"METHOD DEVELOPMENT AND VALIDATION OF NATAMYCIN USING UV-VISIBLE SPECTROSCOPY AND FORCED DEGRADATION STUDIES USING HPTLC"

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IN

PHARMACEUTICAL ANALYSIS

BY

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CERTIFICATE

*This is to certify that the dissertation work entitled "**METHOD DEVELOPMENT AND VALIDATION OF NATAMYCIN USING UV-VISIBLE SPECTROSCOPY AND FORCED DEGRADATION STUDIES USING HPTLC**" submitted by Mr. Pratik Girishbhai Bhatt with Regn. No. (12MPH310) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Analysis" is a bonafide research work carried out by the candidate at the Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University under our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this, or any other university or institution.*

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I hereby declare that the dissertation entitled ***"METHOD DEVELOPMENT AND VALIDATION OF NATAMYCIN USING UV-VISIBLE SPECTROSCOPY AND FORCED DEGRADATION STUDIES USING HPTLC"*** *is based on the original work carried out by me under the guidance of Dr. Charmy s. Kothari, Assistant professor, Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this, or any other university or institution.*

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LIST OF ABBREVIATIONS

ABBREVIATION	FULL FORM
°C	Degree centigrade
±	Plus or Minus
<	Less than
λ	Lambda
%	Percentage
µg	Microgram
µL	Micro liter
Abs.	Absorbance
API	Active Pharmaceutical Ingredient
AR	Analytical Reagent
BP	British Pharmacopoeia
CAS No.	Chemical Abstract Service Number
cm	Centimeter
Cmax	Maximum Plasma Concentration
Tmax	Maximum Plasma Concentration Time
T1/2	Half Life
Conc.	Concentration
FDA	Food and Drug Administration
CDER	Central Drug Evaluation and Research
USP	United States Pharmacopoeia
FT-IR	Fourier Transform Infrared spectrometry
GC	Gas Chromatography
h	Hour
HPLC	High Performance Liquid Chromatography

HPTLC	High Performance Thin Layer Chromatography
i.d.	Internal Diameter
IP	Indian Pharmacopoeia
ICH	International Conference on Harmonization
IUPAC	International Union of Pure and Applied Chemistry
L	Liter
LC	Liquid Chromatography
LOQ	Limit of Quantification
LOD	Limit of Detection
FR	Flow Rate
Max	Maximum
Min	Minimum
mL	Mililiter
min	Minute
mm	Milimeter
MS	Mass Spectrometry
n	Number
No.	Number
ng	Nanogram
Pg.No.	Page number
ppm	Part Per Million
Ref.No.	Reference number
Rf	Retention Factor
RP	Reverse Phase
R.S.D.	Relative Standard Deviation
S.D.	Standard Deviation
Sec.	Second

Sr.No.	Serial number
Std.	Standard
UV/Vis	Ultra violet/Visible
UV	Ultra violet
Vol.	Volume
TLC	Thin Layer Chromatography
v/v	Volume/Volume
w/v	Weight / Volume
Temp.	temperature

LIST OF FIGURES

LIST OF TABLES

INDEX

ABSTRACT

A simple, sensitive, rapid, precise, accurate UV Visible Spectrophotometric method and Stability indicating assay HPTLC Method has been developed for Natamycin in their dosage form and in marketed formulation. The method was developed using methanol in UV and Dimethyl Sulphoxide as solvent. The mobile phase of Chloroform: Methanol: Glacial acetic acid: Water (10: 3: 3:1, v/v/v/v) was used for optimized separation of degradation products. The drug shows different absorption maximas due to presence of strong chromophores in the compound. Natamycin contains chromophores and shows four maxima in its UV absorption spectrum (220, 280, 303 and 318 nm) respectively. The absorption maxima for natamycin was taken at 303 nm as it shows maximum absorbance at this particular wavelength. The Beer-Lambert law was obeyed from the range of 2-10 µg/mL for UV and 1000-3000ng/spot for HPTLC concentration range. The method was able to separate all the degradation peaks formed in ICH prescribed stress conditions with sufficient difference in R_f values. The developed method was validated in terms of linearity, accuracy, precision, repeatability, limit of quantification, limit of detection, robustness, and assay as per International Conference on Harmonization (ICH) Q2 (R1). The utility of this developed method was demonstrated by the assay of commercially available Natamet (natamycin ophthalmic eye suspension) formulation.

1.1 INTRODUCTION TO DISEASE [1-5]

Fungal Keratitis is a type of disease which generally results in the inflammation of the eye's cornea (called keratitis) due to infection caused by the fungal organism. In this disease the cornea which covers the anterior part of the eye which further covers pupil gets affected. It is a great diagnostic and therapeutic challenge for ophthalmologist. Difficulties are generally encountered in relating clinical diagnosis, isolating the etiological organism in the laboratory, and treating the disease with available topical anti-fungal agents. The disease is generally diagnosed lately because of lack suspicion and even though the diagnosis is made accurately, management remains a challenge because of poor corneal penetration and limited commercially available antifungal agents. [1, 2]

Moreover the incidence of fungal keratitis has increased over last 30 years. This effect is due to frequent use of topical corticosteroids and antibacterial agents in treatment of patients of keratitis; the rise in the number of patients who are immune compromised, and better laboratory diagnostic techniques that aid in its diagnosis.[3]

The challenge lies in the fact that treatment of fungal keratitis requires prolonged use of antifungal medications, which are toxic, costly, and usually, not available commercially. Therefore, making an accurate diagnosis is essential to exclusively treat the patients with active fungal keratitis and to avoid exposing others to the cost and toxicity of treatment. However, access to a rapid, inexpensive and accurate diagnostic method is usually limited, especially in the developing countries where generally the rate of fungal keratitis is higher as compared to the developed countries.[4]

Keratitis can be classified by its location, severity, and cause. If keratitis only involves the surface (epithelial) layer of the cornea, it is called superficial keratitis. If it affects the deeper layers of the cornea (the corneal stroma), it is called stromal keratitis or interstitial keratitis. It may involve the center of the cornea or the peripheral part of the cornea (that portion closest to the sclera) or both. Keratitis may affect one eye or both eyes. Keratitis may be mild, moderate, or severe and may be associated with inflammation of other parts of the eye. Keratoconjunctivitis is inflammation of the cornea and the conjunctiva. Kerato-uveitis is

inflammation of the cornea and the uveal tract, which consists of the iris, ciliary body, and choroid.

Keratitis may be acute or chronic. It may occur only once or twice in an eye or be recurrent. It may be limited in its effects on the eye or be progressive in its damage. The various causes of keratitis may result in different clinical presentations, so defining the location, severity, and frequency of the condition can often assist in pinpointing the exact cause. Other helpful facts in establishing the cause of keratitis can include demographic information such as the age, sex, and geographic location of the patient. A medical history is often useful as well in finding the cause of keratitis. Infection is the most frequent cause of keratitis. Bacteria, viruses, fungi, and parasitic organisms may all infect the cornea, causing infectious or microbial keratitis. [5]

Fig 1.1: Eye infection with fungal keratitis

1.1.2 Types of Keratitis [5-8]

Infection is the most frequent cause of keratitis. Bacteria, viruses, fungi, and parasitic organisms may all infect the cornea, causing infectious or microbial keratitis.

If the front surface of the cornea has been damaged by a small scratch and the surface is not intact, almost any bacteria, including atypical mycobacterium, can invade the cornea and

results in keratitis. Ulcerations of the cornea may occur, a condition known as ulcerative keratitis. Before the advent of antibiotics, syphilis was a frequent cause of keratitis. [1]

Viruses that infect the cornea include respiratory viruses, including the adenoviruses and others responsible for the common cold. The herpes simplex virus is another common cause of keratitis. Worldwide, the incidence of HSV keratitus is about 1.5 million, including 40,000 new cases of related blindness each year. The herpes zoster virus (the virus responsible for chickenpox and shingles) may also cause keratitis, particularly when shingles involves the forehead.

Fungi such as Candida, Aspergillus, and Nocardia are unusual causes of microbial keratitis, more frequently occurring in people who are immunocompromised because of underlying illnesses or medications.Fusarium keratitis, a type of fungal infection, occurs primarily in contact-lens wearers. Bacterial co-infection can complicate fungal keratitis.

Contact-lens wearers are also susceptible to acanthamoeba keratitis caused by an amebic parasite. "River blindness," or onchocercal keratitis, is another parasitic infection of the cornea, rarely seen in developed countries, but very common in the Third World. [3]

Physical or chemical trauma is a frequent cause of keratitis. The injury may become secondarily infected or remain noninfectious. Retained corneal foreign bodies are frequent sources of keratitis. Ultraviolet light from sunlight (snow blindness), a tanning light or a welder's arc, contact-lens over wear, and chemical agents, either in liquid form splashed into the eye or in gases in the form of fumes can all result in noninfectious keratitis. Chemical injury or contact lens-related keratitis often causes superficial punctuate keratitis, in which the examiner notices myriads of injured surface cells on the affected cornea. Disturbances in the tear film may lead to changes in the corneal surface through drying of the corneal epithelium. This type of keratitis is usually superficial and is known as keratitis sicca. If the eyes are extremely dry, the surface cells may die and form attached filaments on the corneal

surface, a condition known as filamentary keratitis. Inability to close the eyelids properly can also lead to corneal drying, a condition termed exposure keratitis.

