STABILITY INDICATING RP-HPLC ASSAY METHOD DEVELOPMENT FOR DETERMINATION OF LETROZOLE IN PHARMACEUTICAL FORMULATION

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IN

PHARMACEUTICAL ANALYSIS

BY

JANKI V. ANDHARIYA, B.PHARM.

GUIDED BY

DR. PRITI J. MEHTA, M.PHARM., PH.D.

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

INSTITUTE OF PHARMACY

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CERTIFICATE

This is to certify that the work presented in the thesis entitled Stability Indicating RP-HPLC Assay Method for Determination of Letrozole in Pharmaceutical Formulation by Ms. Janki V. Andhariya . In the partial fulfillment for the requirement of degree of Master of Pharmacy, is carried out under my guidance and to my satisfaction. I recommended this project report ready for examination. This work is carried out by her and has not been submitted earlier either to this university or to any other institutions for fulfillment of requirement of a course of study.

Guided By:

Dr. Priti J. Mehta
H.O.D. of Pharmaceutical Analysis Department,
Institute of Pharmacy,
Nirma University. .

Forwarded Through:

Dr. Manjunath Ghate
D/I, Principal, HOD, Dept. of Pharmaceutical Chemistry,
Institute of Pharmacy,
Nirma University.

DECLARATION

I, **JANKI V. ANDHARIYA**, Final Year M. Pharm student of the Institute of Pharmacy, Nirma University, hereby state that the study entitled "**STABILITY INDICATING RP-HPLC ASSAY METHOD DEVELOPMENT FOR DETERMINATION OF LETROZOLE IN PHARMACEUTICAL FORMULATION**" is my own work and that, to the best of my knowledge, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or Diploma of University or other institute, except where due acknowledgement has been made in this text.

I also here by give my unconditional undertaking that all the rights to use this work, for publication of any kind scientific or commercial purpose, completely rest with my guide.

Date: JANKI V. ANDHARIYA

ACKNOWLEDGEMENT

It gives me immense pleasure today when I, Janki Andhariya, take an opportunity to acknowledge all of those personalities who contributed directly or indirectly to my project. This research would not have been possible without the whole hearted encouragement, guidance, support, and cooperation of my beloved family, teachers, friends, well wishers and relatives. Probably I would have never achieved this without their support and blessings. With profound appreciation, I acknowledge to one and all.

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ABBREVIATIONS

CHEMICALS:

ACN	Acetonitrile
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric acid
KH_2PO_4	Potassium Dihydrogen Phosphate
NaOH	Sodium Hydroxide
MeOH	Methanol
TEA	Tri Ethyl Amine

SYMBOL:

%	Percentage
λ_{max}	Wavelength of maximum absorption
<	Less than
>	Greater than
µg	Microgram (S)
µm	Micro meter (S)
cm	Centimeter (S)
i.d.	Internal diameter
L	Liter (S)
M	Molar
mg	Milligram (S)
Min	Minute (S)
mL	Milliliter (S)
mM	Milimolar
mm	Millimeter (S)
nm	Nanometer (S)
r^2	Correlation coefficient
Sec	Second (S)
Temp	Temperature

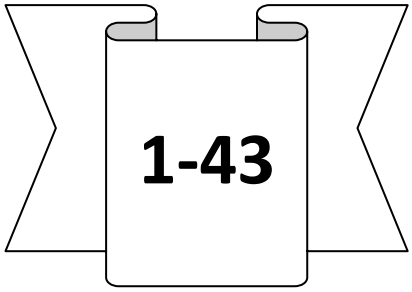
°C	Degree centigrade
No.	Number
Sr.No.	Serial number
N	Normal
v/v	Volume by volume
w/w	Weight by weight
Conc.	Concentration
ppm	Parts per million

OTHERS:

IR	Infrared
CAS No.	Chemical Abstract Service Number
RSD	Relative standard deviation
SD	Standard deviation
USP	United States Pharmacopoeia
UV	Ultraviolet
hrs	hours
ID	Identification
RT	Retention Time
M.P	Mobile Phase
API	Active Pharmaceutical Ingredient
RP-HPLC	Reversed Phase High Performance Liquid Chromatograph
NA	Not Applicable
ICH	International Conference on Harmonization
IUPAC	International union of pure and applied chemistry
LOD	Limit of detection
LOQ	Limit of Quantitation

CHAPTER-1

INTRODUCTION



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1) INTRODUCTION:

1.1 INTRODUCTION TO STABILITY STUDY:

Stability testing of drug products is a requirement from regulatory as well as from industrial point of view, owing to increasing concerns for drug product safety, efficacy and quality. ``Stability behavior, in broad terms, refers to alteration in physical, chemical, microbiological and biological performance of the 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 (1). Isolation and characterization of degradation products is required for validation of SIAM and to investigate mechanisms of degradation (2).

Regulatory requirements specify long term, intermediate and accelerated stability testing(3).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.

1.1.1. Stability Testing

The term 'drug stability' refers to the extent to which a drug substance or product retains, within specified limits throughout its period of storage and use, the same properties and characteristics that it possessed at the time of its manufacture. Drug stability can be categorized as pre-market and commercial (marketed product) stability. Pre-market stability supports the clinical trials where drug products are stored under different conditions for safety and efficacy evaluation. Commercial stability is continuous assurance on the post-approval batches for long term

stability monitoring on drug product. Drug stability assessment generally involves the testing of drug substance or drug product using the stability indicating method in order to establish the retest period (for pre-market stability) and shelf life (for commercial stability) and to help in determining storage conditions.

1.1.1.1. *Factors affecting drug stability*

Stability of drug substances and products is mainly influenced by environmental factors such as temperature, humidity, light and oxygen. Therefore, in order to determine the stability profile of a drug product, it is required that stability testing should be done under specific temperature/ humidity and light conditions.

D. Effect of temperature

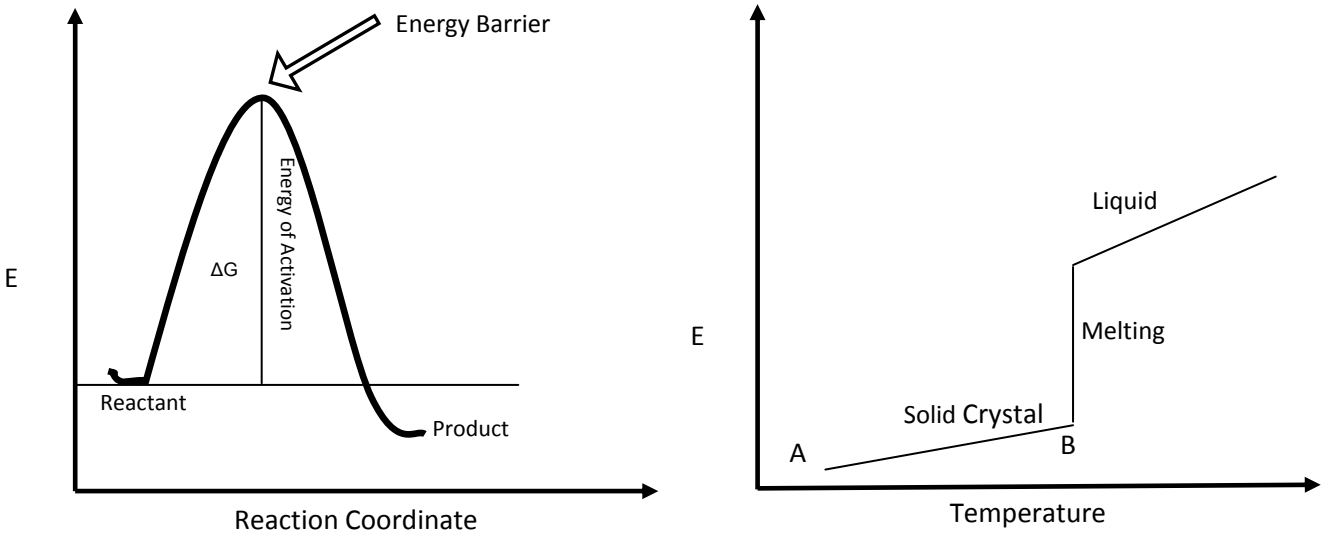


Figure 1.1.1: Energy profile diagram (a) and energy change during heating (b) which shows increase in energy due to increase in temperature, even in solid form before melting. If ΔG is in-between A and B, then degradation product can form easily [4].

This energy profile diagram (Figure 1) shows that for a reaction to occur, there is a minimum threshold energy, which the molecules require to cross. This energy

barrier is known as activation energy. This activation energy can be supplied by increasing the temperature of the reaction, which causes the reaction to proceed, leading to formation of degradation products.

E. Effect of humidity

Humidity is another major factor for degradation. When a dosage form is exposed to high humidity, moisture gets absorbed into it. This moisture soon reaches at its particulate level (Figure1.1.2b) where both excipients and drugs can dissolve (Figure1.1.2c). Dissolution of the dosage constituents creates a microenvironment with differing pH and/ or oxidation potential in which both drug and the excipients exist in their molecular form. Collisions between the molecules increase in such conditions, which help in attainment of activation energy (ΔG), leading to the formation of degradation products (Figure1.1.2d). Presence of high temperature further worsens the condition by exerting a potentiating effect upon the degradation reaction. Exposure to sufficiently high temperatures helps in easy and rapid crossing of threshold energy barrier by the molecules. Due to this reason, simultaneous application of both high humidity and temperature is also indicated by ICH for the stability testing and SIAM development

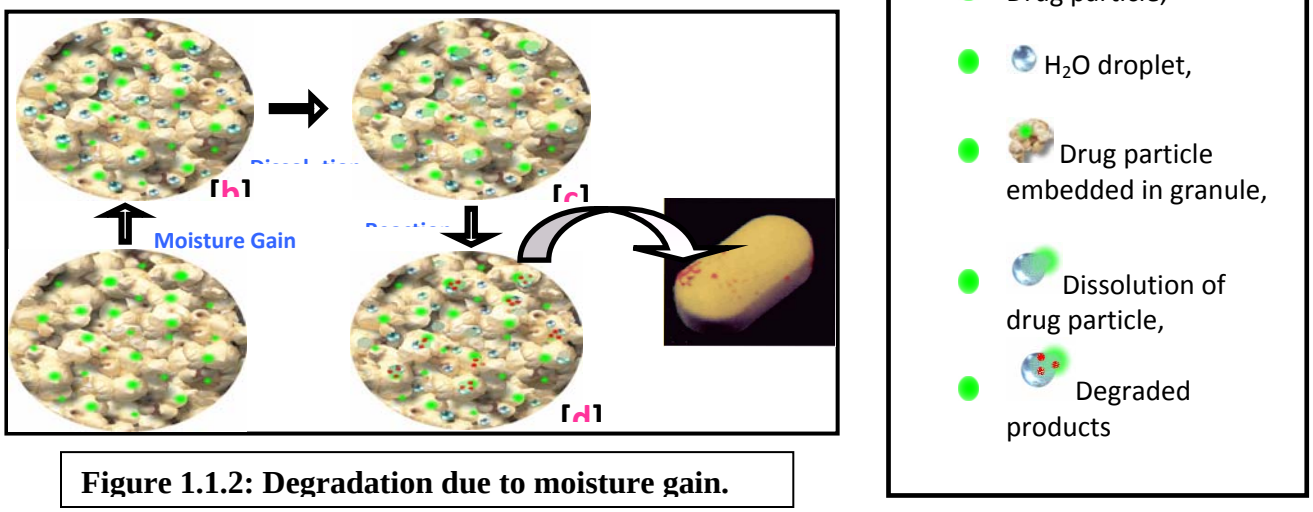


Figure 1.1.2: Degradation due to moisture gain.

F. Effect of Light

Light is a form of energy, which can provide activation energy in the form of certain discrete packets known as photons. When these photons are absorbed by reactants, it often leads to an increase in the reaction rate. Presence of light can result in the attainment of even higher energy states which can enhance the extent of degradation. Generally, light accelerates all the reactions via the formation of certain free radicals, especially radical cations which can result in diverse group of reactions (Figure1.1.3) [6].

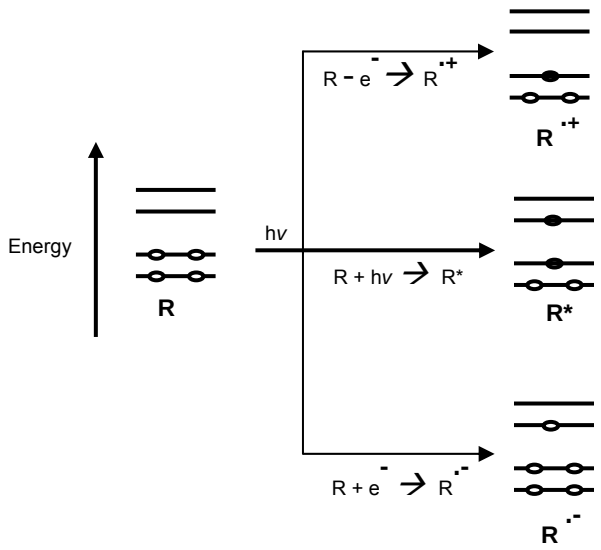


Figure 1.1.3: Mechanisms showing photochemical processes [6]

G. Other factors that influence drug stability in the solid or liquid dosage form include particle size, pH, solvent composition, solution ionic strength, cations and anions, excipients, chemical additives, storage container (primary) and storage conditions. Therefore, information obtained from different stability studies can aid in establishment of the retest period or shelf life of the drug substance and selecting proper excipients, containers and storage conditions.

1.1.1.2. *Stability testing: A Necessity [1, 2]*

- To establish commercial expiry date.
- To determine levels for certain specifications, like active ingredient content (for overage), degradation products, preservatives, etc. and set the acceptance limits for the lot release.
- To support the stability of drug products used in clinical and non-clinical studies.
- Requirements of regulatory agencies.
- To protect the reputation of the manufacturers.
- To provide database for new developing products of the same category.

1.2. Stability Indicating Assay Method (SIAMs)

SIAM is a validated quantitative analytical procedure that can detect changes with time in the pertinent properties of the drug substance and drug product under defined storage conditions. It accurately measures the API without interference from other substances and is sensitive enough to detect and quantify even small amounts of degradation products/ impurities.

Stability-indicating methods according to United States-Food and Drug Administration (US-FDA) stability guideline of 1987 were defined as,

'Quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured.'

To develop a SIAM, stress testing in the form of forced degradation should be carried out at an early stage so that impurities and degradation products can be

identified and characterized. SIAM must be discriminating and properly validated to ensure the accuracy of the long term stability testing study.

1.2.1. *Techniques used in development of SIAM*

Titrimetric, spectrophotometric, and chromatographic techniques have been commonly employed in analysis of stability samples. There are sporadic reports of the use of miscellaneous techniques (Table1.2.1)

Table 1.2.1: Some reported analytical methods [5]

Method	Advantage / Disadvantage	Examples
Spectrophotometric	Low cost Simplicity (Non specific)	Guanabenz (derivative UV)
TLC	Simplicity (Variability, Non quantitative)	Ranitidine HCl
HPTLC	Rapid Accurate Small quantity of mobile phase needed	Nifedipine
GC	Rapid Accurate (Unsuitable for non volatile and	Fluconazole

	thermo labile compounds)	
CE	High efficiency with minimal peak dispersion	Azathioprine
HPLC	High resolution capacity Specificity Sensitivity Best for compounds which are non volatile, ionic and/or unstable at high temperatures	Zidovudine
LC-MS/ LC-NMR/ CE-MS/ LC-MS/MS	Quantitation + Characterization	Losartan (LC-MS)

1.2.2. *Regulatory viewpoint*

Every regulatory guideline and compendia including United States Pharmacopeia (USP) says either emphatically or non-emphatically that samples of the products should be assayed for potency by use of a SIAM. Some of the leading guidelines, which discuss about SIAM, are listed in Table 1.2.2.

Table 1.2.2: Applicability of SIAM in different guidelines

Guideline	Title	Reference
ICH Q1A(R2)	Stability testing of new drug substances and products	[3]
ICH Q1B	Photostability testing of new drug substances and products	[7]
ICH Q3A(R)	Impurities in new drug substances	[8]
ICH Q3B(R2)	Impurities in new drug products	[9]

1.3. Stress Studies

Stress testing is defined as stability testing of drug substances and drug products under conditions exceeding those that are used for accelerated testing. Although, all regulatory guidance documents listed in Table 1.1.2 define the concept of stress testing, they do not provide detailed information about a stress testing strategy. The experimental conditions to conduct stress tests are described in a general way [10] and not exact way. Some articles give guidelines for performing stress tests on a sound scientific basis [2, 5, 10-12]**.** Among them, the approach of Singh and Bakshi [2] is more flexible and realistic. According to them, stress testing should be done for establishment of SIAM under mild conditions, which provide maximum number of degradation products. In other approaches, specific harsh conditions are followed even though they produce non-realistic degradation

products. So, optimum stress conditions should be selected to avoid secondary degradation.

1.3.1. *Practical aspects of stress testing*

The flow chart for carrying out stress testing of drug substances is given in Figure 1.3.1.

