FORCED DEGRADATION STUDY WITH DEVELOPED AND VALIDATED RP-HPLC METHOD FOR SIMULATENEOUS ESTIMATION OF CLINDAMYCIN PHOSPHATE AND KETOCONAZOLE IN BULK AND FINISHED PHARMACEUTICAL DOSAGE FORM

A Dissertation submitted to

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY,

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In partial fulfilment of the award of the degree of

MASTER OF PHARMACY

IN

Branch V – PHARMACEUTICAL ANALYSIS

Submitted by

MOHANA PRIYA S

261930205

Under the Guidance of

Dr. ANANDA THANGADURAI, M.Pharm., Ph.D.,

PROFESSOR

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

J.K.K.NATTARAJA COLLEGE OF PHARMACY

KUMARAPALAYAM – 638183

TAMILNADU.

OCTOBER – 2021

CERTIFICATES

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"FORCED DEGRADATION STUDY WITH DEVELOPED AND VALIDATED RP-HPLC METHOD FOR SIMULATENEOUS ESTIMATION OF CLINDAMYCIN PHOSPHATE AND KETOCONAZOLE IN BULK AND FINISHED PHARMACEUTICAL DOSAGE FORM",** submitted by the student bearing **Reg. No: 261930205** to "The Tamil Nadu Dr. M.G.R. Medical University – Chennai", in partial fulfilment for the award of Degree of Master of Pharmacy in Pharmaceutical Analysis was evaluated by us during the examination held on……

Internal Examiner **External Examiner**

CERTIFICATE

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Dr.ANANDA THANGADURAI,
M.Pharm., Ph.D,
Professor
Department of Pharmaceutical Analysis

Dr. V.SEKAR, M. Pharm., PhD.,
Professor &HOD,
Department of Pharmaceutical Analysis

Dr. R. SAMBATHKUMAR, M. Pharm., PhD.,
Professor &Principal
J.K.K.Nattraja College of Pharmacy

CERTIFICATE

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Place: Kumarapalayam
Date:

Dr.ANANDA THANGADURAI,
M.Pharm., Ph.D,
Professor
Department of Pharmaceutical Analysis

CERTIFICATE

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Place: Kumarapalayam
Date:

Dr. R. Sambathkumar, M. Pharm., PhD.,
Professor & Principal,
J.K.K.Nattraja College of Pharmacy,
Kumarapalayam.

DECLARATON

I do hereby declared that the dissertation **"FORCED DEGRADATION STUDY WITH DEVELOPED AND VALIDATED RP-HPLC METHOD FOR SIMULATENEOUS ESTIMATION OF CLINDAMYCIN PHOSPHATE AND KETOCONAZOLEIN BULK AND FINISHED PHARMACEUTICAL DOSAGE FORM"**submitted to "**The Tamil Nadu Dr. M.G.R Medical University - Chennai",** for the partial fulfilment of the degree of Master of Pharmacy in Pharmaceutical Analysis, is a bonafide research work has been carried out by me during the academic year 2020-2021, under the guidance and supervision of **Dr. ANANDA THANGADURAI, M.Pharm., Ph.D.,** Professor, Department of Pharmaceutical Analysis, J.K.K. Nattraja College of Pharmacy, Kumarapalayam.

I further declare that this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma, associate ship and fellowship or any other similar title. The information furnished in this dissertation is genuine to the best of my knowledge.

Place: Kumarapalayam **Ms. MOHANAPRIYA S**

Date: **Reg.no. 261930205**

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MOHANA PRIYA S

Reg. No. 261930205

Dedicated to

Almighty

My Beloved Parents,

&

MyFamilyMembers

ABSTRACT

A simple, accurate, precise method was developed for the simultaneous estimation of the clindamycin phosphate and ketoconazole in solid dosage form. Chromatogram was run with column C8, 250 X 4.6 mm, 5 µ. Mobile phase containing potassium dihydrogen phosphate with buffer (pH 2.5) and acetonitrile in proportion 50: 50 v/v was pumped through column at a flow rate of 1.0 mL/min. Temperature was maintained at 30°C. Optimized wavelength for clindamycin phosphate and ketoconazole was 210 nm. Retention time of clindamycin phosphate and ketoconazole were found to be 4.846 min and 12.399min. % RSD of method precision for Clindamycin phosphate and Ketoconazole were found to be 0.5 and 0.2 respectively. % RSD of intermediate precision for Clindamycin phosphate and Ketoconazole were found to be 0.1 and 0.4 respectively. % Recovery was obtained as 100.2% and 100.4% for clindamycin phosphate and ketoconazole respectively. The linearity regression coefficient of Clindamycin and Ketoconazole were found to be not more than 0.999. % of y-Intercept were found to be 1.1

Key Words: clindamycin phosphate, Ketoconazole RP-HPLC

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LIST OF ABRIVATION

RP-HPLC	Reverse Phase High Performance Liquid Chromatography
ODS	Octadecylsilane
USP	United State Pharmacopoeia
ICH	International Conference on Harmonization
USFDA	United State Food and Drug Administration
WHO	World Health Organization
HETP	High Equivalent Theoretical Plate
%	Percentage
^{o}C	Degree Celsius
AUC	Area Under Curve
Cmax	Maximum Concentration
Ml	Milliliter
mL/min	Milliliter per minute
^{o}F	Degree Fahrenheit
v/v	Volume per Volume
Nm	Nanometer
UV	Ultraviolet
ng/mL	Nano gram per milliliter

µg/mL	Microgram per milliliter
Mm	Millimeter
Cm	Centimeter
µm	Micrometer
µ	Micron
Mg	Milligram
µL	Microliter
SD	Standard deviation
RSD	Relative standard deviation
LOD	Limit of detection
LOQ	Limit of quantitation

1. INTRODUCTION

1.1 ANALYTICAL CHEMISTRY

Analytical chemistry is the branch of chemistry that deals with the analysis of different substances.Analytical chemistry involves the separation, identification, and the quantification of matter. It involves the use of classical methods along with modern methods involving the use of scientific instruments[1].

Analytical chemistry involves the following methods:

- The process of separation isolates the required chemical species which is to be analysed from a mixture.
- The identification of the analyte substance is achieved via the method of qualitative analysis.
- The concentration of the analyte in a given mixture can be determined with the method of quantitative analysis.

1.1.1 Methods Used in Analytical Chemistry

The methods used to determine the identity and the quantity of the analytes in the field of analytical chemistry can be broadly divided into classical and instrumental methods.

a. Classical Methods

- There exist many classical methods of checking for the presence or absence of a particular compound in a given analyte. One such example is the acid test for gold.
- Another example of a classical method for qualitative analysis is the Kastle-Meyer test which employs phenolphthalein as an indicator to check for the presence of haemoglobin in the given analyte.

- Flame tests can be used to check for the presence of specific elements in an analyte by exposing it to a flame and observing the change in the colour of the flame.
- Gravimetric analysis is a classical method of quantitative analysis, which can be used in analytical chemistry to determine the amount of water in a hydrate by heating it and calculating the weight of the water lost.
- One of the better known classical methods of quantitative analysis is volumetric analysis (also known as titration). In the titration method, a reactant is added to the analyte till an equivalence point is obtained.

b. Instrumental Methods

- Spectroscopy involves the measurement of the interaction between electromagnetic radiation and the atoms or molecules belonging to a sample.
- With the help of electric fields and magnetic fields, the method of mass spectroscopy is used to measure the ratio of the mass of the molecule to its charge.
- A common instrumental method used in the field of analytical chemistry is electrochemical analysis. In this method, the analyte is placed in an electrochemical cell and the voltage or the current flowing through it is measured.
- The interaction between the analyte and energy in the form of heat is studied in the discipline of analytical chemistry known as calorimetry. A calorimeter is an instrument that is used to measure the heat of a chemical reaction.

It can be noted that even biological measurements are made with the help of this branch of chemistry, and this field is known as bio analytical chemistry.

1.1.2 Branches of Analytical Chemistry

a) Quantitative Analysis

Quantitative Analysis is a method of determining the absolute or relative quantity regarding the concentration of one or more substances present in a sample or compound [2].

For example, take a sample of an unknown solid substance. The chemists first use "qualitative" methods to identify what type of compound is present in the sample; then he adopts the quantitative analysis procedure to determine the exact amount or the quantity of the compound present in the sample.

Some Quantitative analysis techniques include Gravimetric Analysis and Volumetric analysis.

b) Qualitative Analysis

Quality means the standard or the feature of one substance. Hence, Qualitative analysis method deals with the determination of the quality of a particular compound, irrespective of its quantity or concentration. In simpler words, the qualitative analysis does not measure the amount of the substance but measures the quality of that material. One of the best examples of this type of method is the observation of a chemical reaction, whether there will be a change in colour or not.

The qualitative analysis method can be measured in different ways such as Chemical tests, flame tests, etc. Several such tests are widely used in salt analysis (identification of the cation& anion of inorganic salts).

1.1.3 Importance of Analytical Chemistry

Analytical chemistry is the branch which is taught in almost all schools and colleges. But the applications of it are made in pharmaceutical industries, food factories, chemical industries, agricultural industries and in

scientific laboratories. The tools used for this purpose are quite expensive which one cannot afford at home.

1.1.4 Applications of Analytical Chemistry

Some important applications of this branch of chemistry are listed below.

- The shelf lives of many medicines are determined with the help of analytical chemistry.
- It is used to check for the presence of adulterants in drugs.
- Soil can be tested to check for appropriate concentrations of minerals and nutrients that are necessary for plant growth.
- It is employed in the process of chromatography where the blood samples of a person are classified.

Table No: 1.1 Analytical method and its properties

Characteristic properties	**Instrumental methods**
Emission of radiation	X-ray, Fluorimetry
Absorption of radiation	UV-Visible, IR, NMR
Scattering of radiation	Turbidometry, Raman spectroscopy
Refraction of radiation	Refractometry, Interferometry
Diffraction of radiation	X-ray and Electron diffraction method
Rotation of radiation	Polarimetry, Optical rotatry dispersion
Electrical potential	Potentiometric
Electrical charge	Colorimetry
Electrical current	Amperometry, Polarography
Electrical resistance	Conductometry
Mass	Gravimetry
Mass-to-charge ratio	Mass spectroscopy
Rate of reaction	Kinetic methods
Thermal characteristics	Thermal gravimetry

1.2 CHROMATOGRAPHY

Chromatography is the process of separation of mixture of components into a single component by using mobile phase and stationary phase.

Stationary phase may be solid or liquid applied on a solid or gel, and may be packed in a column spread as a coating otherwise distributed as a film. The stationary phase may be gases or liquid

1.2.1 Classification of chromatography

1.2.1.1 Based on principle of separation

a) Adsorption chromatography

- Gas solid chromatography
- Thin layer chromatography
- Column chromatography
- Performance liquid chromatography
- Affinity Chromatography
- Hydrophobic interaction chromatography

b) Partition chromatography

- Gas liquid chromatography
- Paper partition chromatography
- Column chromatography

1.2.1.2 Based on nature of stationary and mobile phase

- Solid liquid chromatography
- Liquid liquid chromatography
- Gas solid chromatography
- Gas liquid chromatography

1.2.1.3 Based on modes of chromatography

- Normal phase chromatography
- Reverse phase chromatography

1.2.1.4 Other types of chromatography

- Size exclusion chromatography
- Ion exchange chromatography
- Chiral phase chromatography

1.2.2 Adsorption chromatography

Chromatography on which separation is based mainly on difference between the absorption affinities of the sample components for the surface of an active solid.

A mixture of compounds or dissolved in mobile phase, passes through a column of stationary phase, day travel with relative affinity in the direction of stationary phase.

Compounds having more affinity in the direction of stationary phase travels lower and compounds having a lesser affinity in a direction of stationary phase travels faster.

Hence the compounds of separated. No two compounds have the same affinity for mixture of stationary phase, mobile phase and other conditions.

1.2.3 Partition chromatography

The mixture of compounds are dissolved in mobile phase and passed through a column of stationary phase. The compounds those are more soluble in the stationary face travels slower. Dark compound and those are less soluble in stationary phase travels faster. Bus components of separator mainly on difference in their partition coefficient

Example: GLC, Paper chromatography, Column partition chromatography

1.2.4 Size exclusion chromatography

It comprises porous Matrix as a stationary phase. As they act as molecular sieve gel is to separate the components of a mixture according to their molecular sizes. Diverse gel are used for different molecules. Hence mixture of substances is separated according to their molecular size.

1.2.5 Ion exchange chromatography

The principle of separation is ion exchange means reversible exchange of functional group. Ion exchange chromatography, anion exchange resin is used to separate a mixture of similar charged ions. Cation exchange resin is used for separation of cation and anion exchange resin is used to separate a mixture of anions.

1.2.6 Chiral phase chromatography

In this chromatography, optical isomers can be separated with chiral stationary phase fullstop mostly chemically bonded silica gels are used as stationary phase.

1.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography is now one of the most powerful tools in analytical chemistry. It is also known as high pressure liquid chromatography due to having pressure capability of 500 psi. It as the ability to separate identify and quantify the compounds present in man any sample. Compounds of low concentrations even in parts per trillion may easily be identified. HPLC can be applied to a wide variety of samples such as pharmaceuticals food nutraceuticals cosmetics environmental matrices forensic samples and industrial chemicals

1.3.1 Normal phase chromatography

Normal phase HPLC is the first principle of separation in HPLC. In this analyse or separated based on polarity used for fairly polar analytes. It contains a polar stationary phase and a nonpolar mobile phase.

1.3.2 Reversed phase chromatography

Reversed phase HPLC is commonly used for polar analytes. It consists of nonpolar stationary phase and and aqueous, moderately polar mobile phase. It operates on the principle of hydrophobic interactions, which results from repulsive forces between a polar eluent, the relative Lee nonpolar analyte and the non polar stationary phase.

1.3.3 Isocratic and gradient elution

- In Isocratic mode, mobile phase composition remains constant throughout the separation.
- In gradient mode, mobile phase composition is changed during the separation process.

Eg : separation starting at 10% methanol and ending at 90% methanol after 20 minutes.

1.3.4 Working principle of HPLC

The components of a basic high performance liquid chromatography system.Reservoir holds the mobile phase. Pumps are used for maintaining high pressure with a specified flow rate of mobile phase. Introduced into the continuously flowing mobile phase stream using and injector. Mobile phase carries the sample into the HPLC column.

The column contains ding chromatographic packing material means stationary face which aids the effect of separation. Director is placed to Tum observe the bands from a separated compound is there a load from the HPLC column. As the mobile phase exits the detector, it can be sent to waste or

collector, as desired. Recorder is placed next to them Ekta to record the electrical signals needed and 2 salary then chromatogram on its display which is used for identifying and quantifying the concentration of the sample components. Several types of directors have been developed according to sample characteristics. UV absorbance detector can be used for compounds absorbing UV light. Otherwise a more universal type of detector is used such as evaporative light scattering detector (ELSD). The most powerful approach is usage of multiple detectors in series like UV and ELSD director in combination with mass spectroscopy (MS) to analyse the results of the chromatographic separation [3-5].

Figure 1 A flow diagram of an HPLC system.

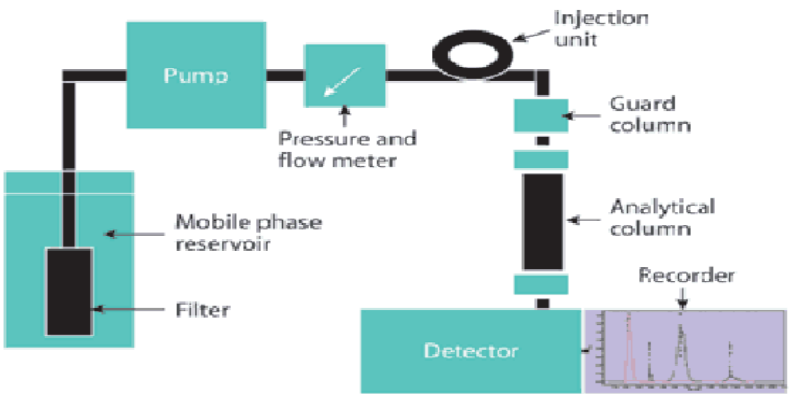


Fig no: 1.1 A Flow diagram of an HPLC system.

1.4 ANALYTICAL METHOD DEVELOPMENT.

The goal of method development is to optimise the solution for the desired and allied in the shortest possible time.

A typical method development involve following steps,

- Mode selection
- Selection of mobile phase
- Selection of column
- Optimize the experimental conditions.

1.4.1 Method development for reverse phase chromatography

1.4.1.1 Mode selection

The selection of HPLC more is centrally decided by the type and solubility of the analyte of interest, its molecular weight, the sample Matrix and the the availability of the appropriate stationary phase and column. Below figure outline the steps for a wall waiting then best choice of mode, based on the molecular weight of compound and solvents using.

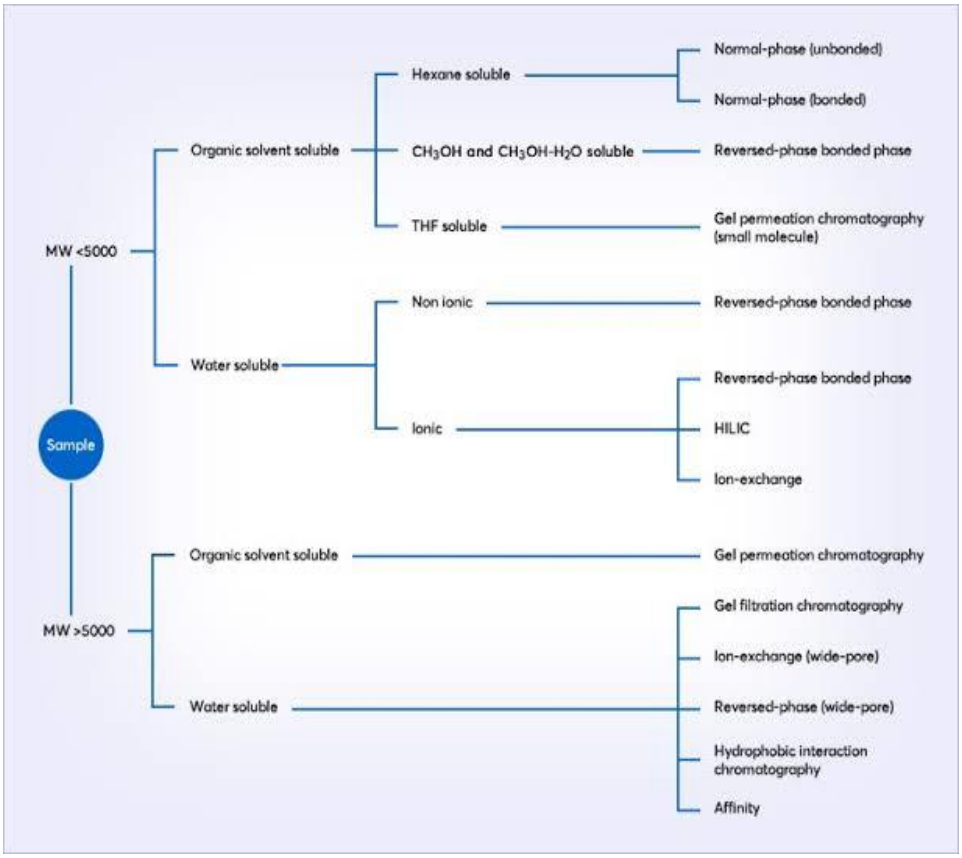


Fig no: 1.2 Columns action by solvent and mode.

1.4.1.2 Selection of mobile phase

The mobile phase consists of two solvents termed as a and b. A is the weak solvent which allows the solute slowly while b is the strong solvent which rapidly loads the solute from the column. In RP-HP,LC solvent A is often water or an aqueous buffer.

While b is an organic solvent miscible with water such as acetonitrile methanol or isopropanol. HPLC grade solvents can be used. Selectively differences and sample retention will vary significantly between mobile phase. Mobile phase in HPLC is selected based on nature of the substances for drug to be analyzed and separated.

The nature of the drug is mainly three types.

I. Hydrophilic / polar: it consists of functional group such as COOH, NH2, OH.
II. Moderately polar: it consists of functional group such as N02,nitrile groups
III. Non polar / hydrophobic: it consists of hydrocarbon chain such as (CH2)n and aromatic compounds such as benzene and its derivatives.

- ✓ Polar substances of separated by polar solvents.
- ✓ Non polar substances of separated by nonpolar solvents.
- ✓ The mobile phase used in the HPLC should be filtered to remove particles greater than 0.45 micrometer.
- ✓ In HPLC grade organic solvents only should be used as mobile phase.

Polarity index of mobile phase:

water> acetic acid > methanol > ethanol > isopropyl alcohol > acetonitrile > propanol THF > dichloromethane > cyclohexane > n-Hexane.

- ➢ Mobile phase is selected based on mode of separation and column used that is in HPLC mobile phase is polar and in NP-HPLC mobile phase is non polar.
- ➢ Acidic substances are separated by using acidic mobile phase that is solvent and suitable buffer.
- ➢ Basic substances of separated by using basic mobile phase that is solvent and basic buffers.

a) Working with mobile phases

Solvents used as mobile phase should be compatible with the shipping solvent full stop to prevent the precipitation of buffer in the column shipped or stored in 100% organic for reverse phase operation. Column should be equilibrated without buffer first, then equilibrating with buffer mobile phase. Both the CN and NH2 columns can be used with normal and reversed phase solvents, so there is no need to check that the solvents or miscible with shipping solvents before equilibration.

b) Solvent miscibility

For HPLC it is important to understand the miscibility of various solvents because in incompatible solvents in the system can cause erratic chromatographic results. Miscibility refers to the ability of a solution to mix with another in all proportions and become a new homogenous solution.

