0.05 %**

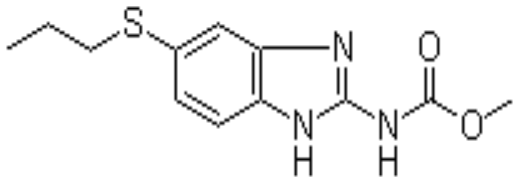
3.3 ALBENDAZOLE [14-16]

a. Category: It is a broad spectrum anthelmintic, effective against: roundworms, tapeworms, and flukes of domestic animals and humans. (ref-1)

b. Molecular formula: C12H15N3O2S

c. Molecular weight: 265.34 g/mole

d. Chemical Structure:



e.Chemical Name: methyl-[5-(propylthio)-1-*H*-benzimidazol-2yl] carbamate Methyl [6-(propylthio)-1*H*-benzoimidazol-2-yl] carbamate

f. Physicochemical Properties:

- **Description:** a white yellowish powder
- **Solubility:** Practically insoluble in water.Freely soluble;e in anhydrous formic acid,very slightly soluble in mythylene chloride,practically insoluble in alcohol.
- **Melting point:** 209°C

g. Official Status: official in U.S. Pharmacopoeia (USP), European Pharmacopoeia, and British Pharmacopoeia,Indian Pharmacopoeia

Marketed Formulations	Company	Composition
BANDY PLUS	**Mankind**	**Albendazole-400mg + Ivermectin-6 mg**
BANDY PLUS	**Mankind**	**Albendazole-400 mg + Ivermectin-12 mg**

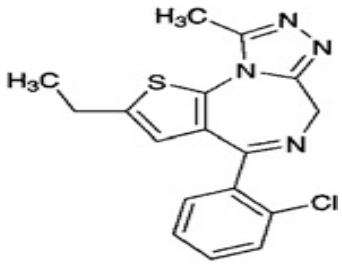
3.4 Etizolam[17-20]

a. Category: It possesses amnesic, anxiolytic, anticonvulsant, hypnotic, sedative and skeletal muscle relaxant properties(ref-1)

b. Molecular formula: $C_{17}H_{15}ClN_4S$

c. Molecular weight: 342.85

d. Chemical Structure:



e. Chemical Name: 4-(2-Chlorophenyl)-2-ethyl-9-methyl-6H-thieno-[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine

f. Physicochemical Properties:

- **Description:** Etizolam occurs as a white to pale yellowish white crystalline powder.
- **Solubility:** It is soluble in ethanol,methanol,dichloromethane and chloroform. sparingly soluble in acetonitrileand in acetic anhydride, and practically insoluble in water
- **Melting point:** 146 – 1499C

g. **Official status**:Etizolam is not official in USP,BP,EP

Marketed Formulations	Company	Composition
ETIZOLA PLUS	**MCLEODS**	**Etizolam 0.5 mg+Escitalopram 5 mg**
EZOLENT PLUS	**TALENT INDIA**	**Etizolam 0.5 mg+Escitalopram 5 mg**

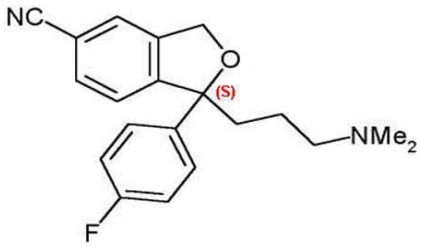
3.5 Escitalopram oxalate[48-51]

a.Category: Escitalopram oxalate is an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class (ref-1)

b. Molecular formula: $C_{20}H_{21}FN_2O \cdot C_2H_2O_4$

c. Molecular weight: 414.43

d. Chemical Structure:



e.Chemical Name: S-(+)-1-[3-(dimethyl-amino) propyl]-1-(p-fluorophenyl)-5-phthalancarbonitrile oxalate.

f. Physicochemical Properties:

- **Description:** Escitalopram oxalate occurs as a fine white to slightly yellow powder
- **Solubility:** freely soluble in methanol and dimethyl sulfoxide (DMSO), soluble in isotonic saline solution, sparingly soluble in water and ethanol, slightly soluble in ethyl acetate, and insoluble in heptane.
- **Melting Point:** 146-149°C

g. Official Status: Escitalopram oxalate is official in Indian Pharmacopoeia

Marketed Formulations	Company	Composition
NEXITO FORTE	**SUN PHARMA**	**Escitalopram oxalate 10 mg+Clonazepam 0.5 mg**
ETIZOLA PLUS	**MCLEODS**	**Etizolam 0.5 mg+Escitakopram 5 mg**

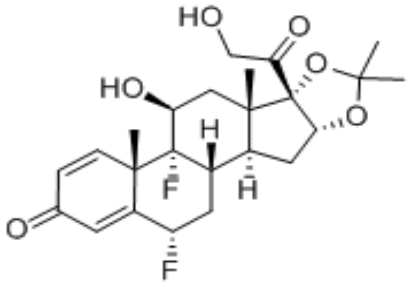
3.6 Fluocinolone acetonide[21-25]

a.Category: Fluocinolone acetonide is a corticosteroid used topically for its glucocorticoid activity primarily in dermatology to reduce skin inflammation and relieve itching.

b. Molecular formula: $C_{24}H_{30}F_2O_6$

c. Molecular weight: 452.49

d. Chemical Structure:



e. Chemical Name: Pregna-1,4-diene- 3,20-dione, 6,9-difluoro-11,21-dihydroxy- 16,17-[(1-methylethylidene) bis(oxy)]-, (6.alpha.,11.beta.,16.alpha.)- (67-73-2)

f. Physicochemical Properties:

- **Description:** Fluocinolone Acetonide is a white or almost white, crystalline powder.
- **Solubility:** Insoluble in water; soluble 1 in 45 of alcohol, 1 in 25 of chloroform, and 1 in 350 of ether; soluble in methyl alcohol.
- **Melting point :** 267-269 °C

g. Official Status: Fluocinolone acetonide is official in U.S. Pharmacopoeia (USP), European Pharmacopoeia, and Indian Pharmacopoeia

Marketed Formulations	Company	Composition
ZOLE-F(Ointment) **ZOLE-F(Lotion)**	**REXEL(RANBAXY)**	**Miconazole nitrate 2% +** **Fluocinolone Acetonide 0.01%**
SUPRICORT-N(Ointment)	**GLENMARK**	**Fluocinolone Acetonide 0.025% +** **Neomycin Sulphate0.5%**

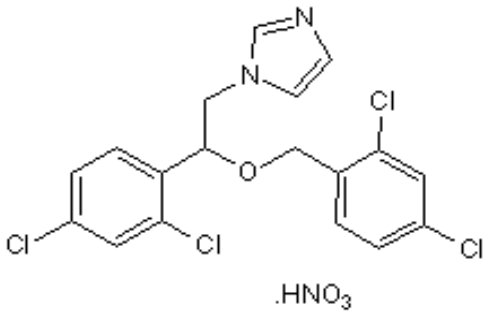
3.7 Miconazole nitrate[26-32]

a.Category: Miconazole Nitrate is an antifungal agent used to treat superficial candidiasis,dermatophytosis and pityriasis vesicolor

b.Molecular formula: $C_{18}H_{14}Cl_4N_2O.HNO_3$

c. Molecular weight : 479.14

d. Chemical Structure:



e.Chemical Name: 1-[2-(2,4-Dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]ethyl]-1*H*-imidazole nitrate

f. Physicochemical Properties

- **Description:** white or almost white cryastalline or microcrystalline
- **Solubility:** Freely soluble in mrthanol,slightly soluble in ethanol(95%) and in chloroform,very slightly soluble in water and ether
- **Melting point :** 170-184°C

g. Official Status: Miconazole nitrate is official in U.S. Pharmacopoeia (USP), European Pharmacopoeia, British Pharmacopoeia, and Indian Pharmacopoeia

Marketed Formulations	Company	Composition
ZOLE-F(Ointment) **ZOLE-F(Lotion)**	**REXEL(RANBAXY)**	**Miconazole nitrate 2% +** **Fluocinolone Acetonide 0.01%**

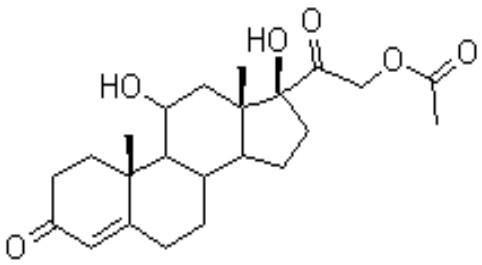
3.8 Hydrocortisone acetate[32-36]

a. Category: Hydrocortisone acetate is used as an anti-inflammatory.

b. Molecular formula: $C_{23}H_{32}O_6$

c. Molecular weight: 404.50

d. Chemical Structure



e. Chemical Name: 11, 17-Dihydroxy-3,20-dioxopregn-4-en-21-yl acetate

f. Physicochemical Properties

- **Description:** white to practically white, crystalline powder; odourless
- **Solubility:** Insoluble in water. slightly soluble in alcohol
- **Melting point:** 222-225 ºC

g. Official Status: Hydrocortisone acetate is official in U.S. Pharmacopoeia (USP),British Pharmacopoeia

Marketed Formulations	Company	Composition
DACTACORT (Gel)	**JOHNSON & JOHNSON**	**Hydrocortisone acetate 1% + Miconazole Nitrate2%**
FUMIC (Cream)	**APURVA BIOPHARM INC**	**Hydrocortisone acetate 1% + Miconazole Nitrate2%**

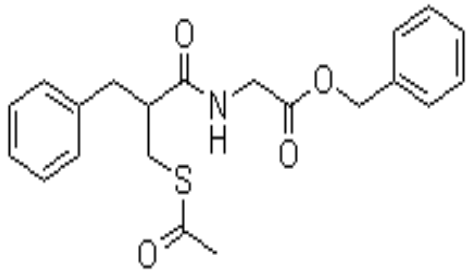
3.9 Racecadotril [37-40]

a.Category: Racecadotril (acetorphan) is an enkephalinase inhibitor used in the treatment of acute diarrhea.

b.Molecular formula: $C_{21}H_{23}NO_4S$

c. Molecular weight: 385.48

d. Chemical Structure:



e.Chemical Name: chemically N-[(R, S)-3-acetylmercapto-2-benzylpropanol]-glycine benzyl ester

f. Physicochemical Properties

- **Description:** white crystalline powder
- **Solubility:** Soluble in methanol and chloroform
- **Melting point :** 75-76°C

g. Official Status: Racecadotril is not official in any pharmacopoeia

Marketed Formulations	Company	Composition
REDOTIL (Capsule)	**DR. REDDY'S LAB**	**Racecadotril 100mg**
ZEDOTT (Powder sachet)	**TORRENT PHARMA**	**Racecadotril 10mg + Racecadotril 30 mg**

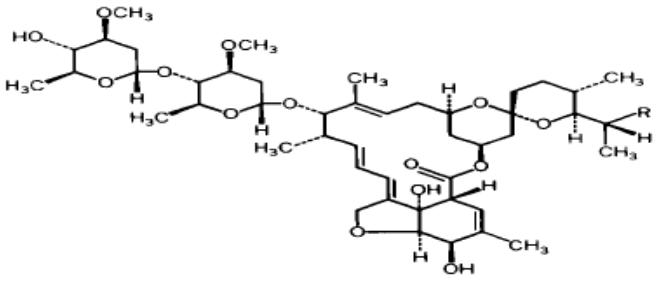
3.10 Ivermectin[41-47]

a.Category: Ivermectin is a semisynthetic, anthelmintic agent for oral administration. Ivermectin is derived from the avermectins, a class of highly active broad-spectrum, anti-parasitic agents isolated from the fermentation product of *Streptomyces avermitilis.*

b.Molecular formula: The respective empirical formulas are $C_{48}H_{74}O_{14}$ and $C_{47}H_{72}O_{14}$.

c. Molecular weight: It is a mixture consisting of ≥80% of compound A and <20% homolog B ,the molecular weight of this drug will range from 872.21 to 875.10

d. Chemical Structure:



Component B_{1a}, R = C_2H_5.....Component B_{1b}, R = CH_3

e.Chemical Name: Ivermectin is a mixture containing at least 90% 5-*O* demethyl-22,23-dihydroavermectin A_{1a} and less than 10% 5-*O*-demethyl-25-de(1-methylpropyl)-22,23-dihydro-25-(1-methylethyl)avermectin A_{1a}, generally referred to as 22,23-dihydroavermectin B_{1a} and B_{1b}, or H_2B_{1a} and H_2B_{1b}, respectively

Description: Ivermectin is a white to yellowish-white, nonhygroscopic, crystalline powder

Solubility: It is insoluble in water but is freely soluble in methanol and soluble in 95% ethanol.

Melting point: 155°C

g. Official Status:

Marketed Formulations	Company	Composition
BANDY PLUS(TABLET)	**MANKIND**	**Albendazole-400 mg + Ivermectin-6 mg**
BANDY PLUS(TABLET)	**MANKIND**	**Albendazole-400 mg + Ivermectin-12 mg**
AZ PLUS(SUSPENSION)	**CURE PHARMA**	**Albendazole-200mg + Ivermectin-3 mg**

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A thorough literature survey has revealed that most of the selected drugs may or may not be official in Pharmacopoeia and a number of analytical methods are available for their individual estimation or in combination with some other drug but no analytical method is reported for the simultaneous estimation of the drugs in standard laboratory mixture or in commercial formulation available in the Indian market. Therefore some selective methods reported for the drugs selected present study are mentioned in the following section

4.1 Butenafine hydrochloride[1.2]

4.1.1 Simultaneous HPLC Determination of Butenafine Hydrochloride and Betamethasone in a Cream Formulation

A fast, specific, accurate and precise reverse phase high performance liquid chromatographic method was developed for the simultaneous determination of butenafine hydrochloride and betamethasone in cream formulation. The determination was carried out on licrocart licrosphere RP-select B (250×4.6 mm, 5 μ) column in isocratic mode, the mobile phase consisting of 50 mM ammonium acetate buffer and acetonitrile in the ratio of 60:40, adjusted to pH 4.5 ± 0.1 with glacial acetic acid. The flow rate was 2.0 ml/min and eluent was monitored at 254 nm. The retention times of butenafine hydrochloride and betamethasone were 4.70 min and 7.76 min, respectively, and the resolution factor was greater than 4.0. Linearity of butenafine hydrochloride and betamethasone were in the range of 100-300 μg/ml and 5-15 μg/ml, respectively. The proposed method is also found to be precise and robust for the simultaneous determination of butenafine hydrochloride and betamethasone in cream formulation.

4.1.2 Stability-Indicating LC Assay for Butenafine Hydrochloride in Creams Using an Experimental Design for Robustness Evaluation and Photodegradation Kinetics Study

A stability-indicating liquid chromatography method for the determination of the antifungal agent butenafine hydrochloride (BTF) in a cream was developed and validated using the Plackett-Burman experimental design for robustness evaluation. Also, the drug photodegradation kinetics was determined. The analytical column was operated with acetonitrile, methanol and a solution of triethylamine 0.3% adjusted to pH 4.0 (6:3:1) at a flow rate of 1 mL/min and detection at 283 nm. BTF extraction from the cream was done with *n*-butyl alcohol and methanol in ultrasonic bath. The performed degradation conditions were: acid and basic media with HCl 1M and NaOH 1M, respectively, oxidation with H_2O_2 10%, and the exposure to UV-C light. No

interference in the BTF elution was verified. Linearity was assessed (r^2 = 0.9999) and ANOVA showed non-significative linearity deviation ($p > 0.05$). Adequate results were obtained for repeatability, intra-day precision, and accuracy. Critical factors were selected to examine the method robustness with the two-level Plackett-Burman experimental design and no significant factors were detected ($p > 0.05$). The BTF photodegradation kinetics was determined for the standard and for the cream, both in methanolic solution, under UV light at 254 nm. The degradation process can be described by first-order kinetics in both cases.

4.2 Betamethasone Dipropionate [3, 4, 5]

4.2.1 Densitometric determination of betamethasone dipropionate and salicylic acid in lotions, and validation of the method

A simple and rapid densitometric method has been developed for determination of betamethasone dipropionate and salicylic acid in lotions. The samples were diluted with 96% ethanol and spotted on precoated silica gel TLC plates which were then eluted with ethanol (96%)-toluene-chloroform-glacial acetic acid, 6.0 + 20 + 14 + 0.5 (*v/v*). Quantitative evaluation was performed by measuring the absorbance reflectance of the betamethasone dipropionate and salicylic acid spots at λ = 250 and 310 nm, respectively. This densitometric TLC method is selective, precise, and accurate and can be used for routine analysis of lotions in pharmaceutical industry quality-control laboratories.

4.2.2 Development and validation of a stability-indicating HPLC method for simultaneous determination of salicylic acid, betamethasone dipropionate and their related compounds in Diprosalic Lotion.

Diprosalic Lotion is an anti-inflammatory drug product that contains salicylic acid and betamethasone dipropionate as active pharmaceutical ingredients (APIs). A reversed-phase high performance liquid chromatography (RP-HPLC) method was developed for simultaneous determination of salicylic acid, betamethasone dipropionate, and their related compounds in Diprosalic Lotion. A 150 mm x 4.6 mm I.D. YMC J'sphere ODS-H80 column at 35 degrees C and UV detection at 240 nm was used. A gradient elution was employed using 0.05% (v/v) methanesulfonic acid solution and acetonitrile as mobile phases. A total of thirty three compounds from Diprosalic Lotion samples were separated in 38 min. The stability-indicating capability of this method has been demonstrated by the adequate separation of all the impurities

and degradation products in expired stability samples of Diprosalic Lotion. The method was validated as per the current ICH guidelines.