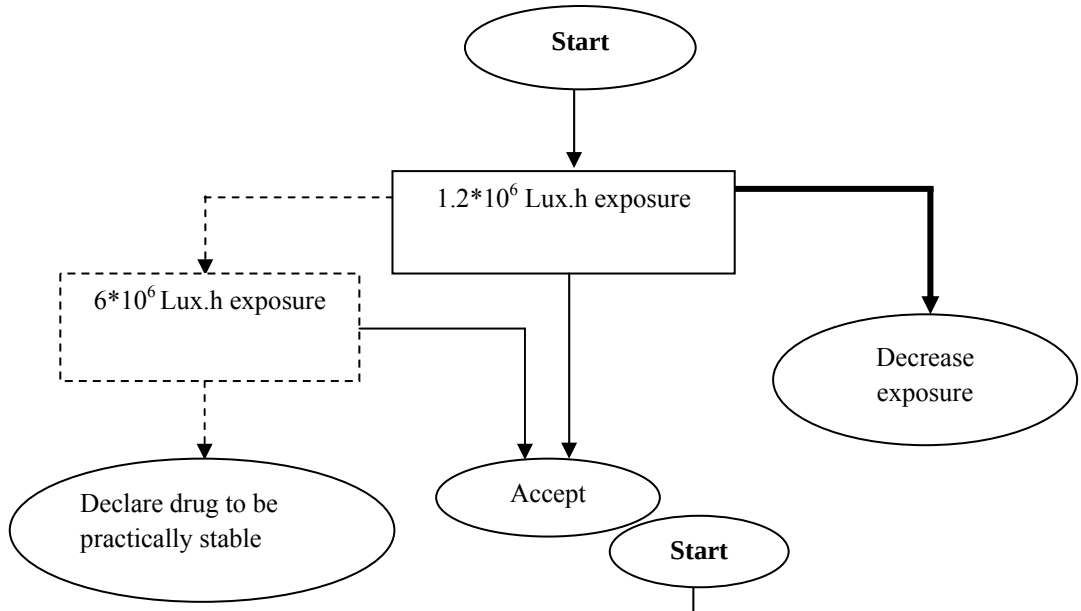


Figure: 1.3.1a Flow chart for photolytic degradation

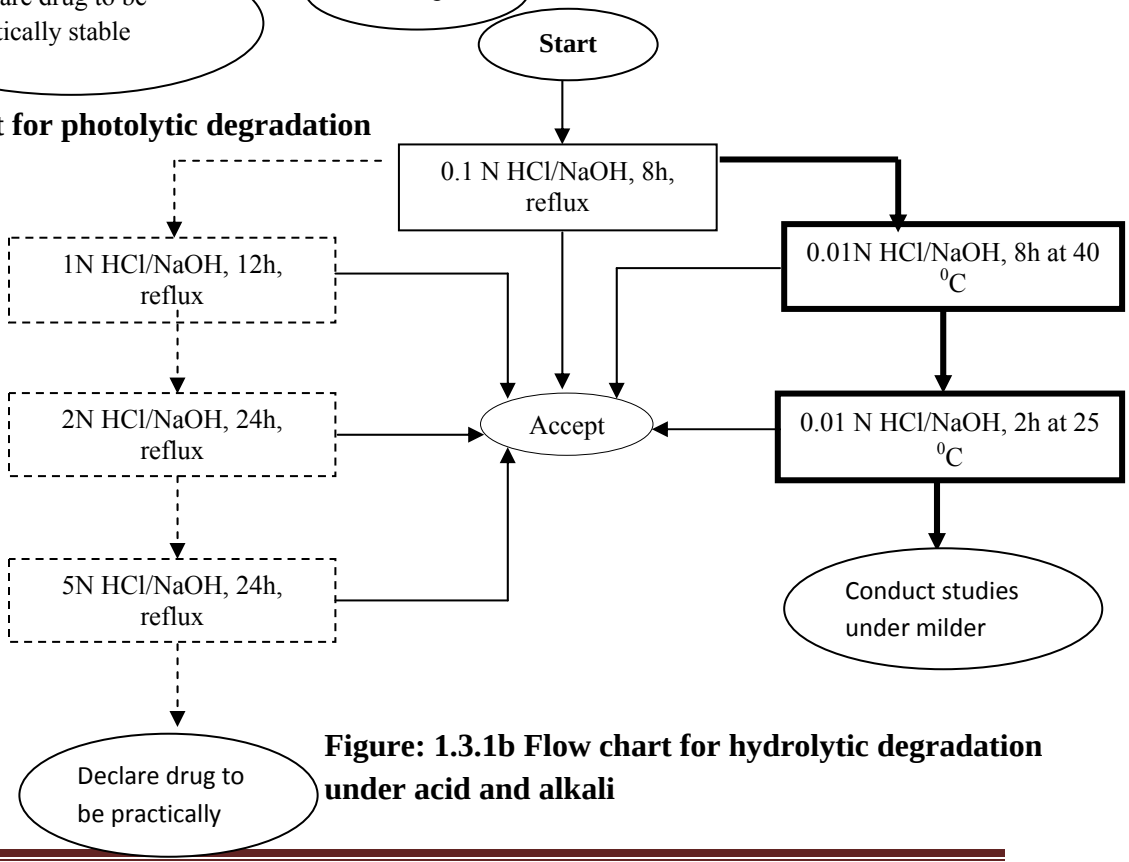


Figure: 1.3.1b Flow chart for hydrolytic degradation under acid and alkali

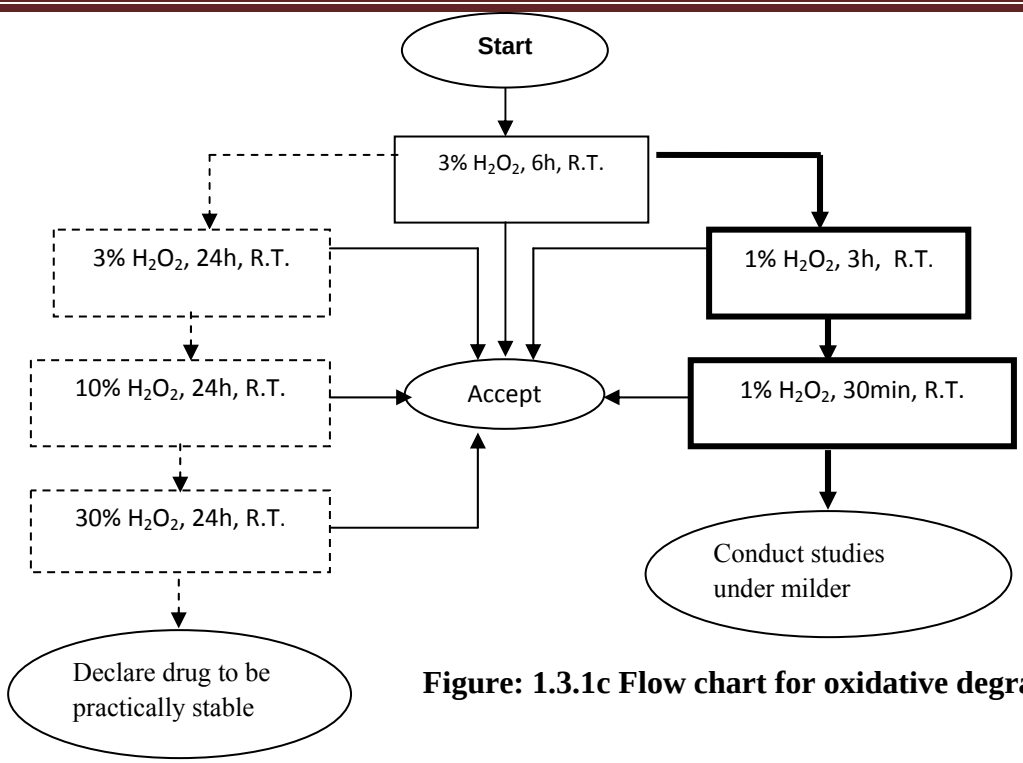


Figure: 1.3.1c Flow chart for oxidative degradation

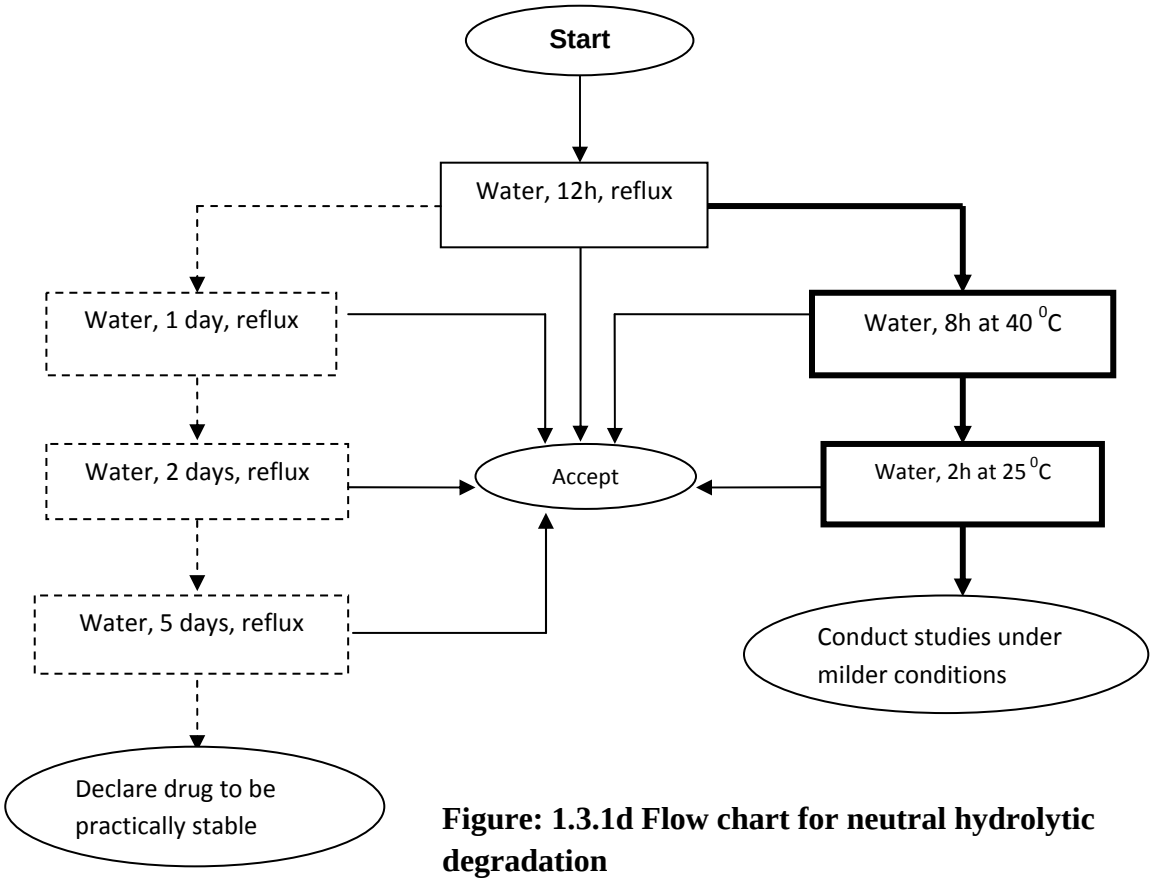


Figure: 1.3.1d Flow chart for neutral hydrolytic degradation

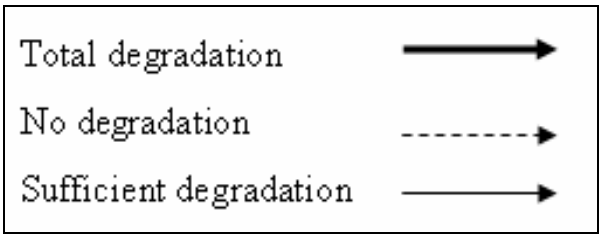


Figure: 1.3.1 Flow chart for stress degradation [5]

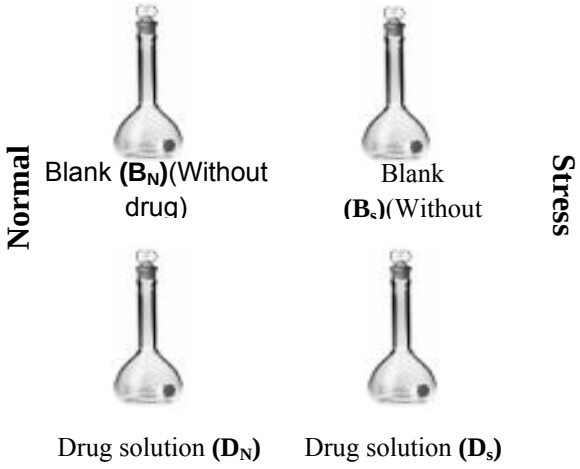


Figure: 1.3.2 Design of study

The studies should be conducted with a concentration greater than or equal to 1 mg/ml depending on the extent of degradation. For every stress study, it is advised to generate four samples as illustrated in Figure 5 and report the results of each [5]. For hydrolytic studies, the reactions can simply be carried out in containers like volumetric flasks or stoppered culture tubes and stored in a water bath with thermostatic device. When studies are being carried out in multiple ampoules at one time, care should be taken on labeling. Best method is to use different color wires for ringing of ampoules. For oxidative stress, studies should be done in a leak proof stopperred container. A caution is that the headspace left above the solution during the study should be small, for which, either the solution volume can be increased or the size of the container could be selected in such a way so that it is just sufficient to accommodate the total volume of reaction solution. For photolytic reaction, if solid drug sample is used, the study should be done in a petriplate, where solid samples are spread evenly as a thin layer. For liquids or dissolved samples, any transparent container can be used which can give maximum exposure.

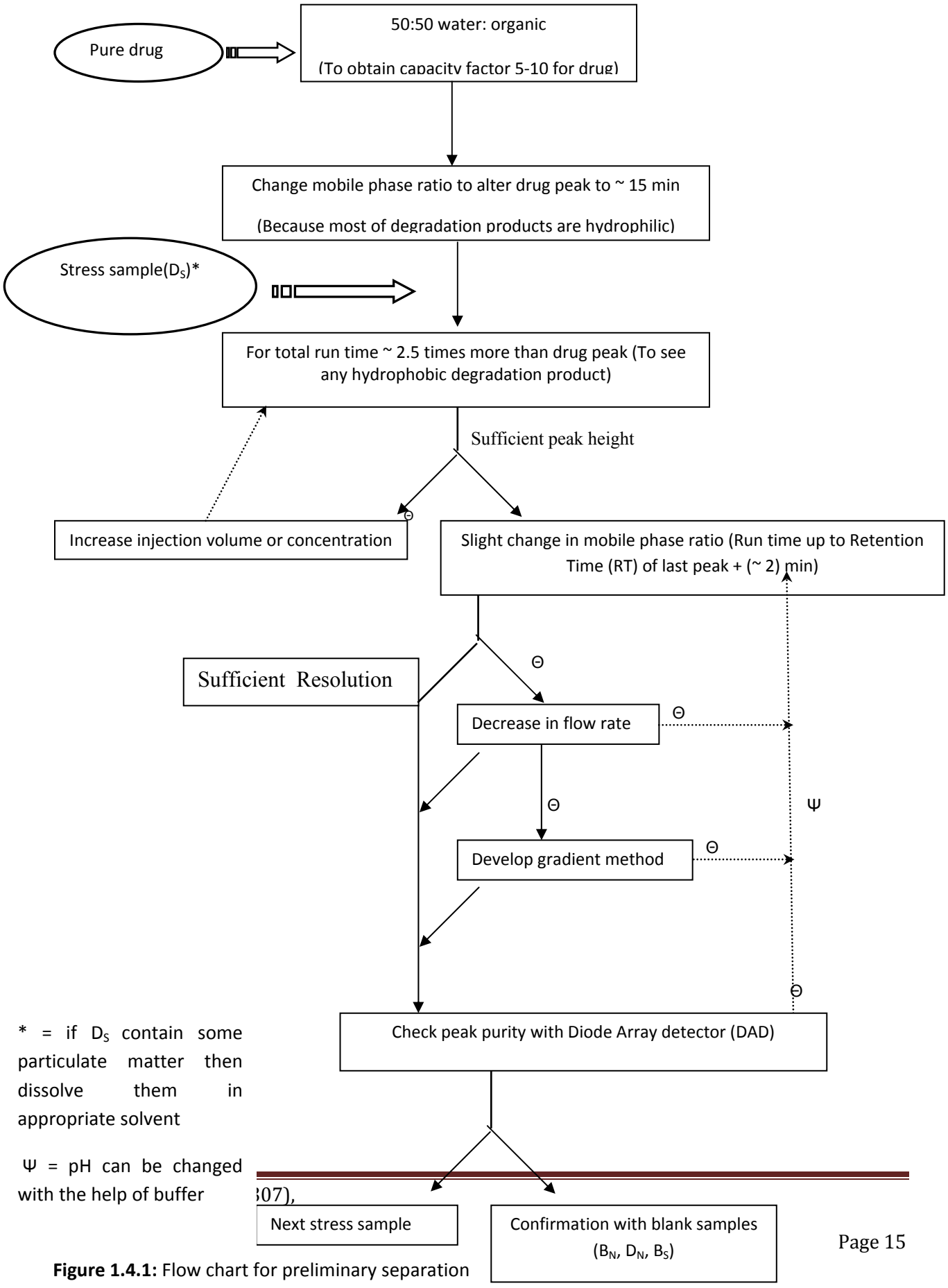
1.4. LC Studies on Forced Decomposition

1.4.1. *Processing of reaction samples for HPLC studies*

Processing is necessary for preparing proper concentrations of drug, acid, alkali or oxidizing agents to make them compatible with HPLC. For this, one approach is to dilute the sample to an extent that reagent concentration falls within the tolerable range. Dilution should be done in the mobile phase. The second approach involves neutralization of acid and alkali solutions to a tolerable pH. However, the dilution is a better method. If stress samples contain solid particles, then these particles are required to be removed and redissolved in appropriate solvents. This treatment helps in detecting insoluble degradation products which can form during stress testing [5].

1.4.2. *Preliminary separation study*

The stress samples so obtained are subjected to preliminary analyses to study the number and types of degradation products formed under various conditions. Use of water-methanol (MeOH) or water-acetonitrile (ACN) as the mobile phase is preferred in the initial stages of HPLC method development. The use of buffers is not suggested at this stage because as is normally required, one can extend the buffer-free mobile phases to preparative LC or LC-MS studies. The general method is shown in Figure 6 [2, 13, 14]. At certain times, a decrease in drug concentration is clearly evident, however no new peak is observed due to the formation of non-chromophoric products. In such situations, the detection at multiple wavelengths or the use of LC-MS becomes necessary.



* = if D_S contain some particulate matter then dissolve them in appropriate solvent

Ψ = pH can be changed with the help of buffer

Figure 1.4.1: Flow chart for preliminary separation

1.4.3. *Final method development and optimization*

Subsequent to preliminary chromatographic studies, the R_T and relative retention times (RR_T) of all products formed should be tabulated for each reaction condition. Special attention is required to be paid to those components whose R_T/RR_T is very close. For such compounds, their PDA spectrum or LC-MS profiles are obtained and critically evaluated to ascertain whether the products are same or different.

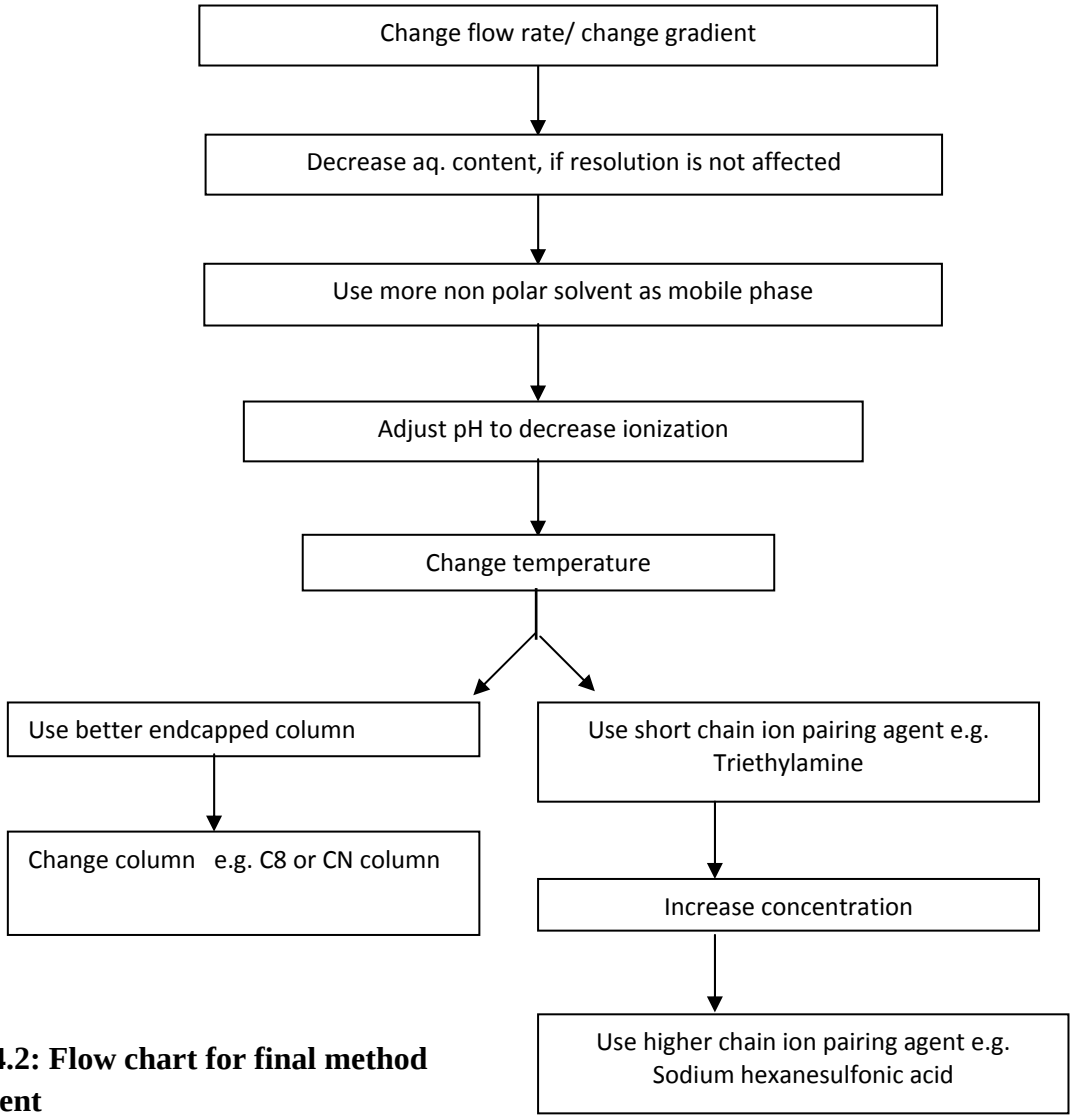


Figure 1.4.2: Flow chart for final method development

If PDA or LC-MS results suggest that any of the products are different but are co-eluting, then suitable modifications should be done in the chromatographic method to achieve a satisfactory resolution by mixing only those solutions where different products are formed in sufficient quantities.

