STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR RELATED SUBSTANCES OF TESTOSTERONE UNDECANOATE IN CAPSULE DOSAGE FORM

A Dissertation submitted to

THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY
CHENNAI – 600 032

In partial fulfillment of the requirements for the award of the Degree of

MASTER OF PHARMACY
IN
BRANCH – III→ PHARMACEUTICAL ANALYSIS

Submitted by
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Under the guidance of
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CERTIFICATE

This is to certify that the project entitled **"STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR RELATED SUBSTANCES OF TESTOSTERONE UNDECANOATE IN CAPSULE DOSAGE FORM"** was submitted by **MOHANA KRISHNAN .S (261930005)** in partial fulfillment for the award of the Degree of **Master of Pharmacy (Pharmaceutical Analysis).** The project was carried out at Steril-Gene Life Sciences (P) Ltd., Villianur, Puducherry and at C.L. Baid Metha College of Pharmacy, Chennai- 600097 under my supervision in the Department of Pharmaceutical Analysis during the academic year 2019 – 2021.

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This is to certify that the project entitled **"ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR RELATED SUBSTANCES OF TESTOSTERONE UNDECANOATE BY RP-HPLC"** submitted in partial fulfillment of the requirement for the Degree of M. Pharm in **Department of Pharmaceutical Analysis in C.L.BAID METHA College of Pharmacy, Chennai 600097**. The above entitled research is a bonafide work carried out by **Mr. MOHANA KRISHNAN.S (Reg.No:261930005)** under my guidance during the academic year 2020-2021.

27/03/2021

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DECLARATION

I hereby declare that this dissertation entitled, **"STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR RELATED SUBSTANCES OF TESTOSTERONE UNDECANOATE IN CAPSULE DOSAGE FORM"** has been originally carried out by me at Steril-Gene Life Sciences (P) Ltd., Villianur, Puducherry and under the guidance and supervision of **Dr. C. N. Nalini, M.Pharm., Ph.D.,** Head of the Department of Pharmaceutical Analysis, C.L. Baid Metha College of Pharmacy, Chennai- 600097., during the academic year 2019 – 2021. This work has not been submitted in any other degree at any other university and that all the sources have used or quoted have been indicated and acknowledged by complete reference.

Date: **MOHANA KRISHNAN .S**

Place: Chennai **Reg. No: 261930005**

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INDEX

ABBREVIATIONS

NMT	:	Not More Than
NLT	:	Not Less Than
% RSD	:	Percentage Relative Standard Deviation
LR	:	Laboratory Reagent
AR	:	Analytical Reagent
GR	:	Guaranteed Reagent
ICH	:	International Council for Harmonization
BP	:	British Pharmacopoeia
TF	:	Tailing Factor
USP- NF	:	United States Pharmacopoeia - National Formulary
TP	:	Theoretical plates
WS	:	Working standard
Ppm	:	Parts per million
Ml	:	Milliliter
S.No	:	Serial number
AVG	:	Average
µg	:	Microgram
µm	:	Micrometer
HPLC	:	High performance liquid chromatography
NAP	:	Not Applicable
Mg	:	Milli gram

API	:	Active pharmaceutical Ingredients
PVDF	:	Polyvinylidenedifluoride
µl	:	Microliter
G	:	Gram
Hz	:	Hertz
Min	:	Minute
Mm	:	Millimeter
Hr	:	Hour
℃	:	Degree Celsius
pH	:	Potential of Hydrogen
RT	:	Retention Time
LOD	:	Limit of Detection
LOQ	:	Limit of Quantitation
%P	:	Percentage Purity
RRT	:	Relative Retention Time
RRF	:	Relative Retention Factor
%W/W	:	Percentage Weight per Weight
IP	:	Indian Pharmacopoeia
TU	:	Testosterone Undecanoate
TD	:	Testosterone Decanoate

1. INTRODUCTION

1.1. Analytical Chemistry

Analytical chemistry is defined as the science and art of determining the composition of materials in terms of the elements or constituents present in them. Analytical chemistry derives its principles from various branches of science like chemistry, physics, microbiology, nuclear science electronics, etc. and it deals with the scientific and technical aspects of measurement of compositional and constitutional features of the sample, etc. The prime concern of Analytical chemistry is quantitative and qualitative analysis. Analytical chemistry is also focused on improvements in experimental designs, chemometrics, and the creation of new measurement tools to provide better chemical information. Analytical chemistry has applications in forensics, bio-analysis, clinical analysis, environmental analysis, material analysis.[1,2]

1.2. Pharmaceutical Analysis

Pharmaceutical analysis can be defined as the series of processes that are used for identification, separation, quantitative estimation, purification, and structure elucidation of the given compound used in the formulation of pharmaceutical products.[3]

1.2.1. Types

There are two main types of chemical analysis.

- Qualitative Analysis
- Quantitative Analysis

1.2.1.1. Qualitative Analysis

To refer identity of the product, i.e. it yields useful clues from which the molecular or atomic species, the structural features, and the functional groups in the sample can be identified.[4]

1.2.1.2. Quantitative Analysis

To refer to the purity of the product, (i.e) the result in the form of numerical data corresponding to the concentration of analytes. In both analyses, the required information is obtained measuring a physical property that is characteristically related to the component of the interest analyte.