For organic compounds the polarity of a solvent determines the compound which is able to dissolve otherwise in which solvents they are miscible. General polar compounds dissolve in polar solvent and non polar compounds dissolve in nonpolar solvents.

For example, water and exiting or not miscible with each other quickly dispersed into two layers although shaken well.

For inorganic compounds the length of the carbon chain of an vitamins miscibility relative to members of homeo logos series.

For example, in the alcohols, ethanol has two carbon atom and is miscible with water, whereas butanol as 4 carbon atoms and is not miscible with water.

c) Miscibility number (M)

- All pairs whose M number differs by 15 units or less are miscible in in all proportions at 15°C.

- Each pair whose number difference is 16 has a critical solution temperature between 25 and 75°C generally about 50°C.
- A difference of 17 or more corresponds to immiscibility or to a critical solution temperature above 75°C.

d) pH adjustment for mobile phase

In reversed phase chromatography for developing rugged methods both PH and ionic strength of Aqueous portion of mobile phases are important. In case of ionic compounds retention of typical species shows significant changes with PH. Controlling pH is very important in search reversed-phase system to stabilize the retention and selectivity full stop for mostsamples including basic compounds and typical week acids, method development is started using recommended pH 2-4. for reproducibility and that pH used to should be 1 unit above or below the pka or pkb of the solutes being separator. Most reversed-phase columns can be used between pH 2-8 and more, Halloween wide range to find the optimum mobile phase PH for separation.

- Non ionized analytes show better retention (i.e.acids at low PH and bases at high pH if possible).

Table No: 1.2 Properties for common HPLC Solvent.

Selection of a mobile phase for a particular LC application can be done by using various tables that summarize properties for common LC solvents:

Solvent	Refractive Index	Viscosity (cP)	Boiling Point (°C)	Polarity Index (P)	Eluent Strength (ε°)
Fluoroalkanes	1.27-1.29	0.4-2.6	50-174	<-2	-0.25
cyclohexane	1.423	0.90	81	0.04	-0.2
N-hexane	1.327	0.30	69	0.1	0.01
1-chlorobutane	1.400	0.42	78	1.0	0.26
Carbon tetrachloride	1.457	0.90	77	1.6	0.18
i-propyl ether	1.365	0.38	68	2.4	0.28
toluene	1.494	0.55	110	2.4	0.29
Diethyl ether	1.350	0.24	35	2.8	0.38
tetrahydrofuran	1.405	0.46	66	4.0	0.57
chloroform	1.443	0.53	61	4.1	0.40
ethanol	1.359	1.08	78	4.3	0.88
Ethyl acetate	1.370	0.43	77	4.4	0.58
dioxane	1.420	1.2	101	4.8	0.56
methanol	1.326	0.54	65	5.1	0.95
acetonitrile	1.341	0.34	82	5.8	0.65
nitromethane	1.380	0.61	101	6.0	0.64
Ethylene glycol	1.431	16.5	182	6.9	1.11
water	1.333	0.89	100	10.2	large

- Usage of extreme pH values, very high or very low should be avoided in order to lengthen the life of column.
- Buffers can be used to adjust pH values.
- To improve reproducibility and maintaining a consistent pH buffers may be used.
- Sometimes and non buffered mobile phase can be selected for pH adjustment.

e) Buffers action

- The buffering ability of every mobile phase is correlated to the prepared molarity and also closeness of the desired eluent PH with reference to the PK of the buffering iron.
- Buffering is typically effective up to 1 pH with reference to the PK of the buffering ion.
- It was not in frequent for acidic analytes to be chromatographed using simple acid solutions do the concentration of acid is adequate to obtain a very low pH than needed.
- Alkaline buffers or limited such as Triethylamine (TEA) and ammonia.
- TEA has my pk and is not freely water soluble.
- Ammonia has at too high pk and used for most columns as itself dissolves freely.
- PH meter is used for adjusting pH of buffer. For most HPLC separations buffers should be filtered using a 0.45 micro meter filter to get rid of any impurities which may be in the water or in the solid buffer.

f) Common buffers for UV detectors.

The choice of buffers strongly impacts the means of detection. Buffer should be transparent at the wavelength of interest for UV detectors. Buffers with UV cut off below 220nm work the best.

Table No: 1.3 HPLC Buffers, Pka value and Useful pH range

Buffer	pKa (25°C)	Useful pH Range
TFA	0.5	<1.5
Sulfonate	1.8	<1-2.8
Phosphate	2.1	1.1-3.1
Chloroacetate	2.9	1.9-3.9
Formaldehyde	3.8	2.8-4.8
Acetate	4.8	3.8-5.8
Sulfonate	6.9	5.9-7.9
Phosphate	7.2	6.2-8.2
Ammonia	9.2	8.2-10.2
Phosphate	12.3	11.3-13.3

1.4.1.3 Selection of column

a) Stationary phase

The stationary face must be chosen mostly a C18 phase (longer chain)

Initially and it is used to for hydrophobic small molecules. Shorter chain C8 or C3 phase should be chosen when there is strong retention and on the c-18 phase. C8 phase show slightly lower retention though it has similar selectivity with a C18 phase. Short chain C3 phase can be preferred for very hydrophobic molecules. An aromatic bonded face like phenyl or die phenyl should be used for analyte molecules of aromatic characters.

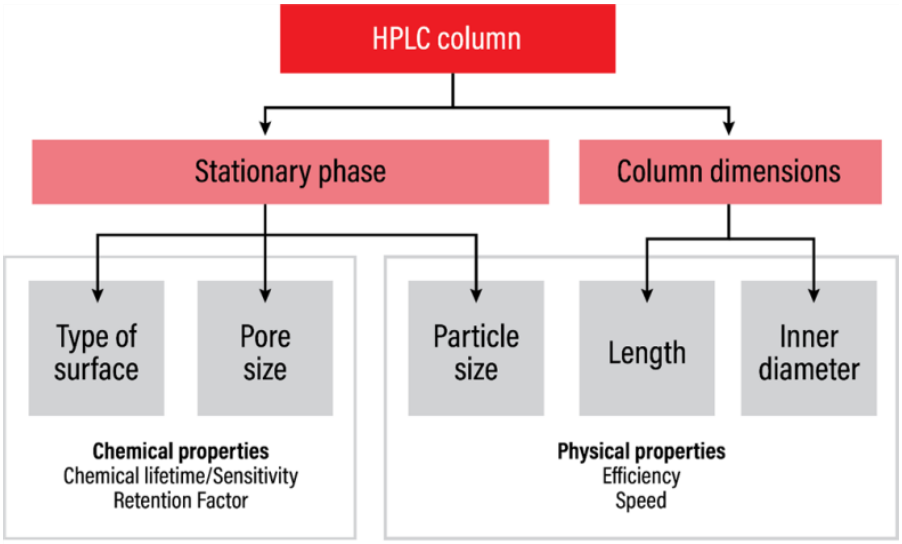


Fig no: 1.3 Selection of Column

Polarity index of functional group in a compound

Carboxylic acid > hydroxyl> amino > Ketone > aldehyde > ester > Nitro > ether > halide phenyl > methylene.

Polarity of stationary phase

Non polar <---------- Moderately polar ---------- polar ---------->

Table No: 1.4 Major HPLC column platform

Silica Gel Column (high density)	Polar Retention Column (low density)	Hybrid column pH (1-12)
SunFireTM	AtlantisR T3	XBridgeTM
StmmetryR	AtlantisR Dc18	XTerraR
Symmetry ShieldTM	AtlantisR HILIC	ACQUITY UPLC TM

b) Chromatographic surface

The particles are very porous like the pores of a sponge. 99% of chromatographic surface is inside the pores.

Mobile phase must be allowed into the PO in order for chromatographic retention of the analyte to take place.

If the pores are dry, the analyte cannot get into the pores and it will not be retained by the chromatographic surface.

c) Pore size

Pore size is important to ensure the analyte molecules of interest penetrating the packing material and interact with the hydrophobic stationary phase within the pores. The pore size of the packing material is very important as the analyte molecules need to fit into the chromatographic surface to make interact with the stationary phase. Smaller fore size packings are best for small molecules weight up to molecular weight of 2000. For larger molecules with molecular weight over 2000 wider pore packing required.

Table No: 1.5 Pore size based on molecular weight

Analyte Molecular Weight	Pore size recommendation
>3000	60-150 at (6-13nm)
3000-10000	125-200 at (12.5-20nm)
>10000	300-1000 at (30-100nm)
Very large	Non-porous

d) Particle size

- Smaller particle(3mm) offer higher efficiency, higher resolution and higher Back pressure.
- Medium particle size (5mm) is commonly used for most separations.

- Large particle size (10mm) offers low back pressure. Hence it is not widely used.
- Efficiency, optimum flow rate and back pressure are inversely proportional to the particle size.

$$N, F_{opt}, \Delta P$$

e) Column length

- Short column with smaller particle size (3mm) is the best choice to perform high throughput analysis.
- For complex separations involving many sample components long columns (10mm) packed with small particle size can be used.
- Medium column length 150 mm is commonly used.
- Long column length 300mm gives high resolution.
- Efficiency is directly proportional to the column length

f) Column internal diameter

- ✓ Column with narrow internal diameter (2.1 mm) offers I sensitivity and low mobile phase consumption.
- ✓ Column having medium internal diameter (4.6mm) is commonly used for most separation.
- ✓ Column with wide internal diameter (22.5 mm) processes high sample load ability.

1.4.1.4 Optimizing the chromatographic conditions for reversed-phase chromatography.

Method should be optimized after selecting the column dimensions, column packing with appropriate stationary phase, mobile phase solvents and buffers. Optimization is dependent on the goal of separation. If the method was developing for quality control, Isocratic separation was carried out so

that there are fewer variables in the method. Longer columns can be used that optimize the method for obtaining best and maximum resolution between all compounds. A shorter column with a fast flow rate can be used to optimize when speed is important. A gradient method must be developed and optimized for complex samples with a number of compounds of interest which may have different degrees of retention [6-8].

Two approaches for condition optimization

- Isocratic (constant mobile phase composition)
- Gradient (changing mobile phase strength as a function of time)

Mostly separations can be carried out with binary solvent systems however the same approach can be used for tertiary or quaternary mobile phases.

1.5 ANALYTICAL METHOD PARAMETERS

1. Retention time (Rt)
2. Void volume or column dead time (to)
3. Retention volume (Rv)
4. Separation factor
5. Capacity factor or retention factor (k)
6. Resolution (Rs)
7. Pressure
8. HETP (High Equivalent to Theoretical Plates)
9. Column Efficiency (N)
10. Asymmetry factor (AF)

1.5.1 Retention time

Retention time is the time required for 50% of a component to be eluted from a column. It is the variation of time among the point of injection and appearance of weak Maxima. It is measured in minutes.

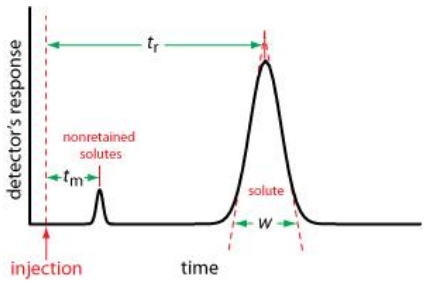


Fig no: 1.4 Chromatographic variable.

1.5.2 Void volume or column dead time

- The volume of the liquid phase in the column is called void volume.
- Time taken for an retarded component to pass through the column.

1.5.3 Retention volume

- Retention volume is the volume of mobile phase required to elute 50% of the component from a column. It is the product of retention time and flow rate.
- Retention volume = retention time × flow rate

1.5.4 Selectivity or separation factor

The separation factor is a measure of a time or distance between the maximum of two peaks.

$$\propto = k2/k1$$

Selectivity can be altered by temperature and also change in the mobile phase components or changing the stationary phase.

1.5.5 Retention factor

Formerly known as capacity factor or Kt.The retention factor measures the period of time that the sample component resides in a stationary phase comparative to the time that the sample component resides in the mobile phase.

If the substance not retained by the stationary phase, tR 0, then t1R = 0

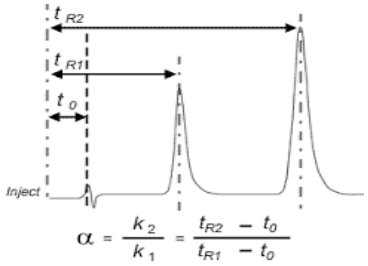


Fig no: 1.5 Chromatographic value.

1.5.6 Resolution

Resolution is the term which explains the quality of separation. It is the capacity of a column to separate the peaks of interest.

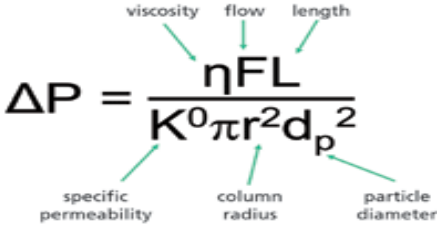
$$R_s = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2'}{1 + k_2'}\right)$$

A value of 1 is required for measurable separation to occur and to allow adequate quantitation. A value of 0.6 is mandatory to separate two equal height peaks.

Values of 1.7 or greater generally or appropriate for Rugged methods. Higher the resolution easier it is to achieve baseline separation between two peaks. Value of 1.6 is deliberated to be a great baseline separation and ensure the most accurate quantitative result.

1.5.7 Pressure.

The pressure equation as shown above identifies five major factors distributing the system pressure, solvent viscosity, flow rate, column length, column radius and particle



As noted in the formula even a small decrease in the particle size as a significant impact on back pressure.

1.5.8 High equivalent to a theoretical plate

Formally theoretical plates are known as functional unit of a column. It is hypothetical unit of a column where distribution of solute among the stationary phase and mobile phase as achieved equilibrium. Theoretical plates or imaginary plates of any hight, defines the efficiency of separation. Column is more efficient of HETP is less and the column is less efficient if HETP is more.

HETP = length of the column / number of theoretical plates.

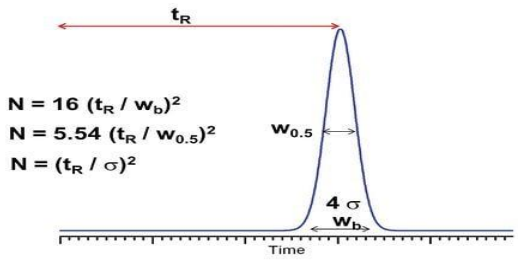


Fig no: 1.6 chromatogram showing column efficiency

HETP is given by Vandeemeter equation

$$HETP = A+B/u+Cu$$

Where,

A = Eddys diffusion term for multiple path diffusion which arises due to the packing of the column. This can be minimised by uniformity of packing.

B = longitudinal diffusion term for molecular diffusion.

C = effect of mass transfer.

u = flow rate or viscosity of mobile phase.

1.5.9 Column efficiency

Column efficiency is a significant parameter of column performance, used to compare the performance of different columns. It is stated as the theoretical plate number, N.

$$N = 16 (t_R/w_t)^2$$

Where, n = no. of theoretical plates

Rt = retention time

W_t = peak width at base

$N = 5.54 (t_R / w_{1/2})^2$

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

Column is said to be highly efficient if number of theoretical plates is high. Longer in the column, more is the number of theoretical plates Vidya high N we'll have a narrower peak at a given retention time than a column with the lower N number.

1.5.10 Asymmetry factor

A chromatographic click must be symmetrical around its centre and leads to follow Gaussian distribution.

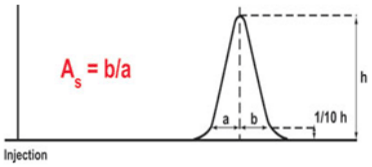


Fig no: 1.7 Chromatographic peak showing asymmetric factor

- But in practice owing to certain factors, the peak is not symmetrical and illustrates tailing for fronting.

- Fronting is due to saturation of stationary phase. Using less quantity of sample avoid fronting.
- Tailing is due to more active absorption sites. It is eliminated by sample pretreatment.

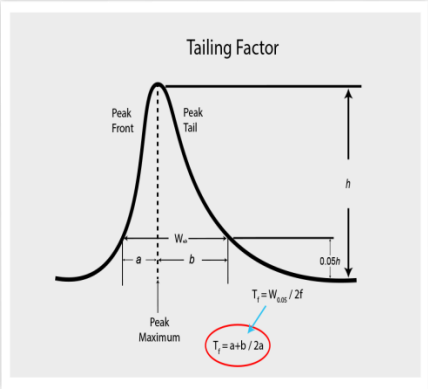


Fig no: 1.8 Chromatographic peak showing fronting and tailing

1.6 ANALYTICAL METHOD VALIDATION

Method validation can be defined as per ICH establishing documented evidence which provides a high degree of assurance that specific activity will consistently produce a desired result for product a desired or product meeting its predetermined specifications and quality characteristics.

The objective of method validation is to demonstrate the analytical method is suitable for its intended use.

1.6.1 Types of analytical procedure to be validated.

- Identification test
- Quantitative test for impurities content.
- Limit test for the control of impurities.

Quantitative test for active moiety in samples of drug substances or drug product aur other components of drug product.

1.6.2 ICH method validation parameters

For chromatographic method used in analytical application there is more consistency in validation related substances are commonly present in the pharmaceutical products but those are always within the limits as specified in ICH (Q2B)

1. System suitability
2. Precision
3. Accuracy
4. Specificity
5. Linearity
6. Range
7. Limit of detection
8. Limit of quantitation
9. Robustness

1.6.2.1 System suitability

System suitability test is an integral part of many analytical procedures. These tests are used to verify that the resolution and reproducibility of the system or adequate to be performed. The purpose of the system suitability test is to ensure that the complete testing system is suitable for the intended application. In general consistency of system performance and chromatographic suitability(tailing factor column efficiency and resolution and detector sensitivity).

1.6.2.2 Specificity

- Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present.
- Three methods were employed for demonstrating specificity. In the first method, the conditions of HPLC method developed, namely, percent of organic solvent in mobile phase, ionic strength, pH of the

mobile phase, flow rate etc. were changed and the presence of additional peaks, if any, was observed.

- The second method involves the peak purity test method using diode array detector. The diode array spectrum and diode array first derivative spectrum of the standard and sample drug peaks were recorded and compared.
- The third method is based on measurement of the absorbance ratio of the drug peaks at two different wavelengths.

1.6.2.3 Precision

Precision is the measure of how close the data values to each other for a number of measurements under the same analytical conditions.

Precision may be considered at three levels according to ICH.

- Repeatability
- Intermediate Precision
- Reproducibility

1.6.2.4 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.

In a method with high accuracy, a sample is analyzed and the measured value should ideally be identical to the true value. Accuracy is represented and determined by recovery experiments. The usual range being 10% above or below the expected range of claim.

1.6.2.5 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.

The linearity is determined from 50% of the ICH reporting level to 150% of the proposed shelf life specifications of the related substance as a minimum.

1.6.2.6 Range

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

The range of an analytical procedure was the concentration interval over which acceptable accuracy, precision and linearity were obtained. In practice, the range was determined using data from the linearity and accuracy studies. Assuming that acceptable linearity and accuracy (recovery) results were obtained as described earlier. The only remaining factor to be evaluated was precision. To confirm the 'range' of any analytical procedure, linearity studies alone are not sufficient, and accuracy at each concentration (minimum three concentration levels covering lower and upper levels) should be proved.

Table No: 1.6 Tests and its acceptance criteria

Test	Level	Range	Acceptance criteria
Assay	5	50%-150%	R>0.999
Dissolution	5-8	10%-150%	R>0.99
Impurity	5	LOQ-2%	R>0.98

1.6.2.7 Limit of Detection (LOD) and Limit of Quantification (LOQ):

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value. Detection limit corresponds to the concentration that will give a signal-to-noise ratio of 3:1.

The limit of quantitation of an individual analytical procedure is the lowest

Amount of analyte in a sample that can be determined quantitatively with suitable precision and accuracy. Limit of quantitation is the concentration of related substance in the sample that will give a signal to noise ratio of 10:1. Both LOD and LOQ are affected by the separation conditions.

1.6.2.8 Robustness:

Robustness is a measure of its ability to remain unaffected by small but deliberate variations in method parameters and offered as sign of its consistency during common usage. Provides and indications of its test method suitability and consistency during typical use. During a robustness study, conditions or intentionally to see if the method results are affected.

Example of typical variations are;

- Stability of analytical solutions
- Extraction time

In case of LC,

- Influence on variations of PH in the mobile phase
- Influence on variations in mobile phase composition
- Different columns
- Temperature
- Flow rate

1.7 FORCED DEGRADATION STUDY:

Forced degradation studies include the degradation of new drug substance and drug product at conditions more severe than accelerated conditions. These studies illustrate the chemical stability of the molecule which further facilitates the development of stable formulation with suitable storage conditions. ICH guidelines demonstrate certain degradation conditions like light, oxidation, dry heat, acidic, basic, hydrolysis etc. ICH

Q1A, QIB and Q2B exemplify the forced degradation studies. This review overviews the strategic approaches and trends in forced degradation studies.

Forced degradation studies offer the following information

a. Determination of likely degradants,
b. Determination of degradation pathways,
c. Determination of intrinsic stability of the drug molecule,
d. Determination of validated stability indicating analytical methods.