Resolution in the mixture is studied closely, to see whether it is similar to that obtained in individual samples. To separate close or co-eluting peaks and for good peak symmetry general approaches may be followed as illustrated in Figure 7 [2, 13, 14].

1.4.4. *Validation of SIAMs*

Validation of analytical methods, in general, has been extensively covered in the ICH guidelines Q2(R1)[15] and by USP (Table 3) [16]. There are two stages in the validation of a SIAM. First stage is early in the development cycle when drug substance is subjected to forced decomposition studies and the SIAM is established based on the knowledge of drug degradation behavior. The main focus of validation at this stage is on establishment of specificity/selectivity, followed by other parameters like accuracy, precision, linearity, range, robustness, etc. The limits of detection and quantitation are also determined for degradation products to help in establishment of the mass balance. This validated method finds application in the analysis of stability samples of bulk drugs for determination of their retest or expiry period. 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. Here only parameters of critical importance like specificity/selectivity, accuracy and precision are revalidated.

So, Strategy for development of stability indicating assay method:

- There are three components necessary for implementing a SIM:

1. *Sample Generation* - as described in stress studies flow-chart untill sufficient degradation obtained

Table 1.4.1: USP/ICH requirements for validation [15, 16]

Analytical Performance Characteristics	**Quantitative**	**Limit Tests**
Accuracy	Yes	*
Precision	Yes	No
Specificity	Yes	Yes
Detection Limit	No	Yes
Quantitation Limit	Yes	No
Linearity	Yes	No
Range	Yes	*

* = Depending on nature of specific test

2. *Method Development* which includes,

   a. Processing of stress samples

   b. Preliminary separation study and

   c. Final method development and optimization

3. *Method Validation* - as described above.

1.5 DRUG PROFILE:[17]

There are three **aromatase inhibitors** which are being used in treatment of metastatic breast cancer and ovarian cancer in post-menopausal women whose disease has progressed despite tamoxifen therapy: Anastrozole (ARIMIDEX, Zeneca), Letrozole (FEMARA, Novartis) and Vorozole (RIVIZOR, Janssen). All belong to the third generation of **non-steroidal aromatase inhibitors**, and each is superior to previous generations in terms of potency and selectivity.(a)

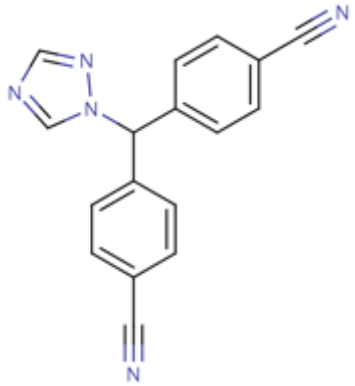
LETROZOLE: Letrozole is approved by the United States Food and Drug Administration (FDA) for the treatment of local or metastatic breast cancer that is hormone receptor positive or has an unknown receptor status in postmenopausal women.

Synonyms: LETROZOL, letrozole**.**

CAS Registry Number: 112809-51-5

1.5.1 PHYSICOCHEMICAL PROPERTIES:

Table 1.5.1: Physicochemical Properties of letrozole

SR.No.	PROPERTY	DESCRIPTION	REFERENCE
1.	Chemical IUPAC Name	4-[(4-cyanophenyl)-(1,2,4-triazol-1-yl) methyl]benzonitrile	18
2.	Chemical Formula	$C_{17}H_{11}N_5$	18
3.	Chemical Structure	 **Figure 1.6.1: letrozole structure**	18

SR.NO.	PROPERTY	DESCRIPTION	REFERENCE
4.	Molecular Weight	285.1014	18
5.	State	white to yellowish crystalline powder	19
6.	Melting Point	184-185°C	18
7.	Experimental LogP Value	2.5	18
8.	Predicted LogP Value	1.86	18
9.	Solubility	Practically insoluble in water Freely soluble in chloroform, dichloromethane, Slightly soluble in methanol,ethanol.	18
10.	Theoretical pKa value	4.28	18
11.	Predicted pKa value	2.87	18

STORAGE:

Store letrozole tablets at room temperature at 77 degrees F (25 degrees C) away from light and moisture. Brief storage between 59-86 degrees F (15-30 degrees C) is permitted.

1.5.2 PHARMACOLOGICAL PROFILE: [19]

Mechanism of Action:

Estrogens are produced by the conversion of androgens through the activity of the aromatase enzyme. Letrozole blocks production of estrogens in by competitive, reversible binding to the heme of cytochrome P450 unit of aromatase enzyme and in this way by inhibiting enzyme for production of estrogen.

The action is specific, and letrozole does not reduce production of mineralo- or corticosteroids.

- **Pharmacokinetics:**
  - **Absorption:** Rapidly and completely absorbed from the gastrointestinal tract and absorption is not affected by food.

- **Metabolism:** Metabolized slowly to an inactive metabolite which get converted to glucuronide conjugate.
- **Excretion:** excreted renally, representing the major clearance pathway.
- **Half life:** Letrozole's terminal elimination half-life is about 2 days.
- **Protien binding**: Letrozole is weakly protein bound.
- **Volume of Dstribution**: Has a large volume of distribution (approximately 1.9 L/kg).

- **Route of Administration:** Oral**.**
- **Indications:**[20]
  - For the adjuvant treatment of postmenopausal women with hormone receptor positive early breast cancer.
  - For the extended adjuvant treatment of early breast cancer in postmenopausal women who have received 5 years of adjuvant tamoxifen therapy.
  - For first-line treatment of postmenopausal women with hormone receptor positive or hormone receptor unknown locally advanced or metastatic breast cancer.
  - For the treatment of advanced breast cancer in postmenopausal women with disease progression following antiestrogen therapy.
- **Letrozole** is being used commonly as an infertility treatment works by inhibiting aromatase thereby suppressing estrogen production, the result is that the pituitary gland produces more of the hormones needed to stimulate the ovaries. These hormones, FSH and LH, can cause the development of ovulation in women who are anovulatory or increase the number of eggs developing in the ovaries of women who already ovulate.(21)
- **Dosage**: 2.5 mg per day.
- **Side Effects:**
  - Hot flashes, aching/painful joints and muscles, unusual sweating at night, weight gain, nausea, tiredness, dizziness, unusual bleeding from the vagina, and vomiting may occur.

1.6 Reverse Phase – High Pressure Liquid Chromatography:

Reverse Phase-High performance or high pressure liquid chromatography (RP-HPLC or RPC) is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds based on their polarities and interactions with the column's stationary phase.

HPLC as compared with the classical technique is characterized by:

- small diameter (2-5 mm), reusable stainless steel columns;
- column packings with very small (3, 5 and 10 μm) particles;
- relatively high inlet pressures and controlled flow of the mobile phase;
- precise sample introduction without the need for large samples;
- special continuous flow detectors capable of handling small flow rates and detecting very small amounts;
- automated standardized instruments;
- rapid analysis;
- high resolution.

Separation mechanism:

RPC operates on the principle of hydrophobic forces, which originate from the high symmetry in the dipolar water structure and allows the measurement of these interactive forces.

Structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a larger hydrophobic surface area (C-H, C-C, and generally non-polar atomic bonds, such as S-S and others) results in a longer retention time because it increases the molecule's non-polar surface area, which is non-interacting with the water structure. On the other hand, polar groups, such as -OH, $-NH_2$, COO^- or $-NH_3^+$ reduce retention as they are well integrated into water. Very large molecules, however, can result in an incomplete interaction between the large analyte surface and the ligand's alkyl chains and can have problems entering the pores of the stationary phase.

Retention time increases with hydrophobic (non-polar) surface area. Branched chain compounds elute more rapidly than their corresponding linear isomers because the overall surface area is decreased. Similarly organic compounds with single C-C-bonds elute later than those with a C=C or C-C-triple bond, as the double or triple bond is shorter than a single C-C-bond.

The retention can be decreased by adding a less polar solvent (methanol, acetonitrile) into the mobile phase to reduce the surface tension of water. Gradient elution uses this effect by automatically reducing the polarity and the surface tension of the aqueous mobile phase during the course of the analysis.

Mobile phase:

Most common solvent used in RP-HPLC is water. Water in combination with other solvents like acetonitrile and methanol is use to provide mobile phase of different polarity to facilitate separation of different polarity compound.

Other mobile phase modifiers can affect analyte retention. For example, the addition of inorganic salts causes a moderate linear increase in the surface tension of aqueous solutions (ca. 1.5×10^{-7} J/cm² per Mol for NaCl, 2.5×10^{-7} J/cm² per Mol for $(NH_4)_2SO_4$), and because the entropy of the analyte-solvent interface is controlled by surface tension, the addition of salts tend to increase the retention time. This technique is used for mild separation and recovery of proteins and protection of their biological activity in protein analysis (hydrophobic interaction chromatography, HIC).

Another important component is the influence of the pH since this can change the hydrophobicity of the analyte. For this reason most methods use a buffering agent, such as sodium phosphate. The buffers serve multiple purposes:

I. They control pH,
II. Neutralize the charge on any residual exposed silica on the stationary phase and
III. Act as ion pairing agents to neutralize charge on the analyte.

Ammonium formate is commonly added in mass spectrometry to improve detection of certain analytes by the formation of ammonium adducts. A volatile organic acid such as acetic acid, or

most commonly formic acid, is often added to the mobile phase if mass spectrometry is used to analyze the column eluent. Trifluoroacetic acid is used infrequently in mass spectrometry applications due to its persistence in the detector and solvent delivery system, but can be effective in improving retention of analytes such as carboxylic acids in applications utilizing other detectors, as it is one of the strongest organic acids. The effects of acids and buffers vary by application but generally improve the chromatography(17)

Stationary phase:[17, 22, 23, 24]

RP-HPLC has a non-polar stationary phase. One common stationary phase is silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. With these stationary phases, retention time is longer for molecules which are more non-polar, while polar molecules elute more readily.

Elution Type:[17]

There are mainly two types of elution pattern:

1. Isocratic elution.
2. Gradient elution.

Table 1.6.1: Comparison of Elution Type

Isocratic elution	Gradient elution
The mobile phase composition remains constant throughout the procedure.	The mobile phase composition is changed during the separation process.
Here, Peak width increases with retention time linearly according to the equation for N, the number of theoretical plates. This leads to the **disadvantages** like 1. Late-eluting peaks get very flat and broad. 2. Their shape and width may keep them from being recognized as peaks.	Gradient elution has **advantage**s like 1. Decreases the retention of the later-eluting components so that they elute faster, giving narrower (and taller) peaks for most components. 2. This also improves the peak shape for tailed peaks, as the increasing concentration of the organic eluent pushes the tailing part of a peak forward. 3. This also increases the peak height (the peak looks "sharper"), which is important in trace analysis.
Advantage: In isocratic elution, the selectivity does not	Disadvantage: In gradient elution, the elution order may

change if the column dimensions (length and inner diameter) change - that is, the peaks elute in the same order.	change as the dimensions or flow rate change.

❖ The HPLC system:

1) MOBILE PHASE RESERVOIR, FILTERING.

The most common type of solvent reservoir is a glass bottle. Most of the manufacturers supply these bottles with the special caps, Teflon tubing and filters to connect to the pump inlet and to the purge gas (helium) used to remove dissolved air. Helium purging and storage of the solvent under helium was found not to be sufficient for degassing of aqueous solvents. It is useful to apply a vacuum for 5-10 min. and then keep the solvent under a helium atmosphere.

2) PUMPS:

The HPLC pump is considered to be one of the most important components in a liquid chromatography system which has to provide a continuous constant flow of the eluent through the HPLC injector, column, and detector.

The two basic classifications are the **constant-pressure** and the **constant-flow** pump.

1. The *constant-pressure pump* is used only for column packing.
2. The *constant-flow pump* is the most widely used in all common HPLC applications.

Standard HPLC pump requirements are:

- Flow rate range: from 0.01 to 10 ml/min
- Pressure range: from 1 to 5000 psi (340 atm)
- Pressure pulsations: less than 1% for normal and reversed phase mode
- Less than 0.2% for size exclusion mode.

1. Constant Pressure Pumps:

Advantages:

- Simplicity;
- Freedom from pulsations, resulting in smooth baselines;
- Cheap and easy to maintain.

Drawbacks:

- Flow rates can and do change!

Flow rate can changes if the solvent viscosity changes due either to a temperature or composition change.

2. Constant Flow Pumps:

Constant-flow systems are generally of two basic types:

1. *Reciprocating piston* and

2. *Positive displacement (syringe)* pumps.

1. Reciprocating piston pump:

Reciprocating piston pump can maintain a liquid flow for indefinitely long time.

The pumping rate is controlled by piston retracts or by the cam rotating speed.

The **main drawback** of this type of pump is sinusoidal pressure pulsations which lead to

the necessity of using pulse dampers.

Dual Piston Pumps

Provides a constant and almost pulse free flow.

Both pump chambers are driven by the same motor through a common eccentric cam; this common drive allows one piston to pump while the other is refilling.

Advantages:

- Unlimited solvent reservoir allowing long-term unattended use;
- Quick changeover and clean out capability;
- Wide flow rate range (0.01 to 10 ml/min) is provided without gear change.

Drawbacks:

- Incompletely compensated pulsations might be observable at high refractive index
- Detector sensitivities, especially at low flow rates;
- Pump reliability depends on the cleanliness of the mobile phase and continued sealing

- Capability of four check valves on each cycle (e.g. several times per minute).

Recent improvements include:

- A computer-designed camshaft is used to achieve maximum overlap of pump strokes,
- Resulting in virtually undetectable pulsation or ripple.
- Small-volume check valves are used to allow the pumps to function reliably at flow
- Rates as low as 0.001 ml/min.

3. **Syringe-Type Pumps**:

   Syringe-type pumps generally consist of a cylinder that holds the mobile phase which is expelled by a piston. The piston is advanced by a motor connected through worm gears, to provide smooth pulse-less flow.

Advantages:

- High pressure capability (up to 78,000 psi);
- Constant flow rate;
- Infrequent maintenance;
- Simple and strong gears.

Drawbacks:

- Limited possibilities to form gradients;
- Limited reservoir capacity.

3) INJECTORS:

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where sample introduction is done with the help of autosamplers and microprocessors.

Sample sizes: Typical sample mass with 4.6 mm ID columns range from the nanogram level up to about 2 mg diluted in 20 ml of solvent.

- The most useful and widely used sampling device for modern LC is the **microsampling injector valve**.

With these sampling valves, samples can be introduced reproducibly into pressurized columns without significant interruption of flow, even at elevated temperatures.

Advantages:

Rapid, reproducible, operator-independent delivery of a wide range of sample volumes (e.g., from 60 nl up to several milliliters), at pressures up to 7000 psi with less than 0.2% error. The valve can be located within a temperature-controlled oven for use with samples that require handling at elevated temperatures (0° - 150°C).

A minor drawback is:

The sample loop must be changed to obtain various sample volumes (but this can often be achieved in a few minutes).

A clockwise rotation of the valve rotor places the sample-filled loop into the mobile-phase stream, with subsequent injection of the sample onto the top of the column through a lowvolume, cleanly swept channel. The minimum injection volume which can be made with the valve-type injectors is 60 nl.

Low-volume switching valves are also available (e.g., Valco, Rheodyne, Siemans) for use in special techniques such as recycle chromatography and column switching. Some of these valves can be operated at pressures up to 7000 psi, and often they can be used at elevated temperatures. The more common valves can be obtained in 3-, 4-, 6-, 8-, or 10- port configurations, for use in either the manual or automated mode.

A specially designed syringe may be used to inject a small volume (e.g. <10 µl) into the loop when required, although in this case the precision in the sample introduction is dependent on the precision of syringe delivery.

Automatic Injectors

With commercially available automatic sampling devices, large numbers of samples can be routinely analyzed by LC without operator intervention. Such equipment is popular for the analysis of routine samples (e.g., quality control of drugs), particularly when coupled with automatic data-handling systems or in unattended analysis.

Most of the autosamplers have a piston metering syringe type pump to suck the pr-eestablished sample volume into a line and than transfer it to the relatively large loop (~100 ml) in a standard six-port valve. The simplest autosamplers utilize the special vials with pressuarization caps. A special plunger with a needle, push the cap down to the vial and displace the sample through the needle into the valve loop.

Most of the autosamplers are microprocessor controlled and can serve as a master controller for the whole instrument.

4) Detectors:[25]

The detector for an HPLC is the component that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. There are many types of detectors that can be used with HPLC. Some of the more common detectors include:

1. ***Refractive Index (RI):***

   Refractive Index (RI) detectors measure the ability of sample molecules to bend or refract light.

2. ***Ultra-Violet (UV:***

   Measures the ability of a sample to absorb light. This can be accomplished at one or several wavelengths:

   A. **Fixed Wavelength** measures at one wavelength, usually 254 nm

   B. **Variable Wavelength** measures at one wavelength at a time, but can detect over a wide range of wavelengths

C. **Diode Array** measures a spectrum of wavelengths simulateneously

Table 1.6.2: UV cutoff wavelengths of different solvents

Solvent	Cutoff wavelength (nm)
Acetonitrile	190
Water	190
Cyclohexane	195
Methanol	205
Dichloromethane	220

3. Fluorescent :

Measures the ability of a compound to absorb then re-emit light at given wavelengths. Each compound has a characteristic fluorescence.