The most important aspect of the analysis is quantitative chemical analysis. In the present age, physical, chemical, and biological analysis involve computerized techniques to facilities better results.[5]

1.3. Types of Analytical Methods

- Chemical
- Physical
- Instrumental

1.3.1. Chemical methods

In these methods, volume and mass are used as means of detection

- Titrimetric methods-viz. acid, base, oxidation and reduction, non-aqueous, complexometric, and precipitation titration.
- Gravimetric and thermo gravimetric methods.[6]

1.3.2. Physical methods

- Refractive index
- X-ray crystallography
- Instrumental methods
- Spectroscopic methods
- Electrochemical techniques
- Thermal methods
- Chromatographic techniques

1.4. Chromatography

Chromatography is a laboratory technique for the separation of a mixture. The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus affect the separation.[7]

The different chromatographic techniques are

- Column chromatography
- Paper chromatography
- Thin layer chromatography
- Gas chromatography
- High Performance Liquid chromatography
- Supercritical Fluid Chromatography
- Affinity chromatography
- Ion exchange chromatography

1.5. High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) was developed in the late 1960s and early 1970s. HPLC is directly derived from classic column chromatography in that a liquid mobile phase is pumped under pressure rather than by gravity flow through a column filled with a stationary phase. Today it is widely applied for separations and purifications in a variety of areas including pharmaceuticals, biotechnology, environmental, polymer and food industries.

High-performance liquid chromatography is used to separate and quantify compounds or a mixture of compounds that have been dissolved in solution. It is a specific form of column chromatography generally used in biochemistry and analysis to separate, identify, and quantify the active compounds.[8]

1.5.1. Principle of HPLC

High performance liquid chromatography (HPLC)[9] is a separation technique utilizing differences in the distribution of compounds in phases called stationary phase and mobile phase. The stationary phase designates a thin layer created on the surface of fine particles and the mobile phase designates the liquid flowing over the particles. Under a certain dynamic solution, each component in a sample has a different distribution equilibrium depending on the solubility in the phases and the molecular size. As a result, the component moves at different speeds over the stationary phase and there by separated by each other.

1.5.2. Theories of HPLC

Two theories have been put forward regarding the rate of migration of solute and the development of peaks in the chromatogram.

1.5.2.1. Plate Theory

According to plate theory developed by Martin and Synge, a chromatographic column consists of a series of discrete yet continuous horizontal layers, which are termed the theoretical plates. The efficiency of separation in the chromatographic column gets increased as the number of theoretical plates increases. If the length of the column is L and the height equivalent of a theoretical plate is H, then N is given by

$$N = L / H$$

The height equivalent of a theoretical plate (HETP) refers to the height of a layer of the column, such that the solution leaving the layer is in equilibrium with the average concentration of the solute in the stationary phase throughout the layer.

1.5.2.2. Rate Theory

The rate theory can explain the effect of variables, such as mobile phase velocity and adsorb abilities, which determine the width of an elution band. It also relates the effects of these variables on the time taken by a solute to make its appearance at the end of the column. Migration of solute particles in a column occurs in a state of confusion, each solute molecule progressing in a stop and go sequence independent of any other molecule.[10]

1.5.3. Instrumentation of HPLC

1.5.3.1. Solvent Reservoir

Mobile phase contents are contained in a glass reservoir. The mobile phase, or solvent, in HPLC is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample.

1.5.3.2. Pump

A pump aspirates the mobile phase from the solvent reservoir and forces it through the system's column and detector. Depending on several factors including column dimensions, the particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressures of up to 42000 kPa (about 6000 psi) can be generated.

1.5.3.3. Sample Injector

The injector can be a single injection or an automated injection system. An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1-100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).

1.5.3.4. Columns

Columns are usually made of polished stainless steel, are between 50 and 300 mm long and have an internal diameter of between 2 and 5 mm. They are commonly filled with a stationary phase with a particle size of 3–10 µm.

Columns with internal diameters of less than 2 mm are often referred to as micro-bore columns. Ideally the temperature of the mobile phase and the column should be kept constant during an analysis.

1.5.3.5. Detector

The HPLC detector, located at the end of the column detects the analytes as they elute from the chromatographic column. Commonly used detectors are UV-spectroscopy, fluorescence, mass-spectrometric and electrochemical detectors.

1.5.3.6. Data Collection Devices

Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret.

Figure 1: Flow diagram of HPLC

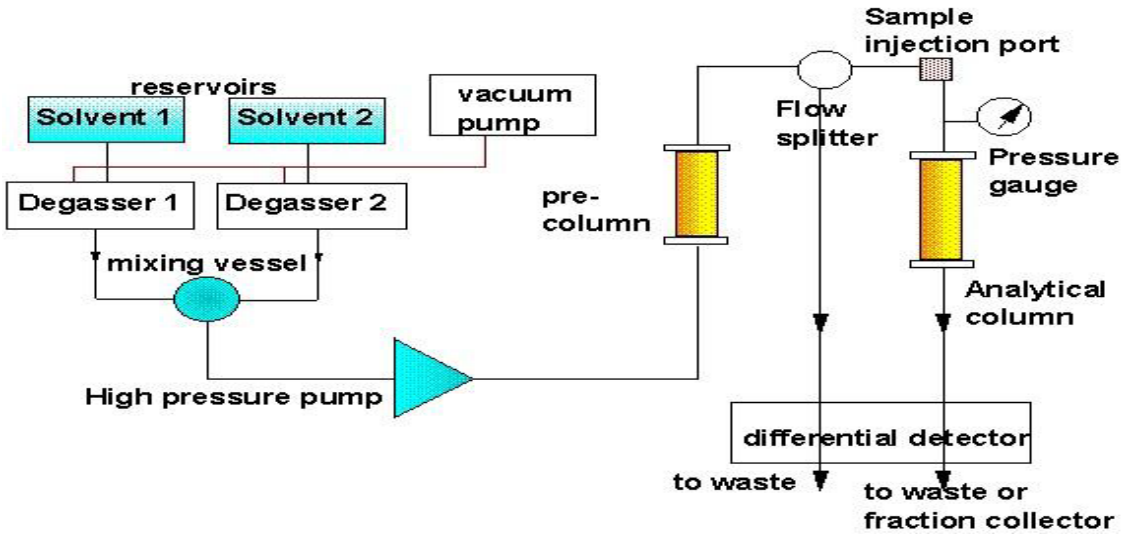


Figure 2: HPLC system

1.6. Analytical Method Development

HPLC is the separation module which contain mainly stationary phase and mobile phase having opposite polarity equipped with high pressure pumps and the separation is achieved by the interaction of stationary phase and the mobile phase. A proper choice of stationary phase and mobile phase is essential to reach desired separation. pH of mobile phase, different types of buffer, column temperature, sample diluents, detection wavelength and many more are the variables which play a major role in method development.[11]