Allergies to airborne pollens or bacterial toxins in the tears may also cause a noninfectious type of keratitis. Autoimmune diseases create a similar appearance, often affecting the periphery of the cornea, termed marginal keratitis or limbic keratitis. [4]

1.1.3 Classification [6-8]

Besides these based on microscopic properties three important types of fungi responsible for causing keratitis include:

1. Filamentous fungi or molds which are generally multicellular organisms joined together to produce tube like filaments or hyphae. They produces cottony or feather like structure in the culture medium. They can be further subdivided into:
   A. Septate (hyphate): they produce cross wall or septa behind the tip of growing hyphae during elongation. These are of two different types
   a. Non-pigmented(monillacea): such as aspergillus
   b. Pigmented(dematiaceae): such as alternaria or curvularia
   B. Non septate organisms: They do not form cross wall such as mucor.
2. Yeasts which produces opaque, creamy, white, pasty colonies on the surface of the culture media such as candida
3. Dimorphic fungi which usually exhibit the properties of yeast when cultivated at 37°C and molds when grown at 25-30°C such as blephonium.[3]

1.1.4 Pathology [7]

The infection generally in eye begins with direct or indirect exposure with the organism which provides a proliferative and vegetative environment for the growth of the fungi. It may be due to vegetative or organic matter which directly comes in contact with the eye or some

unknown pathogenic exposure. After the infection in the eye the fungi further proliferates in the eye's cornea .The infection probably starts when the epithelial integrity is broken either due to trauma or ocular surface disease and the organism gains access into the tissue and proliferates. Proteolytic enzymes, fungal antigens and toxins are liberated into the cornea with the resulting necrosis and damage to its architecture thus compromising the eye integrity and function. [6]

1.1.5 Symptoms [8-10]

The symptoms for fungal keratitis generally include:

- Blurred vision, redness, tearing, photophobia, pain, foreign body sensation and secretions in some cases the lesion are rather indolent which help to delay the diagnosis, white/gray infiltrate with feathery borders, conjunctival injection ,ulcers

1.1.6 Treatment [10]

Generally the line of the treatment includes commercially available topical antifungal preparations or compounded from systemic preparations into eye drops and the instilled into eye or in resistant or severe cases combination of antifungal agents is used or surgical procedure is done for treating fungal keratitis.[6]

1.1.7 INTRODUCTION OF DRUG [11]

Natamycin (INN), also known as pimaricin and sometimes sold as Natacyn, is a naturally occurring antifungal agent produced during fermentation by the bacterium *Streptomyces natalensis*, commonly found in soil. Natamycin has a very low solubility in water; however, natamycin is effective at very low levels. There is an MIC (minimum inhibitory concentration) of less than 10 ppm for most molds. Natamycin is classified as a macrolide polyene antifungal and, as a drug, is used to treat fungal keratitis. It is especially effective against aspergillus and fusarium corneal infections. Other common members of the polyene macrolide antifungal family are amphotericin B, nystatin, and filipin. Natamycin is also used in the food industry as a natural preservative. [7]

1.1.8 Rationale

Natamycin also known as pimaricin was mainly used in food industry as a preservative in dairy products, meats, and other food items. But after its role found as an antifungal it has been used as antifungal in ophthalmic as eye drops and as vaginal tablets for vaginal infection of candidiasis. Natamycin shows very good activity on fungi without affecting bacteria due to its ergosterol binding ability and it has no systemic absorption. The HPLC method for its determination has been reported but stability parameters have not been established and reported. Due to its specific ability to bind to ergosterol and no absorption in GIT, it has a good antifungal potency. As an ophthalmic agent being used in preparation of eye suspension it is necessary to develop its stability indicating parameters for its routine use.

Various stability parameters like pH, photolytic, oxidation, temperature, humidity needed to be studied and its measures must be established for the stability of the drug. The drug intended for ophthalmic activity should be cautiously developed and its stability should be of the relevance in order for the safe use of the drug.

1.2 INTRODUCTION TO DRUG ANALYSIS[12-16]

Analytical chemistry is a branch of chemistry that determines the nature and identity of substance and its composition. In the early twentieth century there were only four accepted branches of chemistry namely, organic chemistry, inorganic chemistry, physical chemistry and biochemistry. Its importance grew, and in the process, absorbed techniques and skills from all other four branches so by the 1950s, analytical chemistry was finally accepted as a branch of chemistry in its own right. The ability to provide timely, accurate, and reliable data is central to the discovery, development, and manufacture of pharmaceuticals. Analytical data are used to screen potential drug candidates, aid in the development of drug synthesis, support formulation studies, monitor the stability of bulk pharmaceuticals and formulated products, and test final products for release. The quality of analytical data is a key factor in the success of a drug development program. Problems increase as additional people, laboratories and equipments are used to perform the method. When the method is used in the developer's laboratory, a small optimization can usually be made to make the method work, but the flexibility to change it is lost, once the method is transferred to other laboratories or used for official product testing, where methods are submitted to regulatory agencies and changes may require formal approval before they can be implemented for official testing. The best way to minimize method problems is to perform adequate validation experiments during development.

The need of the sophisticated analytical instruments and determination using them is almost a routine process for the modern analytical laboratories. It has been vast expanding areas of knowledge as the instrument automation is in ever-increase in power and scope. Further all the manual techniques in the line of analytical studies had steadily been transferred to the instrumental techniques. Analytical methods are generally classified as Physical and Chemical analysis. Physical analysis includes measurement of particle size, dimension, thickens of a solid dosage forms etc. Basically chemical analysis can be divided into three broad categories which are as follow.

1.2.1 Types of Analysis [13-17]

1.2.1.1 Qualitative Analysis

The Qualitative analysis identifies the nature of substance, and if it is mixture, the nature of the components present.

1.2.1.2 Quantitative Analysis

The Quantitative analysis determines the elemental composition of the substance and the quantitative distribution of each component.

1.2.1.3 Instrumental Analysis

The instrumental analysis deals with the use of the instrument for analysis of the particular sample and determining the elemental composition of the particular sample. Various instruments are employed according their use for analytical purposes and highly sophisticated means are used for analysis. The growth of instrumental analysis is related to the developments in the fields of electronic because the generation, transduction, amplification and display of a signal can be done in a convenient manner. Following is the table of list of instruments used for analytical purposes and their major applications in pharmaceutical field.

Table 1.1: Various Instrumental Methods of Analysis [14-17]

Instrumental Method	Property Measured	Application
UV- Visible Spectrophotometry	Absorption of radiation	Identification of the functional group and quantification of Unsaturated compounds.
FTIR Spectroscopy	Absorption of radiation	Quantitative analysis of organic compound at High conc. Level.
Atomic Absorption Spectroscopy	Absorption of radiation	Quantification of metals or Metalloids.
Flame Photometry	Emission of radiation	Quantification of alkali metals or alkaline earth Metals.
X-Ray Diffraction	Diffraction of radiation	Identification of crystal Lattice structure.
Magnetic Resonance (NMR)	Nuclear spin energy level of a mol in an applied	Identifies type of hydrogen and carbon in
LC-NMR	Magnetic field	Organic molecules. Analysis of trace impurity and degradants
Thermal analysis (DTA \ DSC)	Difference in Temperature/heat energy	Determination of melting point, Polymorphism, Drug - excipients compatibility

The use of the instrument generally depends upon the type of analysis to be carried out and instrumental analysis plays a major role in pharmaceutical field for analysis of various samples. Analytical chemistry deals with methods for determining the chemical composition of sample. A compound can be often measured by several methods. The choice of analytical methodology is based on many considerations, such as chemical properties of the analyte and its concentration, sample matrix, the speed and cost of the analysis, type of measurements i.e., quantitative or qualitative and the number of samples.

1.3 INTRODUCTION TO UV-VISIBLE SPECTROMETRIC METHOD[18-22]

Ultraviolet-visible Spectrophotometry (UV/Vis) refers to absorption spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the visible and adjacent (near-UV and near-infrared) ranges. In this region of the electromagnetic spectrum, molecules undergo electronic transitions. This technique is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state. The Determination is usually carried out in solutions.

UV/Vis spectroscopy is routinely used in analytical chemistry for the quantitative determination of different analytes, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules.

Assay as a Single Component Analysis:

The concentration of a component in a sample which contains other absorbing substance may be determined by a simple Spectrophotometric measurement absorbance provided that other substance has a sufficient small absorbance at the wavelength of the measurement. This condition is satisfied only if the concentration of the interfering substances, their absorptivity or the path length are sufficiently small then their product. A systematic error of less than 1% would normally be accepted or ignored.

Fig 1.2: Jasco uv-visible spectrophotometer

The various spectroscopic techniques used for multi-component analysis are as follows:

Simultaneous equation method (Vierodt's method)

Concentration of several components present in the same mixture can be determined by solving a set of simultaneous equation even if their spectra overlap. If Beer's law is followed, these equations are linear.

Two wavelength method

The absorption different between two points on the mixture spectra is directly proportional to the concentration of the component to be determined.

The absorption ratio method

It depends on the property that for a substance, which obeys Beer's law at all wavelength, the ratio of absorbance at any two wavelengths is constant value independent of concentration or path length.

Geometric correction method

It may be applied if the irrelevant absorption is linier at the three wavelengths selected. This procedure is simply algebraic calculations of what the baseline technique in infrared spectrophotometry dose graphically.

Absorption factor method (Absorption correction method)

Quantitative determination of one drug is carried out by E (1%, 1 cm) value and quantification of another drug is carried out by subtraction absorption due to interfering drug using absorption factors.

Orthogonal polynomial method

It involves complex calculation than the three-point correction procedure. The basis of the method is that an absorption spectrum may be represented in terms of orthogonal functions.

Difference Spectrophotometry

Difference Spectrophotometry provides a sensitive method for detecting small changes in the environment of a chromophores or it can be used to demonstrate ionization of a chromophores leading to identification and quantization of various components in mixture.

