CHAPTER 4
4.0. METHODOLOGY, RESULTS and DISCUSSION

4.1 ANASTROZOLE

4.1.1. UV spectra (scan from 400-200nm sample dissolved in methanol)

![UV Spectra of Anastrozole](image)

**Figure 4.1: UV Spectra of Anastrozole**

**Discussion:**

The wavelength maximum of Anastrozole was found to be 202 nm and 215 nm. Also, the wavelength maximum of most of the impurity lie near to 215 nm thus wavelength used in method development was 215 nm.
4.1.2 Infrared Spectra of Anastrozole (Scan from 400 cm\(^{-1}\)-4000 cm\(^{-1}\))

Figure 4.2: I.R. Spectra of Anastrozole

**Discussion:**

The infra red spectra show that the sample IR spectra match with that of reference standard of Anastrozole to 98%. Thus the sample was found to be Anastrozole.
4.1.3 $^1$H NMR Spectra of Anastrozole Sample

**Figure 4.3: $^1$H NMR Spectra of Anastrozole**

**Discussion:**

$^1$H NMR (CDCl$_3$): confirms that drug is Anastrozole and matches with standard.
4.1.4 $^{13}$C NMR Spectra of the Anastrozole Sample

Figure 4.4: $^{13}$C NMR Spectra of Anastrozole

Discussion:

$^{13}$C NMR shows Carbon Skeleton of the compound confirming it to be Anastrozole.
4.1.5 Mass Spectra of Anastrozole Sample

Figure 4.5: Mass Spectra of Anastrozole sample

Discussion:

The mass spectra shows dominant ion peak at m/z 294, which is with expected protonated molecular ion (M+H)^+

4.2. Methodology and Work Plan

HPLC analysis has been selected for the study of impurity profile in Active Pharmaceutical Ingredient (API)s

The work will be done in two phases

a. Method development
b. Validation

Method Development:
In order to develop a HPLC method effectively, most of the effort should be spent in method development and optimization as this will improve the final method.

- Performance
- Design of method development experiment
- Defining method objectives and understanding the chemistry
- Initial HPLC conditions
- Sample preparation procedure
- Final method optimization / robustness
- Method validation

Based on the current ICH guidelines on specifications, the related substances
Method for API should focus on both the API degradation products and synthetic Impurities.
Optimization of HPLC method development needs great emphasis. In order to have an efficient method development process, three critical components for HPLC should be considered.

c. Sample preparation
d. HPLC analysis
e. Standardization (calculation)
Sample preparation involves selection of suitable solvent sample solvent which will ensure that there will not be any compatibility issues the sample solution and the HPLC conditions.

Once the individual components of the method are optimized, perform the final optimization of the method to improve the accuracy, precision and LOQ. Experimental design approach such as Plackett-Burman design can be used to simultaneously determine the main effects of many experimental factors. Some of the typical factors that need to be investigated are
HPLC conditions: % organic, pH, flow rate, temperature, wavelength, column age.
Sample preparation: pH, sonication/shaking, sample size, sample age.
Calculation/standardization: integration, wavelength, standard concentration, response factor correction.

Typical responses that need to be investigated are

Results: Precision(%RSD),%related substance of significant related substance, total related substance

Chromatography: resolution, tailing factor, separation of all related substances.

Standardization may be by area % method or external standard method.

**Wave length selection and relative response factor:**

Photo diode array can be used to investigate the linearity of the active pharmaceutical ingredient and related substances in the proposed concentration range. By comparing the linearity steps of the API and the related substances, one can estimate the relative response factors of the related substances at different wavelengths. Disregard of whether the area % or external standard approach is used if the relative response factors of some significant related substance are from unity, a response factor correction must be applied.

The optimum wavelength of detections is the wavelength that gives highest sensitivity (gamamax) for the significant related substance and minimizes the difference in response factors between those of the API and the related substances.

**VALIDATION:**

**VALIDATION:** Process by which it is established by laboratory studies that the performance characteristics of the method meet the requirements for the intended analytical applications.

**METHOD:** A set of all written procedures and instructions involved n the collections, processing, storage and analysis of chemical matrix for an analyte.