1.7.1 Various degradation conditions

1) Hydrolysis condition

2) Oxidation condition

3) Photolytic condition

4) Thermal condition

5) Humidity condition

1.7.1.1 Hydrolysis:

Over a wide range of pH most common degradation, chemical reactions are Hydrolysis The decomposition of a chemical compound by reaction with water is called Hydrolysis. In acidic and basic hydrolysis the catalysis of ionisable functional groups present in the molecule occurs. Forced degradation of a drug substance occurs when the drug interacts with acid and base. It produces primary degradants in the desirable range. Depending on the stability of the drug substance the class and concentrations of acid or base taken should be decided. For acid hydrolysis hydrochloric acid or sulphuric acids (0.1-1 M) considered to be most suitable whereas sodium hydroxide or potassium hydroxides (0.1-1M) for base hydrolysis are suggested [8,25]. Co-solvents can be used if compounds are poorly soluble in

water. Forced degradation started at room temperature and further temperature increased if there is no degradation .

1.7.1.2 Oxidation conditions:

For oxidative forced degradation, hydrogen peroxide is broadly used. Apart from this as metal ions, oxygen, and radical initiators: azobi-isobutyro-nitrile, AIBN can also be used. Drug structure will allow selecting concentration and condition of oxidizing agents. An electron transfer mechanism occurs in the oxidative degradation of drug substance .

1.7.1.3 Photolytic conditions:

The light exposure does not affect the drug substance for this purpose photo stability is conducted. Photo stability studies are performed to produce primary degradants of drug substance by exposure to UV or fluorescent conditions. In ICH guidelines some recommended conditions for photo stability testing are described.

Samples of drug substance and solid/liquid drug product should be exposed to a minimum of 1.2million lx h and 200 W h/m2 light, 300-800 nm is the most commonly accepted wavelength of light to cause the photolytic degradation . 6 million lxh is the maximum illumination recommended. Photo oxidation can be caused by light stress conditions by the free radical mechanism. Photosensitive groups are carbonyls, nitroaromatic, N-oxide, alkenes, aryl chlorides, weak C–H and O–H bonds, sulfides and polyenes.

1.7.1.4 Thermal conditions:

Thermal degradation (e.g., dry heat and wet heat) should be carried out at more strenuous conditions than recommended ICH Q1A accelerated testing conditions. Samples of solid-state drug substances and drug products should be exposed to dry and wet heat. Liquid drug products should be exposed to dry heat. For a shorter period studies may be conducted at higher

temperatures. Through the Arrhenius equation the effect of temperature on thermal degradation of a substance can be studied.

$k = Ae^{-Ea}$

Where k is specific reaction rate,

A is frequency factor,

Ea is energy of activation,

R is gas constant (1.987cal/deg mol),

T is absolute temperature. Thermal degradation study is carried out at 40-80˚C.

1.7.1.5 Humidity condition:

Humidity is one of the effective factors in establishing the potential degradants in the finished product and active pharmaceutical ingredient. Normally 90% humidity for the duration of one week shall be recommended for the establishment of forced degradation samples.

Table No: 1.7 Conditions for Forced Degradation Studies

Degradation Type	Experimental Conditions	Storage Conditions	Sampling Time (days)
Hydrolysis	Control API (no acid or base)	40˚C, 60˚C	1,3,5
		40˚C, 60˚C	1,3,5
	0.1M HCl	40˚C, 60˚C	1,3,5
	0.1 M NaOH	40˚C, 60˚C	1,3,5
Oxidation	3% H_2O_2	25˚C, 60˚C	1,3,5
	Peroxide control	25˚C, 60˚C	1,3,5
	Azobisisobutyronitrile (AIBN)	25˚C, 60˚C	1,3,5
		25˚C, 60˚C	1,3,5
Photolytic	Light 1 × ICH	NA	1,3,5
	Light 3 × ICH	NA	1,3,5
	Light 3 × ICH	NA	1,3,5
Thermal	Heat chamber	60˚C	1,3,5
	Heat chamber	60˚C /75% RH	1,3,5
	Heat chamber	60˚C	1,3,5
	Heat chamber	60˚C /75% RH	1,3,5

2. DRUG PROFILE

CLINDAMYCIN PHOSPHATE

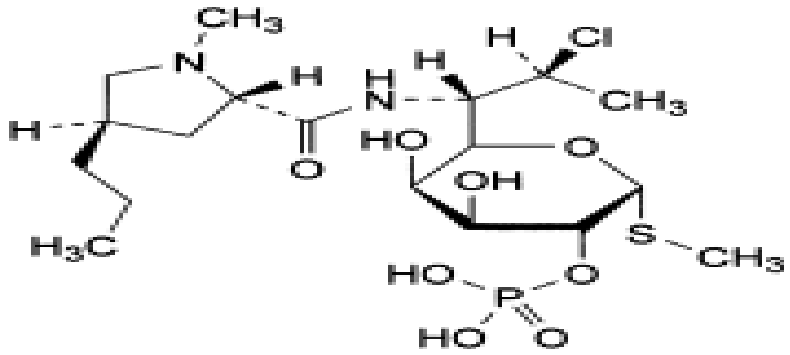


Fig no: 2.1 Structure of clindamycin phosphate.

Molecular formula: $C_{18}H_{34}ClN_2O_8PS$

Molecular weight : 505.0 g/mol

IUPAC name : Methyl 7-chloro-6,7,8-trideoxy-6-(1-methyl-trans-4-propyl- L-2-pyrrolidine carboxamido)1-thio - L- threo- a- D -galacto-octopyranoside-2- (dihydrogenphosphate)

Category : Antibiotics

Trade names : Acanya, Benzaclin, Biacna, Cleocin, Cleocin-T, Clindacin, Clindagel, Clindesse, Clindoxyl, Dalacin, Dalacin C, Duac, Evoclin, Neuac, Onexton, Veltin, Xaciato, Ziana

Physical and chemical properties

A white or almost white powder, slightly hygroscopic, freely soluble in water, very slightly soluble in alcohol, practically insoluble in methylene chloride.

Melting point: 117°C

External IDs

Drugbank: U-21,251

Mechanism of action

Clindamycin inhibits bacterial protein synthesis by binding to 23S RNA of the 50S subunit of the bacterial ribosome. It impedes both the assembly of the ribosome and the translation process The molecular mechanism through which this occurs is thought to be due to clindamycin's three-dimensional structure, which closely resembles the 3'-ends of L-Pro-Met-tRNA and deacylated-tRNA during the peptide elongation cycle - in acting as a structural analog of these tRNA molecules, clindamycin impairs peptide chain initiation and may stimulate dissociation of peptidyl-tRNA from bacterial ribosomes.The mechanism through which topical clindamycin treats acne vulgaris is unclear, but may be related to its activity against *Propionibacterium acnes*, a bacteria that has been associated with acne.

Absorption

Oral bioavailability is nearly complete, at approximately 90%, and peak serum concentrations (C_{max}) of, on average, 2.50 µg/mL are reached at 0.75 hours (T_{max}). The AUC

following an orally administered dose of 300mg was found to be approximately 11 µg hr/mL. Systemic exposure from the administration of vaginal suppository formulations is 40-fold to 50-fold lower than that observed following parenteral administration and the C_{max} observed following administration of vaginal cream formulations was 0.1% of that observed following parenteral administration.

Protein binding

Clindamycin protein binding is concentration-dependent and ranges from 60-94%. It is bound primarily to alpha-1-acid glycoprotein in the serum

Metabolism

Clindamycin undergoes hepatic metabolism mediated primarily by CYP3A4 and, to a lesser extent, CYP3A5. Two inactive metabolites have been identified - an oxidative metabolite, clindamycin sulfoxide, and an N-demethylated metabolite, N-desmethylclindamycin.

Route of Elimination

Approximately 10% of clindamycin bioactivity is excreted in the urine and 3.6% in the feces, with the remainder excreted as inactive metabolites

Half life

The elimination half-life of clindamycin is about 3 hours in adults and 2.5 hours in children. Half-life is increased to approximately 4 hours in the elderly

Clearance

The plasma clearance of clindamycin is estimated to be 12.3-17.4 L/h, and is reduced in patients with cirrhosis and altered in those with anemia.

Volume of distribution

Clindamycin is widely distributed in the body, including into bone, but does not distribute into cerebrospinal fluid. The volume of distribution has been variably estimated between 43-74 L.

Side effects of clindamycin phosphate:

1. Abdominal pain
2. Agranulocytosis
3. Eosinophilia (transient)
4. Diarrhea
5. Fungal overgrowth
6. Pseudomembranous colitis
7. Hypersensitivity
8. Urticaria
9. Hypotension
10. Nausea
11. Vomiting
12. Sterile abscess at IM site
13. Thrombophlebitis
14. Granulocytopenia

KETOCONAZOLE

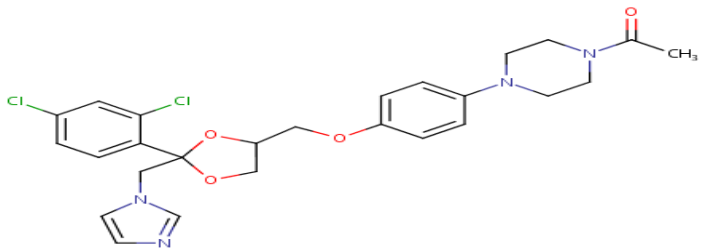


Fig no: 2.2 Structure of ketoconazole.

Molecular Formula : $C_{26}H_{28}Cl_2N_4O_4$

Molecular weight **:** 531.43 $g \cdot mol^{-1}$

IUPAC name **:** 2*RS*,4*SR*)-1-(4-{4-[-2-(2,4-Dichlorphenyl)-2- (imidazol-1-ylmethyl)-1,3-dioxolan-4-ylmethoxy] phenyl}piperazin-1-yl)ethanon

Category **:** Imidazole antimycotic.

Trade *name* **:** *E*xtina, Ketodan, Ketoderm, Nizoral, Xolegel

External IDs **:** KW-1414, R 41,400,R-4140

Physical and chemical properties

Colour: Colorless crystals or powder

Melting Point:148-152 °C

Mechanism of action

Ketoconazole interacts with 14-α-sterol demethylase, a cytochrome P-450 enzyme necessary for the conversion of lanosterol to ergosterol.This results in inhibition of ergosterol synthesis and increased fungal cellular permeability due to reduced amounts of ergosterol present in the fungal cell membrane. This metabolic inhibition also results in accumulation of 14α-methyl-3,6-diol, a toxic metabolite. The increase in membrane fluidity is also thought to produce impairment of membrane-bound enzyme systems as components become less closely packed.

Absorption

Ketoconazole requires an acidic environment to become soluble in water. At pH values above 3 it becomes increasingly insoluble with about 10% entering solution in 1 h. At pH less than 3 dissolution is 85% complete in 5 min and entirely complete within 30 min. A single 200 mg oral dose produces a Cmax of 2.5-3 mcg/mL with a Tmax of 1-4 h.. Administering ketoconazole with food consistently increases Cmax and delays Tmax but literature is contradictory regarding the effect on AUC, which may experience a small decrease. A bioavailability of 76% has been reported for ketoconazole.

Volume of distribution

Ketoconazole has an estimated volume of distribution of 25.41 L or 0.36 L/kg. It distributes widely among the tissues, reaching effective concentrations in the skin, tendons, tears, and saliva. Distribution to vaginal tissue produces concentrations 2.4 times lower than plasma.

Penetration into the CNS, bone, and seminal fluid are minimal. Ketoconazole has been found to enter the breast milk and cross the placenta in animal studies.

Protein binding

Ketoconazole is approximately 84% bound to plasma albumin with another 15% associated with blood cells for a total of 99% binding within the plasma

Metabolism

The major metabolite of ketoconazole appears to be M2, an end product resulting from oxidation of the imidazole moiety.[8] CYP3A4 is known to be the primary contributor to this reaction with some contribution from CYP2D6. Other metabolites resulting from CYP3A4 mediated oxidation of the imidazole moiety include M3, M4, and M5. Ketoconazole may also undergo N-deacetylation to M14, alkyl oxidation to M7, N-oxidation to M13, or aromatic hydroxylation to M8, or hydroxylation to M9. M9 may further undergo oxidation of the hydroxyl to form M12, N-dealkylation to form M10 with a subsequent N-dealkylation to M15, or may form an iminium ion. No metabolites are known to be active however oxidation metabolites of M14 have been implicated in cytotoxicity.

Route of elimination

Only 2-4% of the ketoconazole dose is eliminated unchanged in the urine. Over 95% is eliminated through hepatic metabolism.

Half-life

Ketoconazole experiences biphasic elimination with the first phase having a half-life of 2 hours and a terminal half-life of 8 hours

Adverse effects:

1. Acne.
2. Bleeding from sore in the mouth.
3. Blistering, crusting, irritation, itching, or reddening of the skin.
4. Cracked, dry, or scaly skin.
5. Discoloration of the fingernails or toenails.
6. Eye dryness, irritation, or swelling

3. LITERATURE REVIEW

Sudhakar *et al.*[9] have done RP-HPLC method development and validation for the simultaneous estimation of clindamycin phosphate and clotrimazole in pharmaceutical dosage forms.The aim of this work was to develop and validate a simple Reverse Phase-High Performance Liquid Chromatography method for the simultaneous estimation of Clindamycin and Clotrimazole in pharmaceutical dosage forms.The mobile phase consists of phosphate buffer and Acetonitrile in the ratio of (48:52) with gradient programming, Hypersil BDS (250×4.6 mm,5 µ) column used as stationary phase with a flow rate of 1 mL/min, injection volume 10 µL and the run time was 10 min. Detection wavelength was at 220 nm by using Photo Diode Array detector.The retention times of Clindamycin and Clotrimazole were found to be 2.2 min and 5.7 min respectively. The method was validated according to ICH guidelines. Validation parameters like accuracy, precision, linearity, range, limit of detection, limit of quantification and robustness all were within the limits. The linearity responses of Clindamycin and Clotrimazole were found to be in the concentration ranges of 25-150 µg/mL and 50-300 µg/mL. The percentage recovery for both drugs was found in the range of 99-100%. The LOD & LOQ values for were found to be 1.29 µg/mL and 3.93 µg/mL and Clotrimazole were found to be 1.31 µg/mL and 3.96 µg/mL, respectively. The results obtained are accurate and within the limits. Hence this method can be applicable for the estimation of Clindamycin and Clotrimazole in pharmaceutical dosage forms.

Rajameena's *et al.*[10] developed RP-HPLC method development and validation for estimation of Clindamycin phosphate and Clotrimazole in pharmaceutical dosage forms A simple, efficient and reproducible Reverse Phase-High Performance Liquid Chromatography

(RP-HPLC) method for simultaneous determination of Clindamycin phosphate and Clotrimazole in combined soft gelatin pessaries pharmaceutical formulation has been developed. The separation was carried out on Hypersil BDS C8 (250 × 4.6 mm; 5 µm) column using buffer, 13.6 g Potassium dihydrogen ortho phosphate in 1000 ml of water (adjusted to pH 2.5 ortho phosphoric acid): acetonitrile: l in the ratio of 70:30 v/v as eluent. The flow rate was 1.0 mL / min and effluent were detected at 210 nm. The retention times of Clindamycin phosphate and Clotrimazole were 4.47 minutes, 22.06 minutes and 12.03 minutes respectively. The percentage recovery was within the range between 102.2 % and 103.23 % for Clindamycin phosphate, 98.96 % and 100.54 % for Clotrimazole. The linear ranges were found to be 200 mg/ml (r^2 = 0.9998) for Clindamycin phosphate, 400 mg/mL (r^2 = 0.9895) for Clotrimazole. The percentage relative standard deviation for accuracy and precision was found to be less than 2 %. The linearity was found to be in the range of 80-120 mg/mL and correlation coefficient of were found to be 0.9998, 0.9979, for Clindamycin Phosphate, Clotrimazole respectively. The proposed method is accurate with 102 % recovery and precise (% RSD of Reproducibility repeatability, intra-day and inter-day variations were 0.19, 0.24, 0.44, 0.78, 0.12-0.15, 0.13-0.37, 0.14, 0.22). The method was successfully applied to pharmaceutical formulation because no chromatographic interferences from peccaries excipients were found.

Prakashmodi *et al.*[11] have done Novel Stability-Indicating RP-HPLC Method for the Simultaneous Estimation of Clindamycin Phosphate and Adapalene along with Preservatives in Topical Gel Formulations, A novel stability-indicating RP-HPLC method was developed for the simultaneous estimation of clindamycin phosphate (hydrophilic), adapalene (hydro-

phobic), phenoxyethanol, and methylparaben in topical gel formulations. Optimum chromatographic separation among the analytes and stress-induced degradants peaks was achieved on the X Bridge C18 (50 x 4.6 mm, 3.5 µm) column using a mobile phase consisting of a variable mixture of pH 2.50 ammonium hydrogen phosphate buffer, acetonitrile, and tetrahydrofuran with gradient elution. Detection was performed at 210 nm for phenoxyethanol, methylparaben, and clindamycin phosphate and 321 nm for adapalene. The method was optimized with a unique diluent selection for the extraction of clindamycin phosphate and adapalene from the gel matrix. The developed method was validated for method precision, specificity, LOD and LOQ, linearity, accuracy, robustness, and solution stability as per ICH guidelines. The proposed method can be employed for the quantification of clindamycin phosphate, adapalene, phenoxyethanol, and methylparaben in commercial topical gel formulation.

Rohit Khatri *et al.*[12] have performed a new RP-HPLC method for estimation of clindamycin and adapalene in gel formulation: development and validation consideration This paper describes a validated high-performance liquid chromatographic (HPLC) method for simultaneous estimation of clindamycin (CLI) and adapalene (ADA) in pure powder and gel formulation. The HPLC separation was achieved on a Luna C18 (2) (250 × 4.6 mm i.d., 5 µm particle size) using acetonitrile: phosphate buffer pH 3.0 (60:40, v/v) as a mobile phase delivered at a flow rate of 1.0 mL/min. The calibration curve showed good linear relationship with r^2 = 0.9997 for clindamycin and r^2 = 0.9994 for adapalene in concentration range of 100-500 µg/mL and 10-50 µg/mL respectively. LOD and LOQ were found to be 5.39 and 16.32 µg/mL for clindamycin and 0.89 and 2.72 µg/mL for adapalene, respectively. Assays of clindamycin found to 99.76 ± 0.65 % and adapalene found to 99.58 ± 0.73 %. Clindamycin

undergoes thermal degradation but adapalene was found stable in this condition. The method was validated as per ICH guideline (Q2 R1). The method was successfully applied for routine analysis of clindamycin and adapalene in pure powder and gel formulation.

Martina Mifsud *et al.*[13] have done a simple HPLC-UV method for the determination of clindamycin in human plasma This study describes a simple high performance liquid chromatographic (HPLC) method for the determination of clindamycin in plasma. Analysis was carried out using a Varian Pro Star HPLC unit equipped with an online degasser. A reversed-phase ACE C18 column of dimensions 250x4.6 mm, particle size 5 µm was used. The mobile phase was made up of 0.02M disodiumhydrogen phosphate buffer (pH of 2.9) and acetonitrile at a ratio of 71:29 v/v, running through the column at a flow rate of 1.5 mL/min and with ultraviolet (UV) detection set at a wavelength of 195 nm. Clindamycin was separated from plasma proteins by protein precipitation with ice cold acetonitrile. Clindamycin and the internal standard phenobarbitone eluted after 3.96 and 7 minutes respectively. The method was validated for linearity in the working concentration range of 0.5-20 µg/mL. Linearity was observed with a coefficient of determination (r^2) of 0.990. The recoveries obtained were all above 82% and the limit of quantification and limit of detection were 0.2 µg/ml and 0.1 µg/mL respectively.

MairaPereira *et al.*[14] have Development and validation of a simple chromatographic method for simultaneous determination of clindamycin phosphate and rifampicin in skin permeation studies, Rifampicin (RIF) and clindamycin phosphate (CDM) are the main drugs currently used in combination to treat severe infectious diseases in hair follicles. This work describes a simple, rapid and sensitive method for simultaneous analysis of RIF and CDM in the different skin layers using high performance liquid chromatography (HPLC). The

efficient chromatographic separation of CDM and RIF was succeeded using a C_{18} column (150 mm x 4.6 mm, 5 μm) with gradient elution using a mobile phase composed of 0.01 M phosphoric acid and methanol at a flow rate of 1 mL/min. Determinations were performed using UV–vis detector at 200 nm and 238 nm for CDM and RIF, respectively. The method was precise, accurate and linear ($r^2 > 0.999$) with regression curve in the concentration range from 0.5 to 20.0 μg /mL and recovery rates from the skin layers higher than 85%. The retention times for CDM and RIF were approximately 7.4 and 12.2 min, respectively. The presence of skin components did not interfere with the analysis. The validated method was therefore appropriate for quantification of both CDM and RIF and thus may be feasible to be used in skin permeation studies.

Seetha Lakshmi N *et al.*[15] have done RP-HPLC method development and validation for simultaneous estimation of metronidazole, clindamycin phosphate and clotrimazole in combined pharmaceutical dosage forms. A simple, efficient and reproducible Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) methodfor simultaneous determination of metronidazole, clindamycin phosphate and clotrimazole in combined pharmaceutical dosageforms has been developed. The separation was carried out on Hypersil BDS C8 (250 × 4.6 mm; 5 μm) column using buffer, 13.6 g of potassium dihydrogen ortho phosphoric acid in 1000ml of water (adjusted to pH 2.4 with ortho phosphoric acid): acetonitrile 70:30 v/v as eluent. The flow rate was 2.3 mL/min and effluent were detected at 210 nm. The retention times of metronidazole, clindamycin phosphate and clotrimazole were 4.862 min, 5.712 min and 26.01 min respectively. The percentage recovery waswithin the range between 99.38% and 100.31% for metronidazole,

98.76% and 100.65% for clindamycin phosphate, 99.98% and 99.63% for clotrimazole. The linear ranges were found to be 80-150 µg/mL (r^2 = 0.9983) for metronidazole, 80-150 µg/mL (r^2 =0.9993) for clindamycin phosphate and 80-150 µg/ml (r2 = 0.9984) for clotrimazole. The percentage relative standard deviation foraccuracy and precision was found to be less than 2%. Hence, the method could be successfully applied for routine analysis ofmetronidazole, clindamycin phosphate and clotrimazole in the combined pharmaceutical dosage form.