Derivative Spectrophotometry

Derivative Spectrophotometry is useful means of resolving two overlapping spectra and eliminating matrix interference due to an indistinct shoulder on side of an absorption bands.

Area under curve method

In this method, the absorptivity values (ε1 and ε2) of each of the two drugs were determined at the selected wavelength range. Total area under curve of a mixture at wavelength range is equal to the sum of area under the individual component at that wavelength range.

1.3.1 Theory of Spectrophotometry [22-23]

The absorption and the emission of energy in the electro-magnetic spectrum occur in discrete packets of photons. The relation between the energy of a photon and the frequency appropriate for the description of its propagation is

$E = hv$

Where E = Energy in ergs

v = Represents frequency in cycles per second

h = Plank's constant (6.6256 x 10-27 erg-sec)

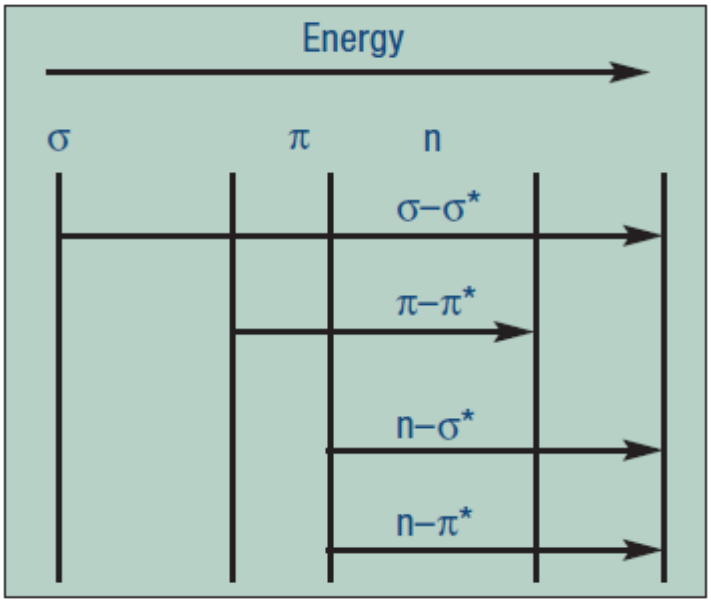


Figure 1.3: Electronic transitions

1.3.2 Beer Lambert's Law [23-24]

When light (monochromatic or heterogeneous) falls upon a homogeneous medium, a portion of the incident light is reflected, a portion is absorbed within the medium and the remainder is transmitted. If the intensity of the incident light is expressed by I, that of the absorbed light by Ia, that of the transmitted light by I_t, and that of the reflected light by I_r, then:

$I = I_a + I_t + I_r$

Limitations of Beers Lambert's Law:

- Beer law obeys at low concentration range.
- Scattering of light occurs due to the particulates present in the sample.
- There is hindrance due to fluorescence in the sample.
- There is change in refractive index at higher concentrations.
- Monochromatic light should be used for analysis.

1.4 INTRODUCTION TO HPTLC METHOD[24-28]

High Performance Thin Layer Chromatography (HPTLC) is a sophisticated and automated form of TLC.

Main Difference between HPTLC and TLC is particle size and pore size of sorbents. Other differences are shown in table 1.2

Table 1.2: Difference between TLC and HPTLC

Criteria	HPTLC	TLC
Layer of Sorbent	100 µm	250 µm
Separations	3-5 cm	10-15 cm
Analysis Time	Shorter migration and less time	Slower
Solid support	Wide choice of stationary phases like silica gel for normal phase and C_8 , C_{18} for reversed phase modes	Silica gel, Alumina & Kiesulguhr
Development chamber	New type that require less amount of mobile phase	More amount
Sample spotting	Auto sampler	Manual spotting

1.4.1 Principle of HPTLC [25-27]

Thin Layer Chromatography (TLC) is a solid-liquid technique in which the two phases are a solid (stationary phase) and a liquid (moving phase). Solids most commonly used in chromatography are silica gel and alumina. HPTLC is a sensitive, fast, simple and inexpensive analytical technique. It is a micro technique; as little as 1-10 ng material can be detected. TLC involves spotting the sample to be analyzed near one end of a sheet of glass, aluminum or plastic that is coated with a thin layer of an adsorbent. The sheet, which can be the size of a microscope slide, is placed on end in a chamber containing a shallow layer of solvent. As the solvent rises by capillary action up through the adsorbent, differential partitioning occurs between the components of the mixture dissolved in the solvent the stationary adsorbent phase. The more strongly a given component of a mixture is adsorbed onto the stationary phase, the less time it will spend in the mobile phase and the more slowly it will migrate up the plate.

1.4.1 Method Development [25-29]

Method development is a common task for laboratories performing chromatography. Generally methods are required, which are rapid, fit for purpose, inexpensive, and reliable.

Steps for Method Development

Selection of Stationary Phase

A scalable TLC plate, preferably that has an identical media as the preparative column should be selected. It is selected based on sample polarity and solubility.

Selection of Mobile Phase

One of the most challenging steps in method development is the selection of the appropriate mobile phase. The criteria for choosing a solvent are based on solubility, affinity and resolution.

Selection of Visualization Technique

If compounds are colored, they are easy to see with naked eye. If not, a UV lamp is used.

Optimization of TLC Separations

The optimum separation of compounds by TLC is usually achieved when R_f values are between 0.3–0.7.

1.4.2 Coupling Techniques of HPTLC [25]

The coupled techniques in HPTLC have the advantage that there is no need to collect fractions and do repeated chromatographic measurements with reference compounds (saving time); the possibility of contamination during collection and storage of fractions is averted and furthermore, it is possible to gain reliable qualitative information for sample identification.

HPLC-HPTLC

Camag has recently introduced a means of coupling HPLC with HPTLC, usually employing reversed phase HPLC stationary phases. The column effluent is transferred to an HPTLC plate. The instrument for this purpose is the Luminat C, which sprays the effluent from the HPLC on to the HPTLC layer. Selected cuts from the column separations can be collected by choice of three methods, manual, triggered by detector or based on time run programs. The undesired parts of the effluent are discarded.

HPTLC-FTIR

The identification of compounds separated by HPTLC can be made by coupling the HPTLC with Fourier transform infrared (FTIR) spectroscopy. The quality of HPTLC-FTIR spectra is sufficient for identification of unknown substances, and the information is extremely valuable when examined in reference to the spectra of pure compounds, on different portions of the spectrum.

HPTLC-MS

A new hyphenated technique that enables coupling of thin-layer chromatography (TLC) with time-of-flight secondary-ion mass spectrometry (TOF-SIMS) has been used. TOF-SIMS has outstanding capabilities for molecular surface analysis, trace metal determination on surfaces, and surface imaging.

HPTLC–RAMAN

Raman spectroscopy depends on the vibrational modes of the molecule of interest. The major reason for coupling of Raman to HPTLC is that, TLC sorbents give weak Raman spectra leading to low background interference.

1.5 INTRODUCTION TO STABILITY INDICATING ASSAY METHODS [25-29]

Stability testing of any drug product is a requirement from the regulatory as well as in the industry, owing of increase concern for the safety of drug product, efficacy, and quality, stability behavior, in the sense refers to alteration in physical, chemical, biological form of a drug substance and drug product.

Availability of a suitable stability-indicating assay method (SIAM) with degradation mechanisms is necessary to study stability behavior of drug substances and drug products

Isolation and characterization of degradation products is required for validation of SIAM and to investigate mechanisms of degradation Regulatory requirements to specify long term, intermediate and accelerated stability testing.

But degradation products formed during long term and accelerated testing may not be present in substantial amounts for isolation and development of SIAM. These drawbacks (long time, less quantity) necessitate forced decomposition by stress testing.

1.5.1 STRATERGY FOR DEVELOPMENT OF VALIDATED SIAMs

Step I: Critical Study of the Drug Structure to Assess the Likely Decomposition Route(s)

This should be the first element whenever one takes up the project on establishment of a SIAMs. Much information can simply be gained from the structure, by study of the functional groups and other key components.

Step II: Collection of Information on Physicochemical Parameters.

It is generally important to know various physicochemical parameters like pKa, log P, solubility, absorptivity and wavelength maximum of the drug in question before the method development in question, before method development is taken up. The knowledge of log P for the drug and the identified degradation products provides good insight into the separation behavior likely to be obtained on a particular stationary phase. The knowledge of pKa is important as most of the pH- related changes in retention occur at pH of the buffer to be used in the mobile phase. The availability of the solubility data in aqueous, organic and commonly used HPLC solvents and their combinations can thus prove to be very useful in the selection of the sample solvents and the mobile phase. For the analysis of the drug or degradation products, it requires that they are soluble in HPLC compatible solvents in the first place.

Step III: Stress (Forced Decomposition) Study

The third step in the development of SIAM is the conduct of stress studies to generate degradation products of the drug. The ICH guideline Q1A suggests the following conditions to be employed:

10°C increments above the accelerated temperature,

Humidity where appropriate E.g. 75 % or greater,

Hydrolysis across wide range of pH,

Oxidation,

Photolysis,

However, the guideline provides no details on how hydrolytic, photolytic and oxidation studies have to be actually performed no specific conditions are mentioned in guidelines. Depending upon the results, Decision is taken on whether to increase the strength of the reaction conditions

Step IV: Preliminary Separation Studies on Stressed Samples

The stress samples are subjected to preliminary analysis to study the number and types of degradation products formed under various conditions. The simplest way is to start analysis with a RP C-18 column, preferably optimized chromatographic conditions. Well-separated and good quality peaks at the outset provide better resolution.