During the preliminary method development stage, all individual components should be investigated before the final method optimization. This gives us a chance to critically evaluate the method performance in each component and to streamline the final method optimization. A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modeling.

There are several reasons for developing new methods of analysis:

- A suitable method for particular analyte in the specific matrix is not available.
- Existing methods may be too error or they may be unreliable (have poor accuracy or precision)
- Existing methods may be too expensive, time consuming.

The various parameters that include to be optimized during method development

- Mode of separation
- Selection of stationary phase
- Selection of mobile phase
- Selection of detector

1.6.1. Selection of mode of separation

In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds, the most preferred mode is reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation. A second factor is the nature of the matrix.

1.6.2. Selection of stationary phase / column

Selection of the column is the first and the most important step in method development. The appropriate choice of separation column includes three different approaches

- Selection of separation system
- The particle size and the nature of the column packing
- The physical parameters of the column i.e. the length and the diameter

The column is selected depending on the nature of the solute and the information about the analyte. Reversed phase mode of chromatography facilitates a wide range of columns like dimethylsilane (C2), butylsilane (C4), octylsilane (C8), octadecylsilane (C18), base deactivated silane (C18) BDS phenyl, cyanopropyl (CN), nitro, amino etc. Columns with 5-µm particle size give the best compromise of efficiency, reproducibility and reliability. Peak shape is equally important in method development. Columns that provide symmetrical peaks are always preferred while peaks with poor asymmetry can result in,

- Inaccurate plate number and resolution measurement
- Imprecise quantitation
- Degraded and undetected minor bands in the peak tail

A column which gives separation of all the impurities, degradants from each other and from analyte peak, which is rugged for variation in mobile phase shall be selected.

1.6.3. Selection of mobile phase

For a given stationary phase, the retention of the given solute depends directly upon the mobile phase, the nature and the composition of which has to be judiciously selected in order to get appropriate and required solute retention. The mobile phase has to be adapted in terms of elution strength (solute retention) and solvent selectivity (solute separation) Solvent polarity is the key word in chromatographic separations since a polar mobile phase will give rise to low solute retention in normal phase and high solute retention in reverse phase LC. Buffer and its strength play an important role in deciding the peak symmetries and separations.

1.6.4. pH of the buffer

pH plays an important role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics.

1.6.5. Mobile phase composition

Most chromatographic separations can be achieved by choosing the optimum mobile phase composition. This is due to that fact that fairly large amount of selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used solvents in reverse phase chromatography are Methanol and Acetonitrile. Experiments were conducted with mobile phases having buffers with different pH and different organic phases to check for the best separations between the impurities. A mobile phase gives separation of all the impurities and degradants from each other and from analyte peak and which is rugged for variation of both aqueous and organic phase by at least ±0.2% of the selected mobile phase composition.

1.6.6. Selection of detector

The selection of detector depends upon some characteristic property of the analyte like UV absorbance, fluorescence, conductance, oxidation, reduction etc. For the greatest sensitivity λmax should be used. UV wavelengths below 200 nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity.[12]

Table 1: Separation goals in brief

Goal	Comment
Resolution	Precise and rugged quantitative analysis requires that R_S be greater than 1.5
Separation time	< 5-10 min is desirable for routine procedures
Quantization	≤ 2% for assays ≤ 5% for less-demanding analyses ≤ 15% for trace analyses
Pressure	< 150 bar is desirable < 200 bar is usually essential (for a new column)
Peak height	Narrow peaks are desirable for large signal/noise ratios
Solvent consumption	Minimum mobile phase use per run is desirable