Olga Popovska *et al.*[16] have done a RP-HPLC Method for the Determination of Ketoconazole in Pharmaceutical Dosage Forms, Background: Ketoconazole is an antifungal drug, available in different pharmaceutical dosage forms. The current published methods for the ketoconazole determination confirmed the primacy of the HPLC method, but high toxic solvents were used, also. A new simple, precise, accurate, and cost-effective RP-HPLC method for the determination of ketoconazole in tablets, topical cream, and shampoo has been developed and validated. The chromatographic separation was conducted on a Lichrospher 100 C18 column (150 mm length x 4.6 mm i.d., 5 µm particle size) using a mixture of methanol and water (90:10 v/v) adjusted to pH 8.90 with a phosphate buffer. The HPLC analysis was carried out at 25 °C column temperature with 1.0 mL/min isocratic flow rate of the mobile phase. The robustness of the method was evaluated changing the flow rate of the mobile phase (0.9 mL/min and 1.0 mL/min) and the column temperature (23°C & 25 °C).The mean recovery data for the ketoconazole pharmaceutical dosage forms were in the range of 92.67–105.23%.The developed and validated method was successfully used for the quantitative analysis of ketoconazole pharmaceutical dosage forms; tablets, topical cream, and shampoo.

Chinmoy Roy *et al.*[17] have performed Stability-Indicating Validated Novel RP-HPLC Method for Simultaneous Estimation of Methylparaben, Ketoconazole, and Mometasone Furoate in Topical Pharmaceutical Dosage Formulation, A simple, specific, precise, and accurate RP-HPLC method has been developed and validated for simultaneous estimation of Methylparaben (MP), Ketoconazole (KT), and Mometasone Furoate (MF) topical pharmaceutical dosage formulation. The separation was achieved by Waters X Terra C18 column using mobile phase consisting of buffer (triethyl amine in water, pH adjusted to 6.5 with glacial acetic acid)-acetonitrile (40: 60, v/v) at a flow rate of 1.5 mL/min and detection at 250 nm. The method showed linearity with correlation coefficient <0.9999 over the range of 0.12–15.2 µg/mL, 0.67–149.4 µg/mL, and 0.42–7.6 µg/mL for MP, KT, and MF, respectively. The mean recoveries were found to be in the range of 99.9–101.1% for all the components. The method was validated as per the ICH guidelines for linearity, limit of detection, limit of quantification, accuracy, precision, robustness and solution stability. Stability indicating capability of the developed method was established by analyzing forced degradation of samples in which spectral purity of MP, KT, and MF along with separation of degradation products from analytes peak was achieved. The method can be successfully applied for routine analysis of quantitative determination of MP, KT, and MF in pharmaceutical dosage form.

Rakesh Kumar J at *et al.*[18] have development and validation of reverse-phase HPLC method for estimation of ketoconazole in bulk drug. A simple, accurate rapid and precise RP-HPLC method has been developed and validated for determination of ketoconazole in bulk drug. The RP-HPLC separation was achieved on Promosil C-18, (250 mm, 4.6 mm, 5µm) using mobile phase water: acetonitrile: buffer PH 6.8 (51:45:4 v/v) at flow rate of 1.0 mL/min

at ambient temperature. The retention times were 2.713 min. for ketoconazole. Calibration plots were linear over the concentration range 1-50 µg/mL. Quantification was achieved with photodiode array detection at 238 nm over the concentration range of 1-50 µg/mL. The method was validated statistically and applied successfully for the determination of ketoconazole. Validation studies revealed that method is specific, rapid, reliable, and reproducible. The high recovery and low relative standard deviation confirm the suitability of the method for the routine determination of ketoconazole in bulk drug.

Shalin Parikh *et al.*[19] have done Stability Indicating RP-HPLC Method for Determination of Ketoconazole in Bulk Drug and in Tablet Dosage Form, A simple, precise, accurate and sensitive isocratic stability indicating RP-HPLC method has been developed and validated for determination of Ketoconazole in bulk drug and pharmaceutical dosage form. Isocratic RP-HPLC separation was achieved on Agilent C8 (150 mm ´4.6 mm, 5 µm particle size) with the mobile phase 0.3 % Triethylamine in 20 mM potassium dihydrogen phosphate buffer pH adjusted to 4.0: Acetonitrile (68:32 % v/v) at a flow rate 1.0 mL/min. The retention time of Ketoconazole was 8.97 ± 0.50 min. The method was validated for specificity, linearity, precision, accuracy and robustness. The linear regression analysis data of calibration curve showed good linearity in concentration range 10-60 mg/mL. The Intraday and Interday precision were 0.59-1.11 % and 0.26-1.73 % RSD respectively. The accuracy was found to be 98.11-99.26 %. The drug was subjected to the stress conditions like hydrolysis, oxidation, photolysis and dry heat. The proposed method is found to be specific with respect to degradation product formed after Acidic hydrolysis, Oxidation, Thermal and Photolytic degradation. The Ketoconazole was found to be stable under neutral hydrolytic, thermal and photolytic stress conditions. Acidic, thermal, photolytic stress conditions showed degradation.

The proposed chromatographic method can be used for estimation of drug during stress testing & formal stability studies.

Patel paresh *et al.*[20] have development and validation of RP-HPLC method for estimation of beclomethasone dipropionate and ketoconazole in combined dosage form, A simple, selective and rapid reversed phase high performance liquid chromatographic (RP-HPLC) method for the analysis of beclomethasone dipropionate and ketoconazole in combined dosage form has been developed and validated. The chromatographic system consisted of a quaternary pump with auto-sampler and PDA detector on a reversed-phase C18 column (250 mm × 4.6 mm i.d, 5 mm particle size) at ambient temperature with a mobile phase consisting of acetonitrile : acidic water(pH is adjusted 6.0 with acetic acid) (57 : 43, v/v) at flow rate of 0.8 mL/min and detection wavelength of 254 nm and the retention time was about 2.81 minutes for ketoconazole(KTZ) and 7.69 minutes for beclomethasone dipropionate(BCD). The method is selective and able to resolve drug peaks. The peaks of beclomethasone dipropionate and ketoconazole were well separated. The linearity of the calibration curves for each analyte in the desired concentration range was good (R^2>0.99). The method was accurate with recoveries in the range of 98-102% for both drugs and precise (%RSD of intra day variation were 0.60 – 0.78 for beclomethasone dipropionate and 0.74 - 1.05 for ketoconazole and %RSD of inter day variation were 0.89 - 1.72 for beclomethasone dipropionate and 0.76 – 1.69 for ketoconazole). The proposed method was found to be highly sensitive, accurate and precise and that's why this method can be used as a more convenient and efficient option for the analysis of beclomethasone dipropionate and ketoconazole in combined dosage form.

JinalHarshadkumar Rathod *et al.*[21] have performed estimation of clotrimazole, clindamycin phosphate and tinidazole by various analytical method, Analytical method development and its validation is an important aspect in drug discovery process. Development of analytical method producing accurate and precise data is necessary to ensure the quality and safety of the drugs. At present, the most common analytical method employed for estimation of drugs is Reverse Phase High Pressure Liquid Chromatography (RP-HPLC) because of its high sensitivity, accuracy and speed. Different types of analytical methods are available for estimation of Clindamycin Phosphate, Clotrimazole and Tinidazole including RP-HPLC. This review article briefly discusses analytical methods available for the estimation of Clindamycin Phosphate, Clotrimazole and Tinidazole individually, in combination with other drugs as well as in combine dosage form.

A S Low et *et al.*[22] have done An HPLC assay for the determination of ketoconazole in common pharmaceutical preparations, An HPLC method is described using octadecyl silica (3 microns) with an acetonitrile phosphate buffer mobile phase containing diethyl amine which is capable of separating ketoconazole [(+/-)-cis-1-acetyl-4-(4[2-(dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl)piperazine] from four related compounds, (R049223, R063600, R053165 and R039519) and from excipients in tablets, cream and shampoo. The method was validated using an external calibration method for tablets, shampoo and creams and a standard addition method for cream. The limits of detection for the related compounds in the presence of ketoconazole are also reported.

Vinay Kumar *et al.* [23] Application of Validated RP-HPLC Method for Simultaneous Determination of Docetaxel and Ketoconazole in Solid Lipid Nanoparticles, Docetaxel has

significant single agent activity in prostate cancer and ketoconazole also has activity as a second line hormonal agent. In vitro, ketoconazole is synergistic with some chemotherapy agents by enhancing the intracellular retention of the cytotoxic agent. A potential drug-drug interaction exists though between docetaxel and ketoconazole because both agents are metabolized hepatically by the cytochrome P-450 system. Hence, a nanoparticulate system was formulated by loading both drugs for tumor targeting. Assay and in vitro release of the formulation were conducted by developing simple, precise, accurate, and validated analytical method for simultaneous determination docetaxel and ketoconazole using reversed-phase high-performance liquid chromatography (RP-HPLC). The RP-HPLC method was developed using Waters Symmetry C18 column (25 cm × 4.5 mm, 5 µm) with a mobile phase consisting of acetonitrile and 0.2% triethylamine pH adjusted to 6.4 (48:52, v/v) at flow rate of 1 mL/min. Intra-day and inter-day variations were less than 2% over the linearity range, 0.5–20 µg/mL. The proposed two methods were successfully applied for the determination of docetaxel and ketoconazole in solid lipid nanoparticles.

Verma Vikrant *et al.*[24] Ketoconazole HPLC method development and validation: a novel approach, The present research article describes the method development and validation of ketoconazole by an innovative HPLC method in dosage form that are solid in nature tablets. Inertsil ODS-C18 column (150 mm,4.6 mm,5 µm) was used for the study and mobile phase consists of buffer (pH 4) and Methanol in the mixture of 30:70. Wavelength for the chromatographic separation used was 274 nm. Injection volume was maintained at 20 µL. A wide variety of mobile phase combinations used for the study and system contains integrated degasser. Flow rates was maintained at 1mL/min throughout the process for mobile phase combinations. In this method ICH guidelines were used for validation studies. After

analysis of different research and review articles it came to light that HPLC method development of ketoconazole has been done in different dosage forms but less work is done on solid dosage forms and hence still there is enormous potential for new methods to be developed in solid dosage form with different mobile phase combinations.

ÉrikaKedor-Hackmann *et al.*[25] have done Determination of Ketoconazole in Pharmaceutical Preparations by Ultraviolet Spectrophotometry and High Performance Liquid Chromatography, The aim of this research was to study and to standardize an ultraviolet spectrophotometric (UVS) and a high performance liquid chromatographic (HPLC) method for the determination of ketoconazole in commercially available pharmaceutical preparations (tablets and creams). The UVS method was both standardized at 222 nm and 269 nm in 0.01M HCl. Beer's law was obeyed in a range of concentration from 4.0 to 13.0 μg/mL at 222 nm and from 100.0 to 280.0 μg/mL at 269 nm. In the HPLC determination were used a Merck Lichrospher 100 RP-18 (5 μm) in Lichrocart (125–4) column, a mobile phase consisting of di-isopropylamine -methanol 1:500/ammonium acetate 1:200 (8:2) and UV detection at 225 nm. There was a correspondence in areas "versus" concentration in a range from 2.0 to 18.0 μg/mL.

MaíraPereira *et al.*[26] have carried out Development and validation of a simple chromatographic method for simultaneous determination of clindamycin phosphate and rifampicin in skin permeation studies, Rifampicin (RIF) and clindamycin phosphate (CDM) are the main drugs currently used in combination to treat severe infectious diseases in hair follicles. This work describes a simple, rapid and sensitive method for simultaneous analysis of RIF and CDM in the different skin layers using high performance liquid chromatography (HPLC). The efficient chromatographic separation of CDM and RIF was succeeded using a

C18 column (150 mm x 4.6 mm, 5 μm) with gradient elution using a mobile phase composed of 0.01 M phosphoric acid and methanol at a flow rate of 1 mL/min. Determinations were performed using UV-vis detector at 200 nm and 238 nm for CDM and RIF, respectively. The method was precise, accurate and linear (r^2 > 0.999) with regression curve in the concentration range from 0.5 to 20.0 μg/mL and recovery rates from the skin layers higher than 85%. The retention times for CDM and RIF were approximately 7.4 and 12.2 min, respectively. The presence of skin components did not interfere with the analysis. The validated method was therefore appropriate for quantification of both CDM and RIF and thus may be feasible to be used in skin permeation studies.

Batzias *et al.*[27] A new HPLC/UV method for the determination of clindamycin in dog blood serum. A new HPLC method for the quantitative determination of clindamycin in dog blood serum at levels down to 80 ng/mL has been developed. Samples were deproteinised with acetonitrile and clindamycin was extracted with dichloromethane. Chromatographic analysis was carried out on a C18 reversed-phase analytical column in the presence of tetra-n-butylammonium hydrogen sulfate (TBA), as an ion-pairing agent. UV detector wavelength was set at 195 nm. The assay was validated for a concentration range from 80 to 6000 ng/mL serum. Good linearity was observed in the entire concentration range. The limit of quantification (LOQ) was 80 ng/mL and the limit of detection (LOD) was 60 ng/ml. Regression of accuracy data yielded an overall mean recovery value (+/-S.E.M.) of 93.98+/-0.42%, while precision data revealed coefficient of variation CV% values lower than 4.41%. The method was successfully applied to determine drug concentrations in serum samples from dogs that had been orally administered clindamycin hydrochloride

Shao-Min Wang *et al.*[28] Separation and characterization of clindamycin phosphate and

related impurities in injection by liquid chromatography/electrospray ionization mass spectrometry, A simple high-performance liquid chromatography/electrospray ionization tandem mass spectrometric (HPLC/ESI-MS/MS) method has been developed for the rapid identification of clindamycin phosphate and its degradation products or related impurities in clindamycin phosphate injection. Detection was performed by quadrupole time-of-flight mass spectrometry (Q-TOFMS) via an ESI source in positive mode. Clindamycin phosphate and its related substances lincomycin, 7-epilincomycin-2-phosphate, lincomycin-2-phosphate, clindamycin B, clindamycin B-2-phosphate, and clindamycin were identified simultaneously by HPLC/ESI-MS/MS results. Based on the MS/MS spectra of their quasi-molecular ions, the fragmentation pathways of clindamycin phosphate and its related substances were compared and proposed, which are specific and useful for the identification of the lincosamide antibiotics and related impurities. The method was rapid, sensitive and specific and can be used to identify clindamycin phosphate and its related impurities in clindamycin phosphate injection without control compounds.

Yenni sriwahyuni *et al.*[29] have performed the effect of storage temperature on phosphate clindamycin stability in emulgel dosage form with hydroxypropyl methyl cellulose (hpmc) as a gelling agentjournal of pharmaceuticsand science, The present study was undertaken to investigate the effect of temperature on the degradation of clindamycin phosphate emulgel. This study aims to determine the rate of decomposition of clindamycin phosphate emulgel was storage at three different temperature of 30˚C, 50˚C, and 70˚C. Determination of percentage clindamycin phosphate was carried out every hour for 5 hours using Spectrophotometry UV-VIS. The results were statistically analyzed using the paired t-test. It was found a decrease in the levels of clindamycin phosphate emulgel at the storage

temperature of 30˚C by 99.08%; 50˚C by 96.93%, and a storage temperature of 70˚C by 96.01%. Therefore, it can be said that clindamycin phosphate in emulgel preparations is chemically stable for 5 hours at a storage temperature of 30˚C, 50˚C, and 70˚C and the kinetics of the degradation rate of clindamycin phosphate in the emulgel preparation is in accordance with the second-order. Based on the statistically processed data result, it can be concluded that the storage temperature of 30˚C, 50˚C, and 70˚C shows that there is a significant difference (<0.005) in the data.

Kumar *et al.*[30] have performed a Novel Method Development and Validation for Related Substances of Adapalene in Bulk Drug Product by HPLC, A simple and inexpensive method was developed with high performance liquid chromatography with PDA detection for determination of Adapalene and related impurities ((3-Adamantyl-4-methoxy) phenyl boronic acid, 6-(toluene-4-sulfonyloxy)-naphtalene -2-carboxylic acid methyl ester, 1-adamantyl-2-methoxybenzene,6-(3-adamantan-1-yl-4-methoxy-phenyl)-naphtalene-2-carboxylic acid methyl ester and 3,3'-di-(1-adamantyl)-4,4'-dimethoxy-1,1'-biphenyl). The chromatographic separations were achieved on (250×4.6 mm), 5.0 µm make: Phenomenex Luna column employing methanol, 0.1% orthophosphoric acid buffer, and Tetrahydrofuran in the ratio of 55:30:15 as mobile phase with gradient programmed at flow rate 1.0 mL/min was chosen. Five impurities were eluted within 35 minutes. The column temperature was maintained at 25^{o}C and a detector wavelength of 260 nm was employed. The method was successfully validated by establishing System Suitability, Specificity, Linearity, Accuracy, limit of detection and Limit of quantification.

Bhavesh Sharma *et al.*[31] A Recent Research Review- Development and Validation with liquid chromatography, nowadays many different methods of HPLC are uses for

Development of Drugs. HPLC are used to able separate, quantify and detection of drug and drug related substance that can form on manufacturing or storage of drugs. Different type of Chromatographic parameters are evaluated in order for optimize the method. A mobile phase, column, temperature of column, gradient and wavelength that are use for stability and comparability of drug and drug substance and also impurities of drugs. HPLC validation method is give all information regarding like Accuracy, precision, specificity, linearity, range and limit of detection, limit of quantification, robustness and system suitability testing as per guidelines of ICH.

Revathi nagalakshmiponnuri *et al.*[32] Development and validation of a stability indicating reverse phasehigh performance liquid chromatography method for simultaneous determination of clindamycin, metronidazole, and clotrimazole in pharmaceutical combined dosage forms. The objective of present work was to develop and validate a simple, fast, precise, selective, and accurate reverse phase high-performance liquid chromatography method for the simultaneous determination of Clindamycin, Metronidazole and Clotrimazole in a pharmaceutical dosage form. The separation of these three drugs was achieved on ODS 250×4.6 mm, 5 mm C18 column. Mobile phase containing 0.1% ortho phosphoric acid buffer and acetonitrile in the ratio of 55:45 v/v was pumped through column at a flow rate of 1mL/minute. Temperature was maintained at 30°C and ultraviolet detection at 238 nm. Results: The retention times were observed to be 2.591, 3.584, and 4.221 minutes for Clindamycin, Metronidazole, and Clotrimazole, respectively. Linearity was found to be 25-150 µg/mL Clindamycin, Metronidazole, and Clotrimazole, respectively. The method was statistically validated for linearity, recovery, the limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision. The stress testing of the drugs individually

and their mixture are carried out under acidic, alkaline, oxidation, photostability, and thermal degradation conditions and its degradation products are well resolved from the analyte peaks. This method was successfully validated for accuracy, precision, and linearity, LOD, and LOQ.

Rajendra Mhaske *et al.*[33] Identification of major degradation Products of Ketoconazole analytical methods were developed for the identification of major degradation products of Ketoconazole, an antifungal agent. The stressed degradation of Ketoconazole drug substance was performed under acid, base, thermal, photo and oxidative stress conditions. The major degradation was observed under acid, base and oxidative stress conditions. The degradation study was performed on Inertsil ODS-3V, length 100 X diameter 4.6 mm, particle size 3 µm column using gradient method. These degradants were identified by LC-MS technique.

Ramavath Mohanbabu naik *et al.*[34] performed Stability Indicating RP- HPLC - DAD Method for the Simultaneous Estimation of Hydrocortisone and Ketoconazole in Tablet Dosage form, A new stability-indicating high performance liquid chromatographic method was developed and validated for the determination of Hydrocortisone and Ketoconazole in a tablet dosage form. The separation was achieved on a reverse phase C18 column (Agilent ODS) (250 mm × 4.6 mm, 5 µ m) with mobile phase consisted of methanol: water (61:39 % v/v containing 0.2% HSA, pH 3.0 adjusted with Orthophosphoric acid) and the eluents were detected at 221nm. The retention time of Hydrocortisone and Ketoconazole was 6.0 min and 26.2 min respectively with the flow rate of 1mL/min. Drugs were subjected to stress conditions of acidic, basic, oxidation, photolytic, neutral and thermal degradation and considerable degradants were detected in all stress conditions. A total of 17 degradation

products were observed and well separated from analyte peaks, hence the method could effectively employed as stability-indicating one. The response of linear was in the range 5-50 µg/mL for Hydrocortisone and 10 to 70 µg/mL for Ketoconazole, respectively. The relative standard deviation values for intra- and interday precision studies were 1.06 % and 1.5 % for Hydrocortisone and 0.46 % and 1.1% for Ketoconazole. Recoveries ranged in between 98-102% for both Hydrocortisone and Ketoconazole.