4.2.3 Development and validation of a stability-indicating RP-HPLC method for simultaneous assay of betamethasone dipropionate, chlorocresol, and for the estimation of betamethasone dipropionate related compounds in a pharmaceutical cream and ointment.

A new stability-indicating reversed-phase HPLC (RP-HPLC) method has been developed and validated for simultaneous assay of betamethasone dipropionate (BD) and chlorocresol and also for the estimation of BD related compounds in a pharmaceutical cream matrix. In addition, this newly developed RP-HPLC method was also demonstrated as suitable for a pharmaceutical ointment product that does not contain chlorocresol. The RP-HPLC method uses a Waters SymmetryShield RP18 analytical column (150 4.6 mm). Water (mobile phase A) and acetonitrile (mobile phase B) were used in the gradient elution with a flow rate of 1.5 mL/min and detection wavelength at 240 nm. A Waters XBridge Shield RP18 analytical column (150 4.6 mm) was identified as an alternate column. The limit of detection (LOD) and the limit of quantitation (LOQ) are 0.02 g/mL and 0.05 g/mL, respectively. The precision of the method for BD is less than 0.3% RSD, and the accuracy of BD ranged from 99.5% to 102.6%. The stability-indicating capability of this method has been demonstrated by analyzing aged samples of the product. This RP-HPLC method was successfully validated per ICH guidelines and proved to be suitable for routine quality control use.

4.3 Escitalopram oxalate[6-14]

4.3.1 Spectrophotometric and reversed-phase high-performance liquid chromatographic methods for simultaneous determination of Escitalopram oxalate and Clonazepam in combined tablet dosage form

Simple, accurate, precise, and sensitive ultraviolet spectrophotometric and reversed-phase high-performance liquid chromatographic (RP-HPLC) methods for simultaneous estimation of Escitalopram oxalate (ESC) and Clonazepam (CLO) in combined tablet dosage form have been developed and validated. The spectroscopic method employs an absorbance correction method using 238.6 and 308 nm as 2 wavelengths for estimation with methanol and water as solvents. Beer's law is obeyed in the concentration range of 10.0-50.0 and 0.5-3.0 micro/mL for ESC and CLO, respectively. The RP-HPLC method uses a Jasco HPLC system with HiQ SiL C18 column

(250 x 4.6 mm id) acetonitrile-0.005 M tetrabutylammonium hydrogen sulfate (55 + 45, v/v) as the mobile phase, and satranidazole as an internal standard. The detection was carried out using an ultraviolet detector set at 287 nm. For the HPLC method, Beer's law is obeyed in the concentration range of 10.0-60.0 and 0.5-3.0 microg/mL for ESC and CLO, respectively. Both methods have been successfully applied for the analysis of the drugs in a pharmaceutical formulation. Results of analysis were validated statistically and by recovery studies.

4.3.2 Simultaneous HPTLC Determination of Escitalopram Oxalate and Clonazepam in Combined Tablets .

A new, simple high-performance thin-layer chromatographic method has been established and validated for simultaneous determination of Escitalopram oxalate and Clonazepam in a combined tablet dosage form. The drugs were separated on aluminum plates precoated with silica gel 60 F_{254}; toluene–ethyl acetate–triethylamine 7:3.5:3 (*v*/*v*) was used as mobile phase. Quantitative analysis was performed by densitometric scanning at 258 nm. The method was validated for linearity, accuracy, precision, and robustness. The calibration plot was linear over the ranges 250–2,500 and 50–500 ng band^{-1} for Escitalopram oxalate and Clonazepam, respectively. The method was successfully applied to the analysis of drugs in a pharmaceutical formulation.

4.3.3 Development and validation of liquid chromatographic method For estimation of Escitalopram oxalate in tablet dosage forms.

A simple, specific, accurate and precise RP-HPLC method was developed and

validated for the determination of Escitalopram oxalate in tablet dosage forms. A hypersil BDS C8, 5- column having 250x4.6mm internal diameter in isocratic mode with mobile phase containing methanol: disodium hydrogen phosphate: acetonitrile (28:44:28v/v, pH 7.0±0.05) was used. The flow rate was 1.5ml/min and effluents were monitored at 226nm. The retention time of Escitalopram oxalate was 8.45 min. The linearity range is 250-1500-g/ml with coefficient of correlation 0.9999. The method was validated in terms of accuracy, precision, repeatability. The percentage recovery for Escitalopram oxalate was found to be 99.0%. The proposed method was successfully applied for quantitative determination of Escitalopram oxalate in single dosage form for routine analysis.

4.3.4 Colorimetric method for the estimation of Escitalopram oxalate in tablet dosage form

A colorimetric method for the analysis of Escitalopram oxalate in pure form and in tablets has been developed based on the formation of chloroform soluble ion associates with bromocresol green acidic dye. The extract of ion associates exhibited absorption maxima at 417 nm obeying Beer«SQ»s law in the range of 2-10 μg/ml. The method is simple, precise and accurate with recovery of 98-102% and does not require any separation of

4.3.5 Zero order spectrophotometric method for estimation of Escitalopram oxalate in tablet formulations

A new, simple, fast and reliable zero order spectrophotometric method has been developed for determination of Escitalopram Oxalate in bulk and tablet dosage forms. The quantitative determination of drug was carried out using the zero order values (absorbance) measured at 238 nm. Calibration graph constructed at 238 nm was linear in concentration range of 2-20 μg/ml with correlation coefficient 0.9999. The method was found to be precise, accurate, specific, and validated as per ICH guidelines and can be used for determination of Escitalopram Oxalate in tablet formulations.

4.3.6 Spectrophotometric Method for Simultaneous Estimation of Escitalopram Oxalate and Clonazepam in Tablet Dosage Form

A simple, accurate and precise spectrophotometric method has been developed for simultaneous estimation of Escitalopram oxalate and Clonazepam in combined dosage form. Simultaneous equation method is employed for simultaneous determination of Escitalopram oxalate and Clonazepam from combined dosage forms. In this method, the absorbance was measured at 238 nm for Escitalopram oxalate and 273 nm for Clonazepam. Linearity was observed in range of 5-100 μg/ml and 5-50 μg/ml for Escitalopram and Clonazepam respectively. Recovery studies confirmed the accuracy of proposed method and results were validated as per ICH guidelines. The method can be used for routine quality control of pharmaceutical formulation containing Escitalopram and Clonazepam.

4.3.7 Study on Pharmacokinetics of Escitalopram Oxalate Tablets in Human Body

Es-citalopram oxalate tablets were administered orally at a single dose of30mg to10healthy subjects respectively,the plasma concentration of Escitalopram oxalate was determined by HPLC method,the pharmacokinetic parameter was fitted with3p97software.

RESULTS: The concentration-time curve of Escitalopram oxalate tablets was in line with the two-compartment model,the main pharmacokinetics parameters of Escitalopram oxalate were as follows:

Cmax was (42.73±10.19) μg·L, tmax was (2.90±0.32) h, t1/2 was (35.34±7.78) h, AUC0～132 was (1241.5±194.3) (μg·h)/L and the AUC0～∞ was (1327.5±210.5) (μg·h)/L.

CONCLUSION: The study on pharmacokinetics can be used as a reference in the clinical medication.

4.3.8 Pharmacokinetics of Escitalopram in healthy volunteers

The volunteers were given a single oral dose of 5,10 or 20 mg Escitalopram for single dose pharmacokinetics, or 10 mg Escitalopram tablets once a day for 10-days multidose and steady-state study. The plasma concentrations of Escitalopram were determined by a validated HPLC-MS/MS method. The pharmacokinetic parameters were calculated by DAS 2.0 software. RESULTS: The main pharmacokinetic parameters of Escitalopram after the single oral dose of 5, 10 or 20 mg were as follows:

AUC(0-144) were (219±s 49), (367±60) and (689±174) (μg·h)/L, respectively;AUC(0-∞) (242±64), (414±79) and (745±207) (μg·h)/L; Cmax (5.5±1.0), (8.8±1.3) and (21±6)μg/L; tmax (4±3), (6±3) and (3.0±1.5) h; t1/2 (40±11), (48±10) and (37±6) h; MRT (56±13), (63±11) and (51±7) h.

The parameters after multidose of 10 mg were as follows:

AUC(0-144) was (914±202)μg·h/L, AUC(0-∞) (993±214)μg·h/L, AUC(ss) (364±78)μg·h/L, Cmax~(ss) (26±4)μg·h/L, Cmin~(ss) (12.4±1.1) μg·h/L, Cav~(ss) (15±3) μg·h/L, DF 0.87±0.22, tmax (2.6±1.7)h, t1/2 (40±7) h.

4.3.9 Method for the estimation of Escitalopram in bulk and in dosage Forms

The present study indicates a simple, accurate and precise RP- HPLC method for the estimation of Escitalopram in bulk and in pharmaceutical formulations. The mobile phase used was phosphate buffer with pH 7.0 and an organic mixture solvent (acetonitrile and methanol in the ratio of 1:1 v/v). Then the mobile phase was prepared by mixing buffer solution and mixture of organic solvents in the ratio of (55: 45 v/v) respectively. The specification of the chromatographic system 150 mm ×4.6 mm Xterra RP 18, 5 μm, flow rate 1.2ml/min, detection 238nm, injection volume 10μl and run time 10 min. Only very few HPLC procedures have been

reported in the literature for the determination of Escitalopram in pharmaceutical formulations and biological fluids. There are no reports for the determination of Escitalopram by HPLC in pure form. Hence I have made an attempt to develop a HPLC method for the determination of Escitalopram in bulk and in pharmaceutical formulations.

4.4 Etizolam[15-18]

4.4.1 Simple method for the determination of benzodiazepines in human body fluids by high-performance liquid chromatography–mass spectrometry

4.4.2 Rapid and sensitive detection of benzodiazepines and zopiclone in serum using high-performance thin-layer chromatography

4.4.3 Effects of different cyclodextrins on the morphology, loading and release properties of poly (DL-lactide-co-glycolide)-microparticles containing the hypnotic agent etizolam

4.4.4 Simultaneous determination of etizolam, triazolam and their metabolites by gas chromatography tandem mass spectrometry.

4.5 Albendazole[19-23]

4.5.1 Developing a Spectrophotometric method for the estimation of Albendazole in solid and suspension forms

A Spectrophotometric method has been developed for the determination of Albendazole in bulk, tablet and suspension dosage forms. Solution of Albendazole in methanolic glacial acetic acid solution shows maximum absorbance at 235 nm. Beer's law was obeyed in the concentration range of 2.5 - 20 _g/ml with molar absorptivity of 1.0815*104 mol^{-1} cm^{-1}. The method was applied for the analysis of the drug in the pure, tablet and suspension forms. The slope and intercept of the equation of the regression line are 0.0310 and 0.00067 respectively. Correlation coefficient was found to be 0.9998. Results of percentage recovery showed that the method was not affected by the presence of common excipients. The proposed method is simple, sensitive, rapid, economical and could find application as an inprocess quality control method for Albendazole.

4.5.2 RP-HPLC Method for Simultaneous Estimation of Levamisole, Mebendazole and Albendazole in Pharmaceutical Products

Single and reproducible RP-HPLC method has been developed for the simultaneous estimation of Levamisole, Mebendazole and Albendazole in pharmaceutical products. Chromatographic

separation was achieved by using Inertsil ODS-3V C18, 250 x 4.6 mm, 5µm column, mobile phase composed of sol-A: Potassium dihydrogen phosphate (1.0 gram in 1000 ml of HPLC Water) buffer and sol-B: Acetonitrile with gradient elution (0-5min- sol-A: 80-80; 5-7min- sol-A: 80-60; 7- 10min- sol-A: 60-30; 10-15min- sol-A: 30-80 and 15-20min- sol-A: 80-80). Flow rate was 1.00 ml per min and measured the absorbance at 210nm. The retention time of Levamisole, Mebendazole and Albendazole are 4.8min, 12.8min and 14.1min, respectively. The linearity of the method was evaluated from 5µg per mL to 100µg per mL for each ingredient and the correlation coefficient result was observed for each ingredient was not less than 0.999. The developed method has wide applicable in the quantification of Levamisole, Mebendazole and Albendazole in pharmaceutical dosage forms.

4.5.3 Quantification of Albendazole in Dewormer Formulations in the Kenyan market

In this study, the amount of active ingredient, Methyl [5-(propylthio)-2-benzimidazolecarbamate] (albendazole) in dewormer formulations was quantified using High Performance Liquid Chromatography (HPLC) and Ultra–Violet/ Visible (UV/ Vis) Spectrophotometer. Dewormer samples were obtained from various drug stores in Nairobi city.The analyses results indicated that in a number of cases the concentrations of albendazole differed with that indicated on the manufacturers' labels. In two cases the concentration of albendazole grossly differed from other samples.

4.5.4 Validated UV Spectrophotometric Method for estimation of Albendazole in Tablet

Albendazole (ABZ) is an oral broad-spectrum anthelmintic, antiparasitic agent generally prescribed for the treatment of tissue infections caused by a variety of nematodes. No UV method is reported for routine analysis of albendazole. Here we have developed simple, accurate and rapid UV spectrophotometric method for estimation of albendazole from Tablet formulation. The drug obeyed the Beer's law and showed good correlation. It showed absorption maxima at 298 nm in Dimethyl Formamide (DMF). The linearity was observed between 2-20 µg/ml. The results of analysis were validated by recovery studies. The recovery was found to be 99.43-101.55%. The method was found to be simple, reliable, rapid, precise, specific and reproducible and can be applied for routine analysis of albendazole in different dosage form and dissolution studies.

4.5.5 A Bioequivalence Study of an Albendazole Oral Suspension Produced in Iran and a Reference Product in Sheep

In a parallel design, a single oral dose ofalbendazole (ABZ), 5 mg/kg, was administered to 2 groups of 8 sheep to study the bioequivalence of an ABZ oral suspension produced in an Iranian pharmaceutical company and a reference product (Valbazen®, Pfizer Inc.). A third group of 8 sheep without dosing was used as control. Blood samplesof all groups were collected at specified times within 0-72 hours post-dosing. The serum levels of albendazole sulphoxide (ABZ-SO), the main metabolite of ABZ, were determined using a high performanceliquid chromatographic method with ultraviolet detection. Peak areas were used for calculatingABZ-SO concentrations and ABZ-SO pharmacokinetic parameters obtained using non-compartmental analysis. Statistical analysis of data pointed out that there were significant differences between the area under the concentration-time curve and peak serum concentration of these products, although there was no significant differencein their time to peak serum. Albendazole is poorly absorbed from the GI tract. This property, which is ideal for its use against luminal parasitic infections, is a problem in the treatment of systemic diseases. After oral administration of ABZ, it is metabolized rapidly to a pharmacologically active metabolite, albendazole sulfoxide (ABZ-SO), and it constitutes the main part of drug in blood.1,4,5 Because the first-pass metabolism for ABZ is extensive and ABZ serum level is negligible after its oral dosing in sheep,1,5 the kinetic profile of ABZ-SO was used for comparison of bioavailability of 2 oral ABZ products in the present study.

4.6 Ivermectin[24-26]

4.6.1 Liquid chromatographic assay of Ivermectin in human plasma for application to clinical pharmacokinetic studies

There is a need for an accurate, sensitive and selective high-performance liquid chromatography (HPLC) method for the quantitation of Ivermectin in human plasma that separates the parent drug from metabolites. Ivermectin and the internal standard, Moxidectin, were extracted from 0.2 ml of human plasma using Oasis HLB solid phase extraction cartridges. After extraction, fluorescent derivatives of Ivermectin and Moxidectin were made by reaction with trifluoroacetic anhydride and *N*-methylimidazole. Separation was achieved on a Alltech Ultrasphere C18 5µ column with a mobile phase composed of tetrahydrofuran:acetonitrile:water (40:38:22 v/v/v). Detection is by fluorescence, with an excitation of 365 nm and emission of 475 nm. The

retention times of Ivermectin and internal standard, Moxidectin are approximately 24.5 and 12.5 min, respectively. The assay is linear over the concentration range of 0.2–200 ng/ml of Ivermectin in human plasma (r = 0.9992, weighted by 1/concentration). Recoveries of Ivermectin are greater than 80% at all concentrations. The analysis of quality control samples for Ivermectin 0.2, 25, and 200 ng/ml demonstrated excellent precision with coefficient of variation of 6.1, 3.6 and 2.3%, respectively (n = 6). The method is accurate with all intra-day (n = 6) and interday (n = 12) mean concentration within 10% of nominal values at all quality control sample concentrations. Storage stability for 30 days at −80 °C and after three freeze–thaw cycles are within acceptable limits. The method separates Ivermectin from multiple less and more polar unidentified metabolites. This method is robust and suitable for clinical pharmacokinetic studies. The analytical procedure has been applied to a pharmacokinetic study of Ivermectin in healthy volunteers and to the analysis of plasma specimens from patients with disseminated strongyloidiasis.