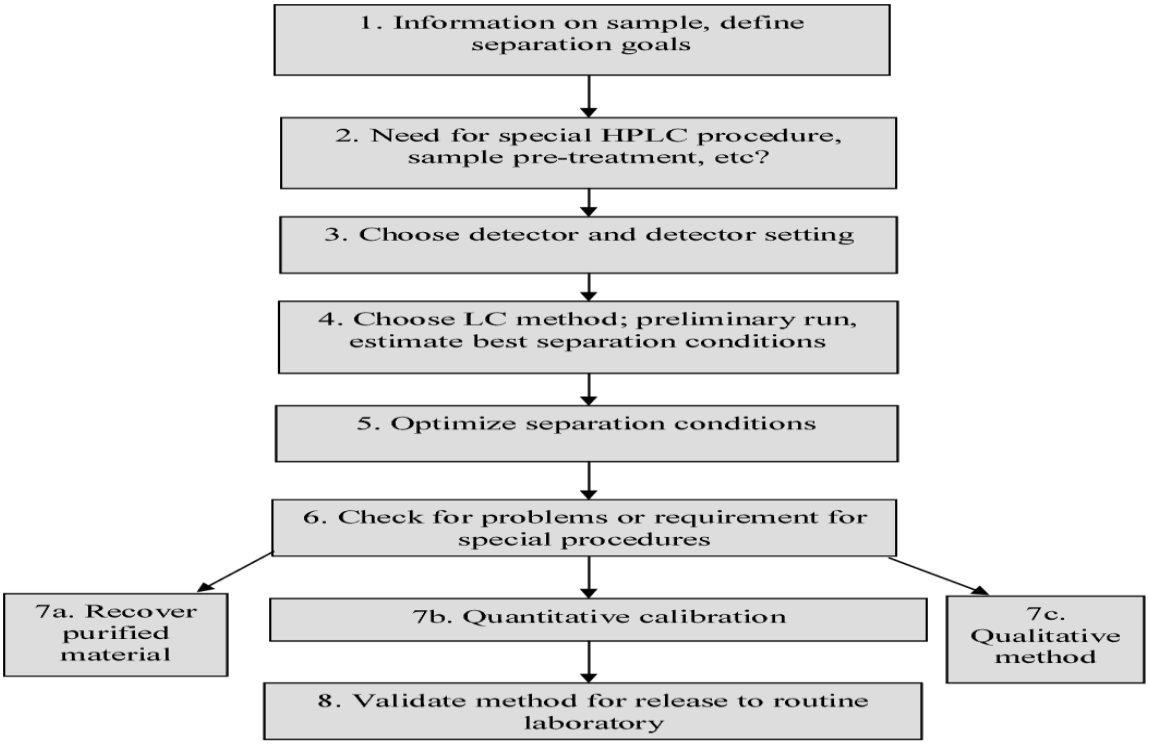


Figure 3: Flow chart of Method development

1.7. System Suitability

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. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated[13]. The parameters that are affected by the changes in chromatographic conditions are,

- Number of theoretical plates or Efficiency (N).
- Capacity factor (K).
- Separation or Relative retention (α).
- Resolution (Rs).
- Tailing factor (T).
- Relative Standard Deviation (RSD).

1.7.1. Number of theoretical plates / Efficiency (N)

In a specified column, efficiency is defined as the measurement of the degree of peak dispersion and it should have the column characteristics. The efficiency is conveyed in terms of number of theoretical plates. The formula of calculation of N is illustrated bellow in the following Figure 4.

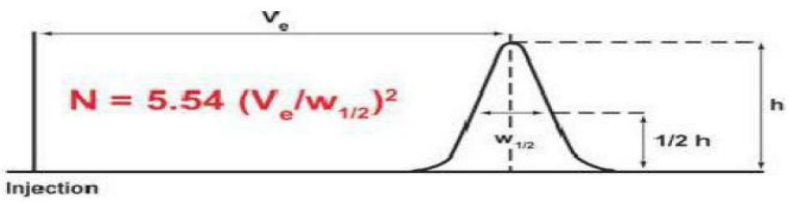


Figure 4: Half height method related to determination of N

Where,

N = Efficiency / Number of theoretical plates

Ve = Retention time of analyte

h = Height of the peak

W ½ = Gaussian function of the peak width at the half- height

The plate number depends on column length. Theoretical plate number is the measure of column efficiency. As stated by plate theory, the analyte will be in instant equilibrium with stationary phase and column has to be divided into number of hypothetical plates and each plate consists of a fixed height and analyte spends finite time in the plate. Height equivalent to theoretical plate (HETP) is given by following formula:

$$\mathbf{HETP = L/N}$$

Where, L = length of column

N = plate number

1.7.2. Capacity ratio (or) Capacity factor (k)

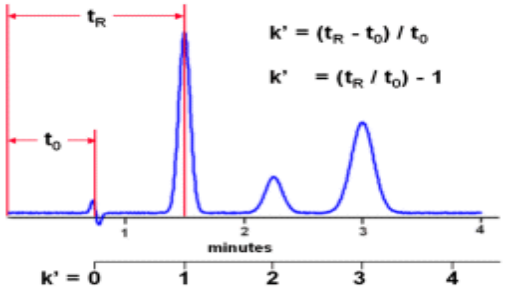


Figure 5: Capacity ratio (or) Capacity factor (k)

It is a measure of the retention of a peak that is independent of column geometry or mobile phase flow rate. The capacity factor is calculated as

$$k' = (t_R - t_0)/t_0$$

Where, t_R is the retention time of the peak

t_0 is the dead time of the column.

1.7.3. Relative retention (or) separation factor (α)

$$\alpha = t_2\text{-}t_a/t_1\text{-}t_a$$

Where,

α = Relative retention

t_2= Retention time calculated from point of injection

t_a= Unretained peak time (Retention time of an inert component not retained by the column)

t_1= the retention time from the point of injection of reference peak defined

(Suppose no reference peak is found, value would be zero)

1.7.4. Resolution (Rs)

Resolution is the capability of the column to separate 2 drugs in 2 individual peaks or chromatographic zones and it is improved by enhancing column length, reduction of particle size and rising temperature, altering the eluent or stationary phase. It can be told in terms of ratio of separation of the apex of two peaks by the tangential width average of the peaks. By using the following formula resolution is calculated.

$$RS = (tR_2\text{-}tR_1)/0.5(tW_1+tW_2)$$

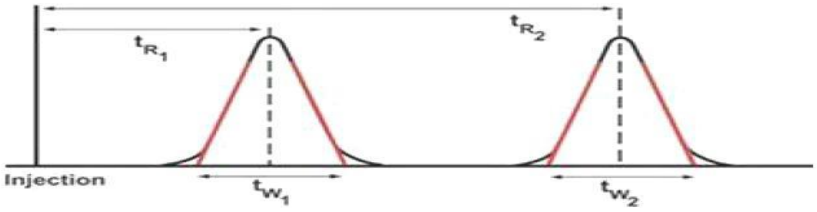


Figure 6: Determination of Resolution between two peaks

Where, tR1 and tR2 are the retention times for the two peaks of components tw1 and tw2 are the baseline lies between tangents drawn to the sides of the peaks. If the peaks are correctly symmetric, provided the valley between the two peaks should touch the baseline Rs is 1.5. Generally good value of resolution is Rs ≥2 should be adequate and preferred normally.