Skiba *et al.*[35] have done Stability assessment of ketoconazole in aqueous formulations, Ketoconazole is an imidazole antifungal agent. It has a wide antifungal spectrum and possesses some antibacterial activity. In inappropriate formulations, especially in aqueous media, ketoconazole molecules may be unsteady. The stability of ketoconazole in aqueous media was assessed as a function of pH, antioxidant and ketoconazole concentrations. It was found that ketoconazole was least stable at pH 1 among the pH values studied (pH 1–9). Since the major degradation pathway was specific acid catalysis, based upon the transition-state theory, the entropy (DS) of the activation was calculated and found to be negative indicating that the activated complex was more constrained than the individual species. The free energy of activation (DG) was estimated to be 30 kcal/mol. The viscosity of the formulation was found to be more stable at high pH because Carbopol is stable at basic pH and protected ketoconazole. It appears that the amount of ketoconazole in the formulation has a low influence on the degradation mechanisms. The increase of the butylated hydroxytoluene antioxidant levels from 0.05 to 0.4%, adversely affected the stability of ketoconazole. In conclusion, the expected shelf life of the final ketoconazole formulation (pH 7, 0.1% butylated hydroxytoluene) was 15 months.

XiaomeiYang *et al.*[36] have done Qualitative and quantitative assessment of related

substances in the Compound Ketoconazole and Clobetasol Propionate Cream by HPLC-TOF-MS and HPLC, Related substances in pharmaceutical formulations are associated with their safety, efficacy and stability. However, there is no overall study already published on the assessment of related substances in the Compound Ketoconazole and Clobetasol Propionate Cream. In this work, a reliable HPLC-TOF-MS qualitative method was developed for the analysis of related substances in this preparation with a quick and easy extraction procedure. Besides the active pharmaceutical ingredients, two compounds named ketoconazole impurity B′ optical isomer and ketoconazole impurity E were identified. Furthermore, a new HPLC method for qualitative and quantitative assessment on related substances and degradation products, which were found in the stability test, was established and validated. The single standard to determine multi-components method was applied in the quantitative analysis, which was an effective way for reducing cost and improving accuracy. This study can provide a creative idea for routine analysis of quality control of the Compound Ketoconazole and Clobetasol Propionate Cream.

4. AIM AND OBJECTIVE

AIM

According to the literature review, there is no HPLC analytical method for estimating Clindamycin phosphate and Ketoconazole in a combination pharmaceutical dose form. Several publications done using various analytical techniques such as UV simultaneous estimation, LC-MS technique, and RP-HPTLC method for Clindamycin phosphate and Ketoconazole in pharmaceutical dose form, either alone or in combination with other drugs, are available.

As a result, an appropriate HPLC approach for routine analysis of Clindamycin phosphate and Ketoconazole in mixed formulations is required. The goal of the study was to establish a simple, quick, and sensitive analytical approach for estimating clindamycin phosphate and ketoconazole in a combined formulation in compliance with ICH Q2B criteria, as well as to extend the method for routine analysis.

OBJECTIVE

Present work is to develop and validate a new simple, rapid, and sensitive method for the simultaneous estimation of Clindamycin phosphate and Ketoconazole in the pharmaceutical dosage form by following 3 methods.

1. Development of HPLC analysis for both drugs.
2. Validation of methods using the formulations.
3. Development of the method to separate the degraded products from the drug under various stress conditions

- Development of HPLC analysis for both drugs**:** Steps involved are,
  - ✓ Selection of suitable wavelength
  - ✓ Selection of stationary phase
  - ✓ Selection of mobile phase
  - ✓ Selection of column
- Validation parameters: steps involved are
  - ❖ Linearity
  - ❖ Precision
  - ❖ Method precision
  - ❖ Accuracy
  - ❖ Range
  - ❖ Robustness
  - ❖ Ruggedness
  - ❖ Specificity
- Development of the method to separate the degraded products from the drug under various stress conditions involves:
  - ❖ Acid hydrolysis
  - ❖ Basic hydrolysis
  - ❖ Oxidation with Hydrogen Peroxide
  - ❖ Heat
  - ❖ Water hydrolysis
  - ❖ Humidity

5. MATERIALS AND METHODS

5.1. Materials and Instruments:

The following materials used were the best possible Pharma grade available as supplied by the manufacturer or supplier without further purification.

Working standards of Clindamycin phosphate and Ketoconazole were obtained from Swapnroop drugs and pharmaceuticals, Aurangabad, Maharashtra, India.

5.1.1 Instruments

- ✓ Afcoset ER-200A Digital balance
- ✓ Adwa – AD 1020 PH Meter
- ✓ HPLC –Waters (Acquity arc).
- ✓ LABINDIA UV 3000^{+-} UV/VIS spectrophotometer

5.1.2 Chromatographic solvents

- ✓ Methanol,
- ✓ Acetonitrile (HPLC grade),
- ✓ Potassium Dihydrogen Phosphate,
- ✓ Ortho Phosphoric Acid

5.2 Optimized chromatographic conditions:

5.2.1 Selection of wavelength

UV spectrum of Clindamycin and Ketoconazole diluent (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 210 nm. At this wavelength both the drugs show good absorbance.

5.2.2 Selection of Stationary phase

As the drugs were polar in nature, non- polar stationary phase was preferred. The C_8 column was selected as reported in the literature.

5.2.3 Selection of mobile phase

Initially the mobile phase tried was methanol: buffer and acetonitrile: buffer with various combinations as well as varying proportions. Finally, the mobile phase was optimized to potassium dihydrogen phosphate with buffer and acetonitrile in proportion 50: 50 v/v respectively.

5.2.4 Procedure

5.2.4.1 Preparation of Phosphate buffer:

Weigh about 1.36 g of potassium dihydrogen phosphate in to a 1000 mL volumetric flask, dissolve and make up with water. Adjust the pH to 2.5 with Orthophosphoric acid.

5.2.4.2 Mobile Phase A: Buffer

5.2.4.3 Mobile Phase B: Acetonitrile

5.2.4.4 Diluent Preparation: Mix 50 volumes of Buffer and 50 volumes Acetonitrile.

5.2.4.5 Preparation of Standard solution-A:

Weigh accurately 80.0 mg of Ketoconazole WS/RS into a 100 mL volumetric flask. dissolve and make up the volume with diluent. Mix well.

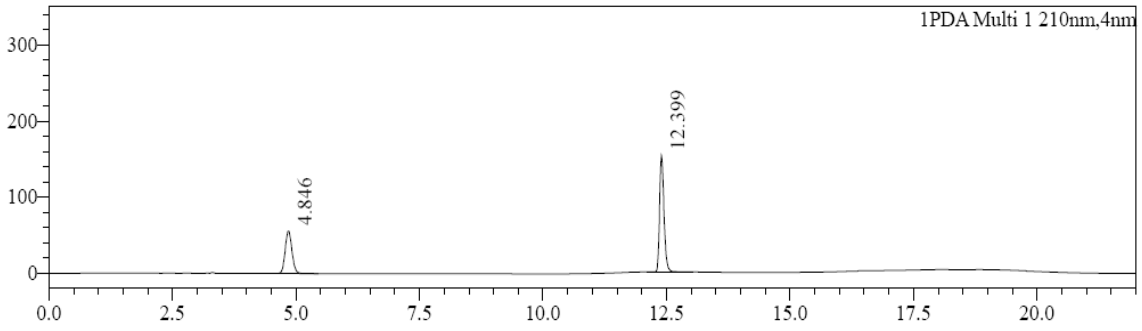
5.2.4.6 Preparation of Standard solution:

Weigh accurately 50.0 mg of Clindamycin Phosphate WS/RS into a 100 mL volumetric flask. Add 20 mL of diluent and Sonicate for 1 minute to dissolve. Add 5 mL of standard solution-A and make upto 100 mL with diluent. Mix well.

5.3 OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

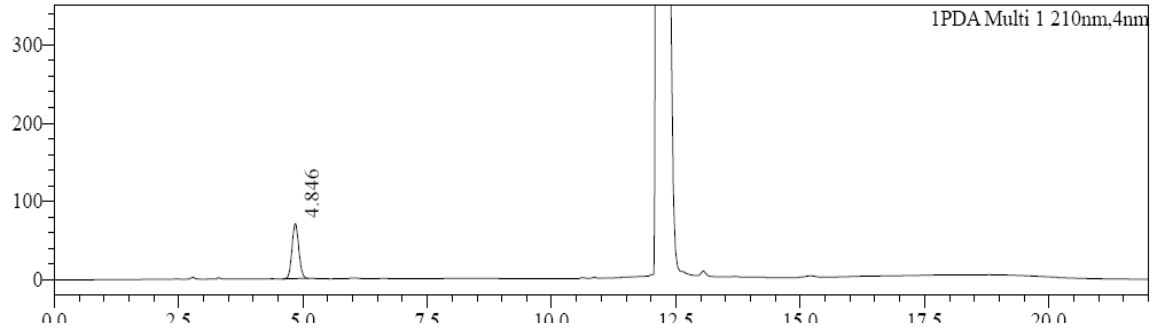
Instrument	: HPLC with UV/PDA detector
Column	: C8, 250 X 4.6 mm, 5µm
Flow Rate	: 1.0 mL/min
Column Temperature	: 30°C
Injection Volume	: 5 µL
Wave length	: 210 nm
Run time	: 22.0 minutes

Standard:



Sample:

Clindamycin phosphate:



Ketoconazole:

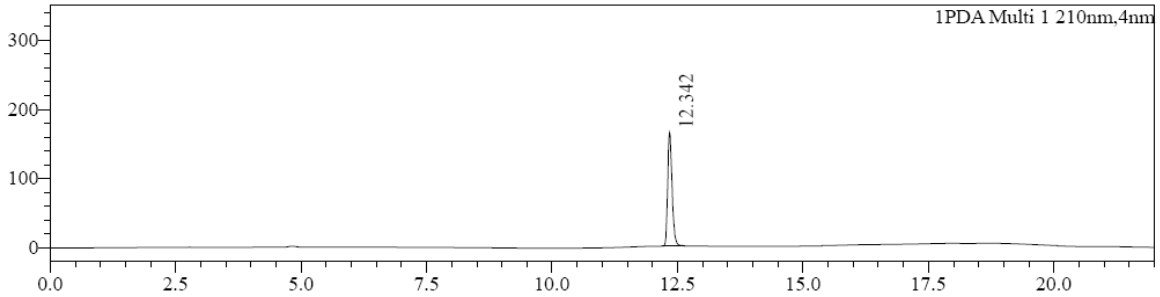


Fig no: 5.1 Optimized chromatograms of standard and sample

Analytical Method Optimization

The present study is to develop a new liquid chromatographic method for simultaneous determination of clindamycin phosphate and ketoconazole Tablet dosage form.

Quantitative determination of the drug by using the developed method

Sample : Clindamycin phosphate and ketoconazole

Label claim : 100 of Clindamycin and 400 mg of Ketoconazole

Assay formula

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{Dt}{WT} \times \text{Average weight of capsule}$$

Where,

AT = Peak Area of sample solution.

AS = Peak Area of standard solution.

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

Acceptance criteria**:** The limit of assay is in between the 98% - 102%. The chromatograms are as shown in Figure 5.1.

5.4 Validation

Validation is a process of establishing documented evidence which provides ahigh degree of assurance that a specific process will consistently produce meeting, itspredetermined specifications and quality attributes.

The objective of the analytical procedure should be clearly understood since this willgovern the validation characteristics which need to be evaluated. Typical validationcharacteristics which should be considered are listed below.

- Accuracy
- Precision
- Specificity
- Linearity & Range

- Robustness
- Ruggedness
- System suitability testing.

After method development, the validation of the current method has been performed in accordance with USP requirements for assay determination (Category-I analytical methods for quantitation of active ingredients in finished pharmaceutical products) which include accuracy, precision, selectivity, linearity and range, robustness and ruggedness.

5.4.1 Method Precision:

5.4.1.1 Preparation of Standard solution-A:

Weigh accurately 80.0 mg of Ketoconazole WS/RS into a 100 mL volumetric flask. dissolve and make up the volume with diluent. Mix well.

5.4.1.2 Preparation of Standard solution:

Weigh accurately 50.0 mg of Clindamycin Phosphate WS/RS into a 100 mL volumetric flask. Add 20 mL of diluent and Sonicate for 1 minute to dissolve. Add 5 mL of standard solution-A and make upto 100 mL with diluent. Mix well.

5.4.1.3 Preparation of Sample Solution:

Take 20 capsules, cut and open the medicament. Collect the medicament in clean dry petri plates. Weigh 1500 mg of medicament (Equivalent to 100 mg of Clindamycin and 400 mg of Ketoconazole) into a 200 mL volumetric flask, add

140 mL of diluent, sonicate for 10 minutes and heat for 10 minutes, make up the volume with diluent. Filter through 0.45 µm nylon filter.

The standard and sample solutions of Clindamycin and Ketoconazole was injected for five times and the peak areas were recorded as below. The mean and percentage relative standard deviation were calculated from the peak areas.

Table No: 5.1 Method precision of clindamycin and ketoconazole

CONTENT	CLINDAMYCIN	KETOCONAZOLE
PREPARATION	% ASSAY	% ASSAY
1	99.1655	99.6916
2	98.1657	100.9281
3	99.6721	99.4652
4	99.1019	99.7382
5	99.2794	99.6652
6	98.1403	101.9047
AVERAGE	98.9	100.2
SD	0.626	0.972
%RSD	0.6	1.0

5.4.2 Intermediate Precision/Ruggedness:

Intermediate precision is demonstrated as per protocol by preparing in 6 replicates the sample from the same Batch (Which is used by Analyst-1) of the product “Clindamycin 100 mg and Ketoconazole 400 mg Suppositories” by a different Analyst, using a different Instrument, Column and on a different day.

Table No: 5.2 Intermediate Precision of Clindamycin phosphate& Ketoconazole

PARAMETER	METHOD PRECISION	INTERMEDIATE
CONTENT	CLINDAMYCIN	CLINDAMYCIN
PREPARATION	% ASSAY	% ASSAY
1	99.1655	98.0893
2	98.1657	100.2656
3	99.6721	98.3331
4	99.1019	99.0505
5	99.2794	99.4774
6	98.1403	99.6211
AVERAGE	98.9	99.1
%RSD	0.6	0.8
CONFIDENCE	0.5	0.7
OVERALL %RSD	0.7	

PARAMETER	METHOD PRECISION	INTERMEDIATE
CONTENT	KETOCONAZOLE	KETOCONAZOLE
PREPARATION	% ASSAY	% ASSAY
1	99.6916	100.3644
2	100.9281	99.0754
3	99.4652	100.8196
4	99.7382	99.9031
5	99.6652	99.2504
6	101.9407	99.3070
AVERAGE	100.2	99.800
%RSD	1.0	0.7
CONFIDENCE LIMIT	0.8	0.6
OVERALL %RSD	0.8	

5.4.3 Accuracy:

For accuracy determination, three different concentrations were prepared separately i.e. 50%, 100% and 150% for the analyte and chromatograms are recorded for the same.

5.4.3.1 Preparation Sample solutions:

Preparation of 50% solution

Weigh accurately 982.0 mg of placebo, 50.0 mg of Clindamycin phosphate and 200.0 mg of Ketoconazole into a 200 volumetric flask.

Add 140 mL of Diluents, sonicate to dissolve and heat for 10 minutes, make up the volume with diluents. Filter through 0.45 µ nylon syringe filter. (Use this filtrate as Clindamycin phosphate sample solution).

Pipette out 2 mL of above solution in to 100.0 mL volumetric flask and dilute to volume with diluent. Mix well. (Use this solution as Ketoconazole Sample solution).

Preparation of 100% solution

Weigh accurately 982.0 mg of placebo, 100.0 mg of Clindamycin phosphate and 400.0 mg of Ketoconazole into a 200 volumetric flask.

Add 140 mL of Diluents, sonicate to dissolve and heat for 10 minutes, make up the volume with diluents. Filter through 0.45 µ nylon syringe filter. (Use this filtrate as Clindamycin phosphate sample solution).

Pipette out 2 mL of above solution in to 100.0 mL volumetric flask and dilute to volume with diluent. Mix well. (Use this solution as Ketoconazole Sample solution)

Preparation of 150% solution

Weigh accurately 982.0 mg of placebo, 150.0 mg of Clindamycin phosphate and 600.0 mg of Ketoconazole into a 200 volumetric flask.

Add 140 mL of Diluents, sonicate to dissolve and heat for 10 minutes, make up the volume with diluents. Filter through 0.45 µ nylon syringe filter. (Use this filtrate as Clindamycin phosphate sample solution).

Pipette out 2 mL of above solution in to 100.0 mL volumetric flask and dilute to volume with diluent. Mix well. (Use this solution as Ketoconazole Sample solution). Results are shown below.

Table No: 5.3 Recovery of Clindamycin Phosphate & Ketoconazole:

SNo	Concentration	Amount of CLN phosphate (ppm)	Amount of CLN phosphate recovered	Recovery %	Avg %	RSD %
01	50.0%	255.0000	254.6352	99.8569	100.9	1.0
	50.0%	249.0000	252.7749	101.5160		
	50.0%	244.5000	248.4752	101.6258		
02	100.0%	544.5000	541.2743	99.4075	99.7	1.8
	100.0%	536.5000	545.4365	101.6657		
	100.0%	510.5000	500.4498	98.0313		
03	150.0%	767.0000	779.8679	101.6776	100.1	1.4
	150.0%	791.5000	783.9639	99.0478		
	150.0%	773.5000	771.3316	99.7196		
				Average	100.2	**NA**
				Overall %RSD	0.6	

SNo	Concentration	Amount of KTZ added (ppm)	Amount of KTZ recovered	Recovery %	Avg%	RSD %
01	50.0%	21.9900	22.2588	101.2223	100.6	0.6
	50.0%	20.8400	20.8592	100.0921		
	50.0%	20.6400	20.7824	100.6899		
02	100.0%	40.4500	39.792	98.3733	99.1	0.6
	100.0%	40.1900	39.9898	99.5018		
	100.0%	40.9000	40.6688	99.4347		
03	150.0%	63.5100	64.7284	101.9184	101.4	0.4
	150.0%	62.6200	63.4883	101.3866		
	150.0%	65.8200	66.5582	101.1215		
				Average	100.4	NA
				Overall %RSD	1.2	

5.4.4 LINEARITY:

Linearity is the ability of the method to obtain test results that are directly proportional to the analyte concentration within a given range.

5.4.4.1 Preparation of sample stock solution:

The linearity of the method is established by performing 5 test concentrations from 50.0% to 150% of working concentrations as per protocol. The standard solutions were prepared with the concentrations of 50%, 75%, 100%, 125% and 150% with respect to 100% working concentration. For each concentration 3 replicates and injections are given into HPLC system.

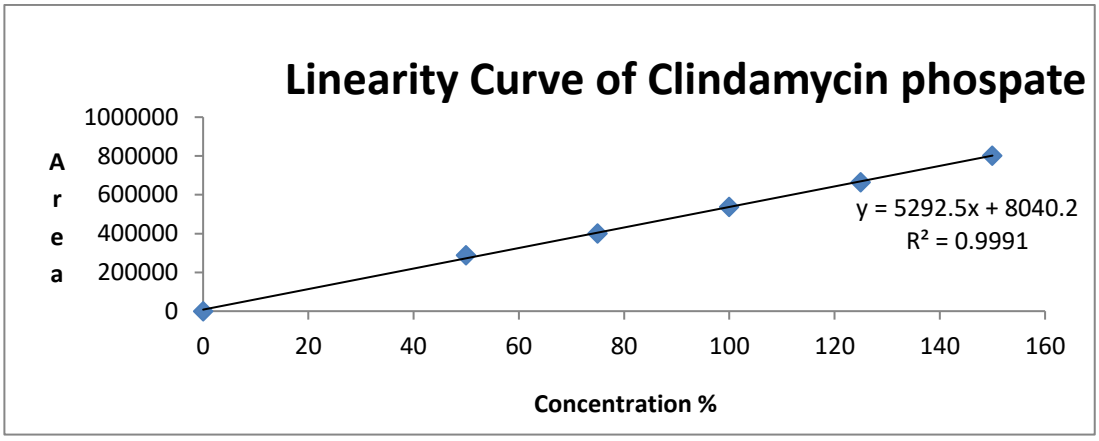


Fig no: 5.2 Linearity curve for Clindamycin phosphate

Acceptance criteria:

System Suitability Parameters	Observed value	Acceptance criteria
Correlation co-eff (r)	**0.999**	**NLT 0.999**
% Of y-Intercept	**1.5**	**±2.0**

Conclusion:

The correlation coefficient and Y-Intercept of the plot is calculated as 0.999 and 1.5 respectively. Hence it is concluded that the method is linear within the concentration of 50 % to 150% with respect 100 % working concentration of Clindamycin.