4.6.2 Simultaneous Analysis of Ivermectin and Clorsulon in Injection Solutions by High Performance Liquid Chromatography with Confirmation by Liquid Chromatography Mass Spectrometry

Ivermectin and Clorsulon are antiparasitic agents used in veterinary medicine, sometimes used in combination. The purpose of this work was to develop a method for the simultaneous analysis of Ivermectin and Clorsulon in injection solutions by high-performance liquid chromatography (HPLC), to differentiate between two Ivermectin analogs, and to confirm structures by ultraviolet (UV) spectroscopy and mass spectrometry.

Methods: Ivermectin and Clorsulon were analyzed by HPLC with detection by UV at 244 nm and by negative ion atmospheric pressure chemical ionization liquid chromatography mass spectrometry (APCI LC-MS). Due to a significant difference in the polarity of Ivermectin and Clorsulon, a mobile solvent gradient and a short (4.6 mm x 50 mm) C-18 column were used to provide separation with a reasonable analysis time.

4.6.3 Determination of Ivermectin stability by high-performance thin-layer Chromatography

A rapid, sensitive and stability signifying high-performance thin-layer chromatographic (HPTLC) method was developed and validated for the quantitative estimation of Ivermectin

(IVM) as a bulk drug and in pharmaceutical formulations. The separation was achieved on Lichrospher TLC aluminum plates pre-coated with silica gel 60F-254 (20cm×10cm×200cm) using *n*-hexane:acetone:ethylacetate (6.5:3.5:0.1 v/v/v) as mobile phase. The densitometric analysis was carried out at 247 nm wavelength.

Compact spots of IVM were found at *R*f = 26.02. For proposed procedure, linearity (*r*2 = 0.9989), limit of quantification (24.9 ng spot^{-1}), limit of detection (8.22 ng spot^{-1}) recovery (98.25–100.16%), and inter as well intra-day precision (≤2.21) was found to be satisfactory. We have synthesized polymeric nanoparticles encapsulated formulation of Ivermectin (IVM-NPs); utilizing micel large aggregates of crosslinked random copolymer Nisopropylacrylamide (NIPAAM) with N-vinyl-2-pyrrolidone (VP) and polyethyleneglycol monoacrylate (PEG-A) for lymphatic targeting and it was also quantified by the developed method. IVM and formulations were subjected to acid and alkali hydrolysis, oxidation and photo-degradation. The drug undergoes degradation under acidic, basic, light and oxidation conditions. This indicates that the drug is susceptible to acid- base hydrolysis, oxidation and photo-oxidation and the developed method is selective for quantifying IVM even in the presence of degradatnts. The method was applicable for routine analysis and stability testing of IVM in pharmaceutical drug delivery systems. As the method could effectively separate the said drug from its degradation products, it can be employed as a stability indicating one

4.7 Racecadotril [27-30]

4.7.1 New spectrophotometric methods for the determination of Racecadotril in bulk drug and capsules

Two simple and sensitive spectrophotometric methods (A and B) for the determination of Racecadotril in bulk drugs and pharmaceutical formulations are described. In method A, methanol was used as solvent and shows absorption maximum at 231 nm. In method B, the solvent used was acetonitrile:water in the ratio of 1:3 and shows absorption maximum at 232 nm. The Beer's law range for method A is 25-100 mg/ml and 20-80 mg/ml for method B. When capsules dosage forms were analyzed, the results obtained by the proposed methods are in good agreement with the labeled amounts and the results were validated statistically.

There is a need for an accurate, sensitive and selective high-performance liquid chromatography (HPLC) method for the quantitation of Ivermectin in human plasma that separates the parent

drug from metabolites. Ivermectin and the internal standard, Moxidectin, were extracted from 0.2 ml of human plasma using Oasis HLB solid phase extraction cartridges. After extraction, fluorescent derivatives of Ivermectin and Moxidectin were made by reaction with trifluoroacetic anhydride and *N*-methyl-imidazole. Separation was achieved on a Alltech Ultrasphere C18 5µ column with a mobile phase composed of tetrahydrofuran:acetonitrile:water (40:38:22 v/v/v). Detection is by fluorescence, with an excitation of 365 nm and emission of 475 nm. The retention times of Ivermectin and internal standard, Moxidectin are approximately 24.5 and 12.5 min, respectively. The assay is linear over the concentration range of 0.2–200 ng/ml of Ivermectin in human plasma (r = 0.9992, weighted by 1/concentration). Recoveries of Ivermectin are greater than 80% at all concentrations. The analysis of quality control samples for Ivermectin 0.2, 25, and 200 ng/ml demonstrated excellent precision with coefficient of variation of 6.1, 3.6 and 2.3%, respectively (n = 6). The method is accurate with all intra-day (n = 6) and interday (n = 12) mean concentration within 10% of nominal values at all quality control sample concentrations. Storage stability for 30 days at −80 °C and after three freeze–thaw cycles are within acceptable limits. The method separates Ivermectin from multiple less and more polar unidentified metabolites. This method is robust and suitable for clinical pharmacokinetic studies. The analytical procedure has been applied to a pharmacokinetic study of Ivermectin in healthy volunteers and to the analysis of plasma specimens from patients with disseminated strongyloidiasis. A simple, precise and rapid RP-HPLC method was developed for the determination of Racecadotril in a pharmaceutical formulation using Gemfibrozil as internal standard. Ratio of the peak area of analyte to internal standard was used for quantification. The chromatographic separation was carried out by using a Reverse Phase C18 column (BDS-Hypersil). The mobile phase consisting of a mixture of 20 mM phosphate buffer (pH 3.5) and acetonitrile in the ratio of (40:60) with detection at 230 nm at a flow rate of 1 ml/min was employed. The method was statistically validated for linearity, accuracy and precision. The elution time was 6.9 min for Racecadotril and 9.8 min for Gemfibrozil. The simplicity and accuracy of the proposed method ensures its use in routine quality control analysis of pharmaceutical formulations.

4.7.2 Development and validation of a rapid RP-HPLC method for the determination of Racecadotril in formulation

A simple rapid specific precise and accurate reverse phase high performance liquid chromatographic method was developed for the determination of Racecadotril (RACE) in sachet dosage forms using Atorvastatin as internal standard. Ratio of the peak area of analyte to internal standard was used to calculate for quantification. A phenomenex-Luna RP-18, 5mm column having 250x4.6 mm i.d. in isocratic mode, with mobile phase containing acetonitrile: 0.05M phosphate buffer (potassium dihydrogen orthophosphate): triethylemine (80:19.95:0.05) adjusted to pH 3.95 using orthophosphoric acid. The flow rate was 1.0ml/min and effluents were monitored at 231nm. The retention times of Atorastatin calcium and Racecadotril were 3.453 min and 4.210 min respectively. Linearity was observed over concentration range of 10-80 mg/ml. The recovery of Racecadotril was found to be in the range of 99.6-100.5%. The proposed method was validated successfully and applied to the estimation of a Racecadotril in sachet dosage forms.

4.7.3 Spectrophotometric and spectrofluorimetric methods for determination of Racecadotril

Two accurate and sensitive spectrophotometric and spectrofluorimetric methods were developed for determination of Racecadotril. In the first method reduction of Fe3+ into Fe2+ in presence of o-phenanthroline by Racecadotril to form a stable orange-red ferroin chelate [Fe- (Phen)3]2+ was the basis for its determination . The absorbance at 510 nm was measured and linear correlation was obtained in the concentration range of 2.5 – 25μg mL^{-1}. In the second method the native fluorescence of Racecadotril in acetonitrile solvent at λ = 319 nm when excitation was at 252 nm is used for its determination. Linear correlation was obtained in the concentration range of 50 to 500 ng mL-1. The proposed methods were applied for determination of Racecadotril in bulk powder with mean accuracy of 100.39±1.239 for the spectrophotometric method and 100.09± 1.042 for the spectrofluorimetric method. The proposed methods were successfully applied for determination of Racecadotril in its pharmaceutical dosage form.

4.7.4 Determination of Racecadotril and its impurities by HPLC

A HPLC method was established for the determination of Racecadotril and its impurities.METHODS A ODS column was used and the mixture of acetonitrile□ potassium

dihydrogen phosphate (KH_2PO_4)solution (70∶ 30) was used as the mobile phase.The detection wavelength was 210 nm.

RESULTS: The liner range of the Racecadotril was 0.08～0.24 $mg \cdot mL^{-1}$ and the regression equation was y=15847x+3873 (r^2=0.9997). The liner range of benzylthiorphan disulphide was 2.40～21.56$\mu g \cdot mL^{-1}$ and the regression equation was Y=1826x+46 (r^2=0.9999). The measurable lowest limit was 1$\mu g \cdot mL^{-1}$.The average recovery was 100.0%.

CONCLUSION: The method was convenient, accurate and specific.

4.8 Fluocinolone acetonide [31-33]

4.8.1 Development of a reversed-phase HPLC method for analysis of fluocinolone acetonide in gel and ointment

Fluocinolone acetonide and additives in gel. Drugs were chromatographed on a C_{18} reversed-phase column with 55:45 (v/v) methanol–water as mobile phase and detection at 238 nm. Solution concentrations were measured on a weight basis to avoid the use of an internal standard. The method was statistically validated for linearity, accuracy, precision, and selectivity. Linearity for assay of fluocinolone acetonide, methyl 4-hydroxybenzoate (nipagin M), and propyl 4-hydroxybenzoate (nipagin P) were confirmed over the ranges 0.5–30, 5–200, and 10–120 $\mu g.mL^{-1}$, respectively.

4.8.2 Determination of fluocinolone acetonide in pharmaceutical preparations by differential-pulse polarography

A simple differential-pulse polarographic method has been developed for the assay of fluocinolone acetonide in cream, gel and ointment. A 0.03 M solution of tetraethylammonium hydroxide containing 50%*V*/*V* of methanol at pH 11 was used as the supporting electrolyte. Commonly used preservatives and bases for the preparations were found not to interfere in the determination. A calibration graph was prepared by the method of standard additions. Results obtained using the proposed and official procedures were comparable.

4.8.3 Analysis of creams (objective reference): Quantitative determination of drugs in creams by UV spectrophotometry

The uv absorbing properties of the components of the cream bases as described in the Formulary of the Dutch Pharmacists were investigated. DirectUV spectrophotometric determinations without any clean-up steps appeared to be possible for a number of drugs (*e.g.* tripelennamine HCl,

tretinoin, salicylic acid, methyl salicylate, resorcinol, clioquinol), with the help of a solvent mixture in which the cream samples dissolved completely to yield clear solutions. Correcting for the contribution to theUV absorbance by the preservative is sometimes necessary and can be achieved by measuring the absorbance at two wavelengths. The determination of chlorhexidine, as an example of a basic drug withUV absorbing properties which prevent direct measurements of the solution of the cream samples, could be achieved after removal of the interfering compounds by a simple liquid-liquid extraction.

4.9 Hydrocortisone acetate [34-37]

4.9.1 Optimization and validation of an RP-HPLC method for analysis of hydrocortisone acetate and lidocaine in suppositories.

An RP-HPLC method has been optimized and validated for the simultaneous determination of hydrocortisone acetate and of lidocaine in suppositories. For the method optimization, response surface methodology was applied, and the obtained model was tested using analysis of variance. The optimal separations were conducted on a Beckman-Coulter 150 x 4.6 mm, 5μm particle-size column at 20 ^{O}C. The mobile phase was methanol-water (65 + 35, v/v), pH adjusted to 2.5 with 85% orthophosphoric acid, with a flow rate of 1.0 ml/min. UV detection was performed at 250 nm. Phenobarbital was used as an internal standard. The method was validated for selectivity, linearity, precision, and robustness.

4.9.2 Analysis of hydrocortisone acetate ointments and creams by high-performance liquid chromatography.

High-performance liquid chromatographic (HPLC) methods for the analysis of hydrocortisone containing ointments and creams have been investigated. A method which uses a silica column and involves a minimum of sample pre-treatment has been shown to compare favourably with the triphenyltetrazolim chloride method of the British Pharmacopoeia. For hydrocortisone ointments the HPLC procedure provides results of equivalent precision and has advantages with respect to the time taken for each analysis and specificity. Application of the method to the analysis of hydrocortisone creams has been explored and the deviation between the HPLC and colorimetric method requires further investigation.

4.9.3 Optimization of a Selective Liquid Chromatography Procedure for Hydrocortisone Acetate, Hydrocortisone Alcohol and Preservatives in a Pharmaceutical Emulsion.

An accurate, reproducible and specific stability-indicating method for the high performance liquid chromatography (HPLC) assay of hydrocortisone acetate, hydrocortisone alcohol, methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate in a pharmaceutical suspension is described. An investigation of several column phases was undertaken and a Zorbax SB-Phenyl column gave the best selectivity and specificity due to the π-π interactions between the analytes and stationary phase. All the components were fully resolved in less than 15 min under isocratic conditions using UV detection at 254 nm with a water-methanol mobile phase. The stability-indicating method was validated over the linearity range of 25% to 150% of the nominal concentrations of each analyte. Nominal concentrations were hydrocortisone acetate (10% *w*/*w*), hydrocortisone alcohol (0.2% *w*/*w* with respect to hydrocortisone acetate), methyl p-hydroxybenzoate (0.1% *w*/*w*) and propyl p-hydroxybenzoate (0.01% *w*/*w*) respectively.

4.9.4 Simultaneous determination of methylparaben, propylparaben, hydrocortisone acetate and its degradation products in a topical cream by RP-HPLC.

A novel reversed-phase high-performance liquid chromatographic method with UV spectrophotometric detection was developed and validated for the determination of compounds in topical cream. The method describes determination of active component hydrocortisone acetate (HCA), its degradation products hydrocortisone (HC) and cortisone acetate (occurring in formulation after long-term stability tests) and two preservatives presented in the cream-methylparaben and propylparaben, using dexamethasone as an internal standard. The chromatographic separation was performed on a 5 μm SUPELCO Discovery C18 125×4-mm ID column. The optimised mobile phase for separation of all the compounds consists of methanol, acetonitrile and water (15:27:58, v/v/v), with the analysis time less than 13 min. The method was applicable for routine analysis (assays and stability tests) of active compound HCA, preservatives and degradation products in pharmaceutical product—topical cream Hydrocortizone cream 1%.

4.9.5 Comparison of HPLC and multivariate regression methods for hydrocortisone and lidocaine analysis of pharmaceutical preparation.

A reverse-phase high-performance liquid chromatographic (HPLC) method to determine hydrocortisone acetate, hydrocortisone hemisuccinate and lidocaine is described in this paper. The separation was made in a LichrCART C_{18} column using a methanol-NaH(2)PO(4)/Na(2)HPO(4) (0.1 mol L(-1)) (pH=4.5) buffer solution as a mobile phase in isocratic mode (60:40 (v/v)). The mobile phase flow rate and the sample volume injected were 1 mL min(-1) and 20 micro L, respectively. The detection was made with a diode-array detector measuring at the maximum for each compound. Quantification limits ranging from 0.18 to 0.84 micro g L(-1) were obtained when the peak area was measured. The method was applied in pharmaceutical formulations that were compared with those obtained by through multivariate regression spectrophotometry and micellar capillary electrophoresis (MEKC). HPLC results are in accordance with the results obtained by MEKC. The spectrophotometric method was suitable only for synthetic samples.