1.7.5. Resolution factor (R)

Resolution is a function of capacity factor, function of selectivity and a function of efficiency (or) number of theoretical plates (N). In order to separate any two peaks you must have right capacity factor ideally between 2 and 10, but appropriate selectivity is required i.e., ideally 1.2 and enough efficiency i.e., number of theoretical plates (more than 2000 theoretical plates). Resolution should be ≥ 1.5.

$$R=k'/1+k' (\alpha-1/ \alpha) (\sqrt{} (N/4)$$

1.7.6. Tailing factor (or) Asymmetry factor

The tailing factor is a measure of peak tailing. It is defined as the distance from the front slope of the peak to the back slope divided by twice the distance from the centre line of the peak to the front slope, with all measurements made at 5% of the maximum peak height. The tailing factor of a peak will typically be similar to the asymmetry factor for the same peak, but the two values cannot be directly converted.[14,15]

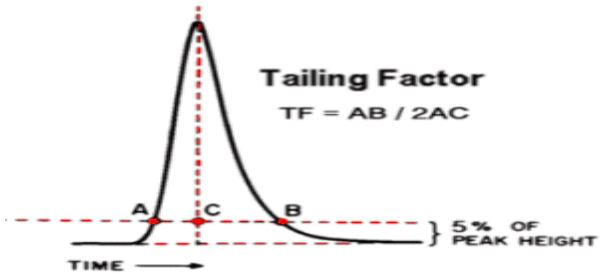


Figure 7: Tailing Factor

1.7.7. Relative standard deviation (RSD)

Relative standard deviation is a measure of the spread of data in comparison of the data. It is simply the standard deviation divided by the mean value.

$$\mathbf{RSD} = \frac{\mathbf{S(xi)}}{\mathbf{X}}$$

Table 2: Acceptance criteria for system suitability parameters

S.No	Parameter name	Acceptance criteria (USP)
1	Number of theoretical plates or Efficiency (N)	> 2000
2	Capacity factor (K)	< 1
3	Separation or Relative retention (α)	> 1
4	Resolution (Rs)	> 1.5
5	Tailing factor or Asymmetry(T)	< 2
6	Relative Standard Deviation (RSD)	< 2

1.8. Analytical Method Validation

Validation is a key process for effective quality assurance. “Validation is established documented evidence, which provides specific a high degree of assurance that a process of equipment will consistently produce a product or result meeting its predetermined specifications and quality attributes”.

USFDA defines validation as “established documented evidence which provides a high degree of assurance that a specific process will consistently produce a product of predetermined specifications and quality attributes”.

EUGMP defines validation as “action of proving in accordance with the principle of Good manufacturing practice (GMP), that any material activity or system actually lead to expected result”.

AUSTRALIAN GMP defines validation as “the action of proving that any material, process, procedure, system, equipment or mechanism used in manufacture or control can and will be reliable and achieve the desire and intended result”.

Analytical method is a process of proving that the method was acceptable for laboratory use to measure the sample concentration within GLP environment and acceptance criteria in ICH guidelines Q2(R1).[16]

1.8.1. Validation parameters

The developed method was validated according to ICH guidelines [17,18]

- Accuracy
- Precision (Method Precision & Intermediate Precision)
- Robustness
- Specificity
- Linearity
- Range
- Recovery
- Solution stability
- Filter integrity

1.8.1.1. Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose "true value" is known) is analyzed and the measured value is identical to the true value. Typically, accuracy is represented and determined by recovery studies. There are three ways to determine accuracy:

- Comparison to a reference standard.
- Recovery of the analyte spiked into blank matrix.
- Standard addition of the analyte.

It should be clear how the individual or total impurities are to be determined.

1.8.1.2. Precision

The precision of an analytical procedure represents the nearness of agreement between a series of measurements got from multiple sampling of the same homogenous sample under the similar analytical conditions and it is divided into 3 categories.

- Repeatability: precision under same operating conditions, same analyst over a short period of time.
- Intermediate precision: method is tested on multiple days, instruments, analysts etc.
- Reproducibility: inter-laboratory studies.

The ICH guidelines suggest that repeatability should be conformed duly utilizing at least 9 determinations with specified range for the procedure (e.g., three concentrations / three replicates each) or a minimum of 6 determinations at 100 % of the test concentration.

1.8.1.3. Linearity and Range

The linearity of a method is a measure of how well a calibration plot of response vs. concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data is then processed using a linear least-squares regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity.

1.8.1.4. Specificity

One of the significant features of HPLC is its ability to generate signals free from interference. Specificity refers to the ability of the analytical method to differentiate and quantify the analyte in complex mixtures. An investigation of specificity is to be conducted during the determination of impurities and validation of identification tests. An ICH guideline defines specificity as ability to assess unequivocally the analyte in the presence of other compounds that may be likely to be present. Typically these might be impurities, degradants, matrix, etc. The definition has the following implications:

- Identification test: Identification tests should be able to differentiate compounds of closely related structure which are expected to be present i.e., to assure identity of an analyte.
- Purity test: To ensure that the analytical procedure performed allows an accurate statement of content of the impurity of an analyte i.e. related substances, residual solvents content, heavy metals, etc.
- Assay: To arrive at an accurate result, this permits a correct report on the potency or content of analyte in a sample.