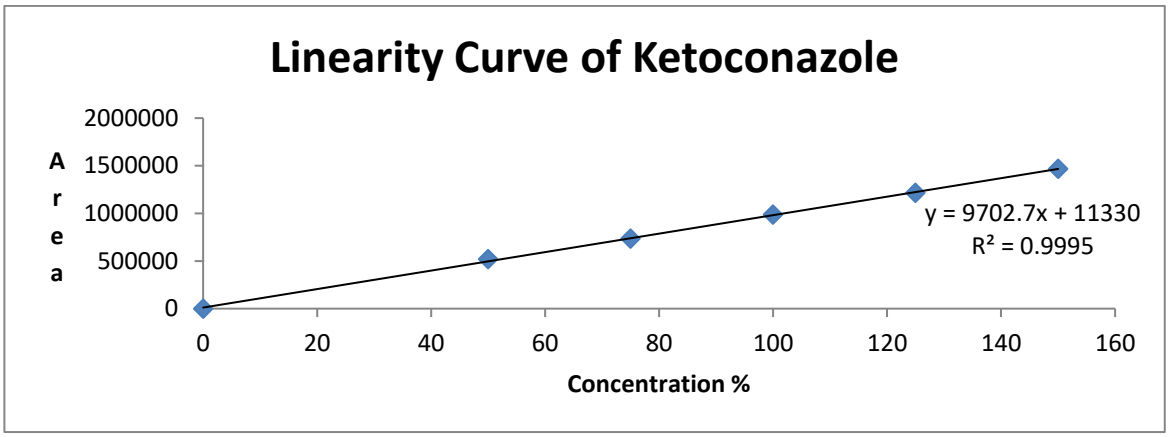


Fig no: 5.3 Linearity curve for Ketoconazole

Acceptance criteria:

System Suitability Parameters	Observed value	Acceptance criteria
Correlation co-eff (r)	**0.999**	**NLT 0.99**
% of y-Intercept	**1.1**	**±2.0**

Conclusion:

The correlation coefficient and Y-Intercept of the plot is calculated as 0.999 and 1.1 respectively. Hence it is concluded that the method is linear within the

concentration of 50 % to 150% with respect 100 % working concentration of Ketoconazole.

5.4.5 ROBUSTNESS:

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

5.4.5.1 Effect of variation in flow rate:

The robustness of the analytical method is demonstrated by small variations in the flow rate (0.8 mL/min, 1.0mL/min and 1.2mL/min) as per protocol. The standard and sample solutions were prepared and injected into HPLC as per the method. The results obtained are listed below.

Table No: 5.4 Variation in flow rate of Clindamycin Phosphate & Ketoconazole

System Suitability Parameters	Observed value			Acceptance criteria
	0.8 mL	1.0 mL	1.2 mL	
The average % of Content of Clindamycin from 2 replicate sample preparation from three different flow rate	**99.5**	**98.7**	**99.7**	90.0-110.0%
The average % of Content of Ketoconazole from 2 replicate sample preparation from three different flow rate	101.9	100.3	102.3	90.0-110.0%

Conclusion:

The results obtained were within the acceptance criteria (NMT 2.0). Hence it is concluded that the method is Robust with respect to small variations in the flow rate

5.4.5.2 Effect of variation in Oven temperature:

The robustness of the analytical method is demonstrated by small variations in oven temperature (28°C, 30°C and 32°C) Temperature as per protocol. The standard and sample solutions were prepared and injected into HPLC as per the method. The results obtained are listed below

Table No: 5.5 Effect of oven temperature of Clindamycin phosphate& Ketoconazole

System Suitability Parameters	Observed value			Acceptance criteria
	28°C	30°C	32°C	
The average % of Content of Clindamycin from 2 replicate sample preparation from three different temperatures.	100.7	98.7	100.2	NLT 90.0-110.0
The average % of Content of Ketoconazole from 2 replicate sample preparation from three temperature	101.7	100.3	101.3	NLT 90.0-110.0

Conclusion:

The results obtained were within the acceptance criteria (NMT 2.0). Hence it is concluded that the method is robust with respect to small variations in the Column oven temperature.

5.4.5.3 Effect of variation in Wavelength:

The robustness of the analytical method is demonstrated by small variations in the wavelengths (208,210 and 212nm) as per protocol.

The standard and sample solutions were prepared and injected into HPLC as per the method. The results obtained are listed below

Table No: 5.6 Effect of variation in Wavelength of Clindamycin phosphate& Ketoconazole

System Suitability Parameters	Observed value			Acceptance criteria
	208nm	210nm	212nm	
The average % of Content of Clindamycin from 2 replicate sample preparation from three different wavelength	98.6	98.7	98.6	90.0-110.0
The average % of Content of Ketoconazole from 2 replicate sample preparation from three different wavelength	100.3	100.3	100.3	90.0-110.0

Conclusion:

The results obtained are within the acceptance criteria (NMT 2.0). Hence it is concluded that the method is robust with respect to small variations in the Wavelengths.

5.5 FORCE DEGRADATION STUDY:

Following stress conditions are generally adopted during forced degradation study, but conditions can be decided and optimized based on physicochemical properties of drug substance, available literature and amount of degradation achieved during study.

Following are the general parameters for different stress conditions.

5.5.1 Acid Hydrolysis:

Transfer 1500.0 mg medicament and 982.0 mg of Medicament placebo, in separate reflux flasks. Add about 25 mL to 50 mL of 0.1 N Hydrochloric acid tothe flask and reflux. After refluxing, cool the sample and add same quantity of 0.1N Sodium Hydroxide so as to neutralize the solution. Transfer contents to a 200 mL volumetric flask, add 140 mL of diluent, sonicate for 10 minutes and heat for 10 minutes, make up the volume with diluent. Filter through 0.45µ nylon syringe filter. (Use this filtrate as Clindamycin phosphate sample solution). Pipette out 2 mL of above solution in to 100.0 mL volumetric flask and dilute to volume with diluent. Mix well. (Use this solution as Ketoconazole Sample solution).

- Prepare the blank solution as per above preparation omitting medicament.

- Inject blank, Placebo, unstressed sample and degradation sample into the HPLC system with photo diode array detector as per the test method and calculate the net degradation.

- Also, main analyte peak shall be confirmed for its purity index.

5.5.2 Base Hydrolysis:

Transfer 1500.0 mg medicament and 982.0 mg of Medicament placebo, in separate reflux flasks. Add about 25 mL to 50 mL of 0.1 N Sodium hydroxide to the flask and reflux. After refluxing, cool the sample and add same quantity of 0.1N Hydrochloric acid so as to neutralize the solution. Transfer contents to a 200 mL volumetric flask, add 140 mL of diluent, sonicate for 10 minutes and heat for 10 minutes, make up the volume with diluent. Filter through 0.45µ nylon syringe filter. (Use this filtrate as Clindamycin phosphate sample solution).

- Pipette out 2 mL of above solution in to 100.0 mL volumetric flask and dilute to volume with diluent. Mix well. (Use this solution as Ketoconazole Sample solution).
- Prepare the blank solution as per above preparation omitting medicament.
- Inject blank, Placebo, unstressed sample and degradation sample into the HPLC system with photo diode array detector as per the test method and calculate the net degradation.
- Also, main analyte peak shall be confirmed for its purity index.

5.5.3 Oxidation with Hydrogen Peroxide:

Transfer 1500.0 mg medicament and 982.0 mg of Medicament placebo, in separate reflux flasks. Add about 25 mL to 50 mL of 1% of Hydrogen peroxide to the flask and reflux. After refluxing, cool the sample and transfer contents to a 200 mL volumetric flask. add 140 mL of diluent, sonicate for 10 minutes and heat for 10 minutes, make up the volume with diluent. Filter through 0.45µ nylon syringe filter. (Use this filtrate as Clindamycin phosphate sample solution). Pipette out 2 mL of above solution in to 100.0 mL volumetric flask and dilute to volume with diluent. Mix well. (Use this solution as Ketoconazole Sample solution).

- Prepare the blank solution as per above preparation omitting medicament.
- Inject blank, Placebo, unstressed sample and degradation sample into the HPLC system with photo diode array detector as per the test method and calculate the net degradation.
- Also, main analyte peak shall be confirmed for its purity index

5.5.4 Thermal:

- Drug product and placebo shall be exposed to heat at 105 ^{0}C.
- Transfer 1500.0mg of medicament and 982.0 mg of Medicament placebo in separate reflux flasks and prepare the samples as per method description.
- Prepare blank as per method description 8.0

- Inject blank, standard solution, unstressed sample and stressed sample into the HPLC system with photo diode array detector as per the test method and calculate the net degradation.

- Also, main analyte peak shall be confirmed for its purity.

5.5.5 Water Hydrolysis:

Transfer 1500.0mg of medicament and 982.0 mg of Medicament placebo in separate reflux flasks. After refluxing, cool the sample and transfer contents to a 200 mL volumetric flask. add 140 mL of diluent, sonicate for 10 minutes and heat for 10 minutes, make up the volume with diluent. Filter through 0.45µ nylon syringe filter. (Use this filtrate as Clindamycin phosphate sample solution). Pipette out 2 mL of above solution in to 100.0 mL volumetric flask and dilute to volume with diluent. Mix well. (Use this solution as Ketoconazole Sample solution).

- Prepare the blank solution as per above preparation omitting medicament.

- Inject blank, Placebo, unstressed sample and degradation sample into the HPLC system with photo diode array detector as per the test method and calculate the net degradation.

- Also, main analyte peak shall be confirmed for its purity index

5.5.6 Humidity

- Drug product and placebo shall be exposed to humidity i.e. 90% RH and 25°C in a desiccator at least for 7 days.

- Transfer 1500.0mg of medicament and 982.0 mg of Medicament placebo in separate reflux flasks. and prepare the samples as per method description.
- Prepare blank as per method description 8.0
- Inject blank, standard solution, unstressed sample and exposure sample into the HPLC system with photodiode array detector as per the test method and calculate the net degradation. Also main analyte peak shall be confirmed for its purity.

Table No: 5.7 Stress conditions of Clindamycin and Ketoconazole

Stressed conditions	Clindamycin		
	% Content	% Degradation	Peak purity index
Unstressed Samples	100.0760		0.999
Acid Hydrolysis (0.1N HCl) reflux for 10 minutes at 60°C Sample	92.1348	7.9412	0.999
Base Hydrolysis (0.1M NaOH) reflux for 10 minutes at 60°C Sample	92.4732	7.6028	0.999
Oxidation reflux (1%H_2O_2) for 10 minutes at 60°C Sample	92.4625	7.6135	0.999
Water Hydrolysis reflux for 10 minutes at 60°C Sample	93.2791	6.7969	0.999
Exposed to heat for a period of 2 hours at 105^0C Sample	92.3684	7.7076	0.999
Exposed to humidity i.e. 90% RH and 25°C in a desiccator for 7 days Sample	96.1336	3.9424	0.999

Stressed conditions	**%Content**	**% Degradation**	**Peak purity index**
Unstressed Samples	100.8941	-	0.999
Acid Hydrolysis (0.1M HCl) reflux for 10 minutes at 60°C Sample	93.1457	7.7484	0.999
Base Hydrolysis (0.1M NaOH) reflux for 10 minutes at 60°C Sample	91.7891	9.1050	0.999
Oxidation reflux (1%H_2O_2) for 10 minutes at 60°C Sample	93.7501	7.1440	0.999
Water Hydrolysis reflux for 10 minutes at 60°C Sample	92.2033	8.6908	0.999
Exposed to heat for a period of 2 hours at 105^0C Sample	91.6560	9.2381	0.999
Exposed to humidity i.e. 90% RH and 25°C in a desiccator for 7 days Sample	89.9326	10.9615	0.999

Conclusion:

The chromatograms of stressed Blank, Placebo and sample solutions shows that there is no interference of Blank and placebo peaks and degradants peaks at the retention time of Clindamycin phosphate and Ketoconazole. The peak purity index values of standard and sample solutions are within the Acceptance criteria.

6. RESULTS AND DISCUSSION

6.1. Optimized chromatograms obtained by following conditions

Trial 1:

Column	:	C18 (4.6 x 150mm, 5µm or equivalent
Mobile phase	:	35% buffer: 65% methanol
Flow rate	:	1.0 mL/ min
Wavelength	:	230 nm
Temperature	:	ambient.
Run time	:	25 min.

Result: From the above mobile phase condition it was observed that the Ketoconazole peak was not detected.

Trial 2:

Column	:	Zodiac C18 (4.6 x 150mm, 5µm)
Mobile phase	:	45% buffer : 55% acetonitrile
Flow rate	:	1 mL/min
Wavelength	:	225 nm
Temperature	:	ambient.
Run time	:	30 min.

Result: From the above chromatogram condition it was observed that the Clindamycin and Ketoconazole peak shape is not good.

Trial 3:

Column	:	Symmetry C8 (4.6 x 150mm, 5µm)
Mobile phase	:	40% buffer : 60% acetonitrile
Flow rate	:	0.6 mL/ min
Wavelength	:	210 nm
Temperature	:	ambient.
Run time	:	6 min.

Result: From the above chromatogram it was observed that Clindamycin and Ketoconazole peaks are fronting and well separated.

Blank:

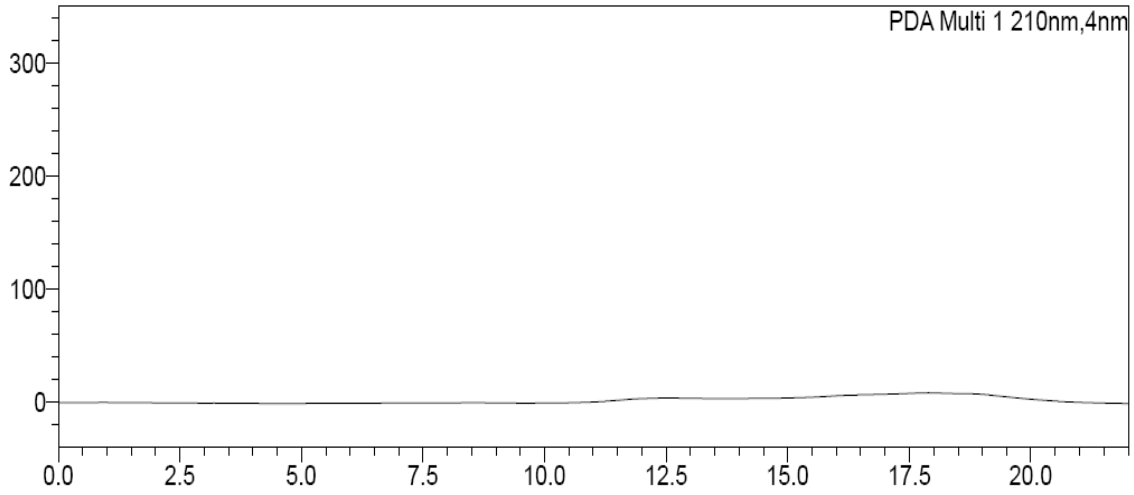


Fig no: 6.1 Chromatogram for blank

From the above chromatogram it was observed that there are no interferences

6.2: SYSTEM SUITABILITY:

Standard:

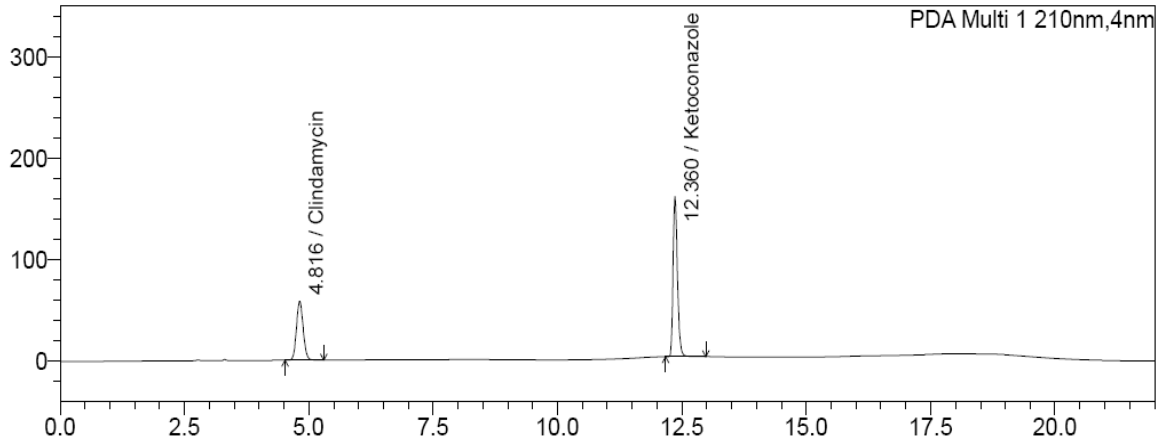


Fig no: 6.2 Chromatogram for system suitability

Table No: 6.1 Results of system suitability parameters for Clindamycin phosphate & Ketoconazole

No of Injection	Retention Time	Standard Area	Tailing factor	Theoretical plates
01	4.816	547891	1.101	21811
02	4.825	549454	1.103	22093
03	4.827	555223	1.104	21898
04	4.809	550633	1.100	21695
05	4.810	549257	1.103	22322
06	4.755	548484	1.102	21646
Average	4.807	550157	1.102	21911
SD	0.027	2651	0.001	257
%RSD	0.6	0.5	0.1	1.2

Table No: 6.2 Results of system suitability parameters for Ketoconazole:

No of Injection	Retention Time	Standard Area	Tailing factor	Theoretical plates
01	12.360	973865	1.280	305339
02	12.351	976225	1.278	298153
03	12.359	979017	1.284	305683
04	12.352	977430	1.274	301038
05	12.314	973688	1.284	307926
06	12.298	975293	1.277	301996
Average	12.339	975919	1.279	303356
SD	0.026	2077	0.004	3592
%RSD	0.2	0.2	0.3	1.2

Acceptance criteria:

- Resolution between two drugs must be not less than 2
- Theoretical plates must be not less than 2000
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

6.3: VALIDATION PARAMETERS:

6.3.1: Precision:

Precision of the method was carried out for both sample and standard solutions as described under experimental work. The corresponding chromatograms and results are shown below.