Step V: Final Method Development and Optimization

The method is optimized, by changing the mobile phase ratio, pH, gradient, flow rate, temperature, solvent type, and the column and its type in such a way that no merging between close or co-eluting peaks.

Step VI: Identification and Characterization of Degradation Products and Preparation of Standards

Before moving to the validation of a SIAM, it necessary to identify the degradation products of drug and arrange for their standards. These are required to establish specificity/selectivity of the method. The work on this aspect can even be initiated once an idea on the nature

and number of degradation products formed under different degradation conditions is obtained from preliminary separation studies. Peak purity of the active substance is checked by photo-diode array detector to verify that the method is selective, and a single component peak is quantified.

Step VII: Validation of Stability Indicating Assay Methods

Validation of analytical methods, in general, has been extensively covered in the ICH guidelines Q2A and Q2B, in the FDA guidance and by USP. Here validation has been carried out as per ICH guidelines Q2A and Q2B. Overall, there are two stages in the validation of SIAM. First stage is early in the development cycle when drug substance is subjected to forced decomposition study and the SIAM is established based on the knowledge of drug degradation behavior .Main focus of validation is on establishment of specificity/selectivity followed by other validation parameters like accuracy, precision, linearity, range, robustness, etc. In the second stage, when the SIAM so developed is extended to formulations or other matrices, the emphasis gets limited to just prove the pertinence of the established validation parameters in the presence of excipients or other formulation constituents.

1.6 METHODOLOGY OF ANALYTICAL METHOD VALIDATION [25-29]

1.6.1 Analytical Method Validation Parameters [28-29]

Before performing validation of analytical method it is necessary to understand the validation parameters. The various Performance parameters, which are addressed in a validation exercise, are grouped as follows.

Table 1.3: Validation Parameters and acceptance criteria

Validation Parameters	Acceptance Criteria
Accuracy/trueness	Recovery 98-102% (individual)
Precision	RSD < 2%
Intermediate precision	RSD < 2%
Specificity/ selectivity	No interference
Detection limit	S/N > 3
Quantification limit	S/N > 10
Linearity	Correlation coefficient r_2 > 0.999
Range	80 –120 %
Stability	> 24 h or >12 h

1.6.2 Selectivity and Specificity

The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix. If an analytical procedure is able to separate and resolve the various components of a mixture and detect the analyte qualitatively the method is called selective.

On the other hand, if the method determines or measures quantitatively the component of interest in the sample matrix without separation, it is said to be specific. Hence one basic difference in the selectivity and specificity is that, while the former is restricted to qualitative detection of the components of a sample, the latter means quantitative measurement of one or more analytes.

Selectivity may be expressed in terms of the bias of the assay results obtained when the procedure is applied to the analyte in the presence of expected levels of other components, compared to the results obtained on the same analyte without a added substances. When the other components are all known and available, selectivity may be determined by comparing the test results obtained on the analyte with and without the addition of the potentially

interfering materials. When such components are either unidentified or unavailable, a measure of selectivity can often be obtained by determining the recovery of a standard addition of pure analyte to a material containing a constant level of the other components.

1.6.3 Linearity and Range

The linearity of an analytical method is its ability to elicit test results that are directly (or by a well defined mathematical transformation) proportional to the analyte concentration in samples within a given range. Linearity usually expressed in terms of the variance around the slope of regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte.

The linear range of detectability that obeys Beers law is dependent on the compound analyzed and the detector used. The working sample concentration and samples tested for accuracy should be in the linear range. The claim that the method is linear is to be justified with additional mention of zero intercept by processing data by linear least square regression. Data is processed by linear least square regression declaring the regression coefficient and b of the linear equation $y= ax + b$ together with the correlation coefficient of determination r^2. For the method to be linear, the r^2 value should be close to 1. The range of an analytical method is the interval between the upper and lower levels of the analyte (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written.

1.6.4 Accuracy

The accuracy of an analytical method may be defined as the closeness of the test results obtained by the method to the true value. It is the measure of the exactness of the analytical method developed. Accuracy may often express as percent recovery by the assay of a known amount of analyte added. Accuracy may be determined by applying the method to samples or mixtures of excipients to which known amount of analyte have been added, both above and below the normal levels expected in the samples. Accuracy is then calculated from the test results as the percentage of the analyte recovered by the assay. The ICH

documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e. three concentrations and three replicated of each concentration).

1.6.5 Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of homogenous samples. This is usually expressed as the standard deviation or the relative standard deviation (coefficient of variation). Precision is a measure of the degree of reproducibility or of the repeatability of the analytical method under normal operating circumstances. Repeatability involves analysis of replicates by the analyst using the same equipment, method and conducting the precision study over short period of time while reproducibility involves precision study at

Different Laboratories

Different Batch of Reagent

Different Analysts

Different Equipments

Determination of Repeatability

Repeatability can be defined as the precision of the procedure when repeated by same analyst under the same operating conditions like same reagents, equipments, settings and laboratory over a short interval of time. It is normally expected that at least six replicates should be carried out and a table showing each individual result provided from which the mean, standard deviation and co-efficient of variation should be calculated for set of n value. The RSD values are important for showing degree of variation expected when the analytical procedure is repeated several time in a standard situation. It should be below 2% for bulk drugs and below 2% for assay in finished product.

The ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e. three concentrations and three replicates of each concentration or using a minimum of six determinations at 100% of the test concentration).

Determination of Reproducibility

Reproducibility means the precision of the procedure when it is carried out under different conditions, usually in different laboratories on separate, putatively identical samples taken from the same homogeneous batch of material. Comparisons of results obtained by different analysts, by the use of different equipments or by carrying out the analysis at different times can also provide valuable information.

1.6.6 Limit of Detection and Limit of Quantification

Limit of Detection

The limit of detection is the parameter of limit tests. It is the lowest level of analyte that can be detected, but not necessarily determined in a quantitative fashion, using a specific method under the required experimental conditions. The determination of the limit of detection of instrumental procedures is carried out by determining the signal-to-noise ratio by comparing test results from the samples with known concentration of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. A signal-to-noise ratio of 2:1 or 3:1 is generally accepted. The signal-to-noise ratio is determined by dividing the base peak by the standard deviation of all data points below a set threshold. Limit of detection is calculated by taking the concentration of the peak of interest divided by three times the signal-to-noise ratio. For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (Sa) which may be related to LOD and the slope of the calibration curve, b, by:

$LOD = 3.3 \sigma / S.$

Limit of Quantification

Limit of quantization is a parameter of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk drugs and degradation products in finished pharmaceuticals. The limit of quantization is the lowest concentration of analyte in a sample that may be determined with acceptable accuracy and precision when the required procedure is applied. It is measured by analyzing samples containing known quantities of the analyte and determining the lowest level at which acceptable degrees of accuracy and precision are attainable, the final assessment is based on an instrumental reading, the magnitude of background response by analyzing a number blank samples and calculating the standard deviation of this response. The standard deviation multiplied by a factor (usually 10) provides an estimate of the limit of quantization. In many cases, the LOQ is approximately twice the limit of detection. σ is the standard deviation of the intercept which may be related to LOQ and the slope of the calibration curve, b, by: LOQ = 10 σ / S

1.6.7 Robustness and Ruggedness

Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. The determination of robustness requires that methods characteristic are assessed when one or more operating parameter varied.

Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay.

DRUG PROFILE OF NATAMTCIN: [42-50]

TABLE 2.1 Chemical Data of Natamycin

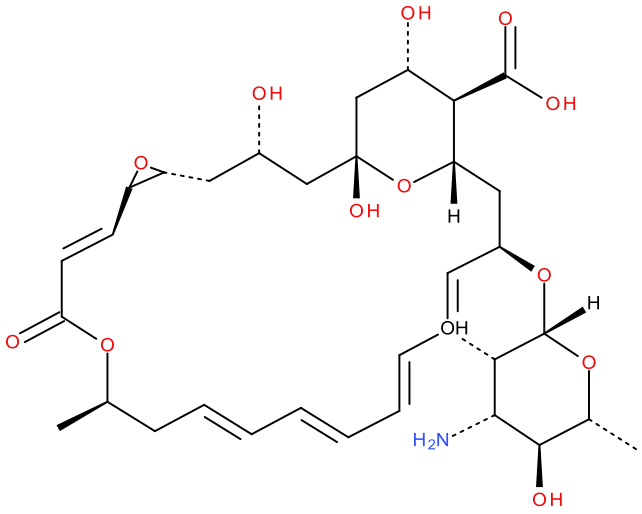
Drug Name	Natamycin
Brand Name	Natacyn
Drug Class	Anti-fungal
Chemical Structure	
Chemical IUPAC Name	methyl-10-oxo-6,11,28-trioxatricyclo[22.3.1.0^{5,7}]octacosa-8,14,16,18,20-pentaene-25-carboxylic acid
Chemical Formula	$C_{33}H_{47}NO_{13}$
Molecular weight	665.72
CAS No.	768-93-8
Melting point	290-292
Dissociation Constant(pK)	
Strongly Acidic	3.58
Strongly Basic	9.11
Partition coefficient(log p)	1.1
Solubility	Practically insoluble in nonpolar solvents, sparingly soluble in methanol, dimethylsulfoxide, dimethyl Formamide, insoluble on water.