4. Radiochemical :

Radiochemical detection involves the use of radiolabeled material, usually tritium (^{3}H) or carbon-14 (^{14}C). It operates by detection of fluorescence associated with beta-particle ionization, and it is most popular in metabolite research. Two detector types:

A. **Homogeneous**- Where addition of scintillation fluid to column effluent causes fluorescence.

B. **Heterogeneous**- Where lithium silicate and fluorescence caused by beta-particle emission interact with the detector cell.

5. Electrochemical:

Compounds that undergo oxidation or reduction reactions. Usually accomplished by measuring gain or loss of electrons from migrating samples as they pass between electrodes at a given difference in electrical potential.

6. Near-Infra Red (Near-IR):

Operates by scanning compounds in a spectrum from 700 to 1100 nm. Stretching and bending vibrations of particular chemical bonds in each molecule are detected at certain wavelengths. This is a fast growing method which offers several advantages: speed (sometimes less than 1 second), simplicity of preparation of sample, multiple analyses from single spectrum, and nonconsumption of the sample.

7. ***Mass Spectroscopy (MS):***

The sample compound or molecule is ionized, it is passed through a mass analyzer, and the ion current is detected. There are various methods for ionization:

A) **Electron Impact (EI)**- An electron current or beam created under high electric potential is used to ionize the sample migrating off the column.

B) **Chemical Ionization**- A less aggresive method which utilizes ionized gas to remove electrons from the compounds eluting from the column.

C) **Fast Atom Bombarbment (FAB)**- Xenon atoms are propelled at high speed in order to ionize the eluents from the column.

8. ***Nuclear Magnetic Resonance (NMR),*** and
9. ***Light Scattering (LS).***

Table 1.6.3: Sensitivity Range of Different Detector

Detector type	Detection limit(gm/ml)
Ultra-Violet (UV)	10^{-8} or 10^{-9}
Fluorescent	10^{-9} to 10^{-11}
Radiochemical	10^{-9} to 10^{-10}
Electrochemical	10^{-12} to 10^{-13}
Mass Spectroscopy (MS)	10^{-8} to 10^{-10}

Table 1.6.4: Different Parameters For Choosing Detector Type:

	RI	UV-VIS	Flour	MS
Response	Universal	Selective	Selective	Selective
Sensitivity	4 microgram	5 nanogram	3 picogram	1 picogram
Flow Sensitive	Yes	No	No	Yes

Temp. Sensitive	Yes	No	No	No

1.7 ANALYTICAL METHOD VALIDATION[15, 16, 26, 27, 29]

1.7.1 TERMINOLOGY

"Doing thorough method validation can be tedious, but the consequences of not doing it right are wasted time, money, and resources."

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.

Method validation is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products. Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose. A successful Validation guarantees that both the technical and regulatory objectives of the analytical methods have been fulfilled.

1.7.2 Type of analytical procedures to be validated

Validation of analytical procedures is directed to the four most common types of analytical procedures.

- Identification test.
- Quantitative test for impurities content.
- Limit test for the control of impurities.
- Quantitative test of the active moiety in samples of drug substance on drug product on other selected components in the drug product.

1.7.3 Objective of validation

The objective of validation of analytical procedure is to demonstrate that it is suitable for its intended purpose. Validation is documented evidence, which provide a high degree of

assurance for specific method. Any developed method may be influenced by variables like different elapsed assay times, different days, reagents lots, instruments, equipments, environmental conditions like temperature, etc so it is expected that after the method has been developed and before it is communicated or transferred from one lab to the other, it is properly validated and the result of validity tests reported.

For analytical method validation of pharmaceuticals, guidelines from the International Conference on Harmonization (ICH), United States Food and Drug Administration (US FDA), American Association of Official Analytical Chemists (AOAC)United States Pharmacopoeia (USP), and International Union of Pure and Applied Chemists (IUPAC) provide a framework for performing such validations in a more efficient and productive manner.

1.7.4 Method validation is required when:

1. A new method is been developed
2. Revision established method
3. When established methods are used in different laboratories and different analysts etc.
4. Comparison of methods
5. When quality control indicates method changes.

1.7.5 Significance of Method Validation:

- To trust the method.
- Regulatory requirement.

Data Elements Required for Assay Validation

Both the USP and ICH recognize that is it not always necessary to evaluate every analytical performance parameter. The type of method and its intended use dictates which parameters needed to be investigated, as illustrated below in Table 1.7.1

Table 1.7.1: ICH Validation Guideline [2]

Type of analytical procedure characteristic	Identifiction	Testing for impurities, quantitate limit.		assay,dissolution (measurement only).content/potency.
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Interm.Precision	-	+(1)	-	+(1)
Reproducibility	-	- (2)	-	- (2)
Specificity (3)	+	+	+	+(4)
Detection Limit	-	-	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- Signifies that this characteristic is not normally evaluated.

+ Signifies that this characteristic is normally evaluated.

(1) Intermediate precision is not needed in some case, when reproducibility is checked.

(2) May be needed in some cases.

(3) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).

(4) May not be needed in some cases.

The different parameters which are to be considered in analytical method validation of an as per USP and ICH guidelines can be summarized as follows.

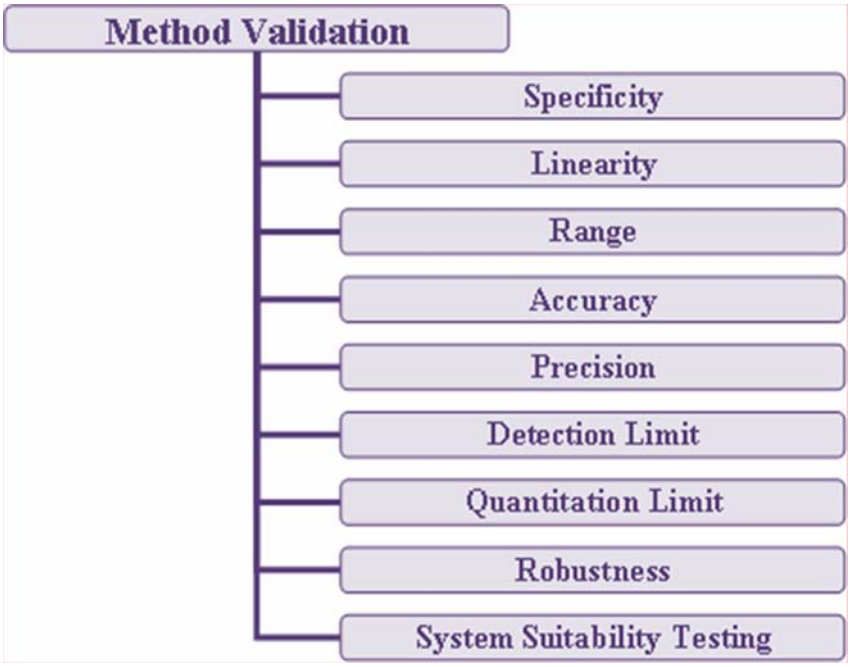


Figure 1.7.1 : The USP and ICH Method Validation Parameters

The different parameters of analytical method development are discussed below as per ICH guideline:-

1) Specificity:

Definition:

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Method:

- When the impurities are available: Spiking of pure substance (drug substance or drug product) with appropriate levels of impurities/excipients and demonstrate the result is unaffected.

- When the impurities are not available: Comparing the test results of sample containing impurities or degradation product to second well-characterized procedure. These comparisons should include sample under relevant stress condition.
- In chromatographic method: Peak purity test to be done by diode array and mass spectrometry.

Expression/calculation:

- Proof of discrimination of analyte in the presence of impurities. e.g. for chromatography chromatogram should be submitted.
- Peak purity test helps in demonstrating that the peak is not attributable to more than one component.
- For assay two results should be compared and for impurity tests two profiles should be compared.

Acceptance criteria:

1. There should not be any interference from blank, placebo peaks with the main peak and known impurity peaks.
2. All impurities should be separated from the main peak.
3. Peak purity for the main peak in standard preparation, sample preparation, sample spiked with known impurities preparation should be more than 0.999.

2) Linearity:

Definition:

The linearity of an analytical procedure is its ability (within given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample.

Method:

Drug (different dilution) and/or separately weighed synthetic mixture. Measurement of response and plot response vs. concentration of analyte and demonstration of linearity by

- Visual inspection of plot
- Appropriate statistical methods

Recommendation:

- Minimum of 5 concentrations are recommended

Expression/calculation:

- Correlation coefficient, y-intercept, slope of regression line, residual sum of squares.

Acceptance criteria:

- The correlation coefficient value should not be less than 0.995 over the working range.

3) Range:

Definition:

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

Method:

Drug (different dilution) and/or separately weighed synthetic mixture. Measurement of response and plot response vs. concentration of analyte and demonstration of linearity by

- Visual inspection of plot
- Appropriate statistical methods

Recommendation:

- Assay of drug/finished product: 80 – 120% of test concentration.
- For content uniformity: 70 – 130% of test concentration.
- For dissolution testing: ± 20% over specified range.
- For impurity: from reporting level to 120% of specification.

Expression/calculation:

- Correlation coefficient, y-intercept, slope of regression line, residual sum of squares.

Acceptance criteria:

- 80-120%.

4) Accuracy:

Definition:

The accuracy of 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. This is sometimes termed trueness.

Method:

- Application of procedure to analyze synthetic mixture of known purity.
- Comparison of result with already established procedure.
- Accuracy may be inferred once precision, linearity and specificity have been established.

Recommendation:

Minimum of nine determinations

- Low concentration of range × 3 replicates
- Medium concentration of range × 3 replicates
- High concentration of range × 3 replicates

Expression/calculation:

- Percent recovery by the assay of known added amount of analyte
- Mean – Accepted true value with confidence interval

Acceptance criteria:

1) % Recovery and % mean recovery at each level should be between 98-102%

2) % RSD of %Recovery at each level should not be more than 2.0%

5) Precision:

Definition:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between the series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Method:

- Determination of % relative standard deviation (RSD) of response of multiple aliquots

Recommendation:

a) Repeatability (Same operating condition over short interval of time):

Minimum of nine determinations

- Low concentration of range × 3 replicates
- Medium concentration of range × 3 replicates
- High concentration of range × 3 replicates (or)
- At target concentration × 6 determinations.

Acceptance Criteria:

- RSD for assay of six determinations should not be more than 2.0%.

b) Intermediate precision (within laboratory variation):

- Different Days
- Different Analysts
- Different Equipment etc.

Expression/calculation:

- Standard deviation, % RSD and confidence interval

Acceptance criteria:

1. RSD of intermediate precision should not more than 2.0%

2. The difference between assay of method precision and intermediate precision should not be more than 2.0%
3. Overall RSD of % assay of six replicates sample preparation of method precision and six replicates sample preparation of intermediate precision should not be more than 2.0%.

6) Detection Limit:

Definition:

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated under stated experimental conditions.

Method:

1. By visual evaluation

2. Based on S/N ratio

   - Applicable to procedure, which exhibit baseline noise.
   - Actual lowest concentration of analyte detected in compared with blank response

3. Based on S.D. of response and slope

LOD = 3.3 σ/s

s = Slope of calibration curve

σ = S.D. of response; can be obtained by

- Standard deviation of blank response
- Residual standard deviation of the regression line
- Standard deviation of the y-intercept of the regression line
- $S_{y/x}$ i.e. standard error of estimate

Expression/calculation:

- If based on visual examination or S/N ratio – relevant chromatogram is to be presented.
- If by calculation/extrapolation – estimate is validated by analysis of suitable no. of samples known to be near or prepared at detection limit.

Acceptance criteria:

- S/N ratio > 3 or 2:1; not specified in other cases

7) Quantitation Limit:

Definition:

- The quantitation limit of an individual analytical procedure is defined as the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.

Method:

1. By visual evaluation
2. Based on S/N ratio
   - Applicable to procedure, which exhibit baseline noise.
   - Actual lowest concentration of analyte detected in compared with blank response
3. Based on S.D. of response and slope

   LOQ = 10 σ/s

s = Slope of calibration curve

σ = S.D. of response; can be obtained by

4. Standard deviation of blank response
5. Residual standard deviation of the regression line
6. Standard deviation of the y-intercept of the regression line
7. $S_{y/x}$ i.e. standard error of estimate.

Recommendation:

8. Limit should be validated by analysis of suitable no. of samples known to be near or prepared at quantitation limit.

Expression/calculation:

9. Limits of quantitation and method used for determining should be presented.
10. Expresses as analyte concentration.

Acceptance criteria:

11. S/N ratio > 10:1; not specified in other cases

8) Robustness:

Definition:

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

Method:

- It should show the reliability of an analysis with respect to deliberate variations in method parameters.
- In case of liquid chromatography, examples of typical variations are
- Influence of variations of pH in a mobile phase,
- Influence of variations in mobile phase composition,
- Different columns (different lots and/or suppliers),
- Temperature,
- Flow rate.

Recommendation:

- Robustness should be considered early in the development of a method.

- If the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement included in the method documentation.

Expression/calculation:

- Effect of these changed parameters on system suitability parameters.

Acceptance criteria:

- The method must be robust enough to withstand slight changes and allow routine analysis of sample.

9) Ruggedness:

Definition:

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by analysis of the same samples under a variety of conditions.

Method:

Analysis of aliquots of homogenous lots in different laboratories by different analysts under different operational and environmental conditions.

Expression/calculation:

- Standard deviation, % RSD and confidence interval

Note: In the guideline on definitions and terminology, the ICH did not address ruggedness specifically. This apparent omission is really a matter of semantics, however, as ICH chose instead to cover the topic of ruggedness as part of precision, as discussed previously.

10) Stability:

During the earlier validation studies, the method developer gained some information on the stability of reagents, mobile phases, standards, and sample solutions. For routine testing in

which many samples are prepared and analyzed each day, it is often essential that solutions are stable enough to allow for delays such as instrument breakdowns or overnight analyses using auto-samplers.

Stability has not been given due importance in ICH guidelines but the USFDA has discussed stability parameters for bio samples. It is important to determine the stability of an analyte in a particular matrix by comparison with freshly prepared standards.

Samples and standards should be tested over at least a 48 h period, and the quantitation of components should be determined. If the solutions are not stable over 48 h, storage conditions or additives should be identified that can improve stability.

11) System Suitability Testing:

The system has to be tested for its suitability for the intended purpose. System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such.

Numerous approaches may be used to set the limits for system suitability tests. This depends on experience with the method, material available and personal preference. Parameters such as plate count, tailing factors, resolution and reproducibility (% RSD retention time and area for six repetitions) are determined and compared against the specifications set for the method.

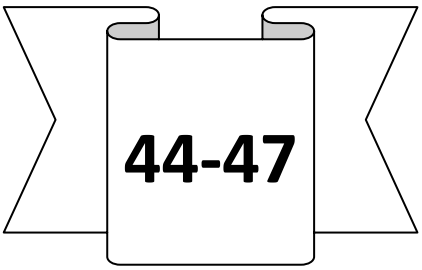
Table 1.7.2: System Suitability Parameters and their recommended limits

Parameter	Recommendation
Capacity Factor (K')	The peak should be well-resolved from other peaks and the void volume, generally K' > 2
Parameter	**Recommendation**

Relative Retention	Not essential as the resolution is stated.
Resolution(R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipients, degradation product, internal standard, etc.)
Tailing Factor(T)	$T \leq 2$
Theoretical Plates(N)	In general should be > 2000.

CHAPTER-2

REVIEW OF LITERATURE



LIST OF CONTENT

2. REVIEW OF LITERATURE

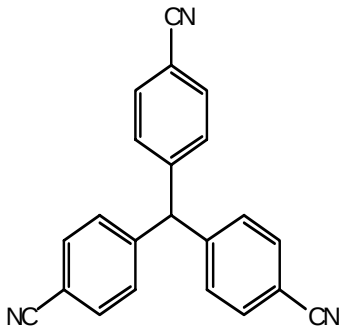
2.1 HPLC ASSAY METHOD OFFICIAL IN USP-2008 [16]

- Column- L1, 5µm packing, 4.6mm*12.5cm.
- mobile phase programming-

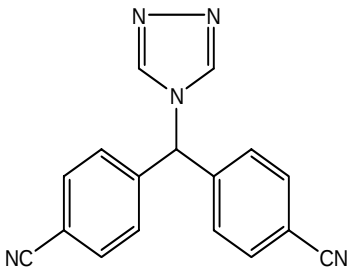
Time	Water%	ACN%
0	70	30
0→25	70→30	30→70

- Flow Rate-1 ml/min.
- Injection volume-20µl.
- Wavelength maxima-230nm.
- RRT of letrozole-1min.
- RRT of letrozole related compound-A-O.68min.
- RRT Of 4,4',4"-methylidenetrisbenzonitrile-1.9 min.
- **Use:** For estimation of letrozole bulk sample, letrozole tablet, letrozole related compound-A, 4, 4', 4"-methylidenetrisbenzonitrile.

- **Structures of two related impurities reported in USP for letrozole are as follow:**



4,4',4'' – methylene-tris benzonitrile
(COM-B)-RT-1.9



4, 4' – (1H – 1, 3, 4 – triazol 1- yl – methylene)dibenzonitrile
(RT-0.68)

Figure – 2.1: Structures of Related Impurities of Letrozole

2.2 VARIOUS ANALYTICAL METHODS REPORTED FOR LETROZOLE:

Table 2.2.1: Various Analytical Methods Reported For Letrozole

Sr. No.	ANALYTICAL METHOD TYPE	DESCRIPPTION	APPLICATION	REFE-RENCE
1.	RP-HPLC Method	S.P.-FinePak C8 column M.P.- deionized water, acetonitrile and methanol (50:30:20 v/v/v) λmax- 240 nm. R.T of letrozole- 9.8 min	To determine letrozole in different pharmaceutical Formulations and its application to studies Of drug release from nanoparticles.	29
2.	Gas Chromatography/ Mass Spectrometry	NA	For identification of letrozole in urine.	30
3.	Micellar Electrokinetic Chromatography	15 mm borate buffer (ph 9.2) containing 20 mm sodium dodecylsulphate and 12% (v/v) 2-propanol used as the background electrolyte. The separation performed through a fused silica capillary at 40 °C with the application of 6 s (3.45 kpa) of hydrodynamic injection and 30 kv of separation voltage. Migration times for all the studied compounds were ranged between 3.0 and 8.0 min. λmax:240nm.	For determination of letrozole, citalopram and their metabolites in human urine	31
4.	RP-HPLC	S.P.- ODS Hypersil C18 M.P- acetonitrilephosphate buffer, pH 7. F.R. - 1.5ml/min. Detection wavelength-	Used for determination of the aromatase inhibitor, letrozole, and its metabolite	32

		λexcitation -230nm λemission – 295nm.	in biological fluids with automated liquid-solid extraction and fluorescence detection.	
5.	High-Performance Liquid Chromatography / Atmospheric Pressure Photoionization (APPI) Tandom Mass Spectrometric Analysis	Column- Zorbax SB-CN, LC-MS column, (2.1 x 50 mm, 5 micron) Mobile phase-acetonitrile/water 40:60v/v Flow rate- 0.2 mL/min with 2.5 min gradient starting at above M.P.composition Internal standard - Letrozole-d4 Interface- Zappi Toluene-ionizing agent.	For determination of letrozole in Human plasma	-
6.	capillary gas chromatography	Clomipramine - as the internal standard Flame ionisation detection (FID) column head pressure -110 kPa time and temperature for the splitless step-0.75 min and 70°C volume injected- 2 L Analysis Time-less than 8 min	Tamoxifen, Anastrozole, Letrozole in pharmaceuti-cal preparations	33
7.	Spectro-photometric method	Wavelength maxima-238nm Beer's law obeyed in the conc. Range of 2–20µg/ml	For estimation in Raw material and tablet.	34

2.3. RESEARCH ENVISAGED

The ICH stability testing guideline, '*Stability Testing of New Drug Substances and Products [Q1A(R2)]'* requires the stress testing to be carried out in order to elucidate the inherent stability characteristics of the active substance, to identify degradation products that may form under a variety of conditions and to establish degradation pathways. It is stated that the testing should

include the effect of temperature, humidity (where appropriate), oxidation, photolysis and susceptibility to hydrolysis.