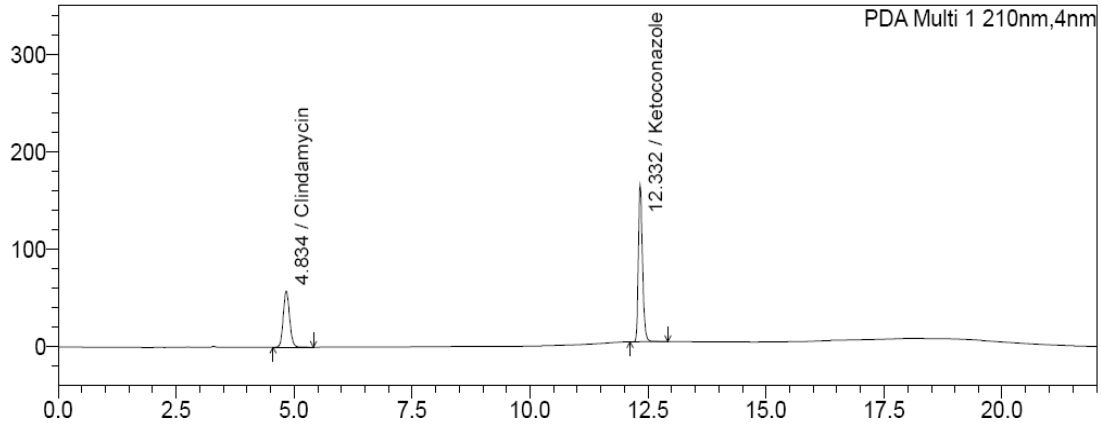


Fig no: 6.3 Chromatogram for standard injection -1

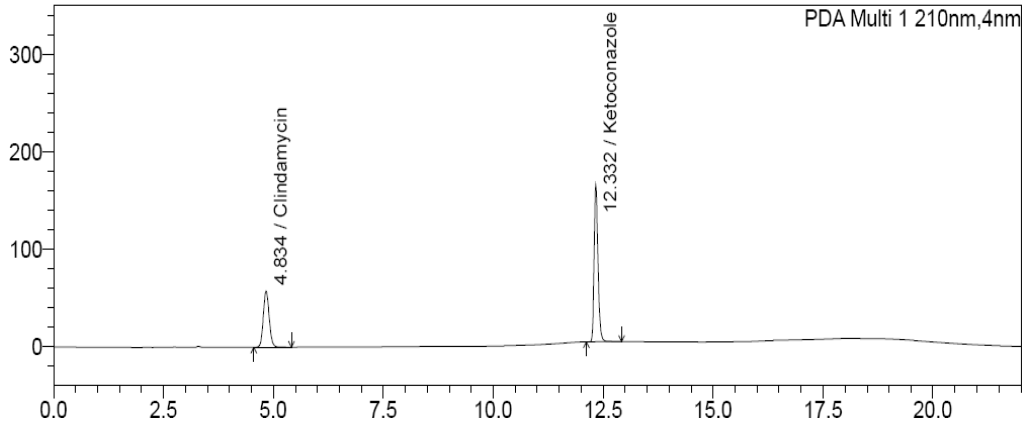


Fig no: 6.4 Chromatogram for standard injection-2

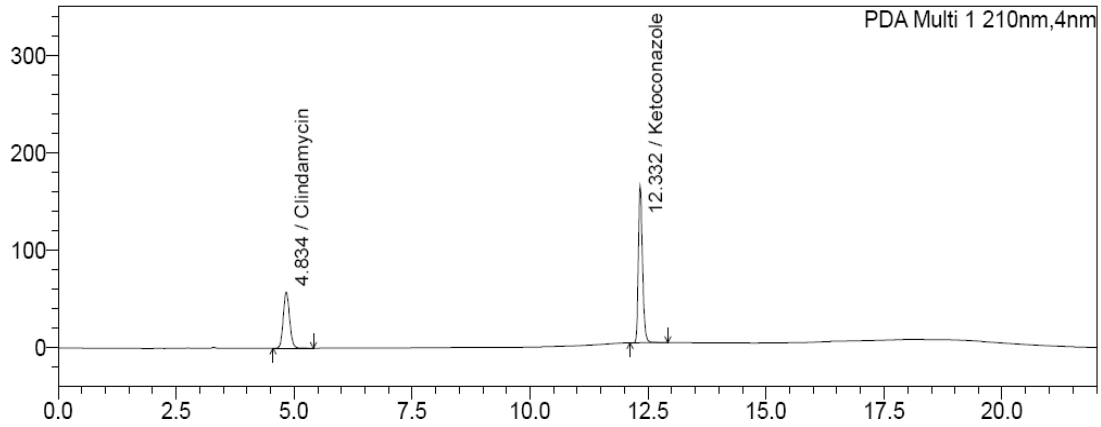


Fig no: 6.5 Chromatogram for standard injection-3

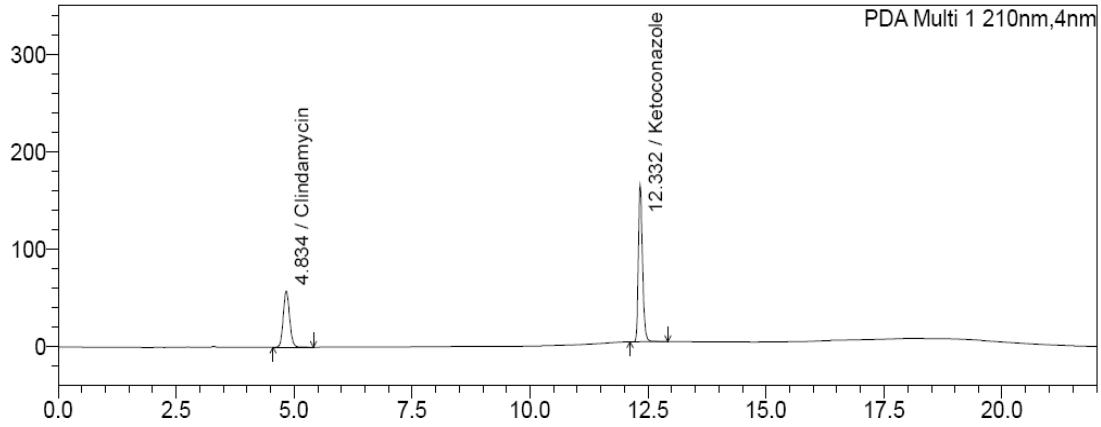


Fig no: 6.6 Chromatogram for standard injection-4

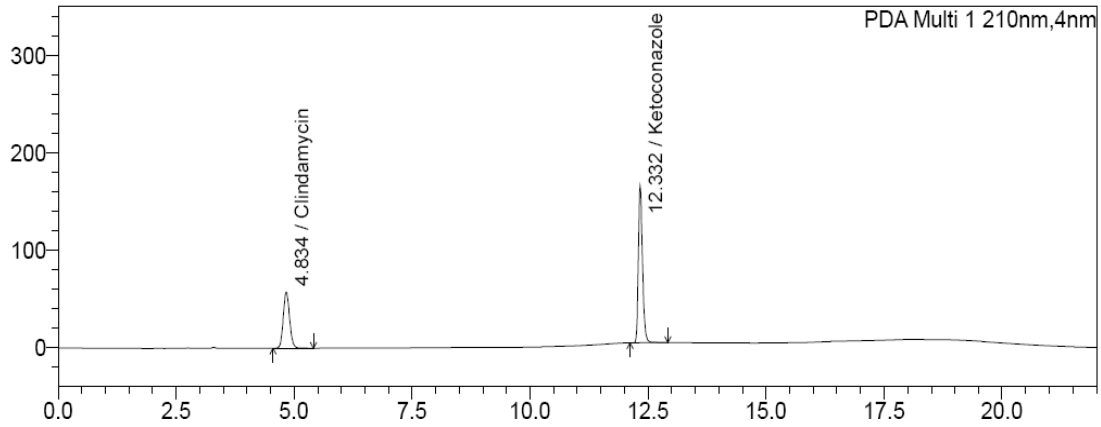


Fig no: 6.7 Chromatogram for standard injection-5

Table No: 6.3 Results of method precision for Clindamycin phosphate

No of Injection	Retention Time	Standard Area	Tailing factor	Theoretical plates
01	4.816	547891	1.101	21811
02	4.825	549454	1.103	22093
03	4.827	555223	1.104	21898
04	4.809	550633	1.100	21695
05	4.810	549257	1.103	22322
06	4.755	548484	1.102	21646
Average	4.807	550157	1.102	21911
SD	0.027	2651	0.001	257
%RSD	0.6	0.5	0.1	1.2

Table No: 6.3 Results of method precision for Ketoconazole

No of Injection	Retention Time	Standard Area	Tailing factor	Theoretical plates
01	12.360	973865	1.280	305339
02	12.351	976225	1.278	298153
03	12.359	979017	1.284	305683
04	12.352	977430	1.274	301038
05	12.314	973688	1.284	307926
06	12.298	975293	1.277	301996
Average	12.339	975919	1.279	303356
SD	0.026	2077	0.004	3592
%RSD	0.2	0.2	0.3	1.2

Acceptance criteria:

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

6.3.2. INTERMEDIATE PRECESION (ruggedness)

There was no significant change in assay content and system suitability parameters at different conditions of ruggedness like day to day and system to system variation.

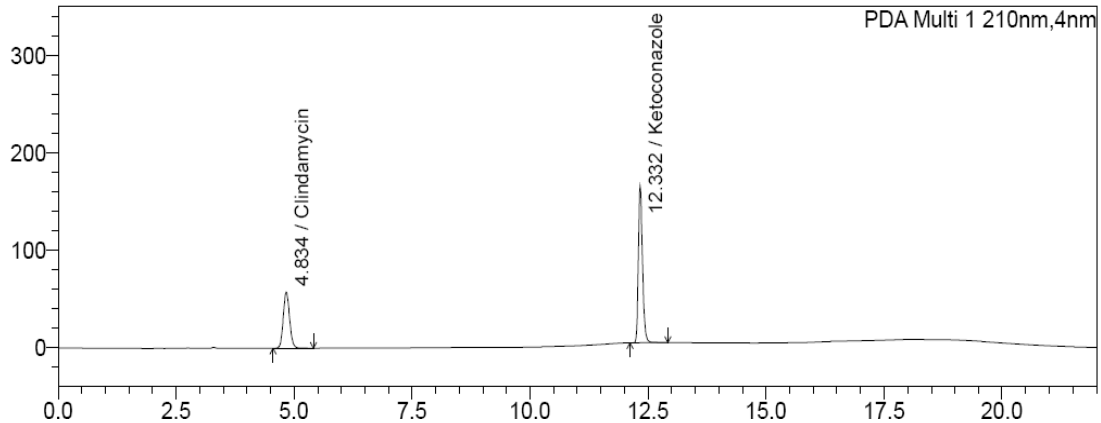


Fig no: 6.8 Chromatogram for sample injectiocn-1

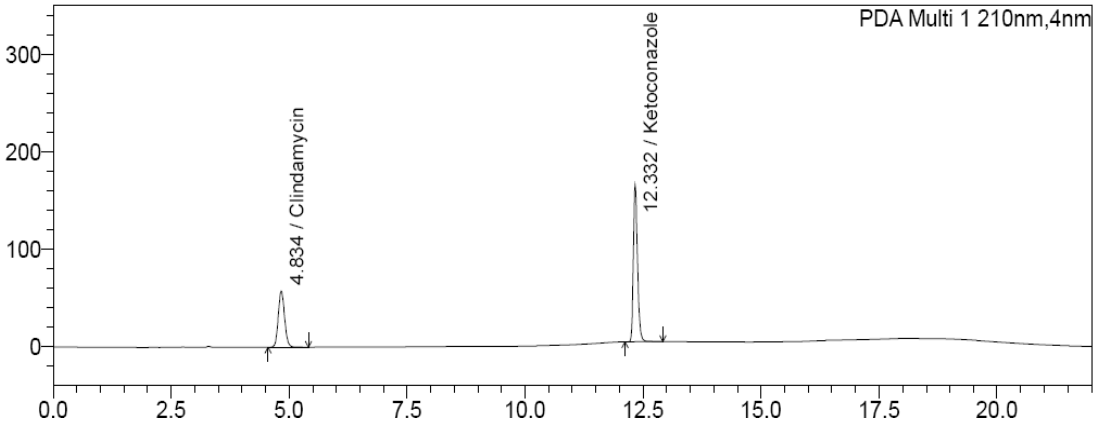


Fig no: 6.9 Chromatogram for sample injection-2

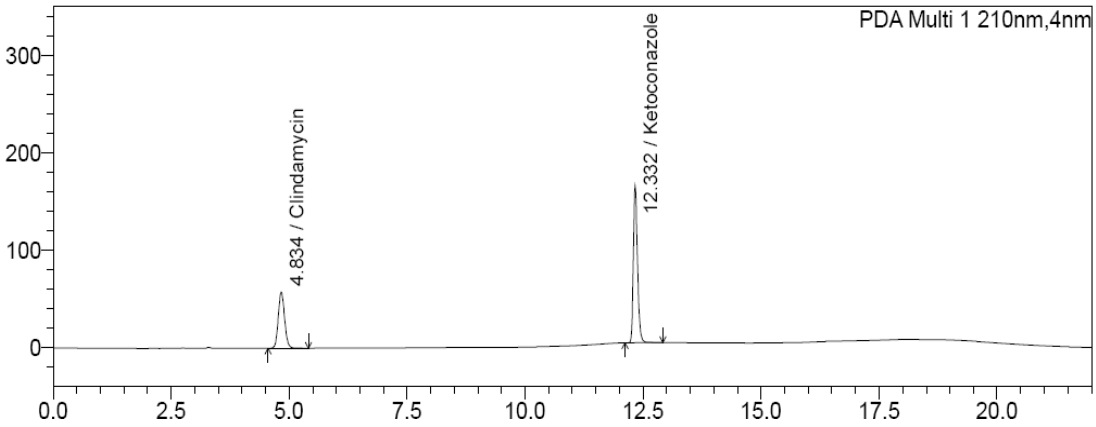


Fig no: 6.10 Chromatogram for sample injection-3

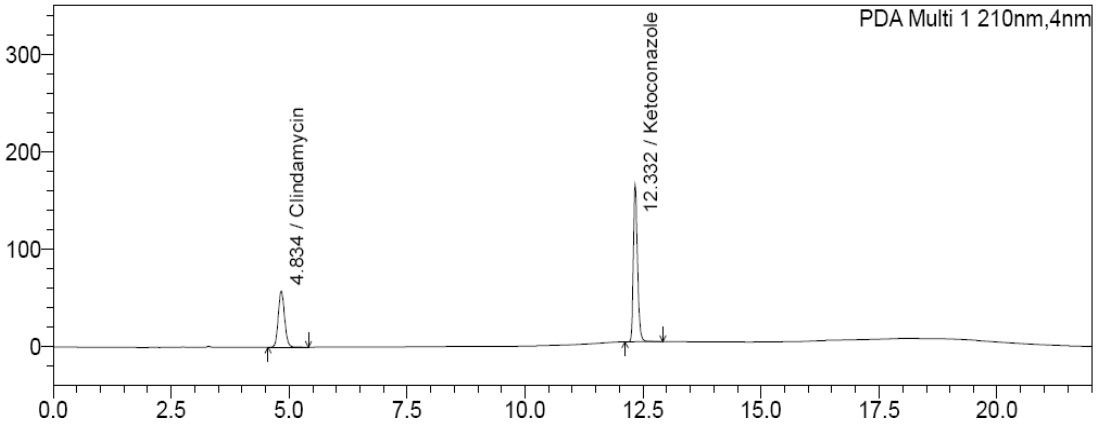


Fig no: 6.11 Chromatogram for sample injection-4

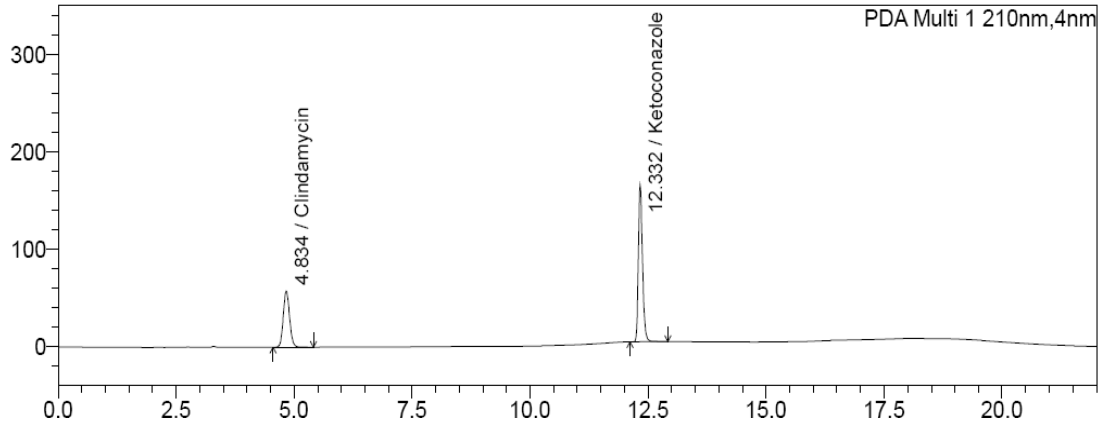


Fig no: 6.12 Chromatogram for sample injection-5

Table No: 6.5 Results of Intermediate precision for Clindamycin phosphate

No of Injection	Retention Time	Standard Area	Tailing factor	Theoretical plates
01	4.980	598184	1.121	5633
02	4.981	597209	1.122	5540
03	4.989	598819	1.118	5584
04	4.993	601012	1.117	5642
05	4.989	602771	1.119	5608
06	5.008	903263	1.129	5640
Average	4.990	600210	1.121	5608
SD	0.010	2513	0.004	40
%RSD	0.2	0.1	0.4	0.7

Table No: 6.6 Results of Intermediate precision for Ketoconazole

No of Injection	Retention Time	Standard Area	Tailing factor	Theoretical plates
01	12.320	1097612	1.320	97540
02	12.318	1089769	1.314	95879
03	12.330	1092849	1.317	95465
04	12.328	1097678	1.315	95830
05	12.334	1100559	1.316	95299
06	12.353	1098268	1.312	95494
Average	12.330	1096123	1.316	95918
SD	0.012	4000	0.002	825
%RSD	0.1	0.4	0.2	0.9

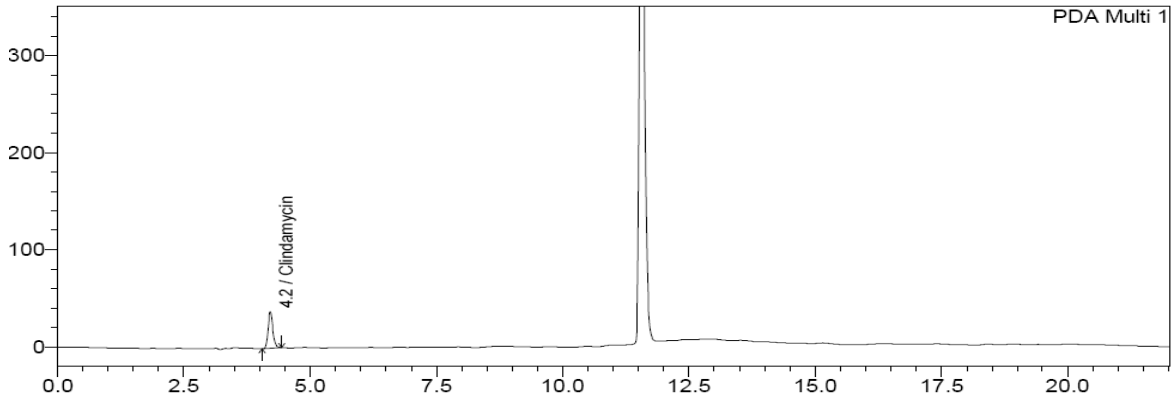
Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

6.3.3: ACCURACY:

Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated.

Clindamycin:



Ketoconazole:

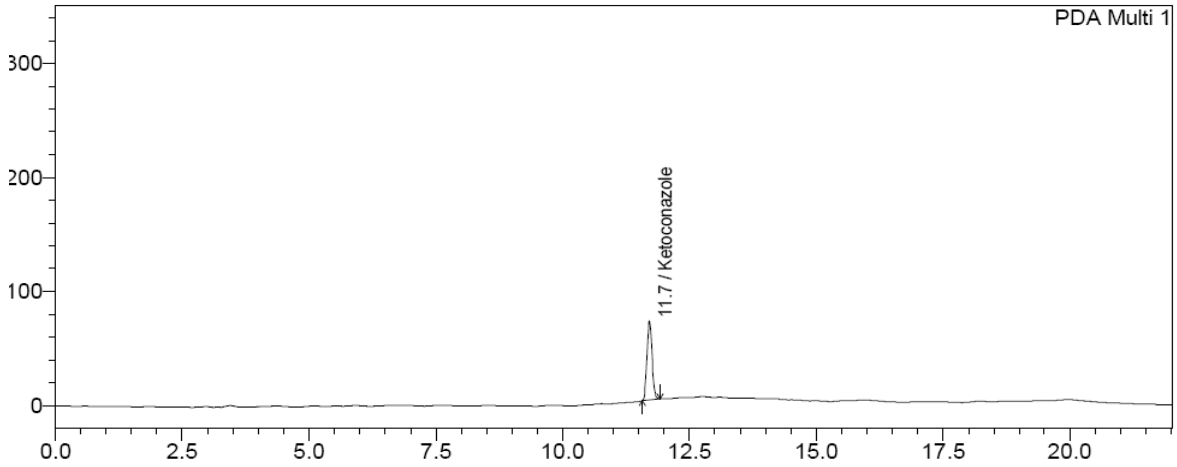
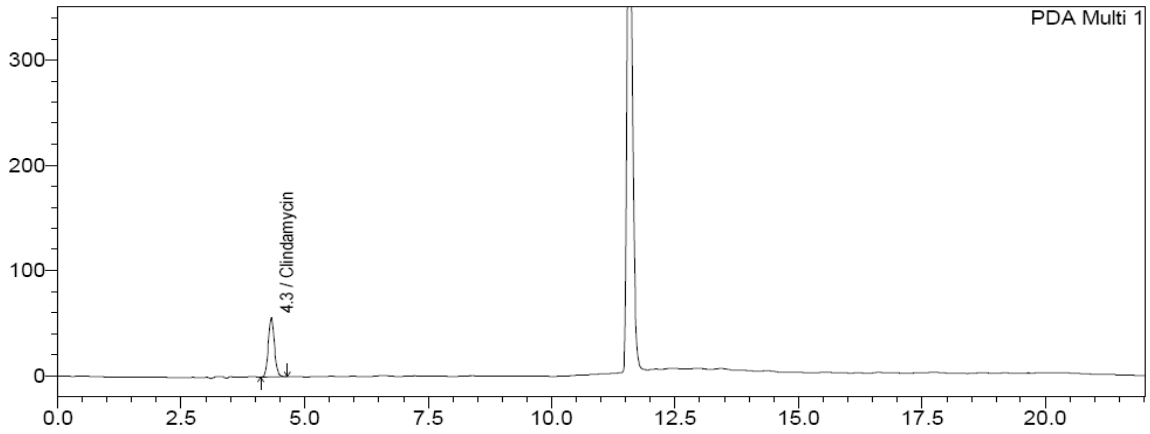


Fig no: 6.13 Chromatogram for sample concentration-50% of Clindamycin and Ketoconazole

Clindamycin:



Ketoconazole:

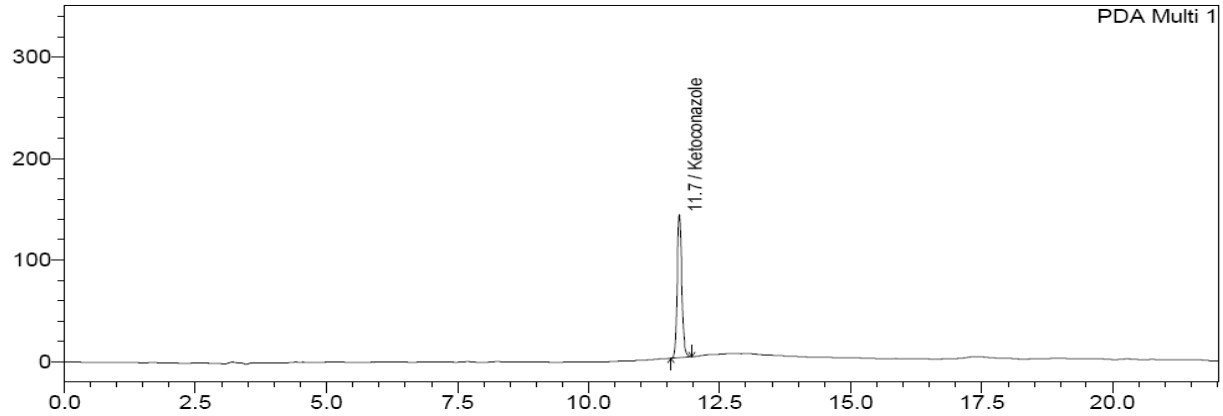
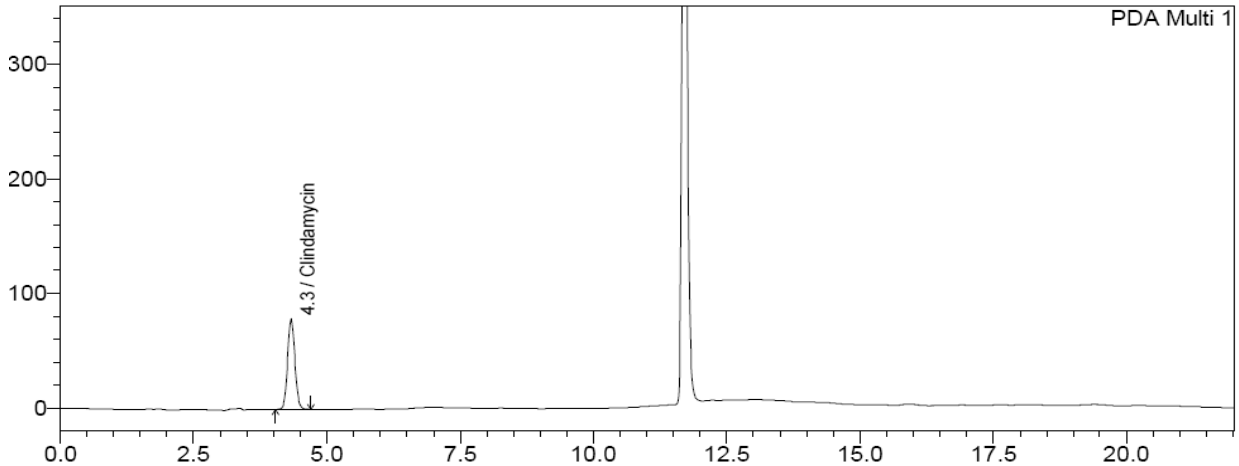