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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 80ºC 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 (25ºC ± 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**

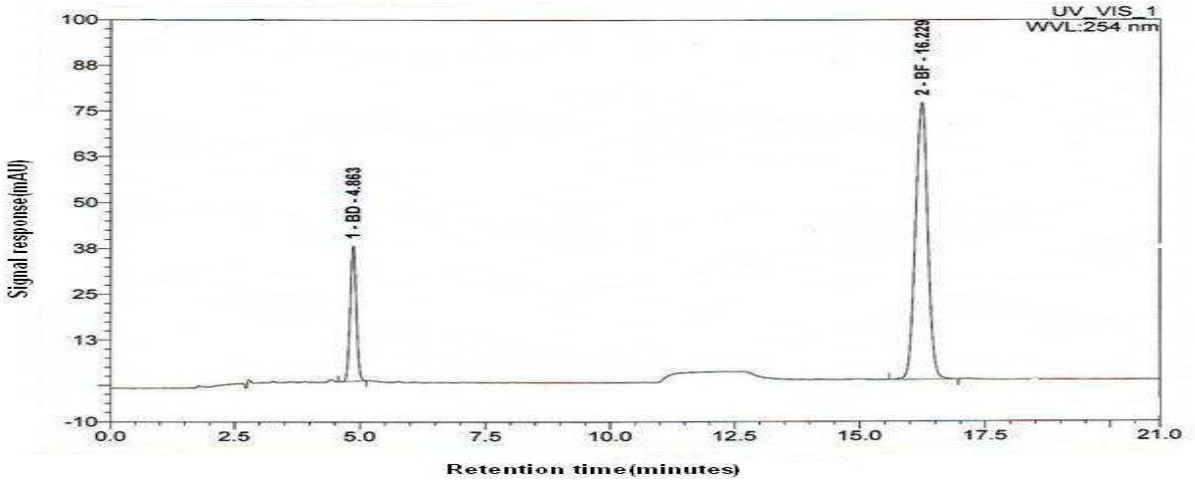

Fig.5.1.1.1: Chromatogram of Betamethasone dipropionate and Butenafine hydrochloride at 254 nm ((Betamethasone dipropionate 10µg/mL and Butenafine hydrochloride 200µg/mL)

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 22ºC and 0.50 and 1.13% for betamethasone dipropionate and butenafine hydrochloride, respectively, at 27ºC.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**

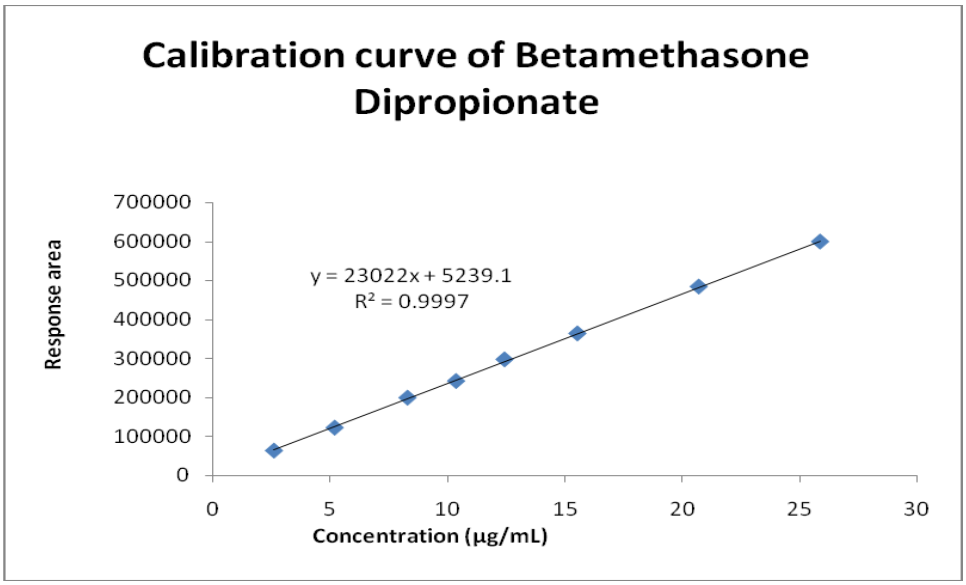

Fig. 5.1.1.2: Calibration Curve for betamethasone dipropionate.(2.5-25µg/ml)

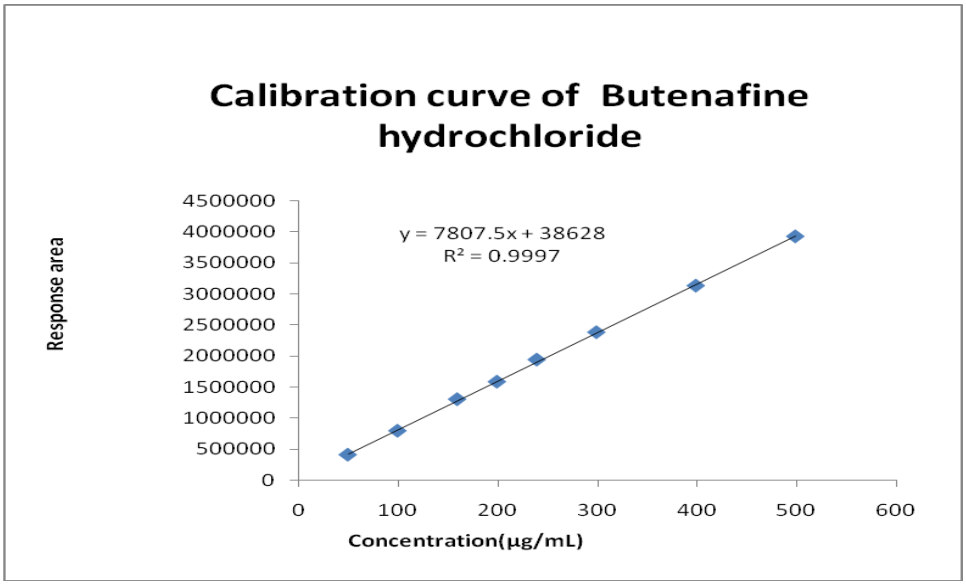

Fig.5.1.1.3 Calibration Curve for butenafine hydrochloride (50-500µg/ml).

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**

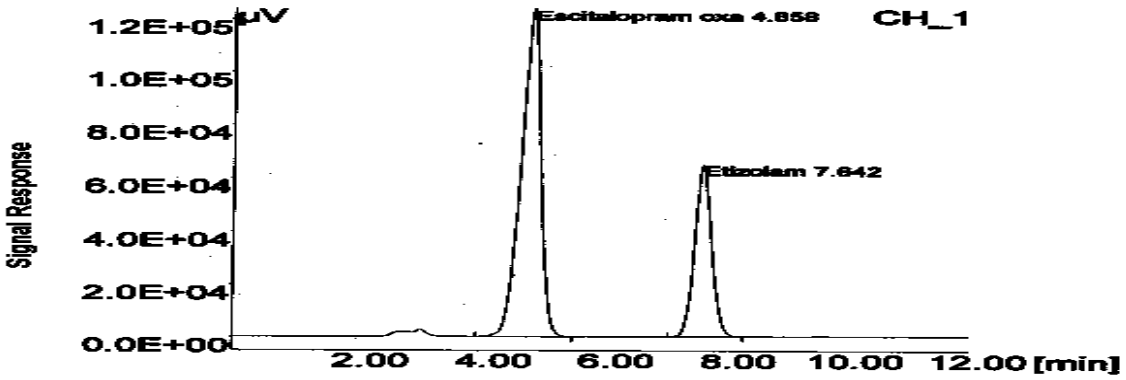

Fig.5.1.2.1Chromatogram of Escitalopram oxalate and Etizolam at 254nm (Escitalopram oxalate 200µg/ml and Etizolam 20µg/ml)

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**

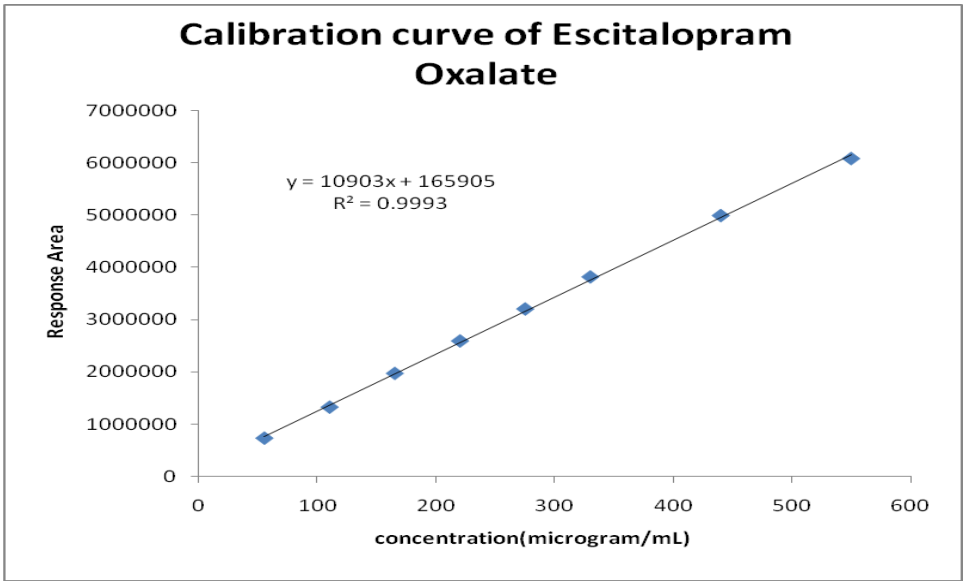

Fig.5.1.2.1 Calibration curve diagram for Escitalopram oxalate(55-500µg/ml).

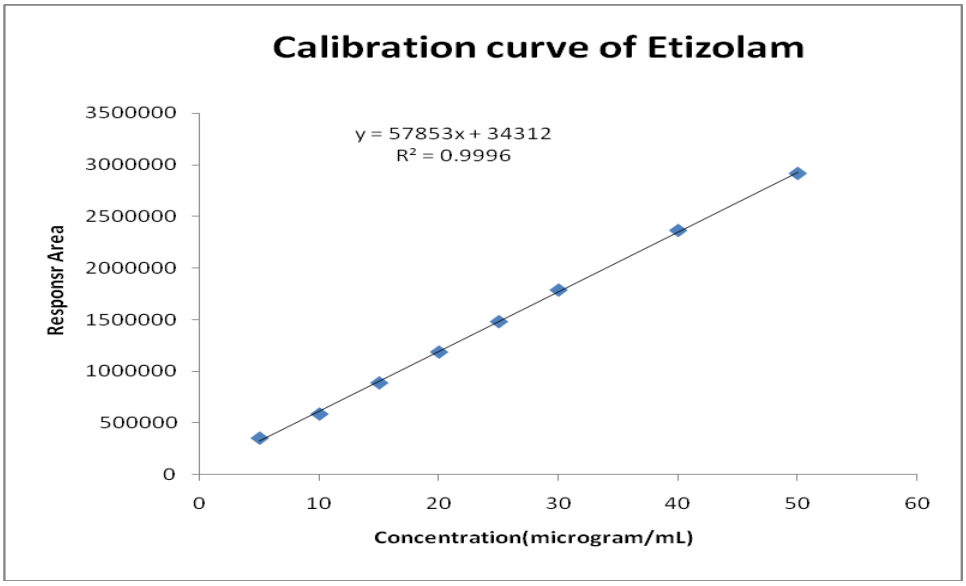

Fig.5.1.2.3 Calibration curve diagram for Etizolam(5-50µg/ml).

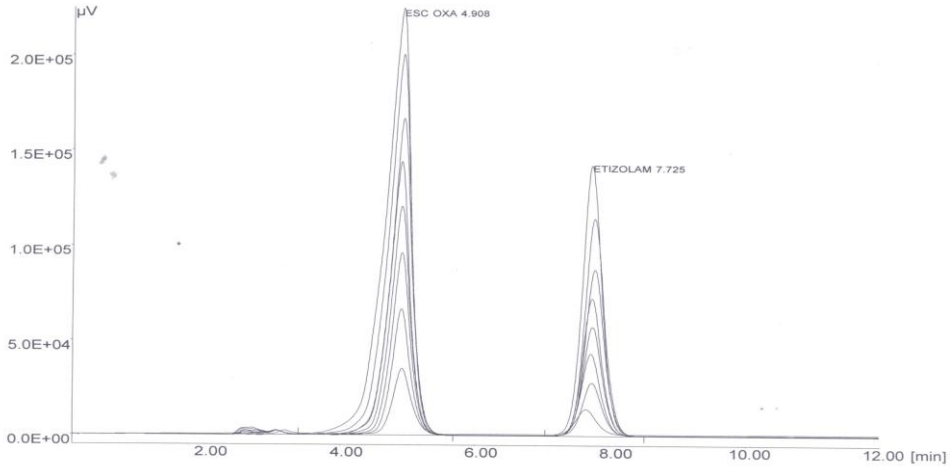

Fig.5.1.2.4 Overlay View of Linearity Chromatograms of Escitalogram Oxalate and Etizolam

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 80ºC 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**

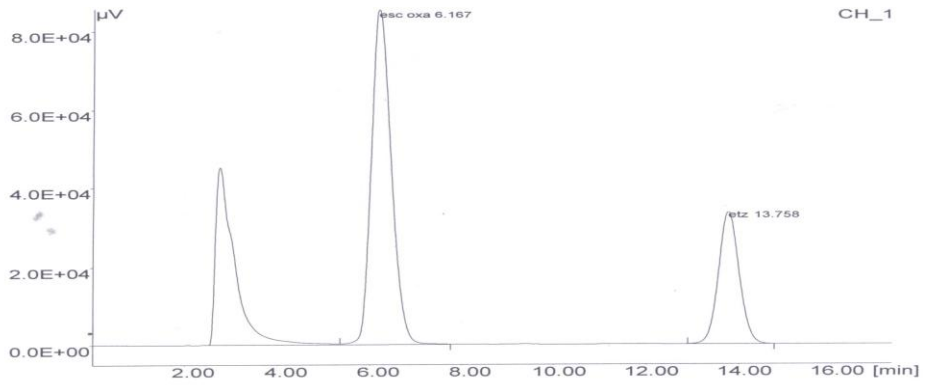

Fig.5.1.3.1Chromatogram of Escitalopram oxalate and Etizolam at 254nm (Esitalopram oxalate 200 µg/ml and Etizolam 20µg/ml)

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.

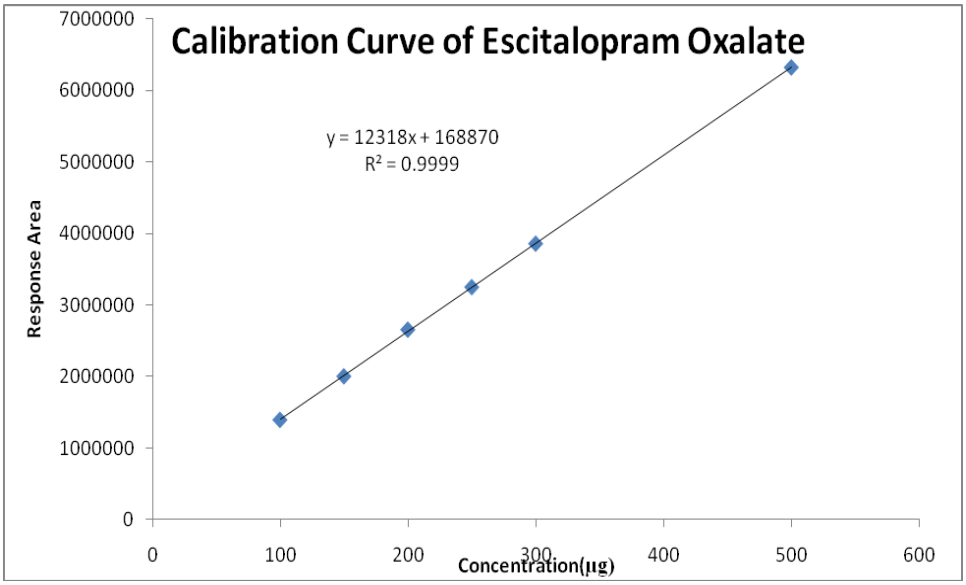

Fig.5.1.3.2 Calibration curve diagram for Escitalopram oxalate(55-550µg/ml)

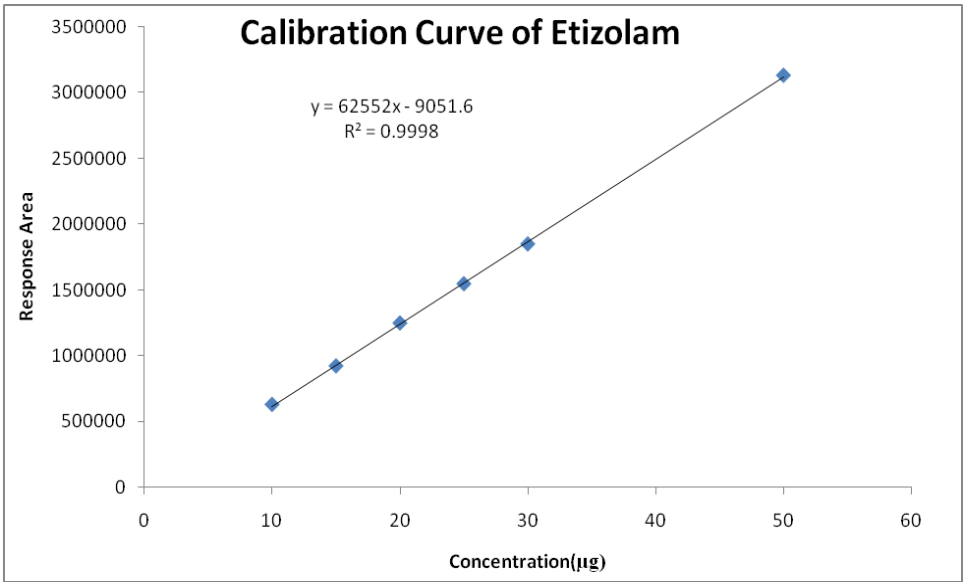

Fig.5.1.3.3 Calibration curve diagram for Etizolam(10-50µg/ml).

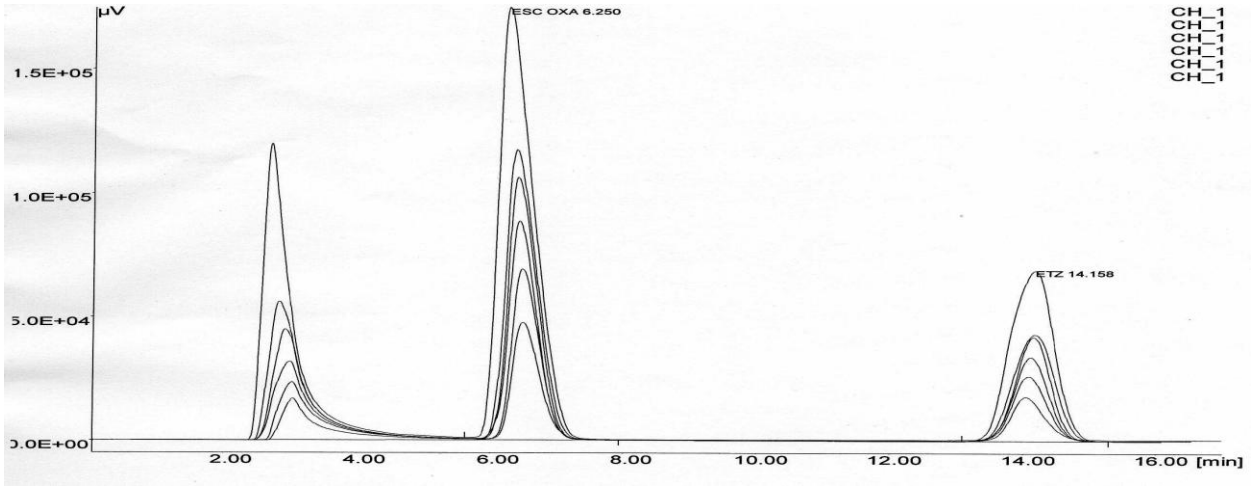

Fig.5.1.3.4 Overlay chromatograms of Escitalopram Oxalate and Etizolam

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	95.09397	94.25155	94.11622	94.03655	93.50348	**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%.