Letrozole is official in USP.till date, only isolated studies exist, which provide structures of a few impurities of the drug. Several LC and LC-MS methods are also reported for analysis of letrozole in blood plasma, in the presence of metabolites well as in the presence of impurities as described in above various method. But there is no study exists so far on the systematic forced decomposition behaviors of letrozole under ICH prescribed stress conditions.

The investigations were carried out under the following heads:

- Forced decomposition studies on letrozole to determine its inherent stability.
- Development of validated SIAMs, which can be extended to analysis of formulations.

CHAPTER-4

IDENTIFICATION OF DRUG

LIST OF CONTENT

4. IDENTIFICATION OF DRUG

Identification of drug sample was carried out by

1) Melting Point Determination,

2) Comparison of UV Spectra and

3) Raman Spectroscopy Studies.

4.1. IDENTIFICATION BY MELTING POINT :

Melting Range Determination –

Melting point of letrozole has been carried out using melting point apparatus. The melting point of compounds was taken by open capillary method and recorded.

Melting Point Apparatus:

- Model: T0603160.
- Manufacturer: EIE Instruments pvt Ltd.

Table 4.1: Comparison of melting point of letrozole with Reported melting point

Drug	Reported melting point (ºC) [26]	Observed melting point (ºC) (n=3)
Letrozole	184 ^{0}C -185 ^{0}C	183 ^{0}C -186 ^{0}C

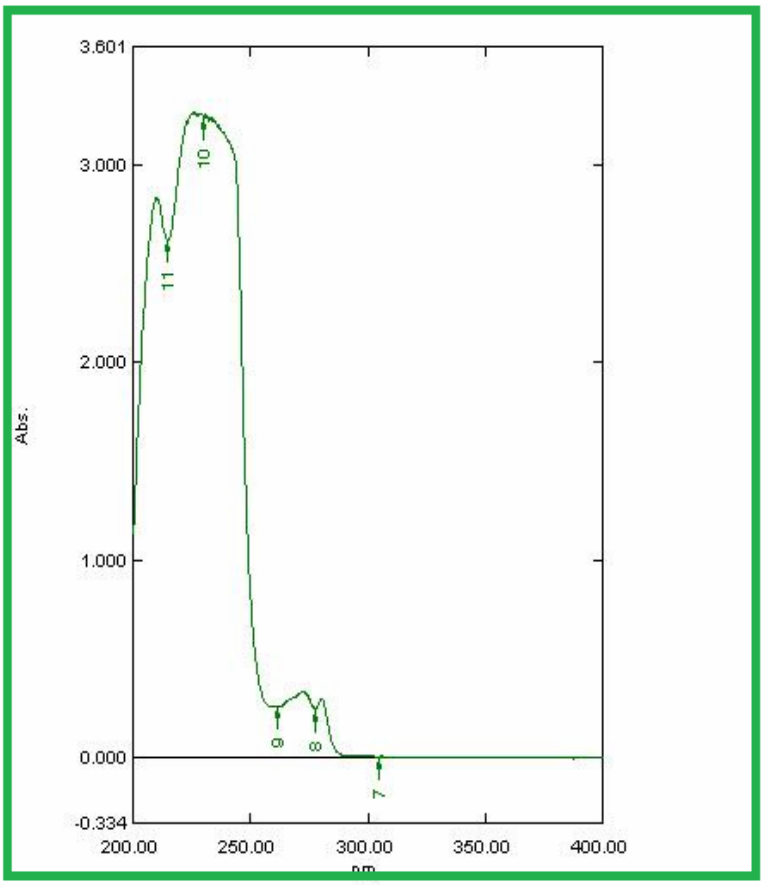
4.2. IDENTIFICATION BY UV SPECTRA:

UV-spectrum of Letrozole (Working standard) (20 µg/ml) in Diluent (Methanol) was taken for the identification of the drug Letrozole (Figure 4.3.1).

Instrument:

A double beam UV visible spectrophotometer

- Manufacturer: Shimadzu.
- Model UV- 2450, Shimadzu, Japan



No.	Wavelength nm.	Abs.
1	305.4	0.004
2	280.4	0.297
3	272.4	0.33
4	230.8	3.255
5	226	3.273
6	209.8	2.831
7	304.6	0.003
8	277.6	0.243
9	261.4	0.255
10	230	3.242
11	214.8	2.618

Figure 4.1.: UV spectrum of Letrozole in methanol (20 ppm) in methanol.

Table 4.2: Comparison of Reported λmax with Obtained λmax of Letrozole Working Standard

Drugs	Reported λmax[16]	Obtained λmax
Letrozole	230,232 nm.	230, 230.8, 226

4.3. IDENTIFICATION BY RAMAN SPECTROSCOPY:

Raman spectrum of Letrozole working standard was recorded in solid form.

Recorded Spectrum is shown below.

Reported wavenumbers are presented in table form over here. Wavenumbers observed were matched with various reported group wavenumber to confirm various group present in the drug structure in order to identify the drug.

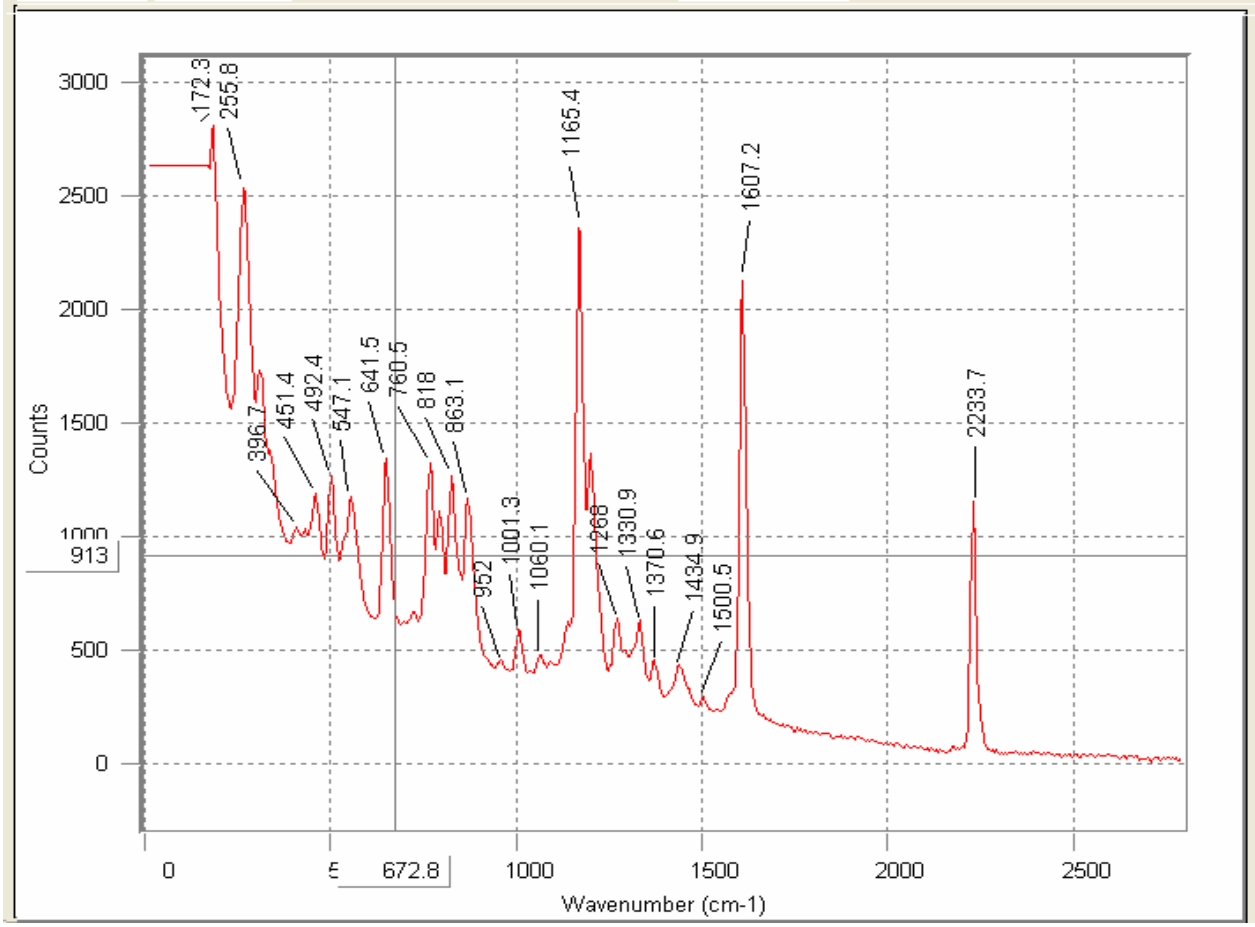


Figure 4.2: Raman spectrum of Letrozole

Table 4.3: Comparisons of Wavenumber (cm^{-1}) Found with reported Wavenumber (cm^{-1}) of Letrozole Working Standard (n==3)

Observed Wavenumber(cm^{-1})	**Reported Wavenumber (cm^{1})[35]**	**Inference**
863.1	830-930	Symmetric CNC stretch
1001.3	990-1010	Mono substituted benzenes
1607.2	1550-1630	Benzene Derivatives
2233.7	2232-2251	CN stretch

INFERENCE:

The Raman spectrum of the API was found to exhibit peaks similar to their respective functional groups. Interpretation of the obtained Raman spectra is displayed in above Table 4.3.

CHAPTER-5

EXPERIMENTAL WORK

LIST OF CONTENT

5. EXPERIMENTAL WORK

5.1. MATERIAL:

1. Pure letrozole (purity-99.8%) was obtained as a gift sample from Astron Research Ltd., Ahemdabad, Gujarat, India.
2. Letrozole tablet with brand name ANOLET (Svizera Healthcare) and LETROZ (Sun Pharma) with labeled claim 2.5 mg of each were purchased from local drug store.
3. Milli-pore water/Triple Distilled Water was used throughout the experimental work.

Reagents and Chemicals

1) Acetonitrile:-HPLC grade, Merck India limited, Spectrochem limited, and Central Drug House.
2) Methanol: - HPLC grade, Merck India limited, Spectrochem limited, and Central Drug House.
3) Di-hydrogen potassium phosphate anhydrous - AR grade,Astro Chemicals(India) Pvt Ltd.
4) Ortho-Phosphoric Acid: - AR grade, Central Drug House.
5) Triethylamine: - AR grade, S D Fine Chemical Limited.
6) Hydrochloric acid: - AR Grade,S D Fine Chem Limited.
7) Sodium hydroxide: - AR Grade,Central Drug House.
8) Hydrogen Peroxide (30%):- AR Grade, S D Fine Chem Limited.

5.2. INSTRUMENTS, APPARATUS AND EQUIPMENT:

1) HPLC system (Jasco, Japan)
   HPLC pump – Jasco PU-2080
   Solvent Mixing module-Jasco MX-2080.31
   Column – Inertsile C_{18}, 4.6*250mm, 5µm packing.
   Detector – jasco UV-2075 Plus
   Software - BORWIN

2) A double beam UV-visible spectrophotometer, UV- 2450, Shimadzu, Japan.
3) Analytical Balance: - CX220, Citizen.
4) pH Meter: - PHCAL,analab scientific Instruments Pvt Ltd.
5) Ultrasonic cleaner, D-compact.
6) Hot air oven: -EIE 108, EIE Instruments Pvt Ltd.
7) Centrifugation Machine: High Speed Research Centrifuge, BL-165 D,BIO-LAB Instruments Mfg. Co.
8) Raman Spectrophotometer, Jasco R-3000
9) Milli Q plus purification system

5.3. DEVELOPMENT AND OPTIMIZATION OF RP-HPLC METHOD ASSAY METHOD:

Preparation of standard stock solution and working standard solution:

Weigh accurately and transfer 25mg of standard drug into the 25 ml volumetric flask. Add about 20ml of diluents (methanol). Sonicate the solution if required for 2 minutes. Make up the volume upto mark with diluents and mix it well. Filter the solution through whatmann filter paper. Sonicate the solution if required for 2 minutes. Store it in a cool place. From this stock solution, prepare various working standard solution as per requirement.

Selection of pH:

The pKa value for letrozole is 2.87.A more rugged mobile phase pH will be nearby pKa value. Low pH has the advantage of creating an environment in which peak tailing is minimized and method ruggedness is, maximized and letrozole is highly labile at basic side pH. For this reasons, operating at low pH is recommended. So the pH of mobile phase was selected 3.0. Resolution between major degradation products and drug was found optimum at buffer pH 3.0 of mobile phase. Ortho phosphoric acid was used to adjust the pH.

5.4. GENERATION OF STRESS SAMPLE:

Drug strength and volume of the stress solution used was similar in all degradation condition i.e. 0.5 mg/ml and 50 ml respectively.

Preparation of stress sample:

Take 25 mg drug in round bottom flask. Add 5 ml of ACN and 45 ml of stressor. Take out 1 ml sample for 0 time study. Heat the solution respective to temperature used in various stress condition. Take out 1 ml sample at different time interval. Cool it. For acid-base hydrolysis sample, neutralize these solutions. Take 0.4 ml of each solution in 10 ml volumetric flask and dilute with methanol upto the mark (20 ppm).

Preparation of Degradation Mixture:

From 20 ppm Solution of all degradation condition showing degradation of drug i.e. acid, base, neural hydrolysis samples and oxidative degradation sample, take out an aliquot of 2.5 ml(5 ppm) from each and transfer it to 10 ml volumetric flask. Dilute the mixture upto the mark methanol which will give final concentration of 20 ppm. Mix it well. Filter the solution through whatmann filter. Sonicate the mixture. Inject the mixture sample and record the chromatogram.

5.5. HPLC STUDIES FOR DEVELOPMENT OF SIAM:

To develop SIAM, HPLC studies were first carried out on all the reaction solutions individually and after getting acceptable peak separation, the same was tried on a mixture of all solutions in which decomposition was observed. In order to achieve good separations with acceptable peak purity, HPLC parameters like different ratios of the mobile phases in isocratic condition, gradient programming of mobile phase composition, gradient programming of flow-rate were attempted. From the results, it was found that mobile phase consisting of water:methanol in a ratio of 45:55 v/v,TEA-0.2%, pH when run at a gradient flow rate programming(as shown in following table) gave best results. The detection wavelength used for analysis was 230 nm.

5.6. VALIDATION OF HPLC METHODS:

The methods were validated for linearity, precision (inter-day, intra-day and intermediate precision), accuracy, specificity and selectivity as per the ICH guideline Q2(R1) [15].

5.6.1. Linearity and range:

The linearity was determined at five levels of sample concentration. Letrozole standard stock solutions were prepared and from these solutions dilution was done with diluent to obtain mixed standard calibration solutions of letrozole having concentration in the range of 5-50 µg/ml as described in section 5.6.1. Each linearity level preparation was injected in duplicate.

Inject diluent as blank, standard solution as per test procedure and above linearity solution preparations at each level in duplicate. Calculate mean area at each level and plot a graph of mean area versus concentration in %. Calculate and record value of correlation co-efficient (r), y-intercept, slope of regression line and residual sum of squares.

Preparation of linearity solution:

Stock solution:

Weigh and transfer accurately about 25mg letrozole working standard into a 25 ml volumetric flask (1000 ppm), Sonicate to dissolve and make up the volume with diluent. Further dilute this stock solution as describe below.

Table 5.6.1:- Dilutions for linearity

Linearity level	Stock solution taken(ml)	Diluted to(ml)	Letrozole conc. (ppm)
1	0.05	10	5
2	0.1	10	10
3	0.2	10	20
4	0.3	10	30
5	0.5	10	50

5.6.2. Precision

Intra-day and Inter-day precision was established by repeating the experiment by analyzing three sample of 20 ppm standard solution under same conditions as per test procedure for Assay using same lot of sample on same day at 3 time interval and on different day respectively. Individual assay value, mean assay value and %RSD shall be calculated for the results obtained and recorded.

5.6.3. Accuracy

Accuracy was determined over the range 80% to 120% of the sample concentration. Calculated amount of Letrozole from standard stock solution was added in 10ppm standard solution to attain 80%, 100% and 120% of sample concentration. Each sample was prepared in triplicate at each level and injected each preparation in duplicate. Blank and standard preparations were injected and the chromatograms were recorded.

Preparation of standard solution:

Weigh and transfer accurately about 10 mg letrozole standard in two different 10 ml volumetric flask, add about 7 ml of diluents(methanol). Filter the solution through whatmann filter paper. Sonicate for about 2 min with occasional shaking. Cool the solution to room temperature and make the volume up to mark with diluent and mix(1mg/ml). Label both the solution as standards solution 1 and 2.Form standard solution 1, prepare 10 ppm standard solution and record the chromatogram.

Level-1 (80%):

From standard solution-1, take 0.1ml (100µl) solution in 10 ml volumetric flask required to prepare 10ppm solution. Then spike this solution with 80% of 100µl i.e 80 µl of standard solution from standard solution-2.Make up the volume with diluent upto the mark and mix it well giving final conc. of 18ppm. Sonicate the solution. Prepare this solution in triplicate. Record the chromatogram.

Level-2 (100%):

From standard solution-1, take 0.1ml (100µl) solution in 10 ml volumetric flask required to prepare 10ppm solution. Then spike this solution with 100% of 100µl i.e 100 µl of standard solution from standard solution-2.Make up the volume with diluent upto the mark and mix it well giving final conc. of 20ppm.Sonicate the solution. Prepare this solution in triplicate. Record the chromatogram.

Level-3 (120%):

From standard solution-1, take 0.1ml (100µl) solution in 10 ml volumetric flask required to prepare 10ppm solution. Then spike this solution with 100% of 100µl i.e 100 µl of standard solution from standard solution-2.Make up the volume with diluent upto the mark and mix it well giving final conc.of 20ppm. Sonicate the solution. Prepare this solution in triplicate. Record the chromatogram.

5.6.4. Specificity:

Specificity Part-A: Interference from blank, placebo and impurities:

Procedure:

Prepared blank preparation, standard preparation, and sample preparation for 2.5 mg tablet as per method.

Blank preparation:

Blank used is HPLC Grade Methanol.