Fig no: 6.14 Chromatogram for sample concentration-100% Clindamycin and Ketoconazole

Clindamycin:



Ketoconazole:

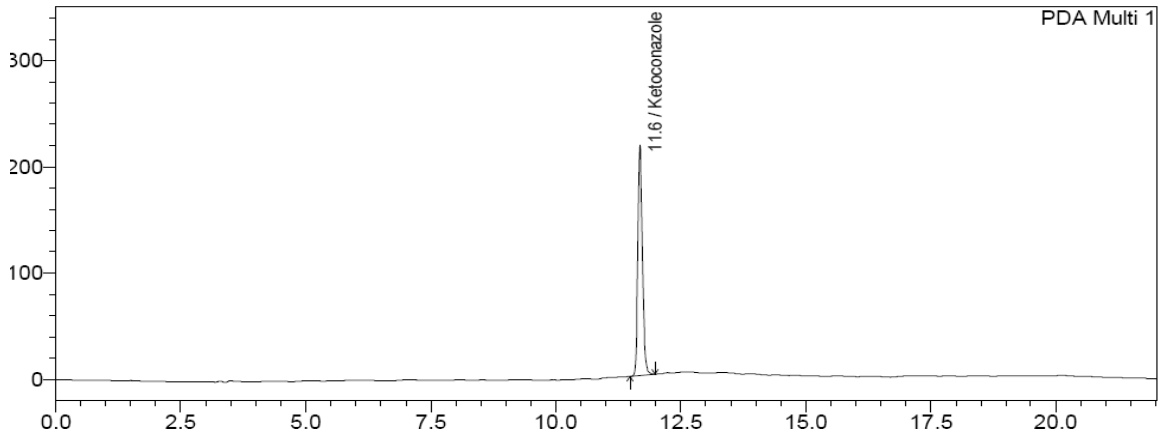


Fig no: 6.15 Chromatogram for sample concentration-150% of Clindamycin and Ketoconazole

Table No: 6.7 Accuracy (recovery) data for Clindamycin phosphate

S No.	Concentration	Amount of Clindamycin phosphate added in ppm	Amount of Clindamycin phosphate recovered in ppm	Recovery in %	Average in %	RSD in %
01	50.0%	255.0000	254.6352	99.8569	100.9	1.0
	50.0%	249.0000	252.7749	101.5160		
	50.0%	244.5000	248.4752	101.6258		
02	100.0%	544.5000	541.2743	99.4075	99.7	1.8
	100.0%	536.5000	545.4365	101.6657		
	100.0%	510.5000	500.4498	98.0313		
03	150.0%	767.0000	779.8679	101.6776	100.1	1.4
	150.0%	791.5000	783.9639	99.0478		
	150.0%	773.5000	771.3316	99.7196		
				Average	100.2	NA
				Overall %RSD	0.6	

Acceptance Criteria:

- The % Recovery for each level should be between 98.0 to 102.0%.

Table No: 6.8 Accuracy (recovery) data for Ketoconazole

S No.	Concentration	Amount of Ketoconazole added in ppm	Amount of Ketoconazole recovered in ppm	Recovery in %	Average in %	RSD in %
01	50.0%	21.9900	22.2588	101.2223	100.6	0.6
	50.0%	20.8400	20.8592	100.0921		
	50.0%	20.6400	20.7824	100.6899		
02	100.0%	40.4500	39.792	98.3733	99.1	0.6
	100.0%	40.1900	39.9898	99.5018		
	100.0%	40.9000	40.6688	99.4347		
03	150.0%	63.5100	64.7284	101.9184	101.4	0.4
	150.0%	62.6200	63.4883	101.3866		
	150.0%	65.8200	66.5582	101.1215		
				Average	100.4	NA
				Overall %RSD	1.2	

Acceptance Criteria:

- The percentage recovery was found to be within the limit 98.0-102.0%

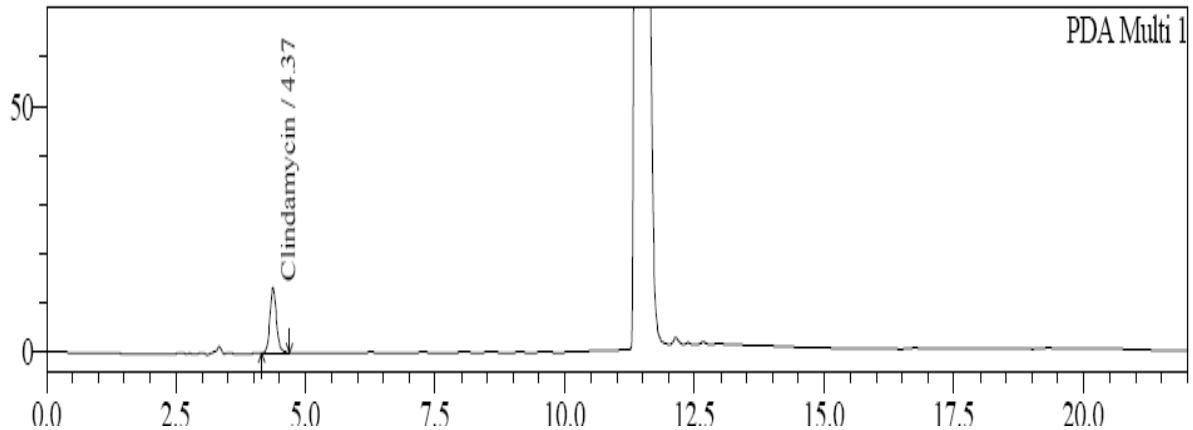
The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate

6.4: STABILITY INDICATING STUDIES

6.4.1 FORCED DEGRADATION:

Acid hydrolysis:

Clindamycin phosphate



Ketoconazole:

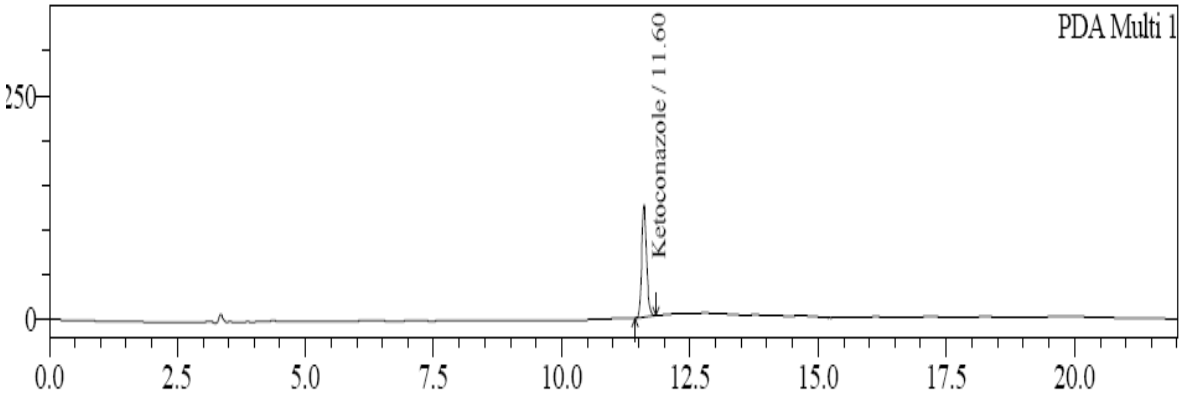
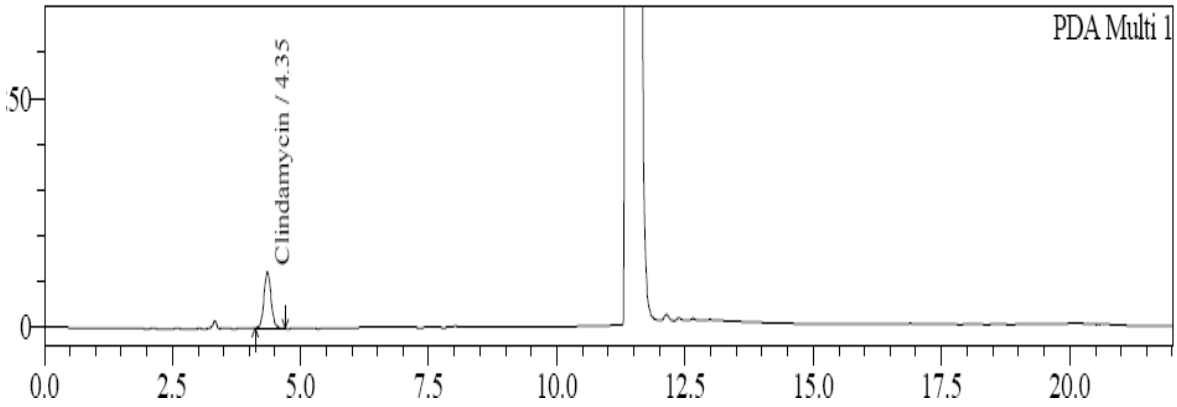


Fig no: 6.16 chromatogram of Acid hydrolysis for Clindamycin phosphate and Ketoconazole

Base hydrolysis:

Clindamycin phosphate



Ketoconazole

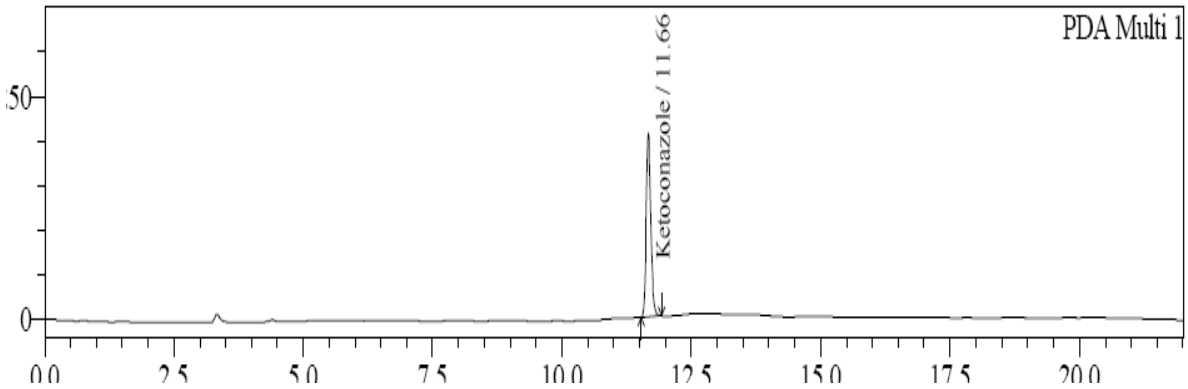
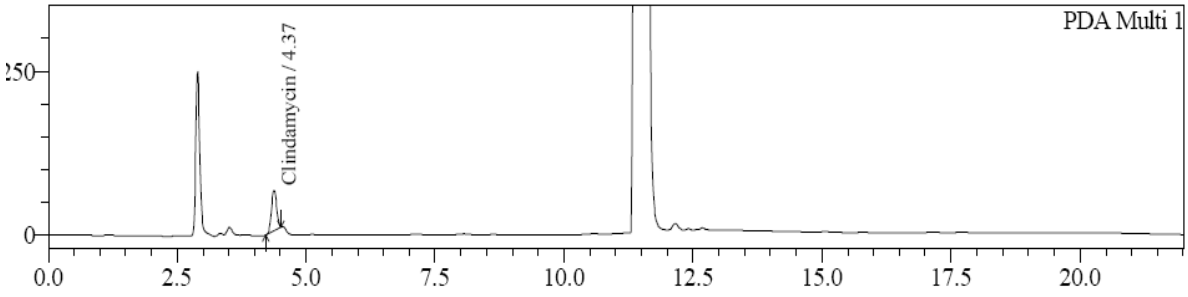


Fig no: 6.17 Chromatogram of base hydrolysis for Clindamycin phosphate and Ketoconazole

Oxidation:

Clindamycin phosphate



Ketoconazole:

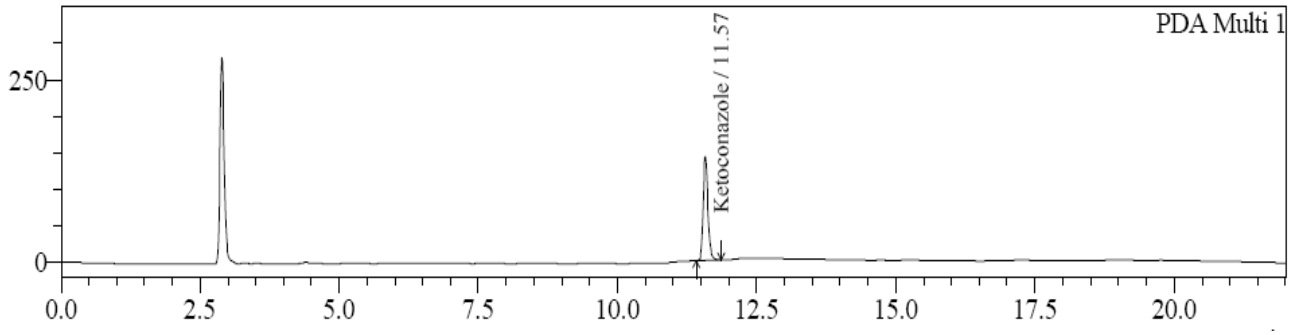
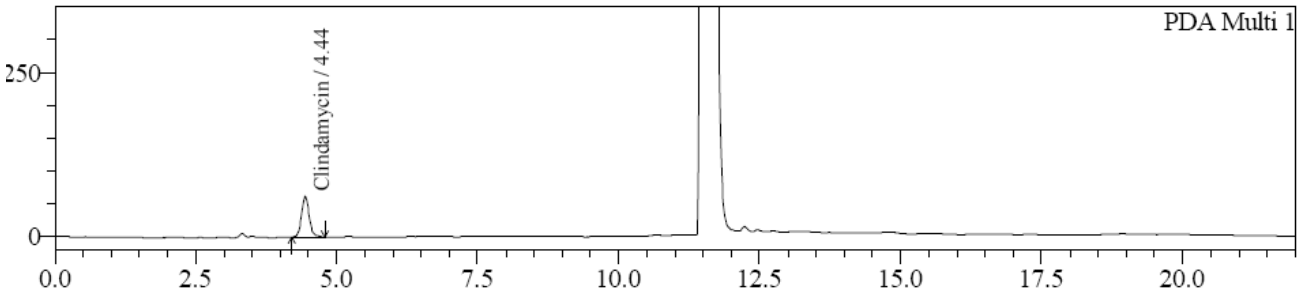


Fig no: 6.18 Chromatogram of oxidation for Clindamycin phosphate and Ketoconazole

Thermal:

Clindamycin:



Ketoconazole:

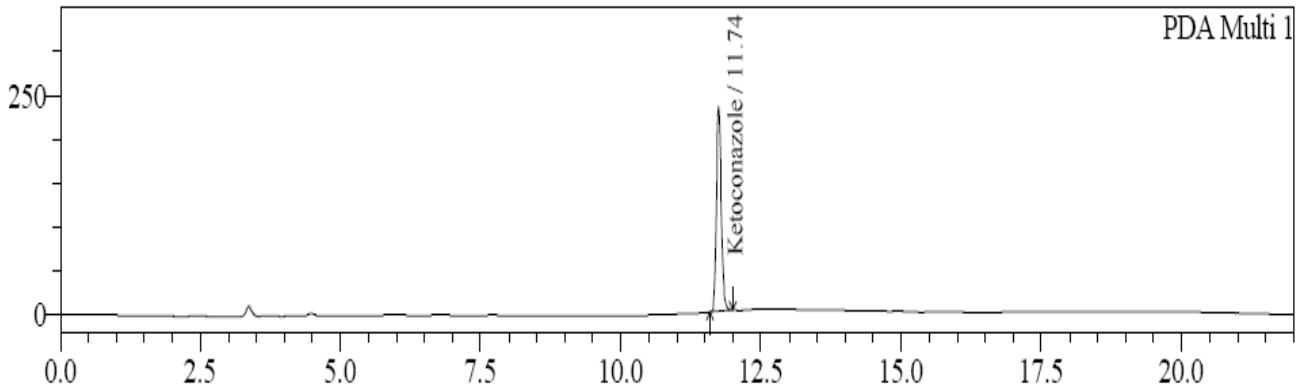
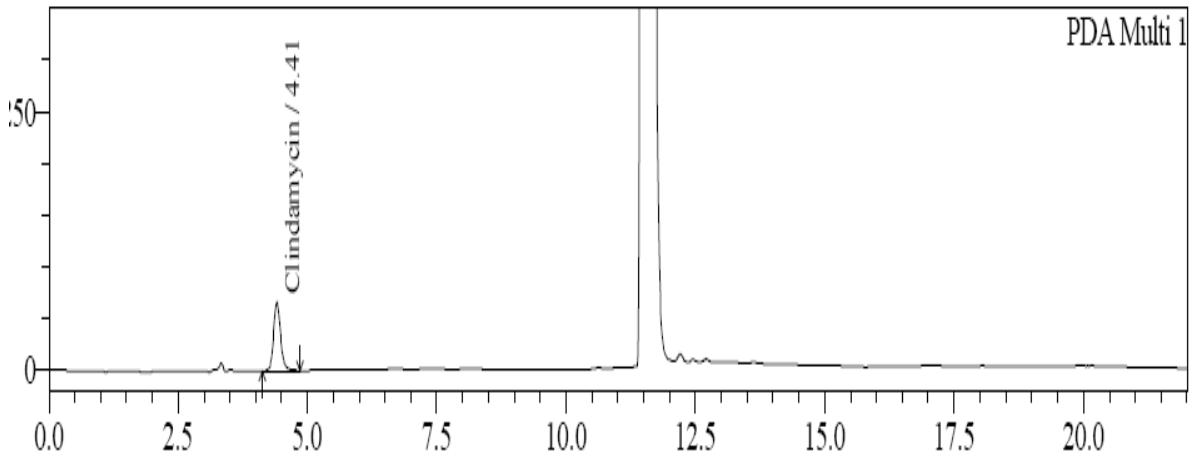


Fig no: 6.19 Chromatogram of thermal condition for Clindamycin phosphate and Ketoconazole

Humidity:

Clindamycin phosphate:



Ketoconazole

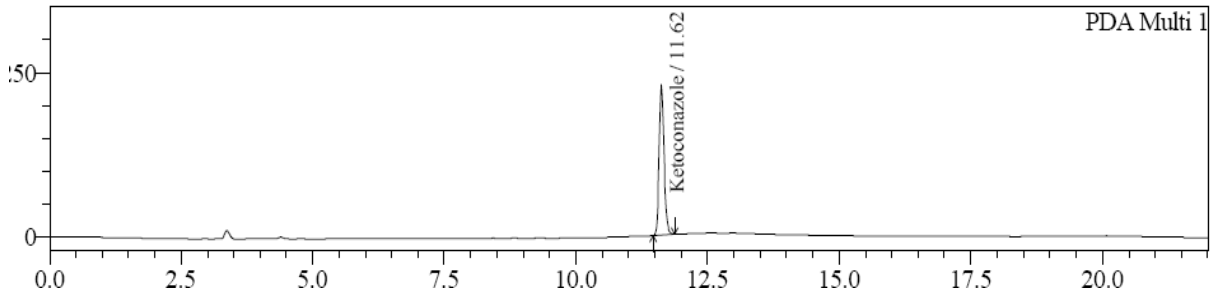


Fig no: 6.20 Chromatogram of humidity condition for Clindamycin phosphate and Ketoconazole

7. SUMMARY AND CONCLUSION

High-performance liquid chromatography (HPLC) is one of the most powerful analytical technologies available today. Clindamycin and ketoconazole concentrations were determined using RP-HPLC. The mobile phase was optimized with a 50:50 percent v/ v mixture of acetonitrile and phosphate buffer. The stationary phase was a C8 column (4.6 x 150 mm, 5 µ) or comparable chemically linked to porous silica particles. The detection was done with a UV detector set to 210 nm. At a steady flow rate of 1.0 mL/min, the solutions were chromatographed. Clindamycin with ketoconazole's linearity regression coefficients were determined to be less than 0.999. The percent RSD values are less than 2%, showing that the approach is accurate and precise. The rate of recovery varies between 98.0 and 102.0 percent.

As a result of forced degradation studies, it may be possible to improve the quality of the pharmaceutical products especially in the areas of formulation, packaging and storage of active pharmaceutical ingredients. The results obtained on the validation parameters met ICH and USP requirements. It inferred the method found to be simple, accurate, precise and linear. Finally, the developed method can be used for the routine analysis of single and combinations in regular drug analysis.

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