**SYSTEM SUITABILITY**

Specific tests to ascertain the suitability and effectiveness of the operating system when employing chromatographic methods such as pressurized liquid chromatography and gas chromatography.
VALIDATION PROTOCOL
Prospective experimental plan that when executed is intended to produce documented evidence that the system has been validated.

SPECIFICITY/SELECTIVITY
The ability of an analytical method to assess or measure unequivocally the analyte in presence of components that may be expected to be present in the sample matrix.

METHOD
Prepare the impurities standard at limit concentration (specification limit). Analyze the impurity standards and detest sample individually to establish the retention times of all components.
Select a sample that meets the specification and spike the impurities at the impurities limit concentration and analyze.

INTERFERENCE BY DEGRADATION PRODUCTS IN TEST SAMPLE
As general guidelines, a target of about 10 to 30% degradation should be aimed while carrying out the degradation study. The higher percent degradation i.e. first degradation impurity degrades further.

a) Acid degradation
b) Base degradation
c) Oxidative degradation
d) Water (hydrolysis) degradation
e) Photolytic degradation
f) Thermal degradation
If no degradation or marginal degradation occurs, continue the study by extending schedule of 12 hours each to a maximum of 48 hours.

LIMIT OF DETECTION (LOD)
Definition
The detection limit of an individual analytical procedure is the lowest amount of an analyte in a sample that can be detected not necessarily quantities as an exact value.

**LIMIT OF QUANTITATION (LOQ)**

**Definition**

The lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions.

**Method**

Establish limit of quantization based on 3.3 times to the limit of detection by using the system software. Analyze six times at limit of quantization concentration level and find out the %Relative standard deviation (RSD).

**ACCURACY**

**Definition**

The accuracy of an analytical method is the closeness of the test results obtained to the true value. It may be expressed as percentage recovery by the assay of known added amounts of the analyte.

**Method.**

- Prepare the concentration of the analyte from LOQ to 150% of the specification at minimum three points i.e. LOQ, 100% and 150%.
- Spike the concentration of the analyte from LOQ to 150% of the specification at minimum three points i.e. LOQ, 100% and 150%.

**i. Related substance and residual solvents:**

Recovery percentage is calculated by the following

\[
\% \text{ Recovery} = \frac{\text{corrected area of impurity} \times 100}{\text{Area of impurity}} \times \frac{\text{Area of impurity in spiked sample}}{\text{area of impurity in as such samples}}.
\]

**ii. Assay:**

Calculate % recovery by the following formula

\[
\% \text{ Recovery} = \frac{\text{Average area of A% solution} \times 100}{\text{Average area of the standard} \times \text{A%}}.
\]

A% = LOQ, 100%, 150%

**LINEARITY**
Definition
The ability to elicit test results that are directly or by well defined mathematical transformation proportional to the concentration of analyte in samples within a given range.
Linearity is usually expressed in terms of the variance around the slope of a regression line from test results obtained by the analysis with varying concentration of the analyte.

PRECISION
It covers i) system precision
   ii) Method precision.

i) System precision:
Prepare standard solution and inject six times the same solution as per the method.
Calculate % RSD for the result of six separate injectors.

ii) Method precision:
Prepare sample solution six times and analyze according to the method.
Calculate %RSD for the results of six individual preparations.

ROBUSTIONESS

Definition
The measure of capacity of an analytical procedure to remain unaffected by small but deliberate variation in method parameters and provides indication of its reliability during normal usage i.e. effects of flow rate, effect of column oven temperature, effect of mobile phase composition, effect of pH variation etc.....

RESULTS AND DISCUSSIONS:
The developed method will be validated as per ICH guidelines. The most fundamental requirements of an analysis are that it should be accurate and precise.
Accuracy is calculated interims of recovery studies.
The % recovery of the analyte in subsequent analysis is a measure of the accuracy of the method.
The precision of an analysis is often expressed as the ± relative standard deviation (±RSD)
\[
RSD = \frac{S}{X} \times 100
\]

**Systems suitability parameters:**
Chromatographic retention times are characteristic of the compounds they represent but are not unique.
Absolute retention times of a given compound vary from one chromatogram to the next comparisons are normally made in terms of relative retention, \( \alpha \) which is calculated by the equation
\[
\alpha = \frac{t_2 - ta}{t_1 - ta}
\]