Standard Preparation:

Prepare standard solution of drug as mentioned earlier in section 5.3.

Sample Preparation:

Weigh accurately tablet powder equivalent to 5 mg API. Transfer it to50 ml volumetric

flask. Add about 35 ml of Methanol. Sonicate in cool water for 20-30 mins. Cool it and make up the volume upto mark with HPLC Grade Methanol (100ppm).Centrifuge at 200 RPM for 10 mins. Take out 10 ml of supernant solution in 50 ml volumetric flask. Make up the volume upto the mark with HPLC Grade Methanol (20 ppm).

Tablets of two brand i.e. ANOLET and LETROZ was used for this study.

Specificity Part –B:

Interference from degradation products by stress study:

API was subjected to acid, base, oxidation, Photo and thermal degradation as mentioned in above section 5.4. For each degradation, blank was prepared accordingly. All the solutions were passed through whatmann filter before making injections. Each was injected and the separation of degraded products peaks from main drug peak were checked with the help of resolution factor.

Note: Minimum 10 to 30% degradation should be achieved in minimum 2 conditions. Final concentration of all the degradation sample should be approximately equal to the test concentration specified in method and final dilution shall be in diluent.

Inject separately blank, Letrozole API (stressed and unstressed), Tablet sample preparation and unstressed) for each degradation. Final concentration for each degradation preparation will remain same as per test procedure.i.e 20 ppm. Check for the separation of degraded products from main peak.

5.6.5. Solution Stability:

Standard and sample preparation were prepared as per test procedure and assay of standard and sample was determined as per method. Standard and sample solution was stored for 48 hours at room temperature. Assay of standard and sample solution after 24 and 48 hours was determined using freshly prepared standard. The assay obtained was compared with the initial assay value and recorded.

5.6.6. Robustness:

Change following parameters one by one and observe their effect on system suitability.

5.7.6.1 Change in the mobile phase composition.

5.7.6.2 Change in pH of the mobile phase.

5.6.7. Limit of Detection/Limit of Quantification:

For determination of LOD/LOQ, all linearity range solution were prepared in triplicate as described in section of linearity. Chromatograms of all these solutions are recorded. 3 linearity curves of Peak area vs concentration(ppm) were plotted. From these curves, mean slop value was calculated from 3 intercept value, SD of intercept was calculated.From all these values, LOD/LOQ were calculated and reported.

5.7. APPLICATION OF DEVELOPED METHOD FOR FORMULATION ASSAY:

- Average Weight of tablets were calculated and reported.
- Preparation of tablet formulation of two brand(Anolet and Letroz) is mentioned in section 5.6.4-specificity part-A under title of sample preparation. These solutions were prepared in duplicate to check weight effect. Total 4 solutions of 2 brands were prepared. Each solution was injected in duplicate. Chromatograms were recorded.
- % assay value was calculated.

5.8. COMPARISION OF RESULTS OF DEVELOPED RP-HPLC SIAM WITH REPORTED RP-HPLC ASSAY METHOD IN PHARMACEUTICAL DOSAGE FORM.

- **Reported RP-HPLC method:**
  1. S.P. – C_{18}
  2. M.P. – ACN: Phosphate Buffer(pH – 7.8) (70:30 V/V)
  3. Flow Rate – 1ml/min
  4. λ_{max} – 232 nm
  5. Internal Standard – Acenapthene
  6. Retention Time of Letrozole – 3.385 min.
- **Preparation of Solution:** same as for formulation assay.
- Chromatograms of all the solutions were recorded using above mentioned method.
- % Assay value was calculated and compared with developed method
- Student-t-test was applied .t-value was calculated and compared with t-table vale.

CHAPTER-6

RESULTS AND DISCUSSION

LIST OF CONTENT

6 RESULTS AND DISCUSSION

6.1 DEVELOPMENT OF RP-HPLC STABILITY INDICATING ASSAY METHOD:

A) Preliminary development of assay method:

Initially chromatographic condition selected was according to USP with little deviation. Afterwards various trials taken are described below in table 6.1.

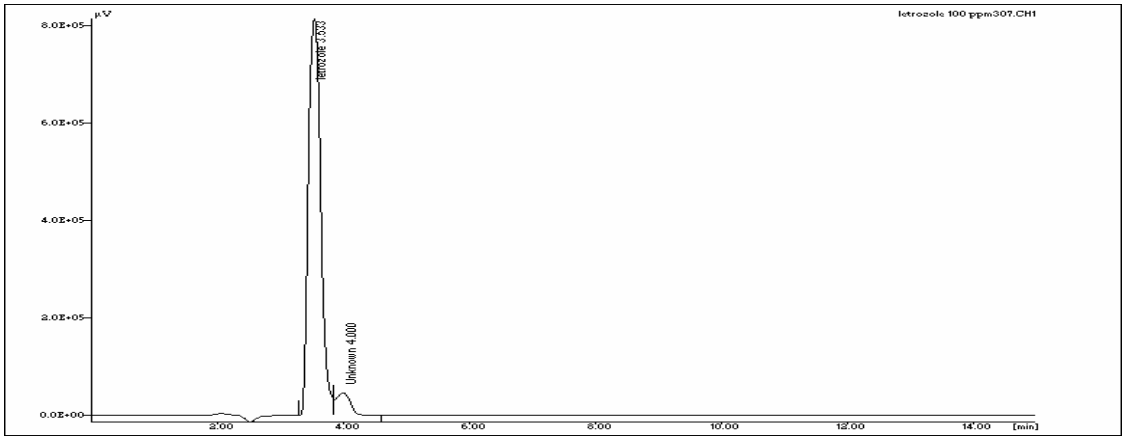


Figure-6.1: Water: methanol (30:70 v/v) > RT of drug(20 ppm) obtained-3.533 min with still good peak shape.

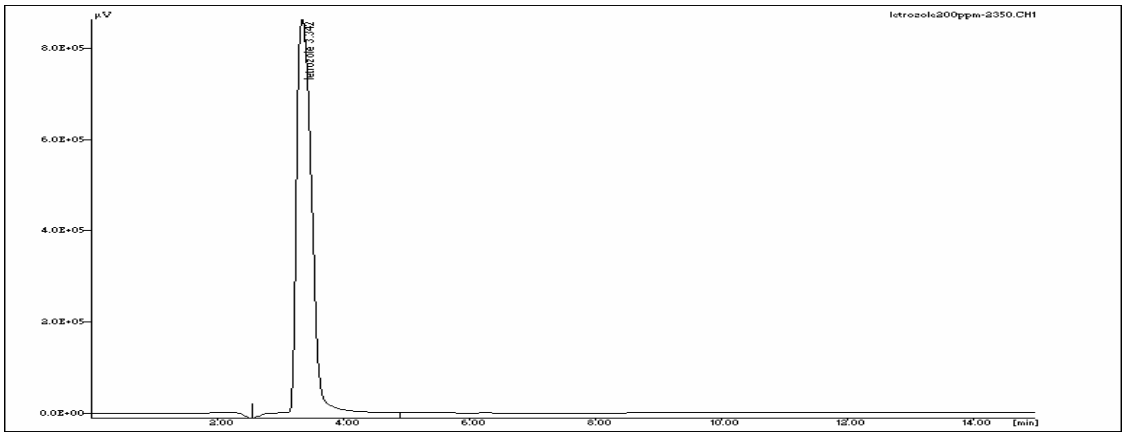


Figure-6.2: Water: methanol (28:72 v/v) > RT of drug (20 ppm) obtained-3.342 min.

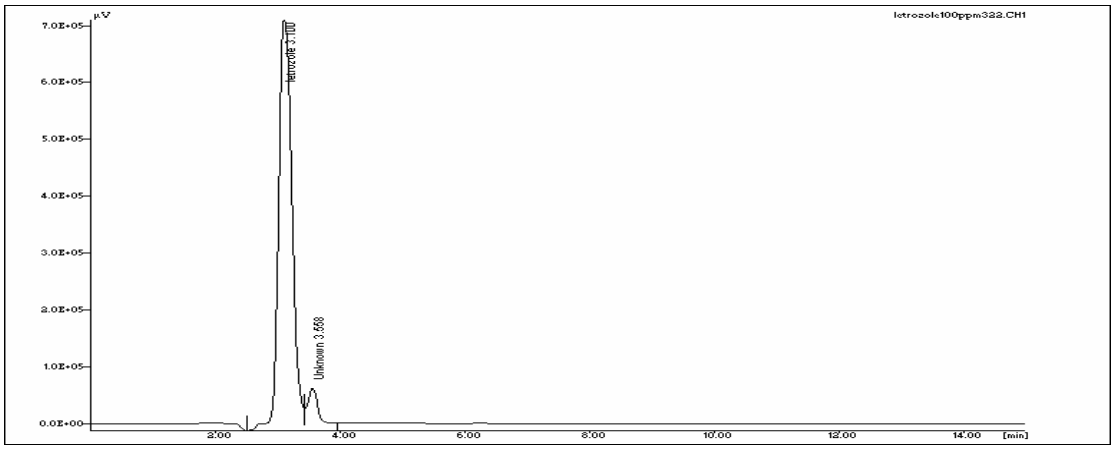


Figure-6.3: Water: methanol (25:75 v/v) at 200ppm> RT of drug (20 ppm) obtained-3.100 min.

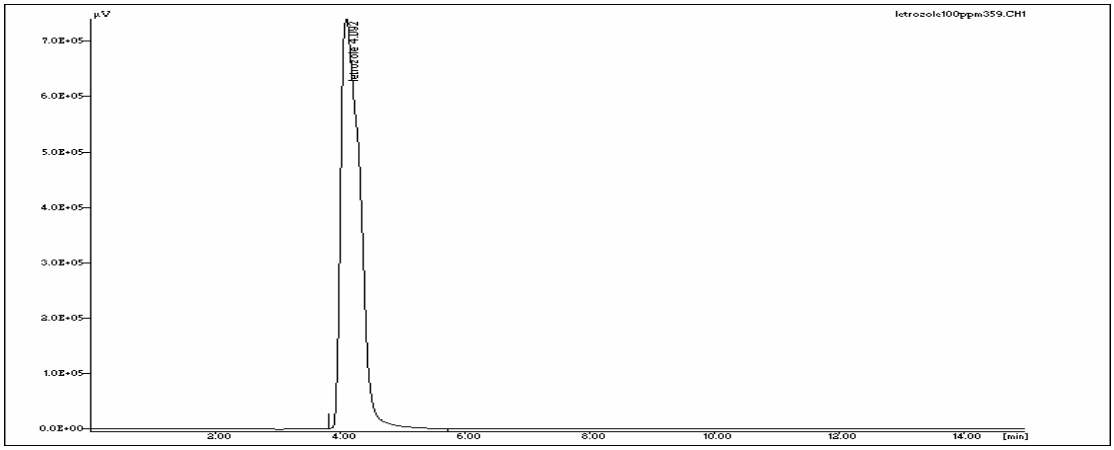


Figure-6.4: Water: methanol (28:72 v/v) > RT of drug (20 ppm) obtained-4.097min at 0.8ml//min flow rate.

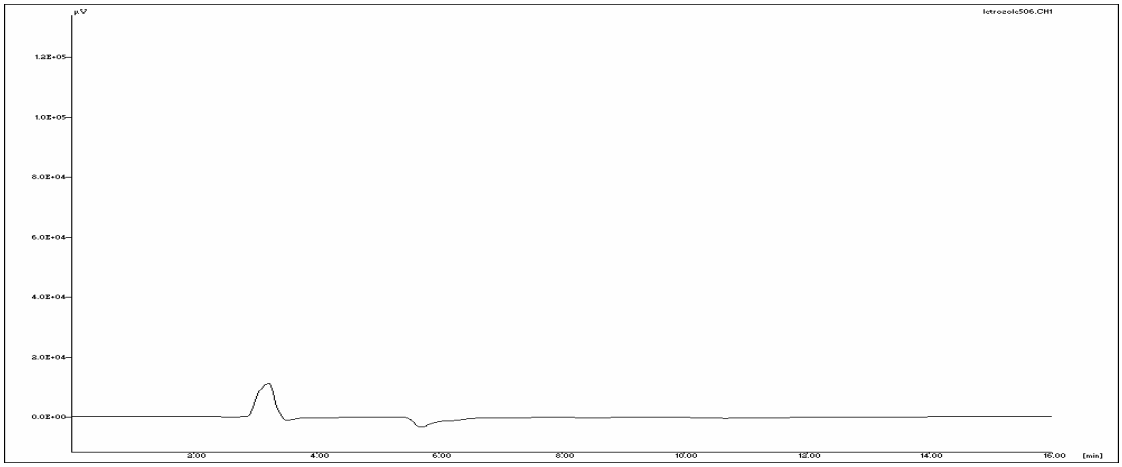


Figure-6.5: HPLC chromatogram of blank preparation

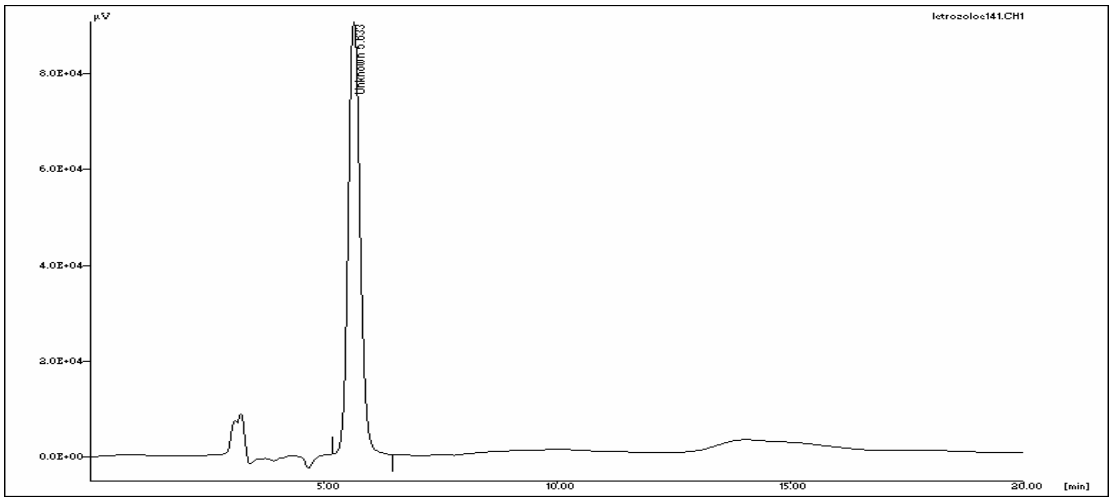


Figure-6.6: Water: methanol (28:72 v/v) > RT of drug obtained-5.63 min at 0.2% TEA, pH-3 and o.8ml//min flow rate.

B) Stress Studies under Different Stress Condition:

Stress studies were carried out under various degradation conditions like acid, base, neutral hydrolysis, oxidation, thermal and photolytic degradation as mentioned in experimental work.

Optimized degradation condition giving 10-30% degradation of main drug under various stress condition are as follow:

Table 6.2: Optimized Degradation Condition Parameter

Degradation Type	Stressor	Time Period	Temperature	% Degradation
Acid hydrolysis	3N HCl	10 Min	90 □C	19.97
Base hydrolysis	0.01N NaOH	10 Min	80□C	19.08
Neural hydrolysis	Water	5.5 hours	100□C	18.52
Oxidation	3% H_2O_2	3 hours	100□C	9.71
Themal degradation	Dry heat	48 hours	100□C	No Degradation
Photolytic degradation	Direct sunlight	4 hours	-	No Degradation

C) Optimization of above developed assay method to get separation of various degradation product peak and main drug peak:

- On degradation study with above mentioned RP-HPLC assay method, it was found that there was no good peak resolution was obtained in case of base degradation.

- Two degradation peaks were getting merged as shown in following chromatograms. So in order to separate that peak, modification in mobile phase was done. Modifications like change in composition ratio, pH changes.

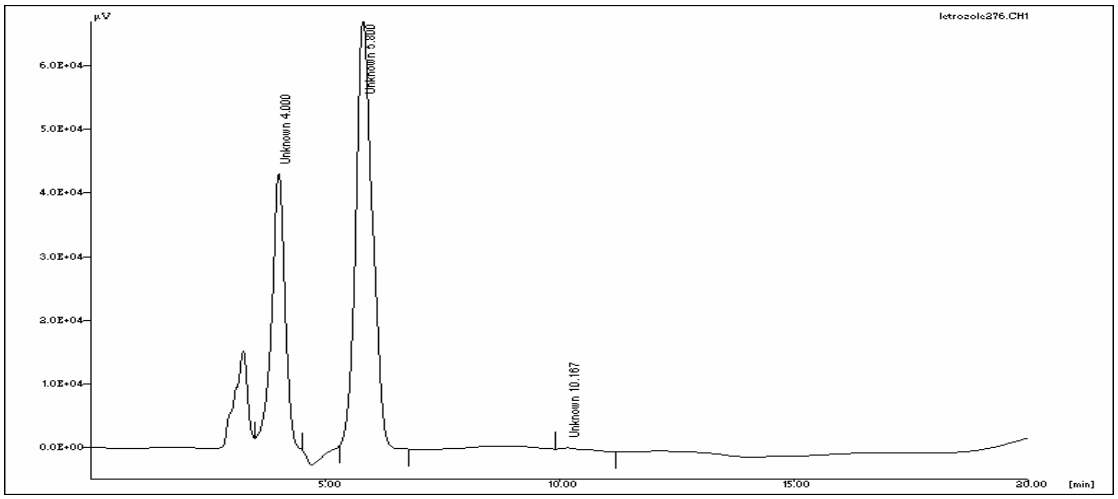


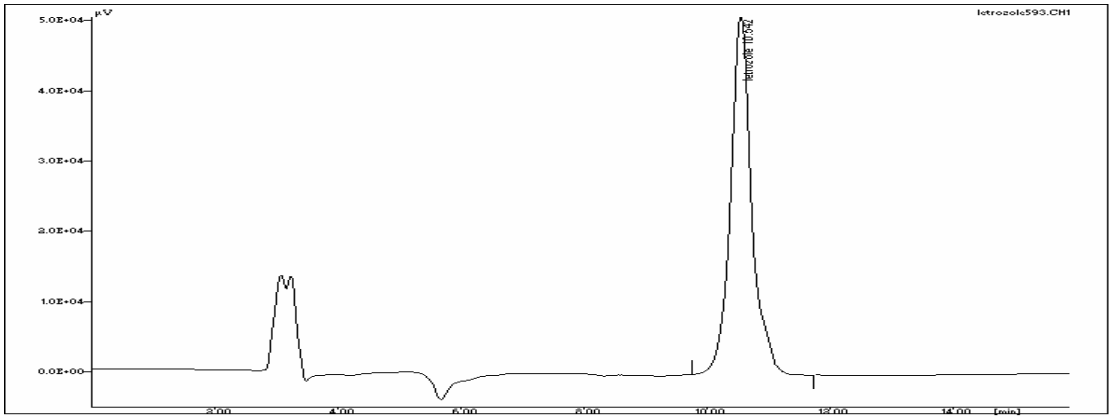
Figure-6.7: Base Degradation (0.01N NaOH, 15 min, 100°C) Chromatogram of letrozole.