Table 2.2: Therapeutic consideration of Natamycin

Drug Category	Antifungal
Dosage and Administration	Ophthalmic suspension 5%, Instil 1 drop in conjunctival sac every 1-2 hours, reduce to 1 drop 6-8 times a day after 3-4 days. Duration of treatment: 2-3 weeks. Ointment 1%
Pharmacology	Acts by increasing cell membrane permeability in susceptible fungi. It is active against Candida and Fusarium
Mechanism of Action	Like other polyene antibiotics, Natamycin inhibits fungal growth by binding to sterols. Specifically, Natamycin binds to ergosterol in the plasma membrane, preventing ergosterol-dependent fusion of vacuoles, as well as membrane fusion and fission. This differs from the mechanism of most other polyene antibiotics, which tend to work by altering fungal membrane permeability instead.
Drug Interactions	Increase spread of fungal eye infection when used with topical corticosteroid

Precautions	Care should be taken during Pregnancy and lactation
Adverse effects	GI disturbances (oral), Irritation (local), Conjunctival chemosis and hyperemia (ophthalmic)
Storage	Sealed in a cool dry place protected from sunlight
Shelf Life	2 Years

AIM

Natamycin is a drug used in treatment of fungal keratitis, it is generally used in the treatment of eye infection, no UV-Visible spectroscopy method for estimation of natamycin in bulk has been reported in any literature hence the method development and validation of natamycin using UV-visible spectroscopy is needed to be developed and simple method must be established.

The purpose of the stability testing is to provide evidence on how quality of a drug substance or drug product varies with time under the influence of variety of environmental factors such as temperature, humidity and light. Stability testing permits the establishment of recommended storage conditions, retest periods and shelf-lives.

Many pharmaceutical substances are known to deteriorate during distribution and storage particularly in hot, humid climates. Nonetheless, little precise information is available on the degradation characteristics of natamycin, relevant data must now be generated. So, in order to attain the detailed behavior of the natamycin in presence of different stress conditions, the stability indicating assay method can be developed.

From the literature survey, it can be accomplished that the several methods are reported for the estimation of Natamycin, but no single stability indicating method has been reported for the drug in bulk and in its dosage form. Hence, it was endeavored to develop an accurate, precise and sensitive stability indicating method for the drug in bulk and in its dosage form.

OBJECTIVE OF THE PRESENT WORK:

- To develop a method for Natamycin using uv-visible spectroscopy and to validate it in terms of Linearity, Range, Accuracy, Precision, Limit of Detection, Limit of Quantification, Robustness, and Repeatability,
- To develop a suitable stability indicating HPTLC method for the Natamycin.

REVIEW OF LITERATURE:

Sr. No.	TITLE	DESCRIPTION	REF. NO
1	LC DETERMINATION OF NATAMYCIN IN DOOGH WITH UV DETECTION	Column: C18(100 × 4.6 mm I.D., 5µm) Solvent: ACN: 30mM Perchloric acid (35:65) Wavelength: 303 nm Flow Rate: 0.5 mL/Min	51
2	USP OFFICIAL ASSAY METHOD	Column: C18 (4.5 × 25cm I.D., 5µm) Solvent system: Tetrahydrofuran: acetonitrile (10:90) Wavelength: 303nm Flow Rate:3mL/min	52
3	ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF NATAMYCIN IN EYE DROP BY RP-HPLC	Column: C18 (250 × 4.6 mm, I.D., 5µm) Solvent system: phosphate buffer pH (pH 4.2; 10 mM): acetonitrile (70:30) Wavelength: 304nm Flow Rate:1 mL/min	53
4	HPLC DETERMINATION OF NATAMYCIN IN FOOD	Column: C18 (4.5 × 25cm I.D., 5µm) Solvent System Phosphate Buffer (pH 5.5; 5 mM): methanol(8:92) Wavelength-303nm Flow Rate-0.8mL/min	54

5	BIOANALYTICAL METHOD DEVELOPEMENT AND VALIDATION OF NATAMYCIN IN RABBIT TEARS AND ITS APPLICATION TO OCULAR PHARMACOKINETICS	Column-Luna Cyano column (100 mm × 2 mm, 3µm) Solvent system-ammonium acetate buffer (pH 4; 3.5 mM) : methanol (10:90) Wavelength: 303 nm Flow Rate-1mL/min	55
6	RAPID METHOD TO DETERMINE NATAMYCIN BY HPLC-DAD IN FOOD SAMPLES FOR COMPLIANCE WITH EU	Column- Kromasil ODS (C18 150 × 3.20 mm ID, 5 µm) Solvent system-acetonitrile: acetate buffer (pH 4; 3 mM) (10:90) Wavelength: 303 nm Flow Rate-1 mL/min	56
7	ANALYTICAL METHOD FOR NATAMYCIN IN WINE USING HPLC	Column:C18 (25 × 4.5 mm I.D., 5µm) Solvent-Tetrahydrofuran :Acetonitrile (12:88) Wavelength: 303nm Flow rate: 0.8 mL/min	57

8	DEVELOPMENT OF AN RP-HPLC METHOD FOR SIMUTANEOUS DETERMINATION OF BENZOIC ACID, SORBIC ACID, AND NATAMYCIN IN PASTAFILATA CHEESE	Column:C18 (150 × 5 mm I.D., 5µm) Solvent – Phosphate buffer (pH 5.5; 20 mM): acetonitrile (30:70) Wavelength: 303nm Flow rate: 1 mL/min	58
9	RAPID ULTRAVIOLET SPECTROSCOPIC NAD CHROMATOGRAPHIC METHOD FOR DETERMINATION OF NATAMYCIN IN LACTOSERUM MATRIX	Column:C18 Luna(100 mm × 2 mm, 3µm) solvent methanol: ammonium acetate buffer ((pH 3; 7 mM) (70:30) Wavelength: 302nm Flow rate: 1 mL/min	59

5. IDENTIFICATION OF NATAMYCIN

Identification of drug natamycin was carried out by Melting point, UV-visible Spectroscopy and FT-IR Spectroscopy.

Instrumentation

- Melting Point Apparatus: T603160, (EIE Instruments, Pvt. Ltd., Ahmedabad, India)
- UV-visible spectrophotometer: UV/vis-2400, (Shimadzu Inc., Columbia, MD)
- FT IR Spectrophotometer: JASCO FT/IR-6100, (Inc. Japan)

5.1 IDENTIFICATION BY MELTING POINT

Melting point of natamycin has been determined using melting point apparatus. The melting point of the pure drug was taken by capillary method.

Table 5.1: Melting point determination data of natamycin

Drug	Reported Melting Point (˚C)[8]	Observed Melting Point(˚C)
NATAMYCIN	290-292˚C	290-291˚C

5.2 IDENTIFICATION BY UV SPECTRA

Solution of 10 µg/mL of natamycin was prepared in methanol and scanned in UV Visible Spectrophotometer in range of 200-400 nm to determine the absorption maxima of drug.

Table 5.2: UV spectra determination data of natamycin

REPORTED ABSORPTION MAXIMA (λ_{max})[8]	OBSERVED ABSORPTION MAXIMA (λ_{max})
303nm	303nm

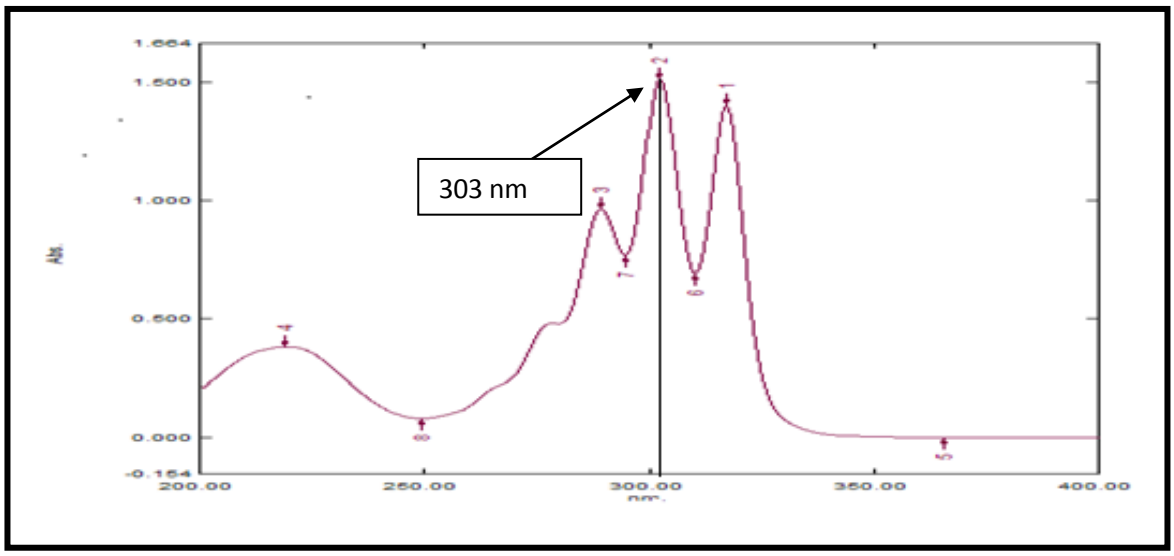


Fig. 5.1: UV Spectra of natamycin (10 µg/mL) in Methanol

5.3. IDENTIFICATION BY INFRARED SPECTROSCOPY

IR Spectra of pure drugs was taken using FT-IR spectrophotometer. IR spectra obtained was verified with the reported IR spectra available in literature.