1.8.1.5. Robustness

Robustness is defined by the measure of the capability of an analytical method to stay unchanged by small deliberate changes in method parameters. The variable method parameters in HPLC technique may involves flow rate, column temperature, sample temperature, pH and mobile phase composition.

1.8.1.6. Solution Stability

The solution stability is stability of standard and extracted sample solution (ready to inject) from the sample or matrix and analyzed as per specified method, and it should be stored properly in room temperature and refrigerated condition depending upon the stability of the sample and standard solution. The stability of standard and sample solution should be established in room temperature and refrigerated, if refrigerated before analyzing it should be thawing to room temperature. The analyzed solutions stored in necessary condition and the stability can be established for two days or solution stability can be established by an hour basis depending upon the nature of the product.[19]

1.9. Stability Indicating Related substances Method

For dosage form monographs, the main purpose of a test for related substances is to control degradation impurities. However, the objective is to limit impurities arising during storage of the drug substance/product.[20]

1.9.1. Related substances

Related substances are structurally related to a drug substance. These substances may be identified or unidentified degradation products or impurities arising from a manufacturing process or during storage of a material.[21]

1.9.2. Impurity

As per ICH guideline Q3A impurity in a drug substance is ―any component of the drug substance that is not the chemical entity defined as the drug substance[22] and as per ICH guideline Q3B impurity in a drug product is ―any component of the drug product that is not the chemical entity defined as the drug substance or an excipient in the drug product[23].

1.9.2.1. Classification of Impurities

As per ICH guidelines Q3A and Q3B Impurities can be classified as:

- Organic impurities
- Inorganic impurities
- Residual solvents

Table 3: Thresholds for reporting Impurities[24]

Maximum daily dose	Reporting threshold	Identification threshold	Qualification threshold
Less or equal to 2 g/day	0.05%	0.10% or 1.0 mg/day (whichever is lower)	0.15% or 1.0 mg/day (whichever is lower)
>2 g/day	0.03%	0.05%	0.05%

1.9.3. Degradation products

Degradation products[25] are defined as ―a molecule resulting from a change in the drug substance brought about over time. Through stability testing of the products, these could occur as a result of storage or processing by means of certain factors (oxidation, deamination, proteolysis & aggregation) and it may also occur as a result of stress testing /forced degradation studies by maintaining stress conditions.

Table 4: Thresholds for reporting Degradation products

Maximum daily dose	Reporting threshold	Identification threshold	Qualification threshold
≤1 mg	-	1.0% or 5 µg TDI (whichever is lower)	0.15% or 1.0 mg/day (whichever is lower)
1 mg–10 mg	-	0.5% or 20 µg TDI (whichever is lower)	0.05%
10 mg–100 mg	-	-	0.5% or 200 µg TDI (whichever is lower)
<10 mg	-	-	1.0% or 50 µg TDI (whichever is lower)
>10 mg–2 g	-	0.2% or 2 mg TDI whichever is lower	-
>100 mg–2 g	-	-	0.2% or 3 mg TDI (whichever is lower)
≤1 g	0.1%	-	-
>1 g	0.05%	-	-
>2 g	-	0.1%	-
>2 g	-	-	0.15%

1.10. Stress testing / Forced degradation studies

The ICH guideline states that stress testing is intended to identify the likely degradation products which further helps in determination of the intrinsic stability of the molecule and establishing degradation pathways, and to validate the stability indicating procedures used. [26]

Forced degradation studies are carried out to achieve the following purposes:[27, 28]

- To establish degradation pathways of drug substances and drug products.
- To differentiate degradation products that is related to drug products from those that are generated from non-drug product in a formulation.
- To elucidate the structure of degradation products. To determine the intrinsic stability of a drug substance in formulation.
- To reveal the degradation mechanisms such as hydrolysis, oxidation, thermolysis or photolysis of the drug substance and drug product.
- To establish stability indicating nature of a developed method. To understand the chemical properties of drug molecules.
- To generate more stable formulations.
- To produce a degradation profile similar to that of what would be observed in a formal stability study under ICH conditions.
- To solve stability-related problems.

Table 5: Conditions mostly used for Degradation studies[29]

Degradation type	Experimental conditions	Storage conditions	Sampling time (days)
Hydrolysis	Control API (no acid or base)	40°C, 60°C	1,3,5
	0.1 M HCl	40°C, 60°C	1,3,5
	0.1 M NaOH	40°C, 60°C	1,3,5
	Acid control (no API)	40°C, 60°C	1,3,5
	Base control (no API)	40°C, 60°C	1,3,5
	pH: 2,4,6,8	40°C, 60°C	1,3,5
Oxidation	3% H_2O_2	25°C, 60°C	1,3,5
	Peroxide control	25°C, 60°C	1,3,5
	Azobisisobutyronitrile (AIBN)	40°C, 60°C	1,3,5
	AIBN control	40°C, 60°C	1,3,5
Photolytic	Light 1 ~ ICH	NA	1,3,5
	Light control	NA	1,3,5
Thermal	Heat chamber	60°C	1,3,5
	Heat chamber	60°C/75% RH	1,3,5
	Heat chamber	80°C	1,3,5
	Heat chamber	80°C /75% RH	1,3,5
	Heat control	Room temp.	1,3,5

2. DRUG PROFILE

Testosterone Undecanoate

IUPAC name : [(8R,9S,10R,13S,14S,17S)-10,13-dimethyl-3-oxo-1,2,6,7,8,9,11,12,14,15,16,17 dodecahydrocyclopenta [a] phenanthren-17-yl] undecanoate