Sr.No.	Name	RT	Area	Asymmetry	Theoretical Plates
1.	Deg-1	4.0	762537	0.91	1476
2.	Letrozole	5.8	1539198	1.09	1547
3.	Deg-2	10.167	54276	2.57	14559

- Final mobile phase composition selected which good separation of all the peaks was as following:
  Mobile Phase: Methanol:Water (55:45 v/v), TEA-0.2%, pH – 3.
- But, this mobile was giving drug peak at higher RR_T -15.533 min.
- So in order to decrease RR_T of drug, gradient flow rate programming was used giving drug at RT-11.8 min.
- Gradient Flow-Rate Programming:

Table 6.3: Gradient Flow-Rate Programming

Time (min)	Flow Rate(ml/min)
0-7	0.8
7-9.5	0.8-1.5
9.5-12	1.5
12-14	1.5-1.2
14-16	1.2-1.0
16-25	1.0-0.8



Figure– 6.8: Chromatogram of letrozole-20ppm in finally optimized RP-HPLC condition.

Sr.No.	Name	RRT	Area	Theoretical Plates	Asymmetry
1.	Letrozole	10.9	1215930	6545	1.35

6.2. DEGRADATION BEHAVIOR:

HPLC studies using above developed assay method on the drug sample under different stress conditions showed the following degradation behavior.

1. Hydrolytic degradation

A. Acid Hydrolysis:

The drug degraded immediately within 10 mins on heating at 90 °C in 3N HCL, forming 1 major degradation product at RT of 4.0 min.

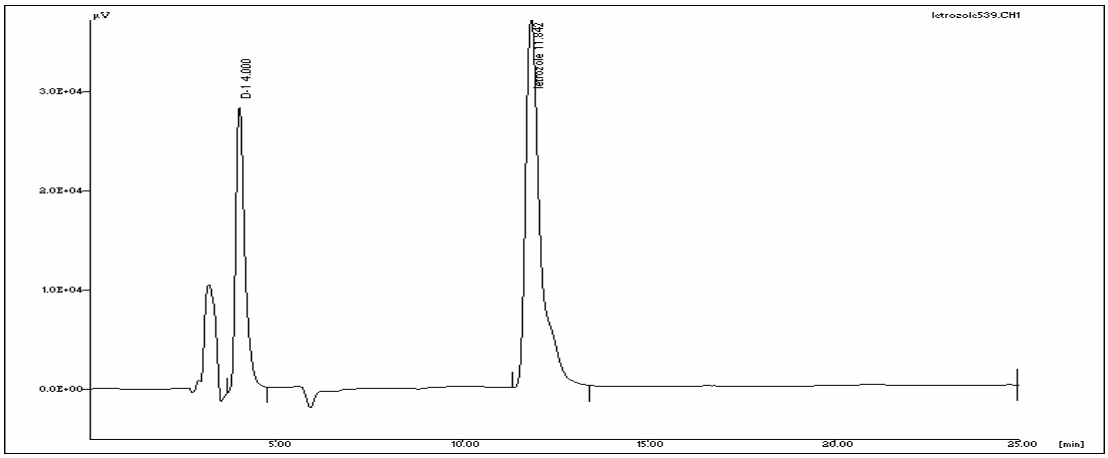


Figure – 6.9: Chromatogram of Acid Degradation Behaviour of Letrozole

Sr.No.	Name	RT	Area(μV.Sec)	Asymmetry	Plates	Resolution
1.	D-1	4	428407	1.53	1995	0
2.	Letrozole	11.842	942599	1.91	7272	17.2032

B. Base Hydrolysis

The drug degraded moderately in basic conditions in 0.01 N NaOH in 10 min at 80°C resulting into a minor degradation product at RR_T of 5.65 and 14.275.

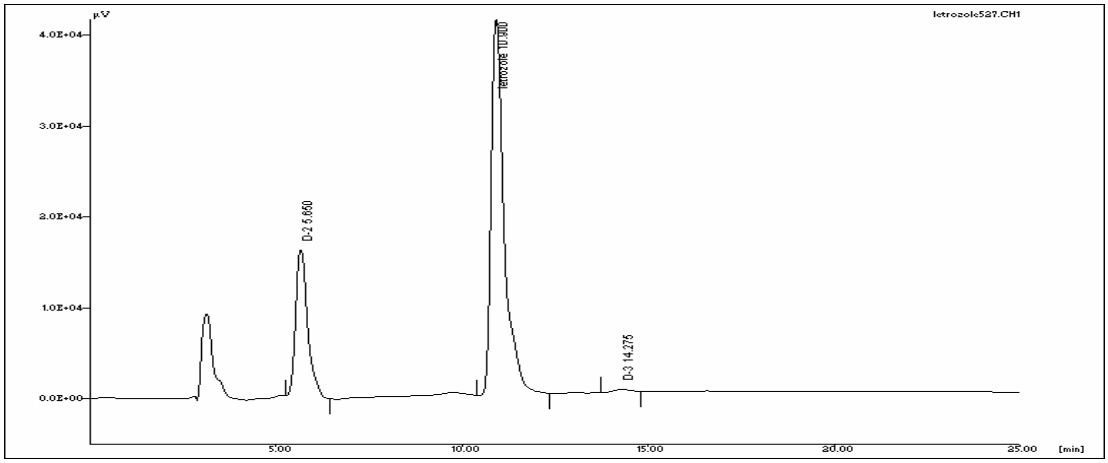


Figure – 6.10: Chromatogram of Base Degradation Behaviour of Letrozole

Sr.No.	Name	RT	Area(µV.Sec)	Asymmetry	Plates	Resolution
1.	D-2	5.65	343087	1.46	2082	0
2.	Letrozole	10.9	953097	1.79	7670	10.5963
3.	D-3	14.275	37772	0.98	5960	5.4668

C. Neutral Hydrolysis

Whereas in neutral condition, degradation was observed gradually within 5.5 hrs on heating at 100 °C, forming one major degradation products at RR_T of 5.725 and one minor degradation product at RT of 6.167.

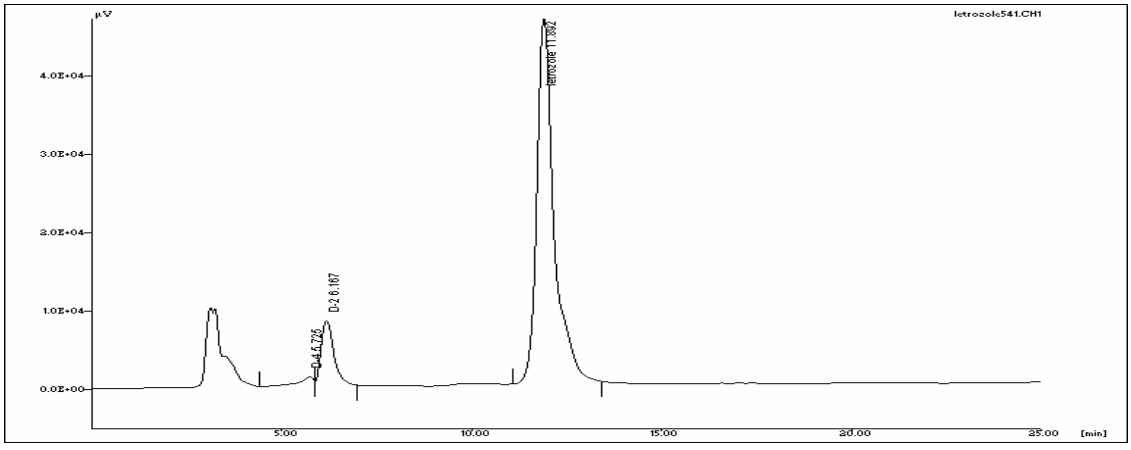


Figure – 6.11: Chromatogram of Neutral Degradation Behaviour of Letrozole

Sr.No.	Name	RT	Area(µV.Sec)	Asymmetry	Plates	Resolution
1.	D-4	5.725	30301	0.56	1833	4.2307
2.	D-2	6.167	177059	1.56	2243	0.8386
3.	Letrozole	11.892	1359630	1.48	5432	9.8396

2. Oxidative degradation

In 3 % H_2O_2, four degradation products were formed after 3 hour at 100 °C at RT of 4.892, 6.175, 9.142, 10.483.

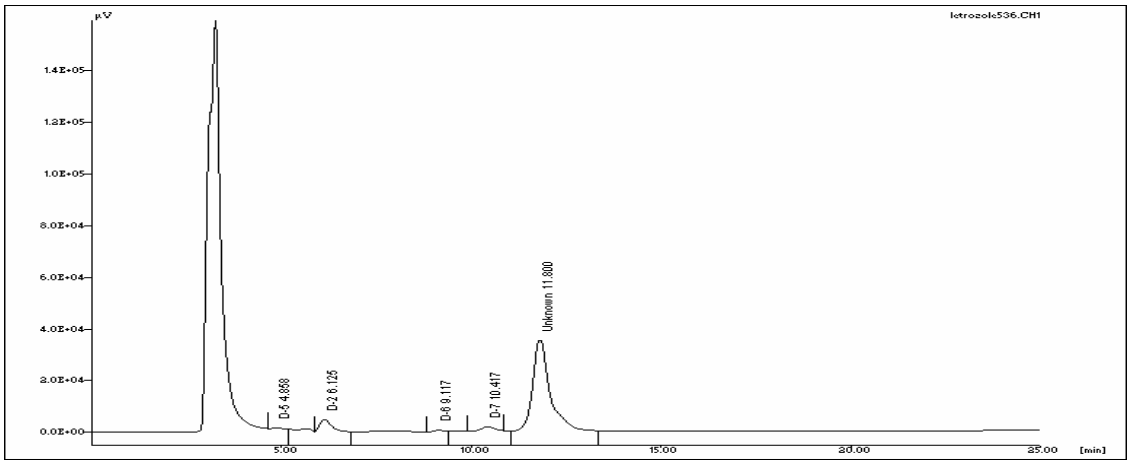


Figure – 6.12: Chromatogram of Oxidative Degradation Behaviour of Letrozole

Sr.No.	Name	RT	Area(µV.Sec)	Asymmetry	Plates	Resolution
1.	D-5	4.858	13375	1.31	2632	1.67
2.	D-2	6.125	90723	1.55	2439	2.902
3.	D-6	9.117	14966	0.92	8179	6.6676
4.	D-7	10.417	59904	1.15	5586	2.7123
5.	Letrozole	11.8	1063365	1.46	5430	2.3144

3. Thermal degradation

In case of thermal stress, drug was exposed to 100°C for 48 hours but no degradation product was formed.

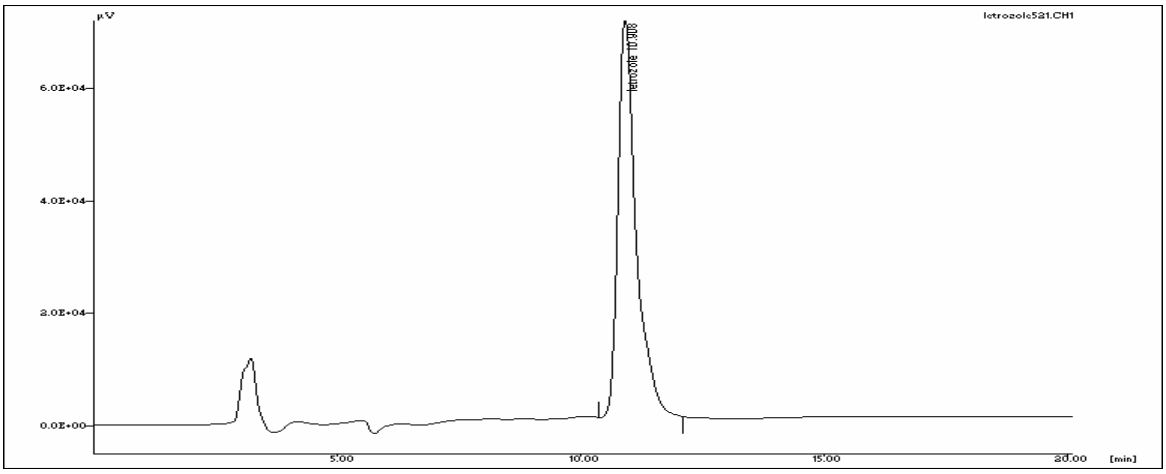


Figure – 6.13: Chromatogram of Thermal Degradation Behaviour of Letrozole

Sr.No.	Name	RT	Area(µV.Sec)	Asymmetry	Plates	Resolution
1.	Letrozole	10.9	1719619	1.6	6124	2.3144

4. Photolytic degradation

No degradation was observed in solid state and solution form of API and its tablet dosage form when exposed to day light for 8 hours.

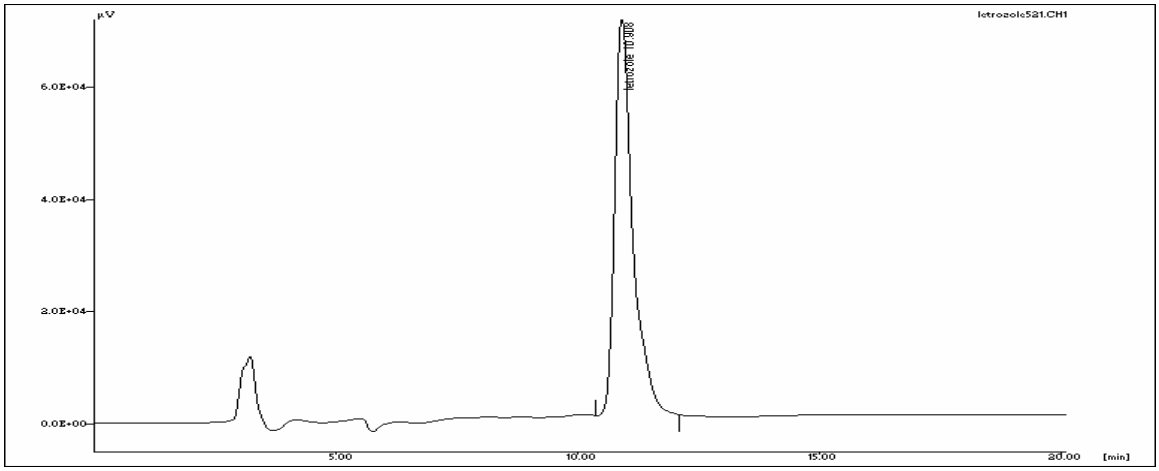


Figure – 6.14: Chromatogram of Photolytic Degradation Behaviour of Letrozole

Sr.No.	Name	RT	Area(µV.Sec)	Asymmetry	Plates	Resolution
1.	Letrozole	10.9	1725391	1.56	5267	2.5

5. Degradation Mixture:

Chromatogram of degradation mixture of all stress condition is shown below which clearly shows that all degradation products and drug peaks are well resolved by proposed method.

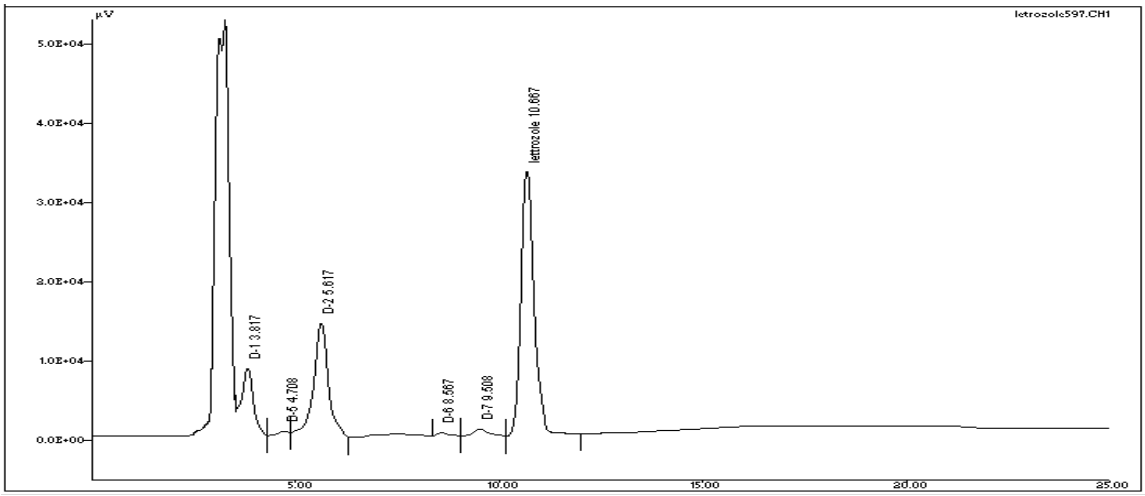


Figure – 6.15: HPLC Chromatogram of degradation mixture

Sr.No.	Name	RT	Area(µV.Sec)	Asymmetry	Plates	Resolution
1.	D-1	3.817	160980	1.19	1777	3.7898
2.	D-5	4.708	10722	0.69	2548	2.4309
3.	D-2	5.617	335660	1.09	1977	2.0728
4.	D-6	8.567	11650	1.71	8691	6.7744
5.	D-7	9.508	26276	1.46	4980	2.0821
6.	Letrozole	10.667	759625	1.33	7471	2.2485

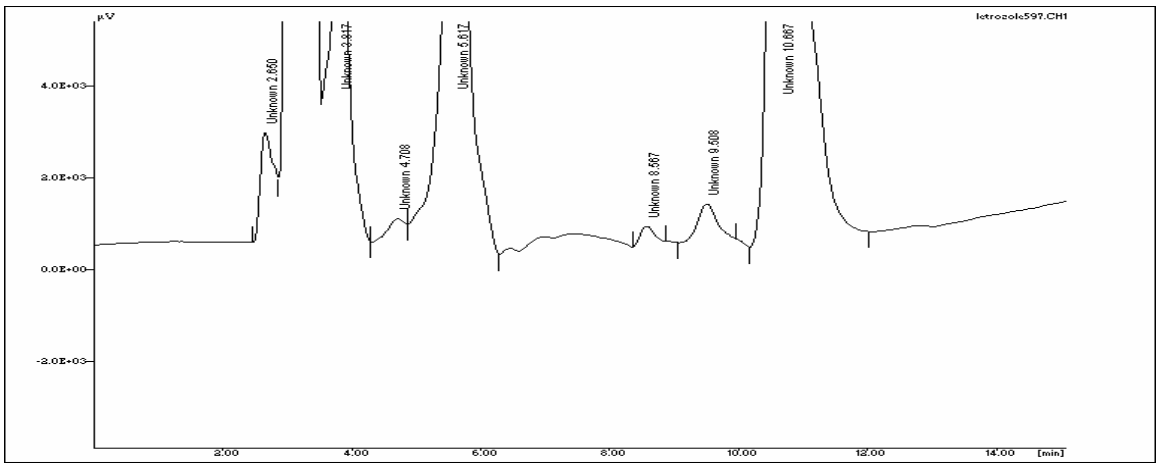


Figure – 6.16: zoomed Chromatogram of Degradation Mixture oh Letrozole in order to show minor peaks.

- D-2 is common degradation product found in base and neutral hydrolysis degradation condition.

6.3. ANALYTICAL VALIDATION OF PROPOSED RP-HPLC SIAM METHOD:

6.3.1. Linearity/Range:

All linearity solutions were prepared as described in experimental work and chromatograms were recorded.