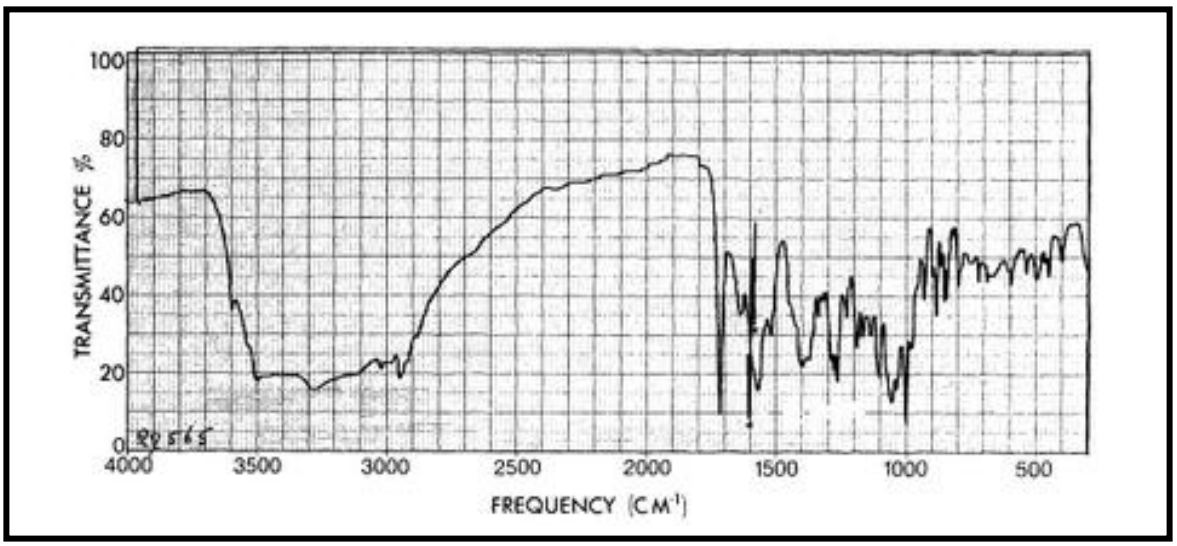


Figure 5.2: Reported FT IR spectra of natamycin[11]

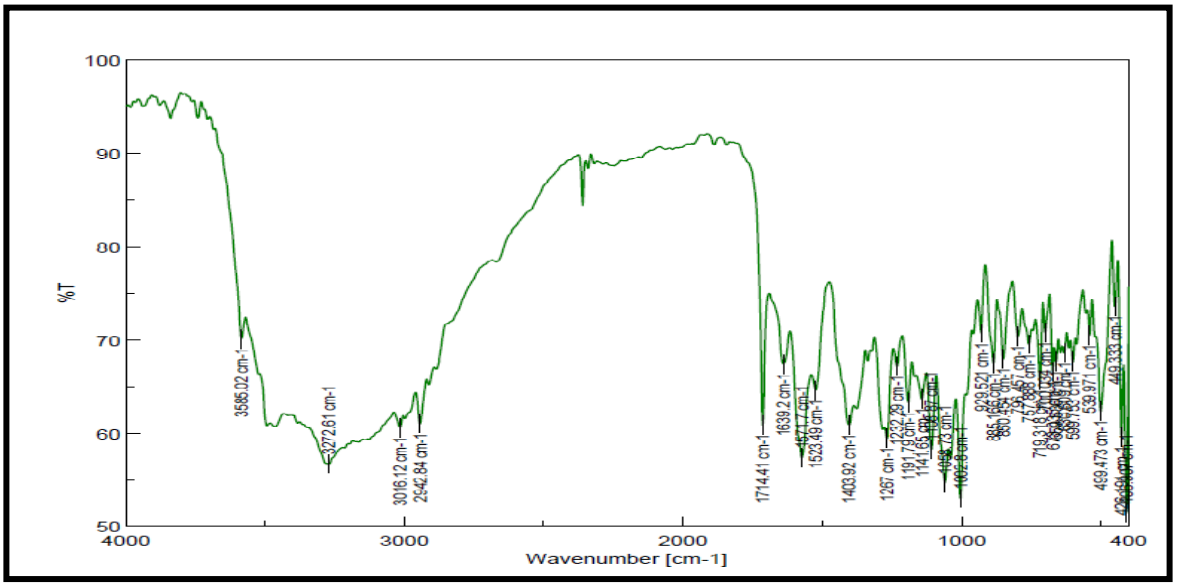


Figure 5.3: Recorded FT IR spectra of natamycin

6.1 DEVELOPMENT AND VALIDATION OF NATAMYCIN USING UV VISIBLE SPECTROSCOPY

6.1.1 Instruments

UV/Vis-2400, Version-2.21 double beam spectrophotometer with spectral width of 2 nm, wavelength accuracy of 0.5 nm, and a pair of 10 mm matched quartz cells (Shimadzu, Columbia, MD),Analytical Balance- Citizen CX 220, Capacity 10 to 220 mg (Citizen Pvt. Ltd, Mumbai, India),Sonicator- D-Compact, Capacity 2 L (Trans-o-sonic, Mumbai, India).

6.1.2 Reagents and Materials

Natamycin standard 98.99% pure was obtained from Bimal Pharma, Mumbai as a gift sample. Analytical grade methanol (Central Drug House Pvt. LTD, Mumbai) was used. Marketed formulation of natamycin (Natamet 5% eye suspension, 3ml) manufactured by Sun Pharma, Halol, Baroda was purchased and analyzed.

6.1.3 Method

6.1.3.1 Selection of Solvent

Owing to the solubility of natamycin in methanol it was selected as a solvent.

6.1.3.2 Preparation of Standard Stock Solution

A 100 mg of standard natamycin was weighed and transferred to 100 mL amber coloured volumetric flask and dissolved in the methanol. The flask sonicated for 15 min and the volume was made upto the mark with methanol to get stock solution of 1000 µg/mL of natamycin. The aliquot 5 mL from this solution was transferred to 50 mL amber coloured volumetric flask and the volume was made upto the mark with the methanol to obtain working standard stock solution of 100 µg/mL natamycin.

6.1.3.3 Preparation of sample Solution

For preparation of sample solution, 0.1 mL of marketed formulation of Natamet 5% eye suspension was taken and transferred into 50 mL amber coloured volumetric flask and the volume was made upto the mark with methanol to get 100 µg/mL stock solution. From the stock solution 1 mL was taken and further diluted upto 10 mL with methanol to get 10 µg/mL.

6.1.3.4 Selection of Analytical Wavelength

From the working standard solution, 1 mL was taken into 10 mL amber coloured volumetric flask and volume was made upto the mark with methanol to get the solution of 10 µg/mL. The solution was scanned in the spectrum range of 200-400 nm. The spectrum is shown in the figure 5.1.From the spectrum of the drug the wavelength maxima was selected as 303 nm for analysis.

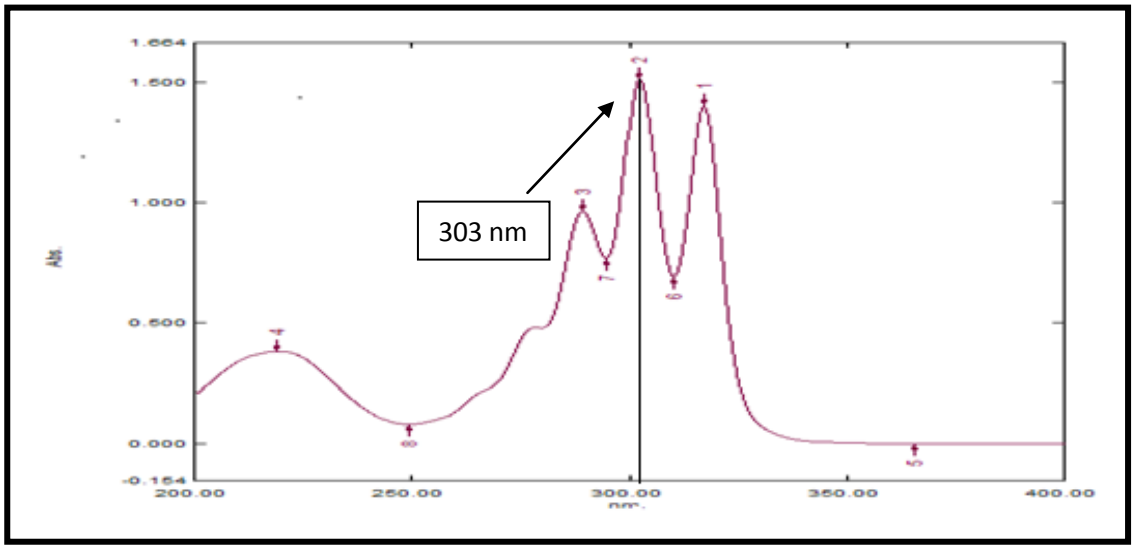


Figure 6.1: UV Spectra of Natamycin (10 µg/mL) in methanol at 303 nm

6.1.4 Method Validation

6.1.4.1 Linearity

Appropriate volume of aliquots (0.2, 0.4, 0.6, 0.8, and 1.0 mL) from working standard Natamycin stock solution were transferred to different amber coloured volumetric flask of 10 mL capacity, the volume was made up to mark with methanol to obtain concentration of 2, 4, 6, 8, 10 µg/mL. Absorbance of each solution using methanol as blank at 303 nm was measured and plot of Absorbance vs. concentration at this wavelength was plotted. The %RSD of the slope and intercept of the calibration curve was calculated.

6.1.4.2 Precision

The precision of the method was verified by repeatability and intermediate precision studies. For precision studies, solutions were prepared by taking appropriate aliquots of 0.2, 0.4 and 0.6 mL from working standard stock solution and diluting with methanol upto 10 mL in amber coloured volumetric flask. Repeatability studies were performed by taking absorbance of 5 µg/mL six times at λ_{max} 303 nm. The intermediate precision of the method was checked by repeating studies on three different days and 3 times on single day for 3 different concentrations (2, 4, 6 µg/mL)

6.1.4.3 Accuracy

Accuracy was determined in terms of percentage recovery using standard addition method. For accuracy studies, 0.4 mL of the sample stock solution was taken in three 10 mL amber coloured volumetric flask in triplicate and 0.32, 0.4, 0.48 mL of working standard stock solution was spiked, and the solutions were scanned at 303 nm.