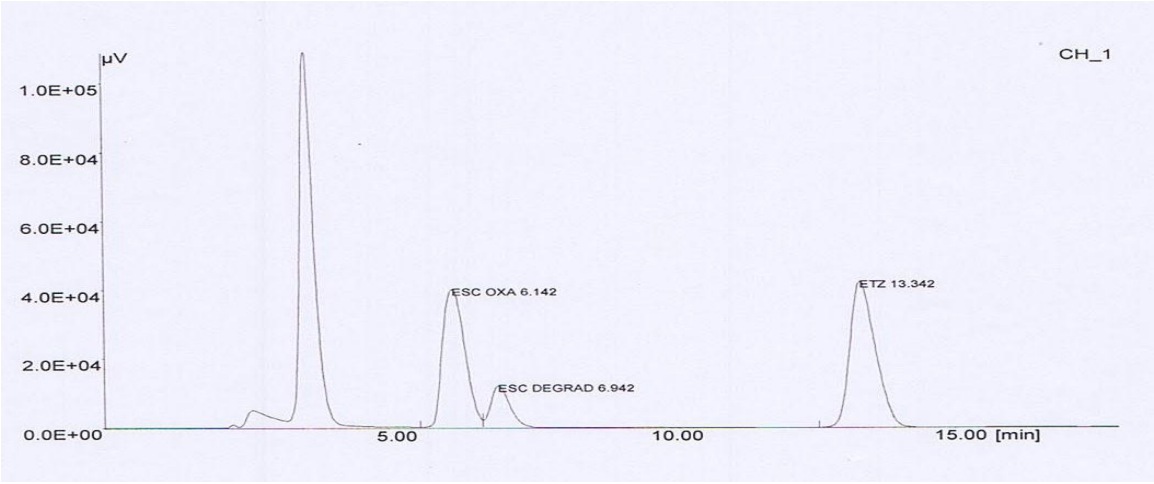

Fig 5.1.3.5 Degradation of Escitalopram under basic condition (1N NaOH)

Oxidative condition

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

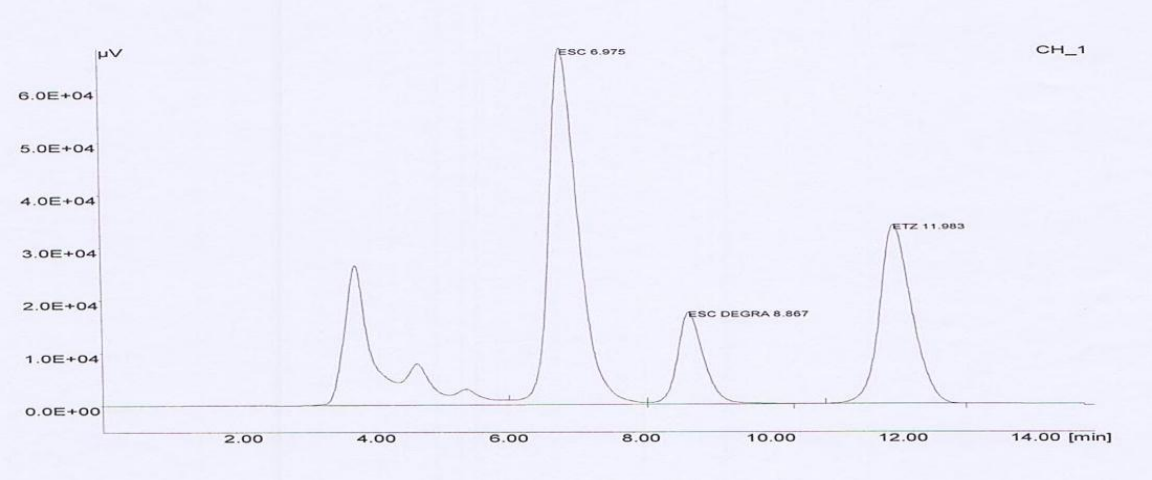

Fig 5.1.3.6 Degradation of Escitalopram under oxidative condition

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.

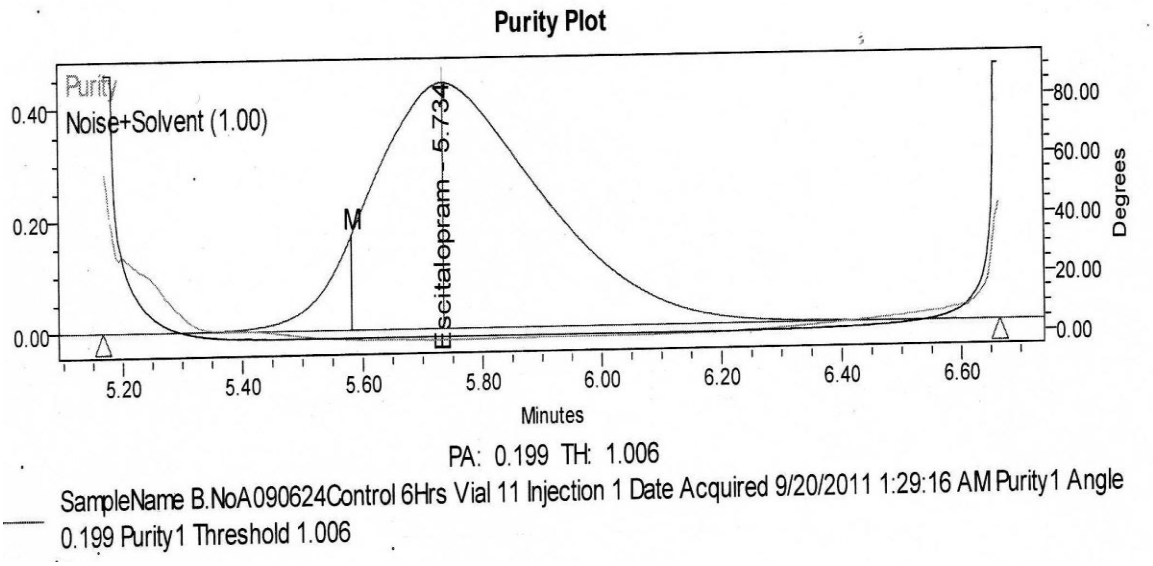

Fig.5.1.3.7 Peak Purity of Esitalopram Oxalate in Control Sample.

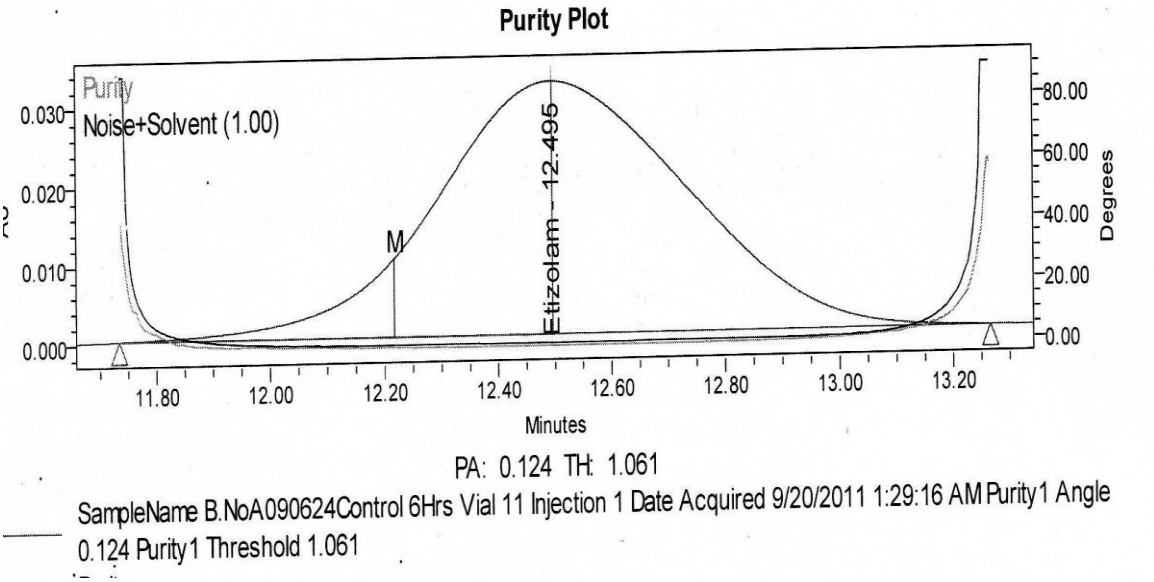

Fig.5.1.3.8 Peak Purity of Etizolam in Control Sample

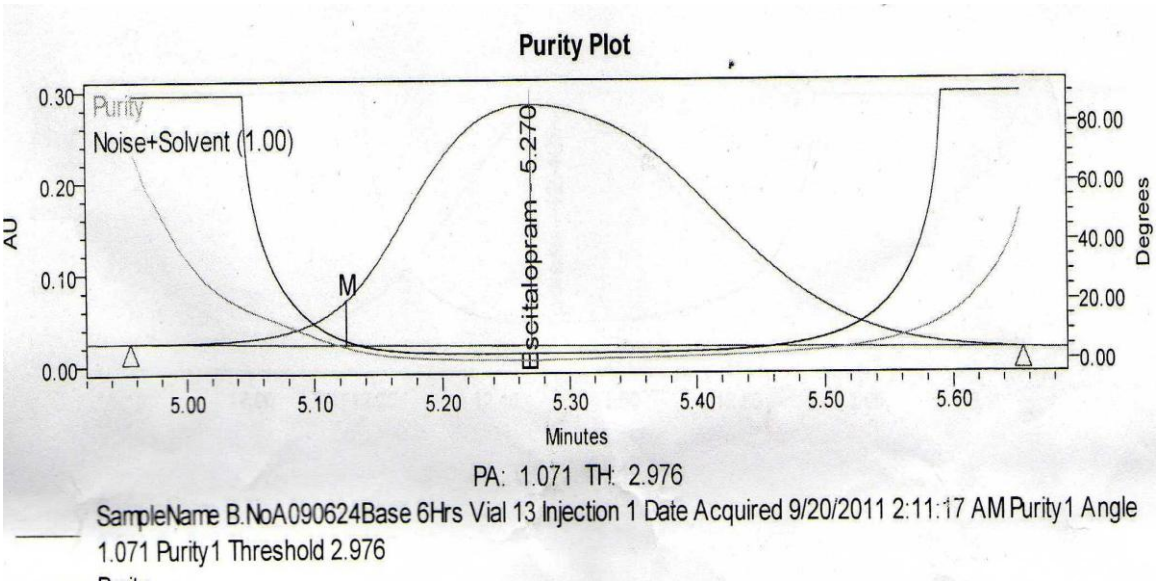

Fig.5.1.3.9 Peak Purity of Escitalopram Oxalate in alkaline condition

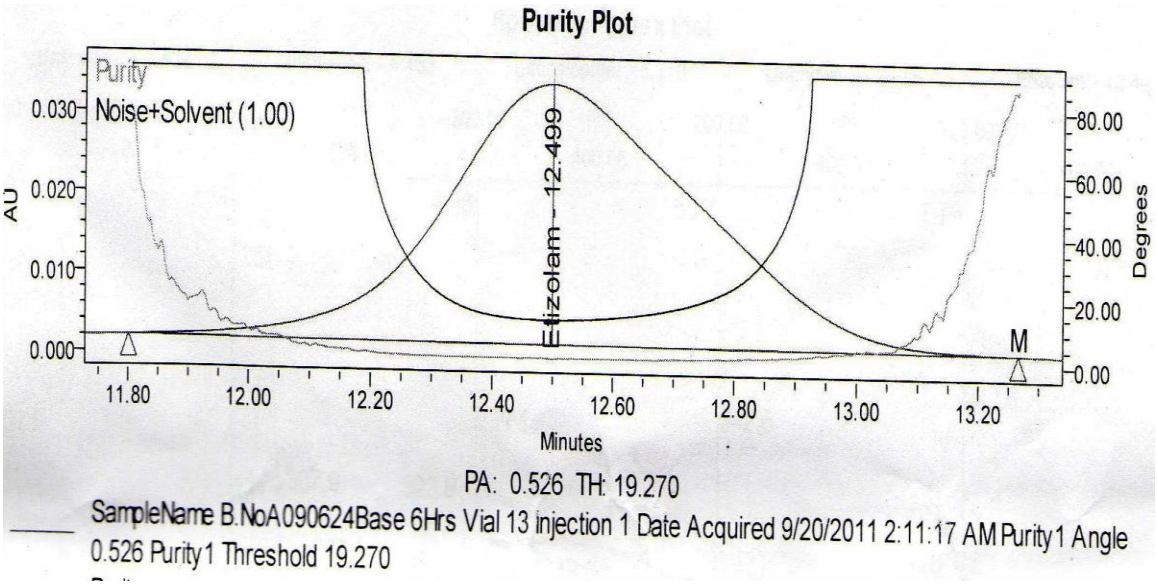

Fig.5.1.3.10 Peak Purity of Etizolam in alkaline condition

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:

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

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**

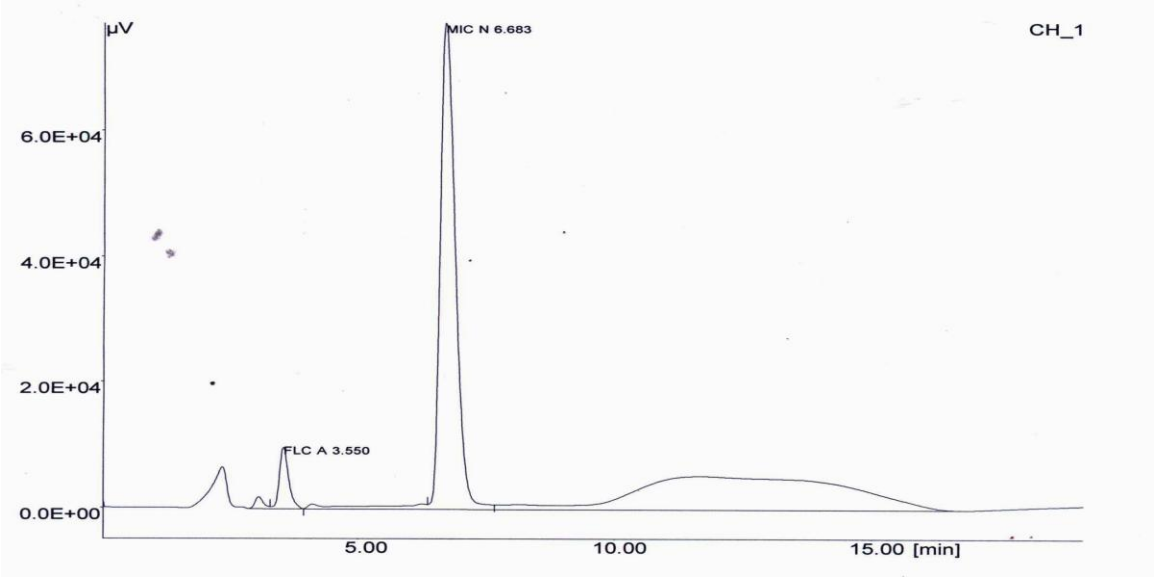

Fig.5.1.5.1: Chromatogram of Fluocinolone Acetonide and Miconazole Nitrate 238 nm (Fluocinolone acetonide 2µg/ml andMiconazole nitrate 400µg/ml)

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

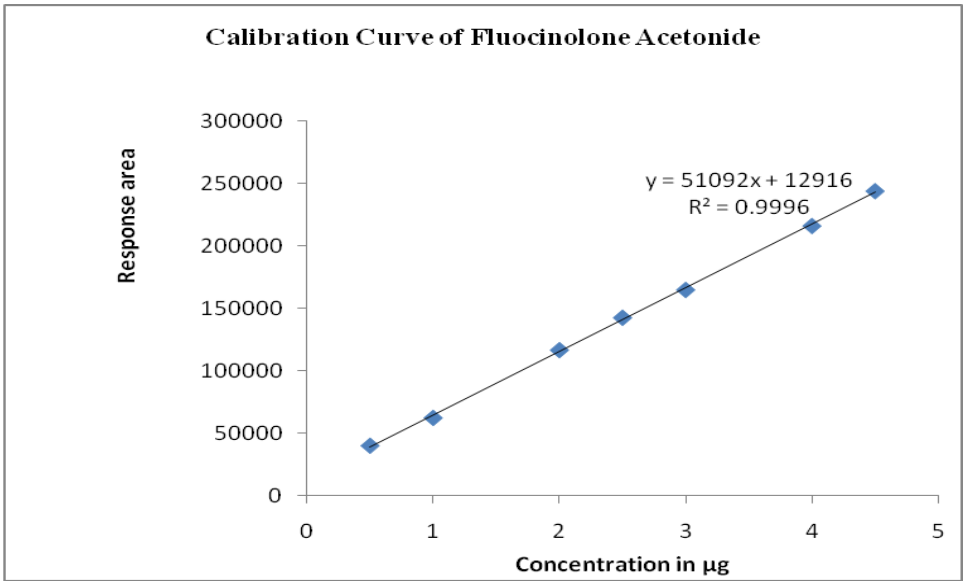

Fig.5.1.5.2:Calibration Curve for fluocinolone acetonide (0.5-4.5µg/ml))