Synonyms : Andriol, Nebido, Pantestone, Restandol, testosterone undecylate, Undestor

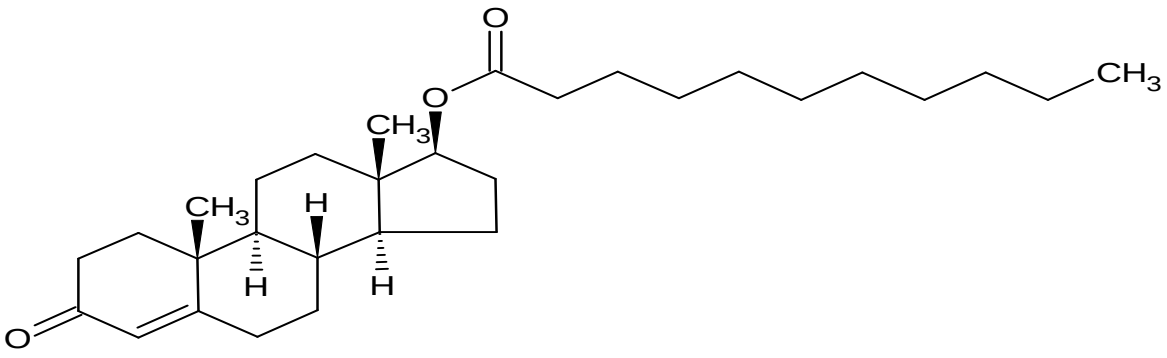
Molecular formula : $C_{30}H_{48}O_3$

Molecular Weight : 456.711 g/mol

CAS number : 5949-44-0

Description : Testosterone Undecanoate is the undecanoate ester form of the androgen testosterone, with gonadotropin-secretory inhibiting & hormone replacement activity[30].

Structure:



Therapeutic group : Androgen, Anabolic steroid[31]

Dosage forms : Injection, Capsule[32]

Route of administration : Oral, Intramuscular[31]

Physical & Chemical Properties

Appearance	:	White to off white crystalline powder
Solubility	:	Practically insoluble in water[30,32], soluble in methanol, freely soluble in chloroform, dioxane and methylene chloride
Melting point	:	64 °C
Dissociation constant (pKa)	:	11.1
Log P	:	9.15[33]

Pharmacokinetic Properties

Absorption

The absorption of Testosterone Undecanoate varies based on the formulation. The intramuscular formulation of testosterone esters is suspended in oil and is absorbed from the lipid phase. Testosterone is released when tissue esterases cleave the undecanoic acid side chain. The oral formulation of Testosterone Undecanoate is also formulated as a prodrug, is best absorbed with food and ideal absorption occurs when taken with a meal containing at least 30 g of fat.[30,32]

Protein binding

Circulating testosterone is highly protein bound with about 40% bound to sex hormone-binding globulin (SHBG), and a large percentage of the remaining hormone loosely bound to albumin and other plasma proteins. Only about 2% of testosterone is unbound.[32]

Bioavailability: 6.83±3.32%[34]

Metabolism

The side chain of Testosterone Undecanoate is cleaved by non-specific esterases when it enters circulation and the undecanoic acid side chain is metabolized by the beta-oxidation pathway. The resulting Testosterone molecule is then metabolized to dihydrotestosterone (DHT) by the enzyme 5-alpha reductase. DHT is reduced by 3-alpha-

hydroxysteroid dehydrogenase (major) and 3-beta-hydroxysteroid dehydrogenase prior to being glucuronidated and cleared by the kidneys. It should be noted that testosterone is metabolized to several other 17-keto steroids in the body.[30]

Hepatic clearance

The hydrolysis–time profiles of TU incubation in human liver microsomes and Caco-2 cell homogenate were used to predict hepatic first-pass metabolism. By applying the "well-stirred" model, the fraction of TU that could escape hepatic first-pass metabolism was predicted as 0.915 ± 0.009. Hence 91% of the absorbed fraction of Testosterone Undecanoate can escape through hepatic metabolism.[35]

Excretion

The majority (~90%) of an intramuscularly administered dose of testosterone is conjugated and eliminated in the urine. Approximately 6% of the dose is eliminated primarily unconjugated in the faeces.[30]

Elimination half-life

Testosterone Undecanoate has a very long elimination half-life and mean residence time when given as a depot intramuscular injection. Its elimination half-life is 20.9 days and its mean residence time is 34.9 days in tea seed oil, while its elimination half-life is 33.9 days and its mean residence time is 36.0 days in castor oil.[31]

Pharmacodynamic properties

Testosterone plays a key role in male sexual differentiation and is involved in regulation of hematopoiesis, body composition, and bone metabolism. As a result, testosterone replacement therapy in males with hypogonadism can result in improved sexual function, increased lean body mass, bone density, erythropoiesis, prostate size, and changes in lipid profiles.[32,36]

Mechanism of action

Testosterone is produced by Leydig cells and exert its effects by binding to androgen receptors throughout the body. Testosterone affects the voice, genitalia, mood, and influences muscle growth and protein expression. Accordingly, males with low levels of testosterone

often experience decreased libido, fatigue, mood changes and dysphoria. Exogenous sources of testosterone are designed to mimic the effects of endogenous testosterone.[32,37]