The mean area at each level was calculated and a graph of mean area versus % concentration was plotted. The correlation co-efficient, Y intercept and slope of regression line were calculated and recorded in Table 6.5.

Table 6.5: Linearity Results of Letrozole

Linearity level	Conc. (µg/ml)	Area	Mean Area ± R.S.D
Level – 1	5	396716.7	397370.4 ± 0.21
		397898.0	
Level – 2	10	809578.6	809784.3 ±0.03
		809990.0	
Level – 3	20	1365513.0	1372314 ± 0.7
		1379114.0	
Level – 4	30	1966921.5	1963619 ± 0.23
		1960317.1	
Level – 5	50	3156613.0	3147264 ± 0.42
		3137914.0	
Correlation coefficient		0.998	
Slope of regression line		60122	
Y-intercept		15625	

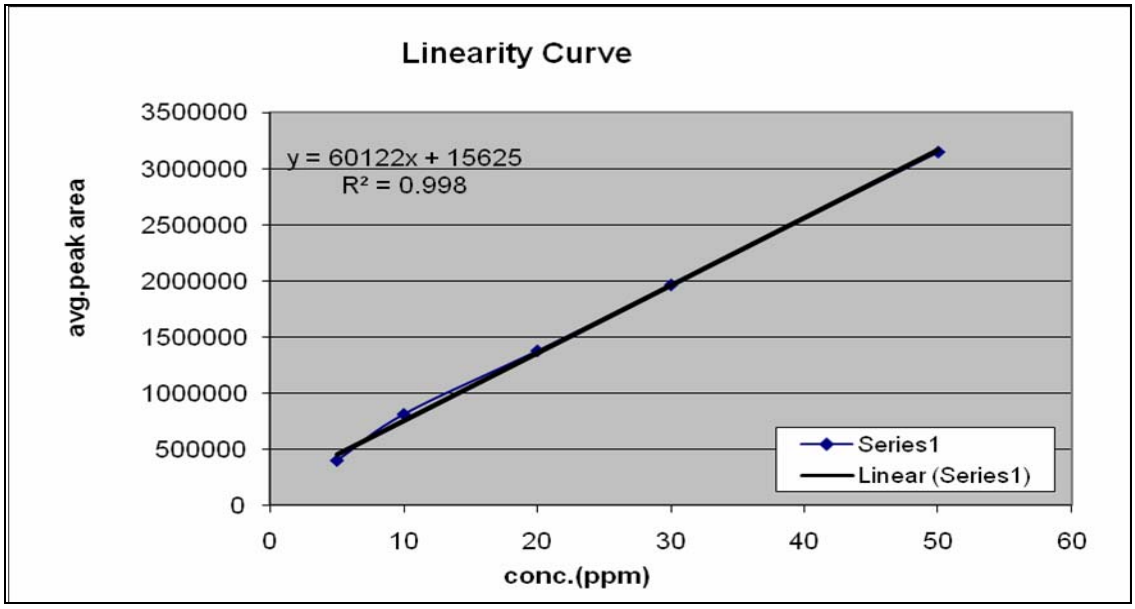


Figure - 6.17: Linearity Curve of Letrozole

6.3.2. Accuracy:

Accuracy study was performed on standard solution of 10 ppm at 3 level 80%, 100%, 120% in triplicate and results were recorded as shown below in Table 6.6.

Acceptance Criteria:

1) % Recovery and % mean recovery at each level should be between 98-102%

2) % RSD of %Recovery at each level should not be more than 2.0%.

Result and Calculation:

% recovery, mean% recovery and %RSD were calculated at each level and recorded in Table 6.5.

Table 6.6: Data Indicating Recovery Study of Letrozole in Tablet Formulation

Level of Recovery	Mean Area	Total Amount (µg/ml)	Recovered Amount (µg/ml)	% Recovery	Mea % Recovery	% RSD
80%	1976271.33	18	17.70	98.32	98.5	1.07
	1945338.39	18	17.82	99.0		

	1936423.90	18	17.86	98.2		
100%	2220028.52	20	19.94	99.4	99.97	0.72
	2228962.24	20	19.97	99.7		
	2251296.53	20	20.08	100.8		
120%	2425528.75	22	21.85	98.73	98.94	0.36
	2439856.77	22	21.92	99.4		
	2423481.89	22	21.84	98.7		
Mean					99.14	0.72

6.3.3. Precision:

6.3.3.1 Method Precision (Repeatability):

Acceptance Criteria:

RSD for assay of six determinations should not be more than 2.0%.

Result and Calculation:

Individual % assay, mean % assay and % RSD were calculated and recorded in Table 6.7.

Table 6.7: Results of Repeatability Study

Sample No.	Repeatability Results		
	Area	Mean Area ± R.S.D	% Assay
1	1297525 1327446	1312486±0.81	99.74711452
2	1319938 1339500	1329719±1.04	101.0568371
3	1328902 1319619	1324261±0.49	100.6419985
Mean Assay			100.48

SD	0.67
% RSD	0.67

6.3.3.2 Intermediate Precision (Ruggedness):

Acceptance Criteria:

1. RSD of intermediate precision should not more than 2.0%
2. The difference between assay of method precision and intermediate precision should not be more than 2.0%
3. Overall RSD of % assay of six replicates sample preparation of method precision and six replicates sample preparation of intermediate precision should not be more than 2.0%.

Result and Calculation:

The mean % assay value was calculated and compared with the mean % assay value obtained in method precision study. The difference of the mean assays obtained was calculated and recorded in Table 6.8.

Table 6.8: Intermediate precision data for analysis of Letrozole

Sample No.	**Intermediate Precision Study Result**		
	Area	Mean Area ± R.S.D.	% Assay
1	1327771	1325109 ± 0.28	100.7064454
	1322446		
2	1315930	1317715± 0.19	100.1445494
	1319500		
3	1293270	1305445± 1.31	99.21200809
	1317619		
Mean Assay			100.021001
SD			0.754840289
% RSD			0.754681798
Overall % RSD			0.324457974
Difference			0.08

6.3.4. Specificity:

Specificity Part-A: Interference from blank, placebo and impurities:

Results of this study are shown below in form of HPLC Chromatogram (Figure 6.17-6.19). It shows that there is no interference from blank and tablet excipient with main drug peak using proposed RP-HPLC method.

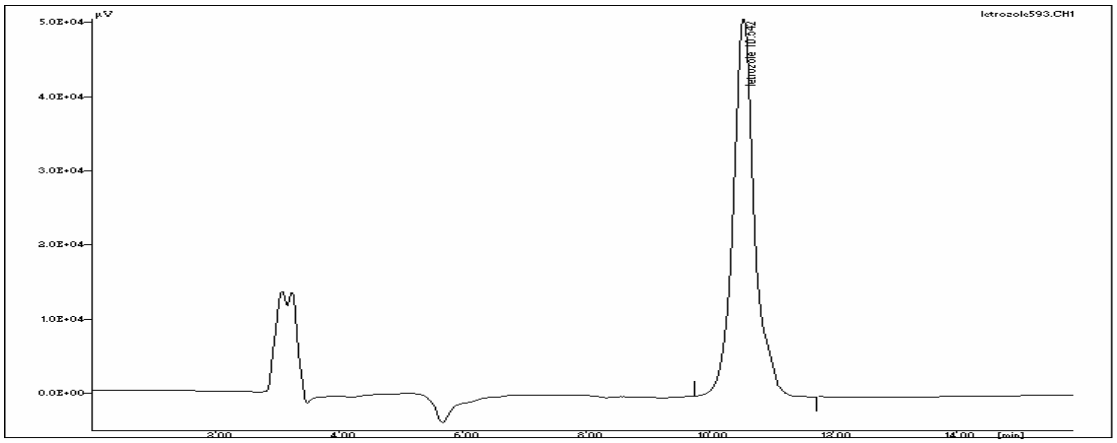


Figure - 6.18: HPLC chromatogram of standard preparation.

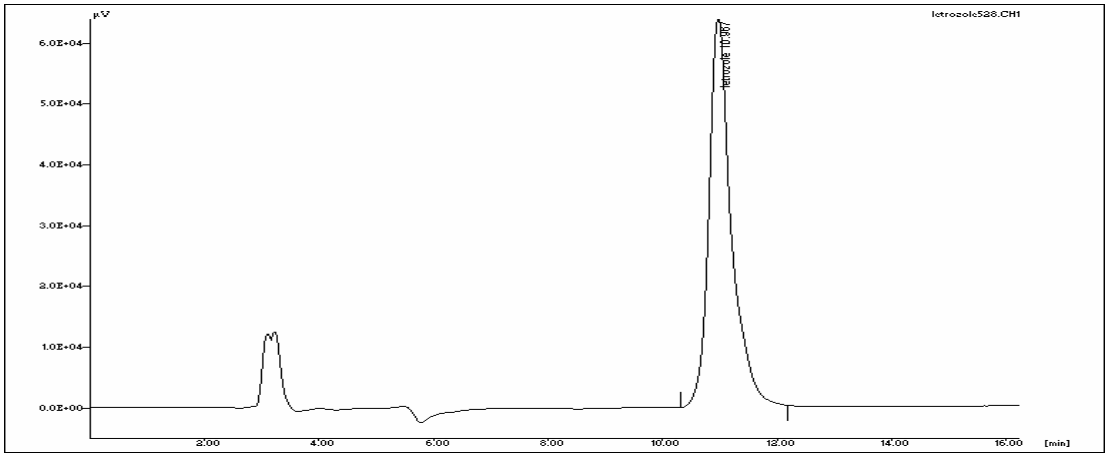


Figure – 6.19: HPLC chromatogram of sample preparation (ANOLET).

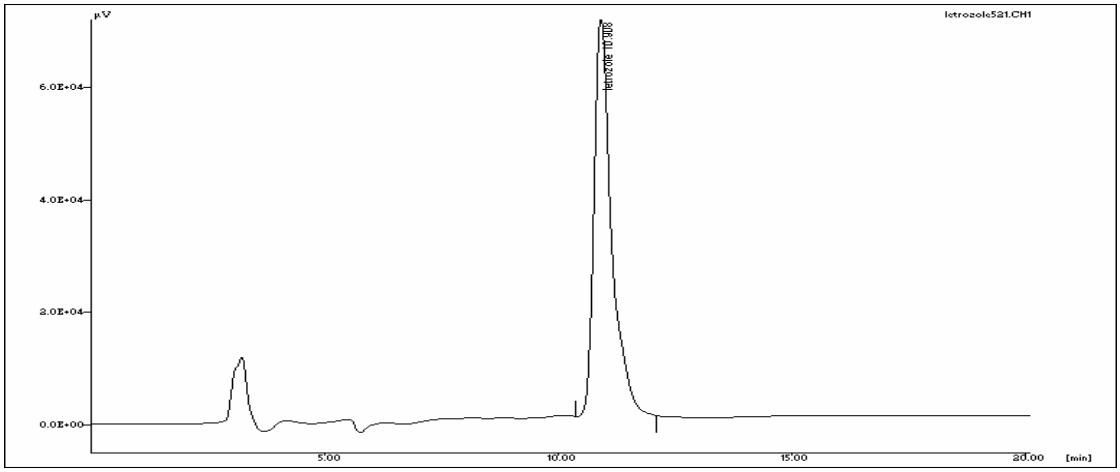


Figure – 6.20: HPLC Chromatogram of sample preparation (Letroz).

Specificity Part-B:

Results of this study are shown above in section 6.2 under various degradation studies in form of chromatogram. These forced degradation study results shows that proposed method is specific and there is no interference of any degradation product peak with main drug.

6.3.5. Solution Stability:

Results of solution stability study are shown below which shows that all solutions are stable.

Acceptance criteria:

1. The difference in the response of standard preparation should not be more than ± 2.0% from the initial value at any time interval.
2. The absolute difference in the assay value of sample should not be more than ± 2.0% from the initial value at each time point.

Result and Calculation:

Solution stability period for sample preparation was determined and recorded in Table 6.9.

Table 6.9: Data Indicating Solution stability for Standard and Tablet preparation

Time	Standard			Tablet					
	Average Area	% Assay	Absolute Difference	Average Area		% Assay		Absolute Difference	
				A	L	A	L	A	L

Initial	2267492	100.0	-	2333405	2333331	102.88	102.88	-	-
After 24 hrs	2230209	98.36	1.64	2319246	2286347	103.99	102.51	1.11	0.37

A = Anolet, L = Letroz

6.3.6. Robustness:

Acceptance criteria:

1. Theoretical plates: Not less than 2500.
2. Asymmetry (tailing factor): Not more than 2.0.
3. S.D. should not be more than 2.

Result and Calculation:

The effect of changes was observed on system suitability values and recorded in the Tables 6.10.

Table 6.10: Data Indicating Robustness of the Method by Changing Various Parameters.

Factor Changed	% Change	RT	Asymmetry Factor
Organic Phase composition	-5	11.902	1.33
	0	11.817	1.21
	+5	11.755	1.27
	S.D.	0.07	0.06
pH	-0.2	11.45	1.23
	0	11.80	1.28
	+0.2	11.62	1.33
	S.D.	0.17	0.05

6.3.7. Limit of Detection:

LOD was calculated based on the standard deviation of intercept using equation,

$$LOD = 3.3*\partial/S$$

Where ∂ = Standard deviation (SD) of intercept

S = slop of the linearity curve.

Results and Calculations:

Results of LOD are shown below in table 6.11.

Table 6.11: Statistical data required for calculating LOD value.

Parameter	Value calculated
Average of intercept	15312
SD of intercept	1962.131
Slop of linearity curve	60122
LOD calculated	0.11µg/ml

6.3.8. Limit of Quantification:

LOQ was calculated based in the standard deviation of intercept using equation,

$$LOQ = 10*\partial/S$$

Where ∂ = Standard deviation (SD) of intercept
S = slop of the linearity curve

Results and calculation:

Results of LOQ determination are shown below in table 6.12.

Table 6.12: Statistical data required for calculating LOQ value.

Parameter	Value calculated
Average of intercept	15312
SD of intercept	1962.131
Slop of linearity curve	60122
LOD calculated	0.33µg/ml

6.5. COMPARISON OF RESULTS PROPOSED RP-HPLC SIAM METHOD WITH REPORTED RP-HPLC ASSAY MEHTOD:

Comparison of the developed Chromatographic method was done with reported chromatographic method by applying student-t Test (paired-t-test).

Table 6.14: Assay Results of Marketed Formulation of Letrozole for comparison of Methods:

Brand Name	Assay Results of Two Analytical Methods	
	Developed RP-HPLC Method	Reported RP-HPLC Method
Anolet	102.88	102.66
	102.56	104.22
	102.33	99.23
Letroz	99.23	98.89
	98.87	98.56
	98.34	99.57

Table 6.15: Results of Student-t-Test (Paired-t-Test)

Parameter	Anolet		Letroz	
	Developed Method	**Reported Method**	**Developed Method**	**Reported Method**
Mean	102.59	102.04	102.14	100.67
Variance	0.0763	6.52	0.7	1.35
No. of Observation	3	3	3	3
Degree of Freedom	2	2	2	2
Pearson Correlation	0.599		-0.724	
Hypothesized Mean Difference	0		0	
t-cal two tail	0.727		0.304	
t-table value	3.18		3.18	
	t-cal < t-table value		t-cal < t-table value	

t-cal = t-calculated value.

Inference from Student t-Test:

The t-cal value was found to be less than t-table value in case of both brands. Hence it was concluded that both method do not differ significantly and can be satisfactorily applied for the routine analysis of letrozole in pharmaceutical tablet dosage form.

CHAPTER-7

CONCLUSION

7. CONCLUSION

Degradation study of Letrozole shows that it is stable to thermal and photolytic degradation while unstable to acid, base, neutral hydrolysis and oxidative degradation. It forms total 7 degradation products. One degradation product is common in base and neutral hydrolysis and oxidative degradation. Degradation studies also show that Letrozole is highly unstable to base hydrolysis.

Developed RP-HPLC Method is able to separate all the degradation products and drug from each other with very good resolution. Thus, the developed method can be used to separate, quantify and study all the degradation products that may be formed under normal stress condition and so it is stability indicating assay method.

Results of assay of 2 brands of letrozole tablet shows that the proposed Stability indicating RP-HPLC method can be used for formulation assay.

Method is validated in terms of linearity, accuracy, precision, robustness, specificity, solution stability, sensitivity.

Table: 7.1: Summary of Validation Parameters

Validation Parameters		**Statistical Parameter**	**Value**
Linearity		Range	5-50 ppm
		Regression Line Equation	y= 60122x + 15625
		R^2	0.998
Accuracy/Recovery		Mean % Recovery	98.54-99.97 %
Precision	Intra-day	%RSD	0.67
	Inter-day	%RSD	0.75
LOD		-	0.11 µg/ml
LOQ		-	0.33 µg/ml

CHAPTER-8

FUTURE SCOPE

8. FUTURE SCOPE

- Proposed method can be extended to characterization of degradation products under various stress condition through LC-MS/MS by slight modification in mobile phase composition. Use formic acid in place of ortho-phosphoric acid used for pH adjustment as volatile component must be used to make mobile phase compatible to ms studies .
- By determining structures of various degradation products, prediction of degradation mechanism can be done.
- By knowing potent degradation condition or mechanism, we can use stabilizer to ultimately make the formulation of drug more stable to various normal stress condition that the marketed formulation going to face during its entire life period to the expiry date.

CHAPTER-9

SUMMARY

RP-HPLC method developed for determination of letrozole and its degradation products in pharmaceutical dosage form.

RP-HPLC method has shown adequate separation for Letrozole from their degradation products. Separation was achieved on a Inertsile C-18, 4.6 mm *250mm, 5µm column by using a mobile phase consisting Methanol: Water (55:45 v/v), TEA – 0.2%, pH – 3 (adjusted with ortho-phosphoric acid) and UV detection at 230 nm. Methanol was used as diluent. Gradient flow-rate was used as shown below:

Gradient Fl0w-Rate Program:

Time Point(min)	0→7.0	7.0→9.5	9.5→12	12→14	14→16	16→25
Flow-Rate (ml/min)	0.8	0.8→1.5	1.5	1.5→1.2	1.2→1.0	1.0→0.8

Present research work describes comprehensive stress testing of Letrozole carried out according to ICH guideline Q1A (R2). The specificity of the method was determined by assessing interference from the excipient in tablet formulation and by stress testing of the drug (forced degradation).There were no other coeluting, interfering peaks from excipients, impurities, or degradation products due to variable stress conditions, and the method is specific for estimation of Letrozole and it is stability indicating as it can be used for separation and quantification of degradation products of letrozole.

The developed RP-HPLC method was validated according to ICH guidelines in terms of linearity, precision, accuracy, specificity, robustness and solution stability. The linearity of the proposed method was investigated in the range of 50-50ppm of letrozole working standard solution in methanol with $R^2 = 0.998$. Specificity of the method was established by determining the resolution of the peaks of the drugs in a stress samples. Data of method validation suggest that proposed method is simple, accurate, precise, robust, and specific for the estimation of Letrozole.The method was applied for formulation of two brands of letrozole tablet i.e. Letroz and Anolet.

CHAPTER-10

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