6.1.4.4 Sensitivity

The sensitivity of the analytical method was evaluated by determining the Limits of Detection (LOD) and Limits of Quantification (LOQ). The LOD and LOQ of the drug were calculated using the following equation as per ICH guidelines.

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

6.1.4.5 Robustness

The robustness was performed on concentration of 10 µg/mL. Robustness of the method was determined by making changes in λ_{max} of the drug by ± 2nm. The assay values were compared and calculated with that of standard and the results were reported in terms of % RSD

6.1.4.6 Assay of marketed formulation

For the assay of the marketed formulation, 1mL was taken from the above prepared sample stock solution in a 10 mL amber coloured volumetric flask and the volume was made by the methanol. The solution was scanned at absorbance maxima of 303 nm.

6.2 FORCED DEGRDATION STUDIES OF NATAMYCIN USING HPTLC METHOD

6.2.1 Instrumentation

- Precoated TLC aluminum silica gel plate 60 f_{254} (10 X 10 cm, 0.2 mm layer thickness) E Merck Ltd India.
- Camag 100 µL applicator syringe.
- Camag linomat V applicator with N_2 pressure.
- Camag twin trough chamber (10 X 10) with stainless steel lid.
- Camag TLC scanner III.
- UV cabinet with dual wavelength (254 and 366).
- Software- Wincats IV.

6.2.2 Materials and Reagent

6.2.2.1 Materials

Natamycin 98.99% pure was obtained from Bimal Pharma, Mumbai.

6.2.2.2 Reagents

Methanol, DMSO, chloroform, glacial acetic acid, sodium hydroxide, hydrochloric acid, hydrogen peroxide (30%v/v) was used of AR grade (S.D. Fine chemicals Ltd., Mumbai, India) and distilled water

Marketed formulation Natamet 5% eye suspension.

6.2.3 Optimization of Method for HPTLC

6.2.3.1 Optimization of mobile phase

Mobile phase was optimized by several trials. From the trials Chloroform: methanol: glacial acetic acid: water (10:3:3:1) was optimized as the mobile phase, it showed optimum separation of drug and degradation products. Chromatogram of standard Natamycin (2000ng/spot) was taken in optimized mobile phase, it shows good peak shape and R_f value (fig 4.2)

Table 6.1 Trials for optimization of mobile phase

Sr. No.	Trial	Observation	Remark
1	Methanol	High R_f value	Not satisfactory
2	Hexane	Spot do not travel	Not satisfactory
3	Water	High R_f value	Not satisfactory
4	Methanol+ water (5:1)	Good R_f value but zig zag solvent front	Not satisfactory
5	Chloroform	Spot do not travel	Not satisfactory
6	Chloroform+ methanol (6:2)	Low R_f value	Not satisfactory
7	Chloroform+ methanol (8:2)	Low R_f value	Not satisfactory
8	Chloroform + methanol + ammonia solution (8:2:1)	Low R_f value and zig zag solvent front	Not satisfactory
9	Chloroform + Methanol (10:3)	Low R_f value with diffusion of spot	Not satisfactory
10	Hexane + methanol (8:2)	Spot do not travel	Not satisfactory
11	Chloroform + methanol water (10:3:0.5)	Low R_f value with diffusion of spot	Not satisfactory
12	Chloroform + methanol + water (10:3:0.7)	Good R_f value with diffusion of spot	Not satisfactory

13	Chloroform + methanol + water (10:3:0.7)	Good R_f value but broad peak	Not satisfactory
14	Chloroform + methanol + water (10:3:1)	Good R_f value but broad peak	Not satisfactory
15	Chloroform + methanol + glacial acetic acid + water (10:3:1:1)	Good R_f value but broad peak	Not satisfactory
16	Chloroform + methanol + glacial acetic acid + water (10:3:1:2)	Good R_f value but broad peak	Not satisfactory
17	Chloroform + methanol + glacial acetic acid + water (10:3:1:3)	Good R_f value and good peak shape	Satisfactory

6.2.3.2 Selection of wavelength for quantification

Standard Natamycin solution (1000 µg/mL) 2µL was applied on a TLC plate. The plate was developed with the optimized mobile phase, dried and scanned by scanner III using UV detector in the range of 200-400 nm. The UV scanned spectra is shown in figure 5.2 the spectrum of natamycin showed maximum absorbance at 303 nm, and hence 303 nm was selected as detection wavelength.

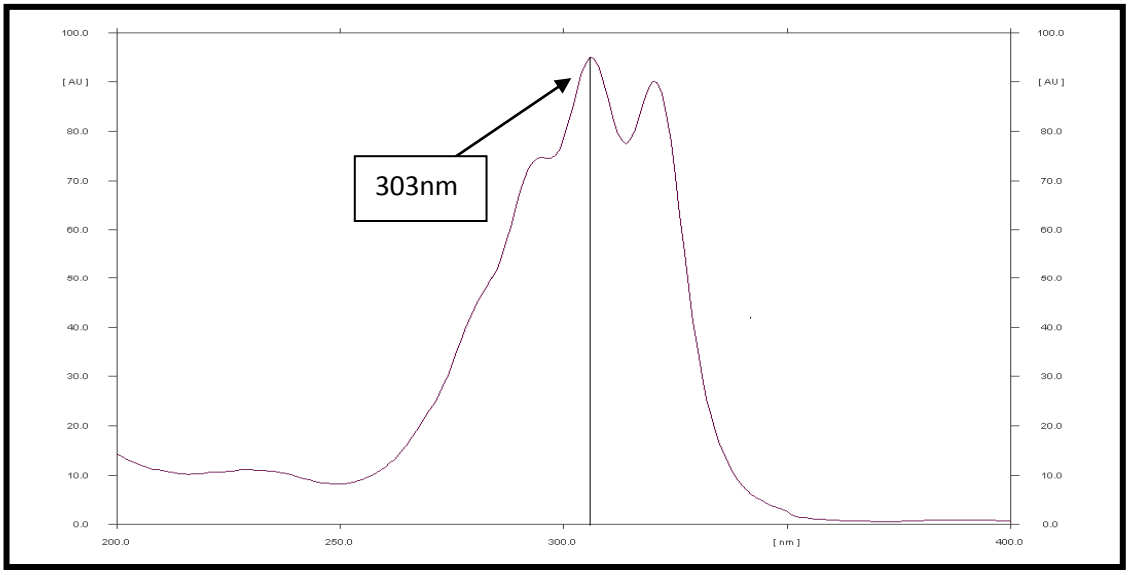


Figure 6.2: UV spectra of Natamycin (1000µg/mL) in DMSO

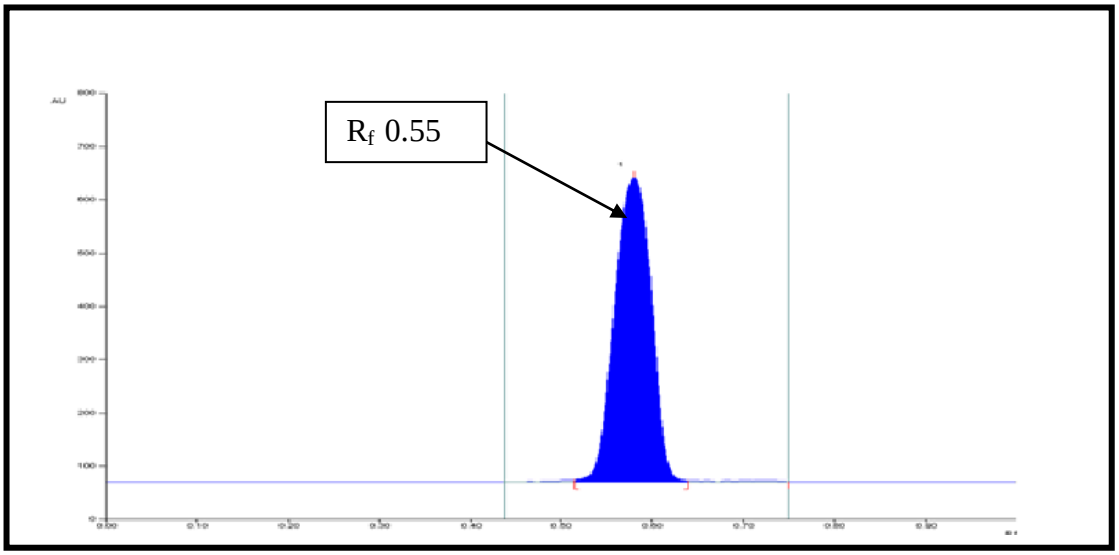


Figure 6.3: HPTLC chromatogram of Natamycin Standard in optimized condition (Conc. 2000 ng/spot, R_F 0.55)

6.2.3.3 Optimized HPTLC conditions

Stationary phase: Precoated silica gel on aluminum plate 60F254, (10cm x 10cm), pre-washed by methanol and activated at 105 ℃ for 15 minutes prior to chromatography.

- Mobile phase: **Chloroform: Methanol: Glacial acetic acid: Water (10:3:3:1, v/v/v/v)**
- TLC chamber saturation time: 20 min
- Run length: 70 mm
- Development Time: 15 min
- Application rate: 0.1 µL/s
- Scanner band width: 5 mm
- Slit dimension: 4 mm x 0.45 mm (micro)
- Scanning speed: 20 mm/s
- Detection: Densitometrically using a UV detector at 303 nm
- Temperature: Ambient
- Evaluation mode: Absorbance mode

6.2.4 Preparation of solutions

6.2.4.1 Preparation of standard stock solution

Standard Natamycin (200 mg) was accurately weighed and transferred to 100 mL volumetric flask, and dissolved in 50 mL DMSO. The flask was sonicated for 15 min in sonicator, and the volume was made upto the mark with DMSO to obtain stock solution of concentration 2000 µg/mL.