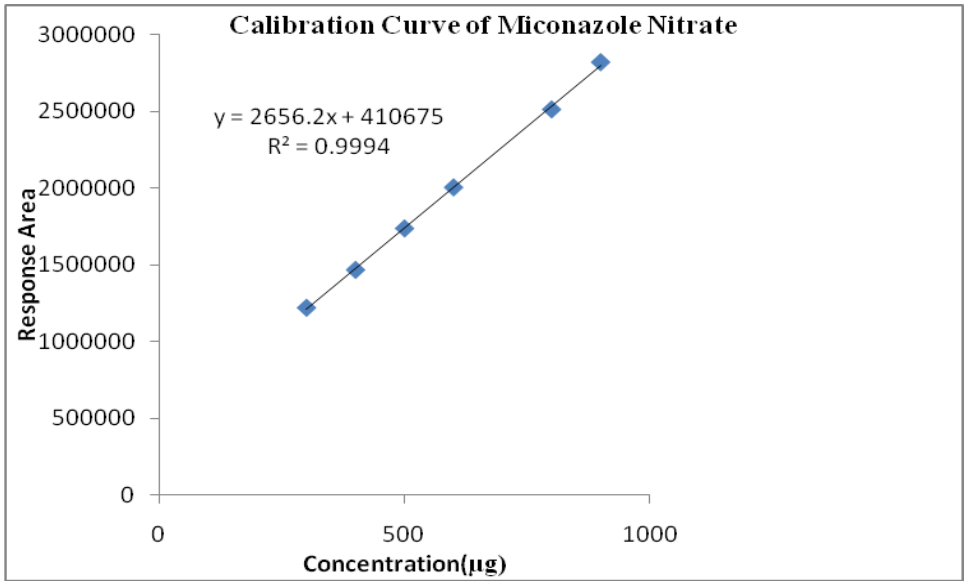

Fig. 5.1.5.3: Calibration Curve for Miconazole Nitrate (300-900µg/ml)

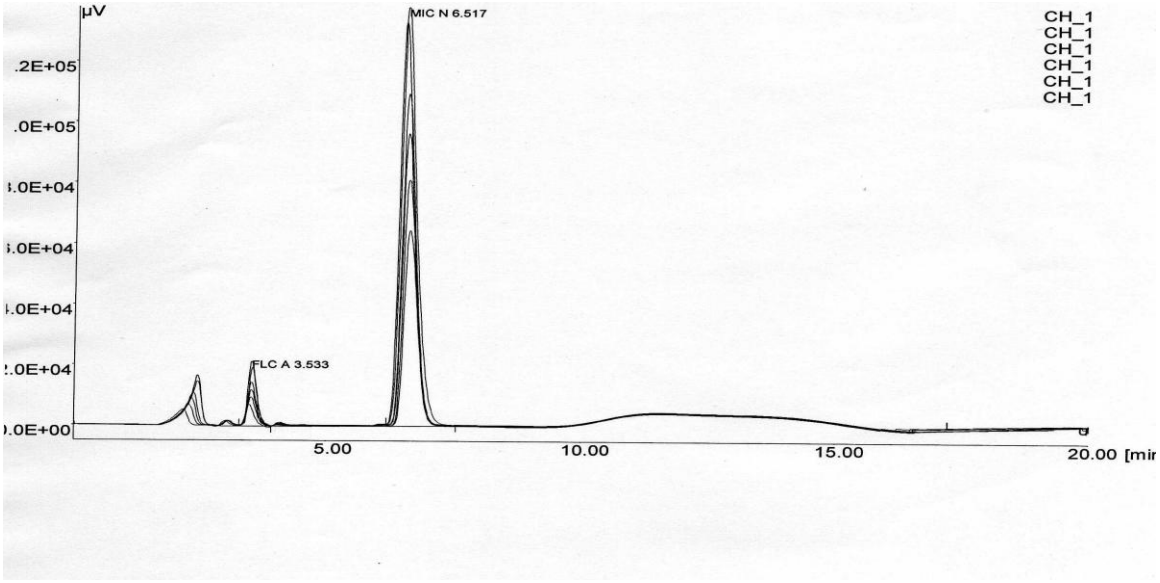

Fig:5.1.5.4 Overlay chromatogram of fluocinolone acetonide and miconazole nitrate

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**

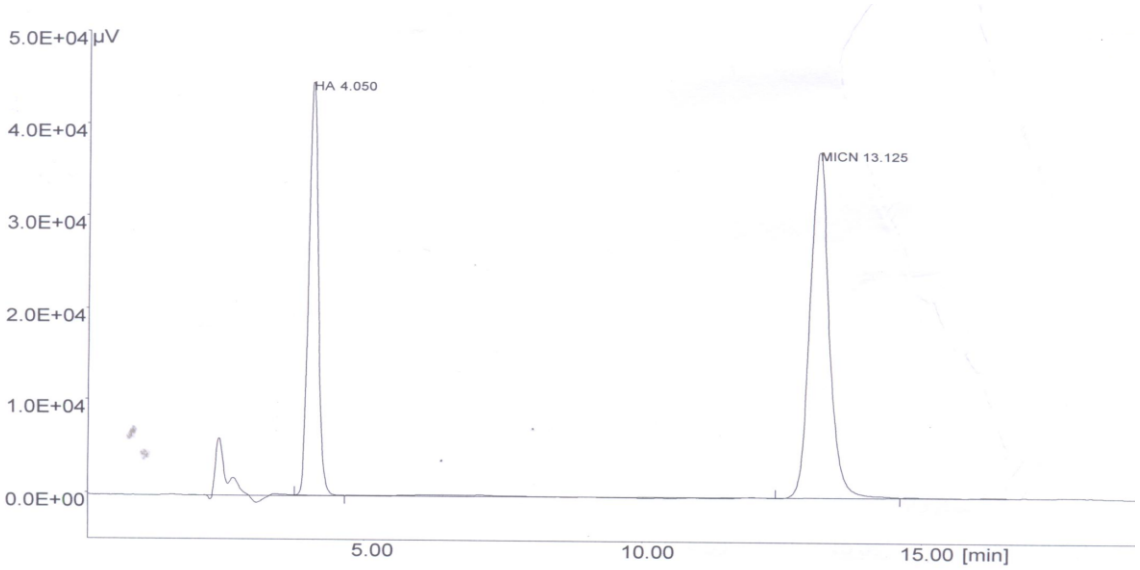

Fig.5.1.6.1 Chromatogram of Hydrocortisone acetate and and Miconazole nitrate at 225nm (Hydrocortisone acetate 10µg/ml,miconazole nitrate 20µg/ml)

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

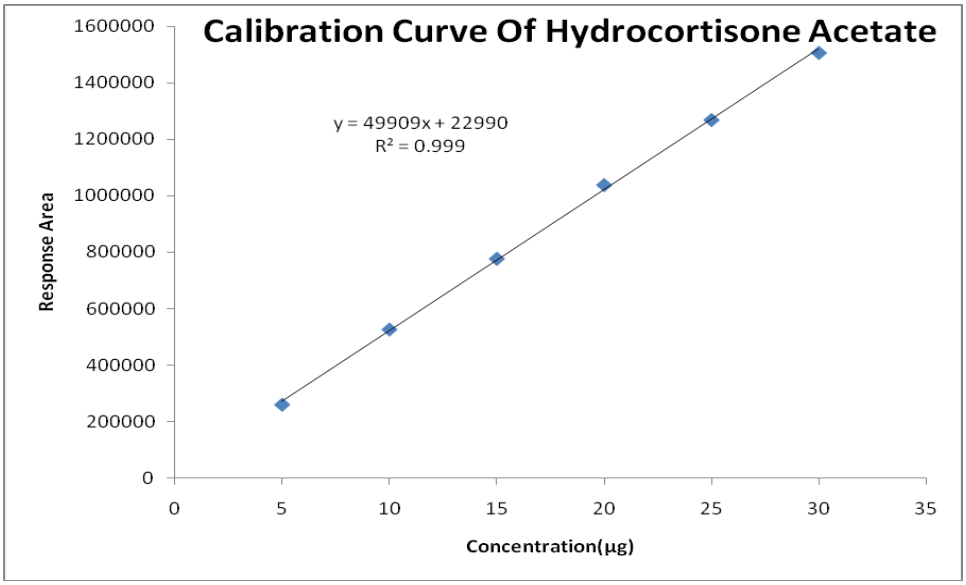

Fig. 5.1.6.2 Calibration curve diagram for Hydrocortisone acetate(5-30µg/ml)

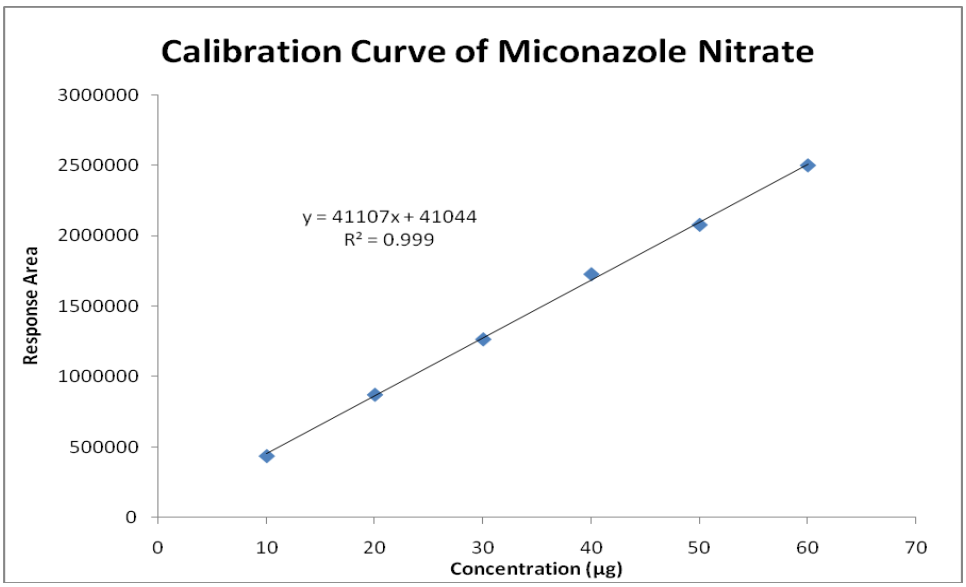

Fig. 5.1.6.3 Calibration curve diagram for Miconazole nitrate (10-60µg/ml)

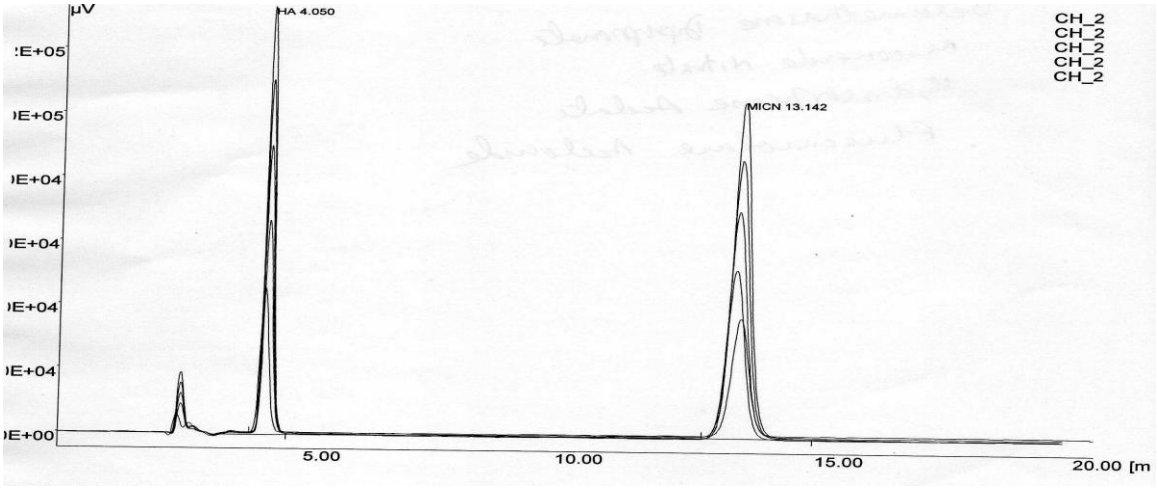

Fig. 4.1.6.3 Overlay calibration Chromatogram for Miconazole nitrate and hydrocortisone acetate

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 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 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**

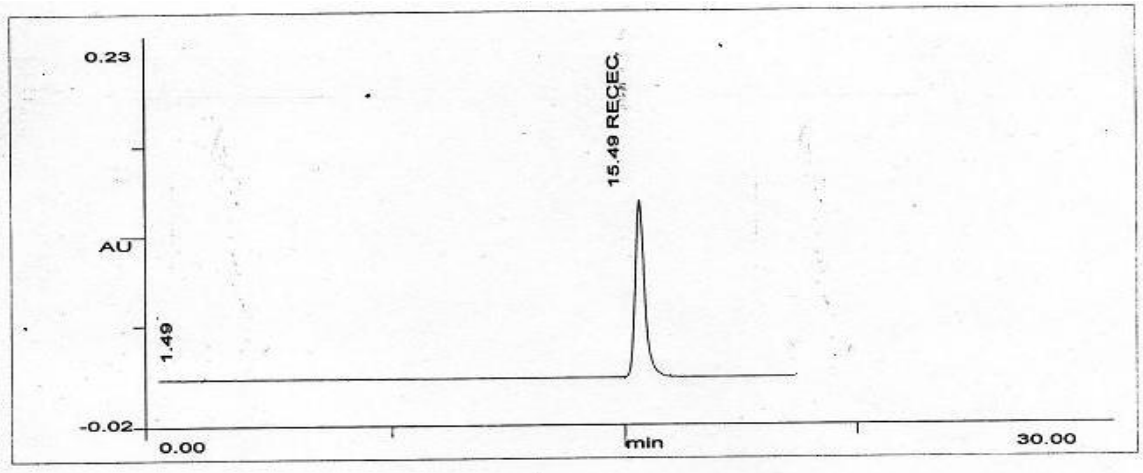

Fig:5.1.7.1 Representative chromatogram of racecadotril.