6.2.4.2 Preparation of sample solution

From the marketed formulation of Natamet 5% eye suspension 1 mL was taken into 50 mL volumetric flask and diluted with the diluent to get concentration of 1000 µg/mL.

6.2.4.2 Preparation of mobile phase

Chloroform (10 mL), methanol (3mL) and glacial acetic acid (3mL) was mixed and then water (1mL) was added to the above solution and mixed properly and sonicated for 10 minutes to get optimum mobile phase.

6.2.5 Method validation

6.2.5.1 Linearity

For linearity, from the above prepared standard stock solution 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7 and 7.5 mL was withdrawn and transferred in 10 mL amber coloured volumetric flask to get concentration ranging from 500-1500 µg/mL. The same was repeated for six times. The plate was developed in optimized conditions. Peak areas were plotted against corresponding concentrations to obtain the calibration graph.

6.2.5.2 Precision

The precision of the method was verified by repeatability and intermediate precision studies. For precision studies, solutions were prepared from the stock solution of 800, 1000, 1200 µg/mL to get concentration of 1600, 2000, 2400 ng/spot, and the spots were applied on the TLC plate in three replicates. Repeatability was performed by analysis of concentration of Natamycin (2000ng/spot) eight times. The intermediate precision of the method was checked by repeating studies on three different days and three times on a single day for three different concentrations of Natamycin (1600, 2000, 2400 ng/spot)

6.2.5.3 Accuracy

Accuracy was performed in terms of percentage recovery by standard addition method. For accuracy of Natamycin, from the above prepared sample stock solution 0.5 mL is withdrawn and transferred to three 10 mL volumetric flask in triplicate and from the standard stock solution 0.2 mL, 0.25 mL and 3 mL were added to the volumetric flask to get 80%, 100% and 120% of the standard concentration and the volume was made upto the mark with diluent. From this above prepared solution the spot of 2µL was applied on TLC plate and analyzed by running the chromatogram in optimized conditions.

6.2.5.4 Specificity

Interference from degradation products is formed by stress condition, was checked by specificity study. Purity of the Natamycin and degradation products was checked by scanning in the range of 200-400 nm with the help of spectra scanner model of the WinCATS software. The peak purity of Natamycin and degradation products were determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M), and peak end(E) positions which confirms that the peak represents pure single component for Natamycin and also for its degradation products.

6.2.5.5 Sensitivity

The sensitivity of the analytical method was evaluated by determining the Limits of Detection (LOD) and Limits of Quantification (LOQ). The LOD and LOQ of the drug were calculated using the following equation as per ICH guidelines.

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

Where σ = Standard deviation of the response, S = Slope of the calibration curve.

6.2.5.6 Robustness

Following the introduction of small change in the mobile phase composition, the effects on the results were analyzed. The detection wavelength was varied by ± 2 nm. The robustness of the method was determined at three different concentration range 1600, 2000, 2400 ng/spot. The percentage assay values were calculated and compared with that of the standard. Results were reported in terms of %RSD.

6.2.5.7 Analysis of marketed formulation

For the analysis of the marketed formulation the above prepared sample stock solution was taken and spot was applied of 2 µL volume (2000 ng/spot) and the peak area was recorded and compared with the standard and reported in terms of percentage drug present in the formulation**.**

6.2.6 Generation of stress samples

6.2.6.1 Optimization of stress conditions

Acid hydrolysis:

Acid hydrolysis was carried out in different concentration (0.1N, 0.5N, 1N) of HCl at 25ºC to get sufficient degradation of the drug. The sample after degradation period was neutralized with NaOH and subjected for analysis.

Base hydrolysis:

Base hydrolysis was carried out in different concentration (0.1N, 0.5N, 1N) of NaOH at 25ºC to get sufficient degradation of the drug. The sample after degradation period was neutralized with HCl and subjected for analysis.

Neutral hydrolysis:

Neutral hydrolysis was carried out in water by heating at 70ºC for different time intervals to get sufficient degradation. Aliquots were withdrawn periodically and checked for degradation.

Oxidation:

The oxidative stress studies were carried out in 3%, 10% and 30% to check the degradation of the drug, the aliquots were withdrawn periodically to analyze for degradation of the drug.

Thermal degradation:

The drug was subjected for dry heat at 80ºC for different time interval to get sufficient degradation of the drug the samples were withdrawn periodically and were analyzed for degradation of the drug.

Photolytic degradation

Photolytic studies were performed by exposing the drug solution under sunlight for different intervals of time and the samples were withdrawn and were analyzed for degradation.

6.2.6.2 Preparation of stress samples

The standard Natamycin drug was accurately weighed (10 mg) and transferred to 10 mL volumetric flask and 5mL of the diluent was added to dissolve the drug and the solution was sonicated for 10 minutes, after sonication 1ml of the stressor was added to the above solution and kept for degradation and after optimized time and incase of acid and base hydrolysis the sample was neutralized with counter acid or base and the final volume was made upto the mark with the diluent and the solution was analyzed and the chromatogram was recorded.

6.2.7 Optimized Stress conditions

Table 6.2: optimized degradation condition parameters

Degradation Type		Stressor	Time Period	Temperature
Acid hydrolysis		0.1N HCl	3 Hours	25ºC
Base hydrolysis		0.1N NaOH	10 Minutes	25ºC
Neutral hydrolysis		Water	48 Hours	70ºC
Oxidation		30% H_2O_2	24 Hours	25ºC
Thermal degradation		Dry heat	24 Hours	80 ºC
Photolytic degradation	Solution	Direct sunlight	15 Minutes	-----
	Solid	Direct Sunlight	15 minutes	-----

Comparison:

Comparison of developed UV-Visible spectroscopic and HPTLC methods was performed by applying unpaired t- test the result are shown in table 9.2.

Table 8.1: Assay Results of the Proposed Methods

Drug	% Assay	
	UV Method	HPTLC
Natamycin	100.11	100.18
	100.14	100.19
	100.13	100.17

Table 8.2: Results of Unpaired t-test

Natamycin		
Parameters	**UV Method**	**HPTLC Method**
Mean % Assay	100.13	100.18
Variance	0.0152	0.011
Observations	3	3
Degree of freedom	2	2
P value	0.09	

Output of unpaired t-test at 95% confidence interval shows that there is no significant difference with respect to % assay between the proposed UV-Visible spectroscopic and HPTLC method.

P value obtained for natamycin unpaired t-test for Comparison between UV-Visible spectroscopic method and HPTLC method was found to be more than 0.05 so indicates that there is no significant difference in both the methods.

CONCLUSION:

The developed UV-visible and HPTLC method was simple, rapid, accurate, precise, specific and HPTLC method has the ability to separate natamycin natamycin and other degradation products formed during different stress conditions.

The results of validation parameters are satisfactory which indicates the reliability of proposed method of estimation of natamycin in routine analysis.

The method allows for the application in the quality control laboratory for routine analysis as well as for the stability studies of the drug substance.

The result of the t-test reveals that UV-Visible Spectroscopy method and HPTLC method for analysis of the bulk drug are same as there in no significant difference between both the methods

7.1 SUMMARY

Analytical method for natamycin using UV-Visible spectroscopy was developed and was validated in terms of different validation parameters. The λ_{max} was selected by taking spectra of the standard drug and it was found to be 303 nm. Owing to the solubility of the drug, methanol was selected as a solvent for this method development. The linear regression analysis data for calibration plot show good linear relationship with concentration range of 2-10µg/mL with linearity equation y = 0.156x - 0.054 with correlation coefficient of 0.9999. The method is validated for specificity, accuracy, precision, linearity, repeatability and robustness as per ICH guideline Q2 (R1) method validation. The limit of detection and quantification were calculated with the signal to noise ratio of 3.3 and 10 respectively and the values were found to be for limit of detection it was 0.0713µg/mL and for limit of quantification was found to be 0.2162 µg/mL.

A stability indicating HPTLC method was developed and validated for determination of Natamycin in presence of its degradation products. The optimization of mobile phase was done by doing different trials using various solvents. Optimized mobile phase was Chloroform: Methanol: Glacial acetic acid: Water (10: 3: 3: 1 v/v/v/v). The method is based on high performance thin layer chromatography using precoated silica Gel 60 F_{254} plates with 250 µm thickness. Detection was carried out at 303 nm wavelength. The R_f value of Natamycin was found to be 0.55 ± 0.02.

Forced degradation was carried out according to ICH guidelines Q1A (R2) and Q1B. Stability indicating capability is established by forced degradation of Natamycin to acid, alkali, oxidation, thermal and photo degradation. The peaks of degraded products were well separated.

The linear regression analysis data for calibration plot shows good linear relationship with concentration range of 1000-3000 ng/spot with linearity equation of y = 3.545x + 10076 with Correlation co-efficient of 0.9989.

The method is validated for specificity, accuracy, precision, linearity, repeatability and robustness as per ICH guideline Q2 (R1) method validation. The limit of detection and quantification were calculated with the signal to noise ratio of 3.3 and 10 respectively. As the

method could effectively separate the drug from its degradation products, it can be employed as a stability indicating method. The developed method is also useful for the assay of marketed formulation of Natamycin and in routine analysis of API.

7.2 FUTURE SCOPE

- Develop HPLC method for forced degradation studies of Natamycin and to develop, LC-MS, GC-MS, UPLC methods for the determination of Natamycin.

- Isolation of the degradation sample after the forced degradation studies using preparative HPLC method for the structural elucidation of the Natamycin.

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