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

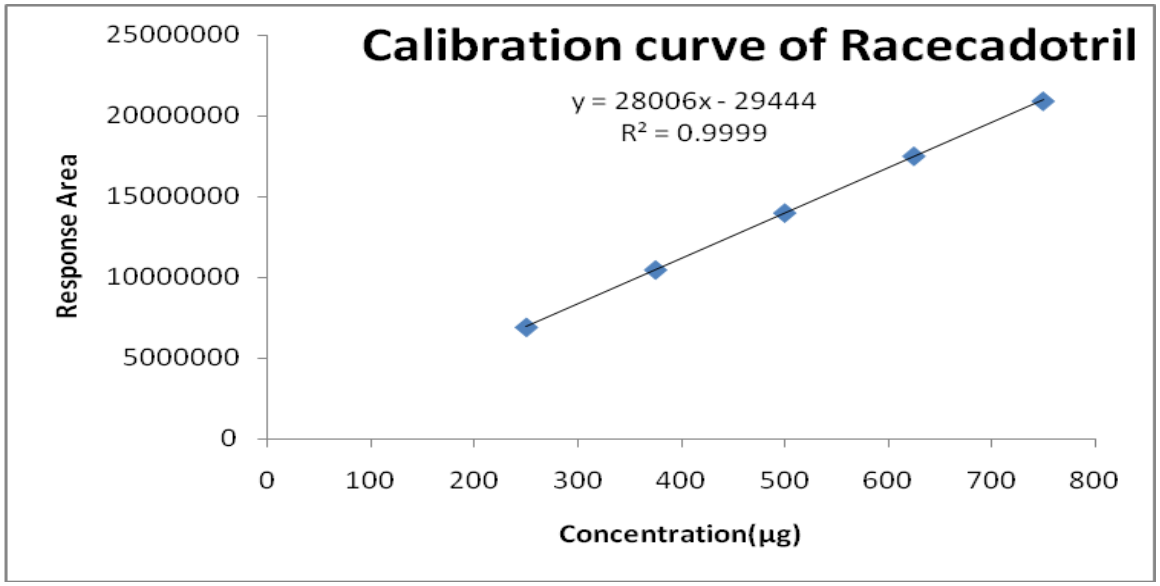

Fig:5.1.7.2 Calibration curve of Racecadotril (250-750µg/ml)

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	Sample 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^oC)	48	98.60	01.40
Heat ($105\ ^0C$)	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

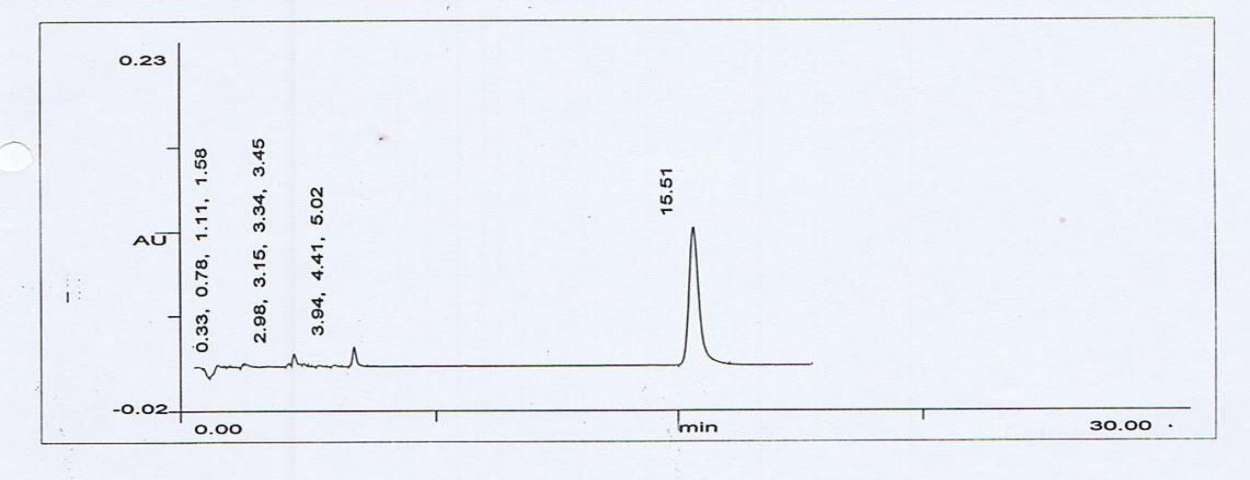

Fig: 5.1.7.3 Degradation of Racecadotril in alkali (0.1 N Na OH)

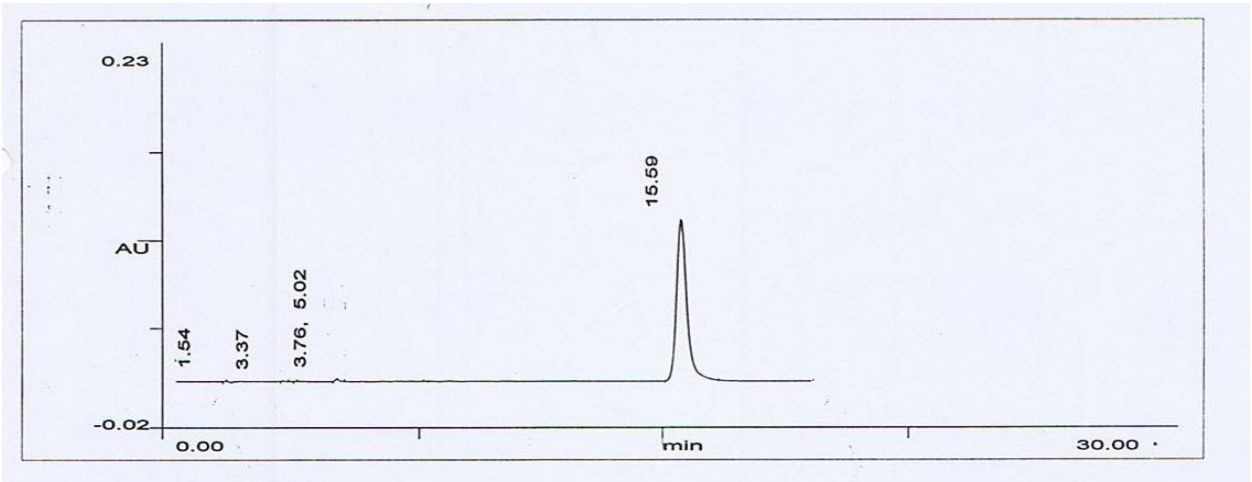

Fig:5.1.7.4 Thermal Degradation of Racecadotril

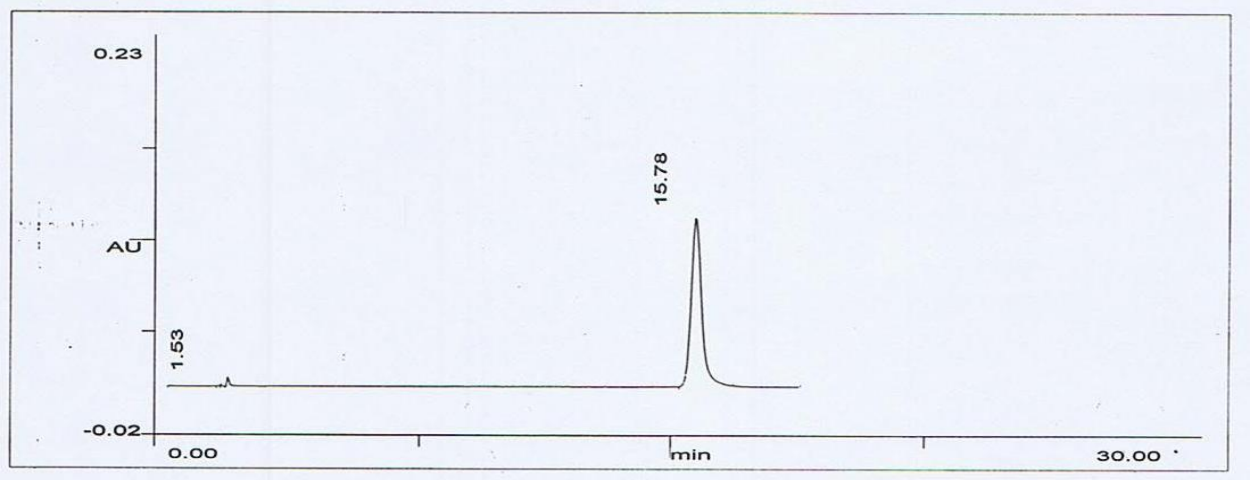

Fig:5.1.7.5 Degradation of racecadotril with hydrogen peroxide

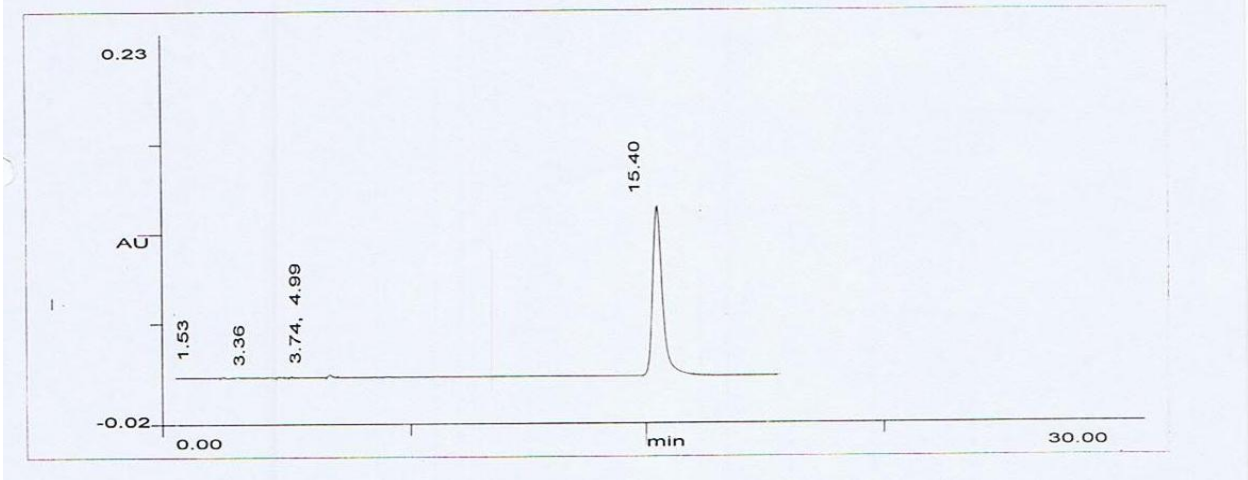

Fig:5.1.7.6 Degradation of racecadotril in UV light

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 x σ/S LOQ = 10 x σ/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

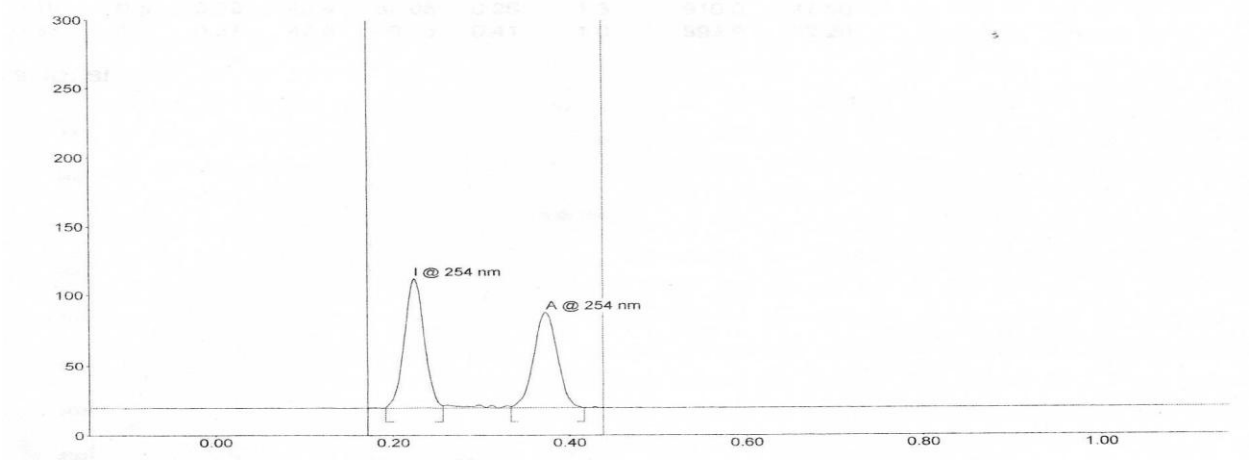

Fig.5.2.1.1 HPTLC Chromatogram of Ivermectin and Albendazole at 254 nm (Albendazole 160 ng/µl and ivermectin 260ng/µl)

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

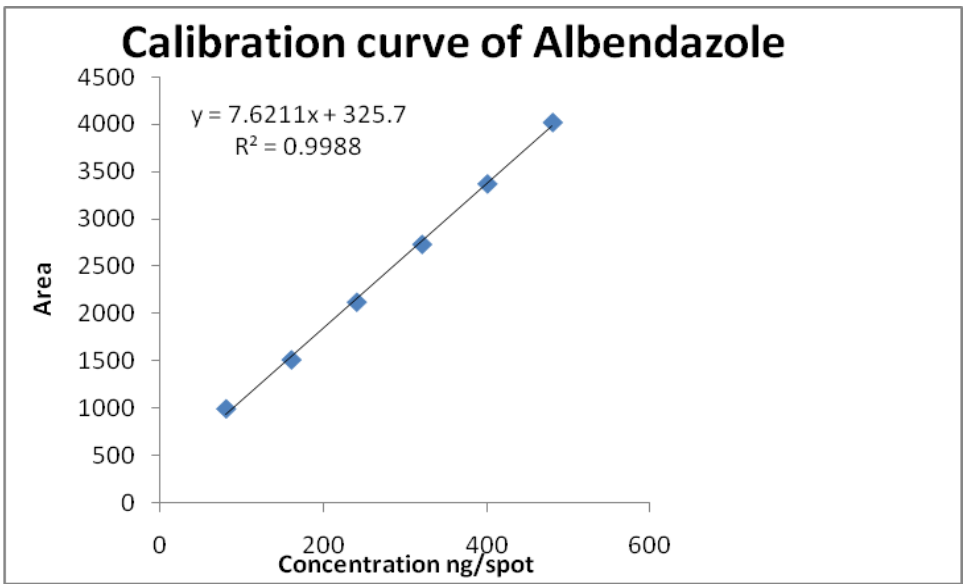

Fig.5.2.1.2 Calibration Curve for Albendazole(80-480 ng/µl)

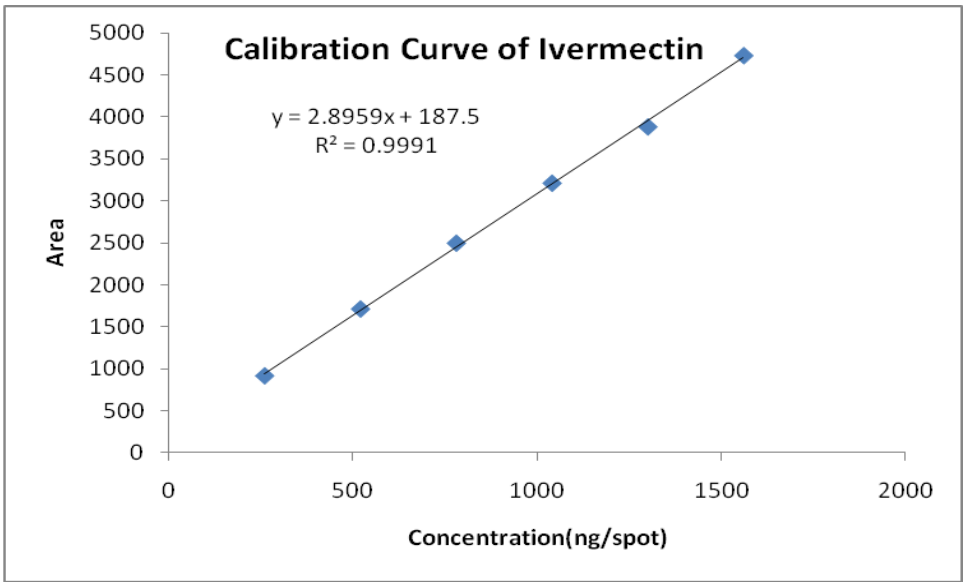

Fig.5.2.1.3 Calibration Curve for Ivermectin.(260-1560 ng/µl)

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 x σ/S LOQ = 10 x σ/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**

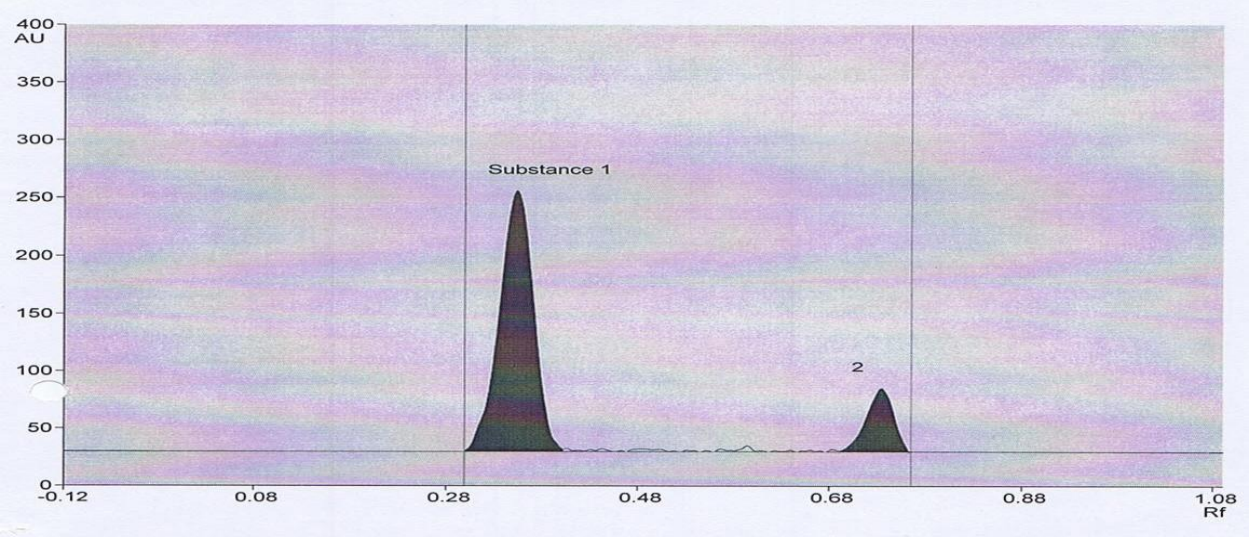

Fig. 5.2.2.1Chromatogram of Butenafine hydrochloride and Betamethasone dipropionateat 254 nm

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.

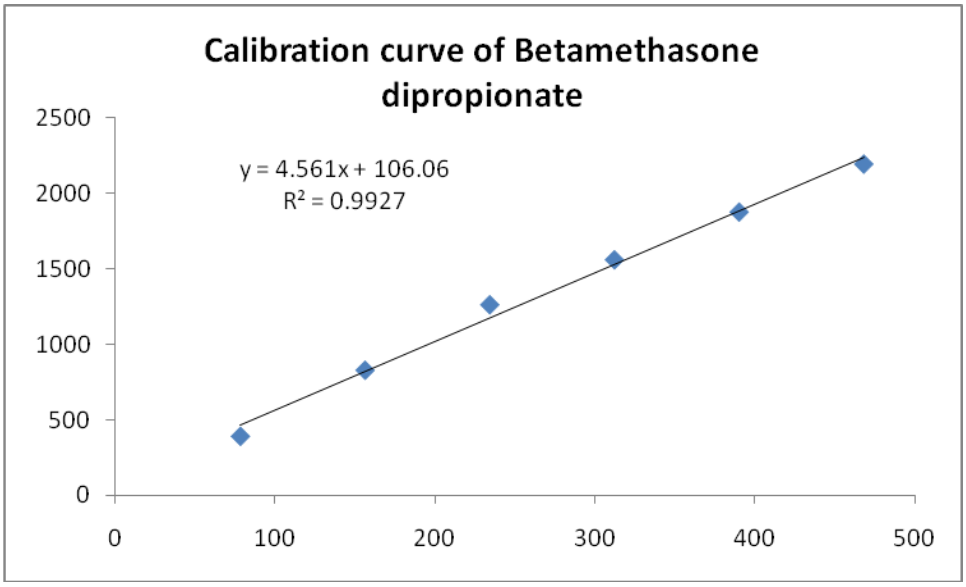

Fig. 5.2.2.2 Calibration Curve for Betamethasone dipropionate (100-600 ng/µL)

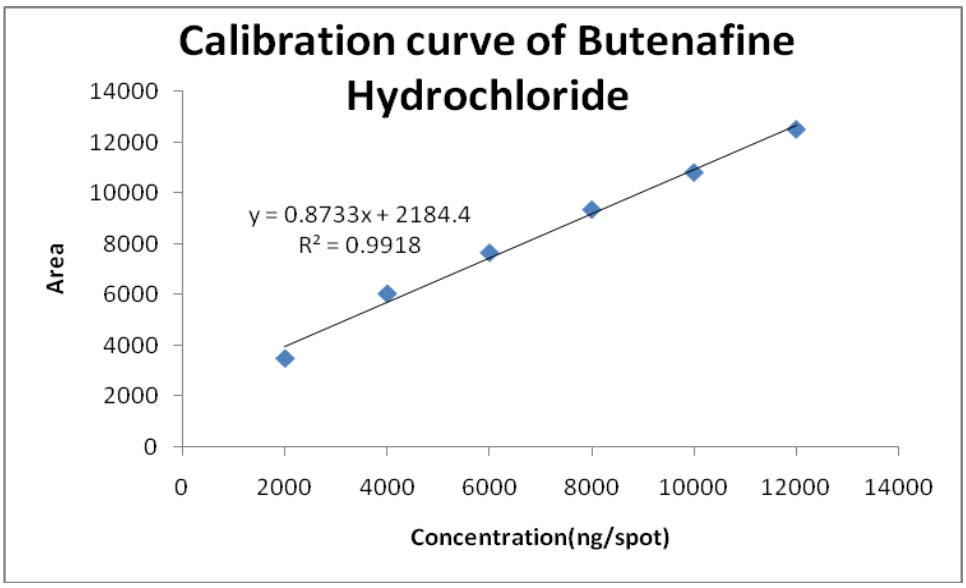

Fig. 5.2.2.3 Calibration Curve for Butenafine hydrochloride(2000-12000ng/µL)

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

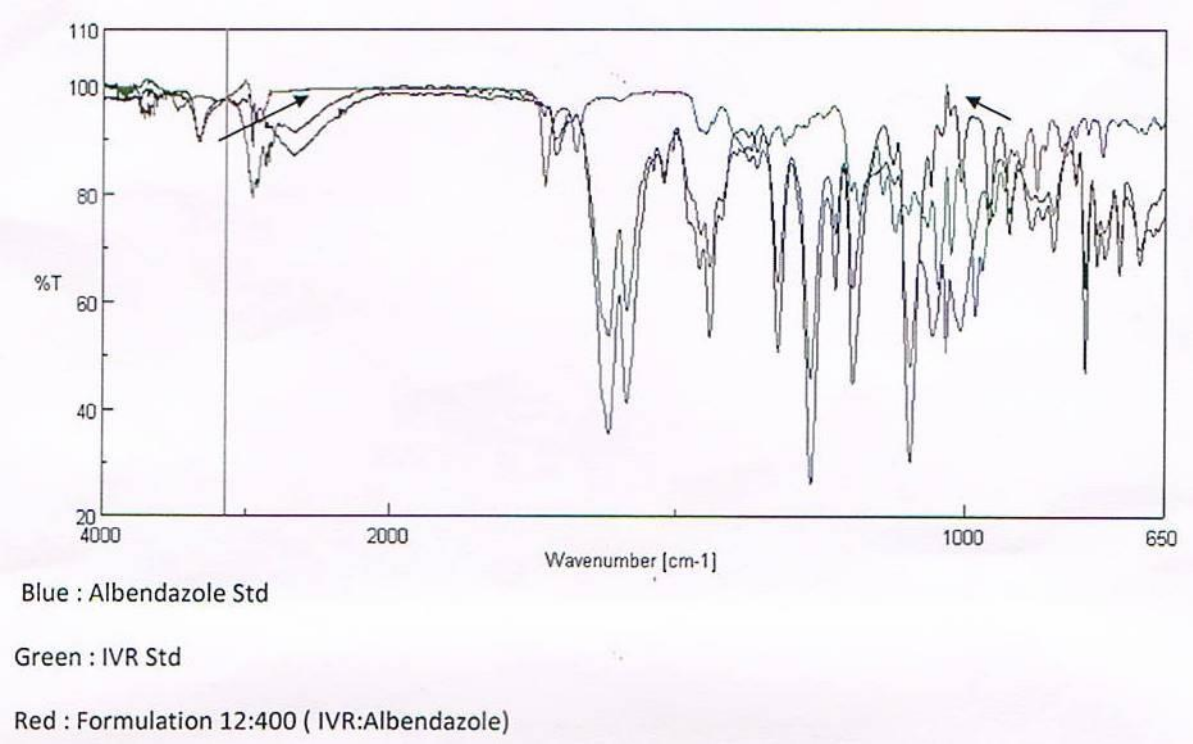

Fig. 5.3.1 IR spectrum of Albendazole standard, Ivermectin Standard and Tablet Formulation

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.

6. Summary and Conclusion

A patient taking a pharmaceutical product expects the product to be safe and efficacious.

Due to abundance of pharmaceutical agents available in the pharmaceutical market in various dosage forms either as a single drug component or in combination with other drugs and also due to potency of the most of the drugs, it becomes necessary to quantitate these agents in their formulations in a precise manner.

Pharmaceutical regulatory agencies worldwide demand that the product retains its quality, purity, and potency for the time the product is commercially available and also expect to see the stability data supporting the proposed expiration date of the product in the marketing submission. Therefore there is always a need to develop validated analytical methods which are precise, accurate, selective, and sensitive and can be used for routine analysis and stability studies of the drug products.

In the present work validated analytical methods and stability indicating methods were developed as per ICH guidelines for different drugs in bulk and in their formulations.

Mainly validated Reverse Phase High Performance Liquid Chromatography (RP-HPLC),High Performance Thin LayerChromatography (HPTLC) methods were developed. After a thorough literature survey, it was realized that cream formulations are difficult to analyze, therefore it was decided to analyze certain cream formulations, which are mainly multicomponent formulations and for which no analytical method was reported at the time of development. Accordingly, validated HPLC methods were developed for three semisolid dosage forms namely cream formulation containing Butenafine hydrochloride 1% w/w and betamethasone dipropionate 0.05 % w/w(Butenaskin BM),cream formulation containing Hydrocortisone acetate 1%w/w and miconazole nitrate 2%w/w(Fumic) and an ointment containing Fluocinolone acetonide0.01%w/w and and miconazole nitrate 2% w/w (Zole –F). It was also found that for the combination of etizolam and escitalopram oxalate in a tablet dosage form no analytical method was reported, therefore a validated HPLC method for simultaneous estimation of this combination of drug was developed(Etizola Plus 5 tablet containing 0.5 mg of etizolam and 5 mg of escitalopram oxalate) .The developed method was further optimized and validated to study its application as a stability indicating method. It was also applied for dissolution testing of tablet containing etizolam and escitalopram oxalate.Racecadotril is one of the the drugs selected for method development as it was newly introduced in the Indian market and no analytical method

for its estimation was available in the literature at the time of development.Therefore a validated HPLC method was developed for its estimation in bulk and in oral powder dosage form. The method was used further, to determine racecadotril in presence of any degradation product due to its forced degradation.

A validated HPTLC method was developed for estimation of Butenafine hydrochloride and betamethasone dipropionate in a cream formulation. A validated HPTLC method was also developed for simultaneous determination of albendazole and ivermectin in a tablet dosage form.An attempt was made to quantitate albendazole and ivermectin by using IR spectroscopic technique. All these developed methods are summarized in the following section.

6.1 HPLC methods

Various developed HPLC methods are as follows

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

A RP-HPLC method has been developed for the simultaneous determination of butenafine hydrochloride and betamethasone dipropionate on an Inertsil C18 column (250 ±4.6 mm id) using a mobile phase gradient consisting of methanol and water at a flow rate of 1 mL/min. Detection was carried out at 254 nm. Retention times of betamethasone dipropionate and butenafine hydrochloride were 4.82 (±0.80) and 16.18(±0.17) min, respectively. The method was validated with respect to specificity, linearity, precision, ruggedness, and robustness.

Linearity of the method was found to be more than 0.999, %RSD of precision,ruggedness and robustness was study was below 2% for both the drugs. System suitability parameters were within the limit.This method is simple, precise, and sensitive, and applicable for the simultaneous quantification of butenafine hydrochloride and betamethasone dipropionate in a cream formulation.

6.1.2 RP-HPLC method for simultaneous determination of Escitalopram Oxalate and Etizolam in bulk and in a Tablet Formulation.

An isocratic reversed phase HPLC method has been developed for the simultaneous determination of escitalopram oxalate and etizolam on a HiQ-silC18HS (250 x 4.6 mm) column using a mobile phase consisting of methanol:phosphate buffer pH-5 (70:30,v/v) at a flow rate of 1 mL/min and the detection was carried out at 254 nm. The retention times of escitalopram oxalate and etizolam were found to be 4.85 (± 0.31) min and 7.65 (± 0.56) min

respectively. The method was validated with respect to specificity, linearity, accuracy, precision, ruggedness and robustness.This method is simple, precise, and sensitive and is applicable for simultaneous quantification of escitalopram oxalate and etizolam in a tablet formulation. Linearity of the method was found to be more 0.999 .%RSD of precision,ruggedness and robustness was study was below 2% for both the drugs.System suitability parameters were within the limit and the method is simple, precise, and sensitive, and applicable for the simultaneous quantification of etizolam and escitalopram oxalate in a tablet dosage form.

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

The method developed in 1.2 was further optimized with respect to mobile phase composition to separate the drug peaks from the degradation product peaks.The optimized method was also validated with respect to specificity, linearity, accuracy, precision, ruggedness and robustness. Except the change in mobile phase composition from Methanol: Phosphate Buffer pH-5 (70:30 v/v) to Methanol: Phosphate Buffer pH-5 (62:38 v/v) all other experimental requirements were same. The retention times found to be 6.178 (±) 0.11 and 13.71± 0.10 for escitalopram oxalate and etizolam respectively.

This method is simple, precise, and sensitive and is applicable for simultaneous quantification of escitalopram oxalate and etizolam in a tablet formulation. Individual standard drugs ,their binary mixture and, tablets were forced to degrade under acidic(1N HCL)),basic(1NaOH),oxidative(3%H_2O_2),thermal, photolytic(UV),and humidity conditions.Escitalopram oxalate was found to be sensitive to basic and oxidative conditions,but on significant degradation was obtained for it in rest of the stress conditions.No significant degradation was obtained for etizolam under any of the above mentioned stress conditions. Degradation product were properly resolved from the escitalopram oxalate peak. All the validation parameters of the developed method are within the limit. Thus, this method was found to simple, accurate, precise, and sensitive and is applicable for simultaneous quantification of escitalopram oxalate and etizolam in a tablet formulation.

6.1.4 Application of developed RP HPLC method for the dissolution studies of etizolam and escitalopram oxalate in a tablet dosage form.

RP-HPLC method developed as **1.2** was applied for the dissolution studies of ETIZOLA PLUS-5 tablets 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. This study was a single point technique and samples were drawn only once after 45 min.More than 70% of the drug release was obtained in 45 minutes.Thus,developed method can be applied successfully for the dissolution studies of etizolam and escitalopram oxalate in a tablet dosage form.

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

Ointment used for this method contain the fluocinolone acetonide and miconazole nitrate in the ratio of 1:200.This ratio was found to be a challenging one for method development. A gradient RP-HPLC method has been developed for the simultaneous determination of fluocinolone acetonide and miconazole nitrate on HiQ-silC18HS (250 x 4.6 mm) using a mobile phase gradient consisting of methanol and water and at a flow rate of 1 mL/min. Detection was carried out at 238 nm. Retention times of fluocinolone acetonide and miconazole nitrate were 3.52 (±032) and 6.55(±1.10) min, respectively. The method was validated with respect to accuracy, specificity, linearity, precision, ruggedness, and robustness.

This method is simple, precise, and sensitive, and applicable for the simultaneous quantification of miconazole nitrate and fluocinolone acetonide in an ointment formulation.

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

A RP-HPLC method has been developed for the simultaneous determination of hydrocortisone acetate and miconazole nitrate on an HiQ-sil C18HS column (250 ±4.6 mm id) using a mobile phase gradient consisting of acetonitrile and 0.1 M ammonium acetate (80:20 v/v) at a flow rate of 1 mL/min. Detection was carried out at 225 nm. Retention times of hydrocortisone acetate and miconazole nitrate were 4.05(± 0.141) and 13.14 (± 0.31) min, respectively. The method was validated with respect to accuracy,specificity, linearity, precision, ruggedness, and robustness.

Linearity of the method was found to be more than 0.999, recovery of added drugs was within 98-102 %, %RSD of precision,ruggedness and robustness was study was below 2% for both the drugs.system suitability parameters were within the limit is method is simple,accurate, precise, and sensitive, and applicable for the simultaneous quantification of hydrocortisone acetate and miconazole nitrate in a cream formulation.

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

An isocratic reversed phase HPLC method has been developed for the quantitation of Racecadotril on a Prochrome C-18 (250x4.6mm) column using a mobile phase consisting of water: acetonitrile: glacial acetic acid (49:50:1) at a flow rate of 1.2ml/min and detection at 254nm. Retention time of Racecadotril has been found to be 15.49 min. Stress degradation studies on Racecadotril were also carried out under stress testing conditions of hydrolysis, oxidation, photolysis and thermal decomposition. The method was validated with respect to linearity, precision, accuracy, specificity and ruggedness.Racecadotril was found to be susceptible to degradation mainly in basic condition.All the validations parameters were found to be within limits prescribed as per ICH guidelines.

6.2 HPTLC METHODS

6.2.1 Simultaneous HPTLC Determination of Albendazole and Ivermectin in bulk and in tablet dosage form.

A validated HPTLC method was developed for albendazole and ivermectin in a tablet dosage form.Mobile phase composition was Toluene:Diethyl ether:Ethanol:Formic acid(03:05:0.2:0.5v/v) and determination was carried out at 254nm.Linearity,recovery,precision and ruggedness study were carried out.Linearity was found to be more than 0.999 for both the drugs ,accuracy studies revealed that recovery of added standard drugs was within the limit of 98-100%.Assay values for albendazole and ivermectin in marketed formulation were within 90%-110%.

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

A validated reverse phase HPTLC method has been developed for betamethasone dipropionate and and butenafine hydrochloride in a cream formulation. Mobile phase composition was methanol:ammonia(9:1v/v) and determination was carried out at 254nm. Rf value for butenafine hydrochloride and betamethasone dipropionate was found to be 0.26± 1.93 and 0.645± 1.62 respectively Linearity,recovery,precision and ruggedness study were carried out. Linearity was found to be more than 0.999 for both the drugs ,accuracy studies revealed that recovery of added standard drugs was within the limit of 98-100%.Assay values for butenafine hydrochloride and betamethasone dipropionate in a marketed cream formulation were within 90%-110%w/w.

6.3 IR Method

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.But as the proportion of ivermectin to albendazole in tablet is 12:400,the ivermectin peaks were masked,due to which correlation between the area of peak or height of the peak with concentration of albendazole in the tablet could not be established.

6.4 Conclusion

As discussed above all the proposed HPLC methods and HPTLC methods were successfully developed and validated as per ICH guidelies. Also their applicability in estimation of the selected drugs in their formulations was proved.

Publication

A paper has been published in Journal of AOAC International.

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

SURYAKANT D. BHOSALE and SADHANA J. RAJPUT

The Maharaja Sayajirao University of Baroda, Quality Assurance Laboratory, Pharmacy Department, Faculty of Technology and Engineering, Vadodara-390 001, India

An RP-HPLC method has been developed for the simultaneous determination of butenafine hydrochloride and betamethasone dipropionate on an Inertsil C18 column (250 _ 4.6 mm id) using a mobile phase gradient consisting of methanol and water at a flow rate of 1 mL/min. Detection was carried out at 254 nm. Retention times of betamethasone dipropionate and butenafine hydrochloride were 4.82 (±0.80) and 16.18(±0.17) min, respectively. The method was validated with respect to specificity, linearity, accuracy, precision, ruggedness, and robustness. This method is simple, precise, and sensitive, and applicable for the simultaneous quantification of butenafine hydrochloride and betamethasone dipropionate in a cream formulation.