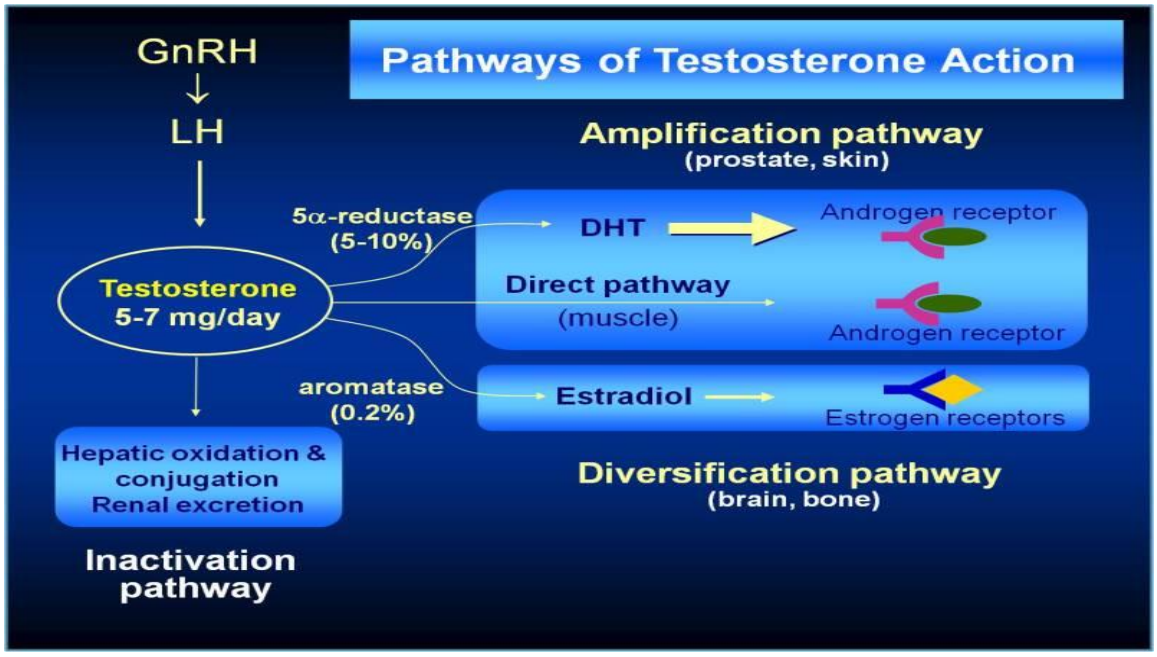


Figure 8: Mechanism of Action

Adverse effects

Virilization or Masculinization.[31,38]

Common side effects

Nausea, vomiting, Headache, skin color changes, increased/decreased sexual interest, oily skin, hair loss, and acne.

Uses

This medication is used by men who have low or no testosterone due to certain medical conditions (such as hypogonadism). It helps the body to develop and maintain male sexual characteristics (masculinity), such as a deep voice and body hair. It also helps to maintain muscle and prevent bone loss, and is needed for natural sexual ability/desire.[39]

3. REVIEW OF LITERATURE

Atheer Zgair, et al. (2021) predicted the esterase-inhibitory effect of natural fruit extract of strawberry (STW) on the intestinal degradation of Testosterone Undecanoate as a potential approach of increasing the oral bioavailability of Testosterone. The hydrolysis of TU was assessed in fasted state simulated intestinal fluid with added esterase activity (FaSSIF/ES) and Caco-2 cell homogenates in the presence of STW extract. The determination of TU concentrations was performed using Waters Alliance HPLC-UV system. Analytes were separated on ACE C18 (4.6 mm ID × 10 cm) column at 50°C. The mobile phase was an isocratic mixture of ACN and water (96:04; v/v). The flow rate was set at 0.5 mL/min and the absorbance was monitored at 240 nm for 20 mins. Empower TM 2 software was used for data processing. The elution time of TU was 11.3 min. The analytical method was validated for selectivity, accuracy, and precision in accordance with the FDA Guidance for Bio-analytical Method Validation. The lower limit of quantification was 0.2 µM. Inter- and intra-day precision and accuracy were below 15% RSD and RE, respectively.[40]

Monica Butnariu, et al. (2020) developed a Spectrophotometric and chromatographic strategies for exploring the nanostructure pharmaceutical formulations containing Testosterone Undecanoate. The chromatography study was made using JASCO HPLC apparatus. For this study of the parent compound and possible impurities, a reverse C18 (250 × 4.6 mm) stationary phase column was used. As mobile phase absolute methanol purity from MERCK has been used. Flow passage of mobile phase through the column was 1 mL/min. Monitoring of the eluent was carried out at 240 nm, due to the fact that, in alcoholic environment at this wave-length the parent compound presents a maximum optical absorption. In the working conditions choosing the eluent was justified because the interest component showed a good retention (6.7 min). Quantification was done with injection volume of 10 µL sample with concentration 4000 mg. The experiments were carried out in triplicate.[41]

Ronald S. Swerdloff, et al. (2020) determined a new Oral Testosterone Undecanoate Formulation that restores Testosterone to normal Concentrations in Hypogonadal men using LC-MS/MS. Patients were randomized 3:1 to oral TU, BID (JATENZO® ; n=166) or a topical T product QD (Axiron® ; n=56) for 3-4 months. Dose titration was based on average T levels (C_{avg}) calculated from serial pharmacokinetic (PK) samples. T was assayed by LC-MS/MS. Patients had two dose adjustment opportunities prior to final PK visit. Safety was

assessed by standard clinical measures, including ambulatory BP. 87% of patients in both groups achieved mean T C_{avg} in the eugonadal range. NaF-EDTA plasma T C_{avg} for oral TU group was 403 ± 128 ng/dL (~14 ± 4 nmol/L; mean ± SD) [serum T equivalent ~ 489 ± 155 ng/dL (17 ± 5 nmol/L)] and for topical T was 391 ± 140 ng/dL (~14 ± 5 nmol/L). Modeling/simulation of T PK data demonstrated that dose-titration based on a single blood sample 4-6 hours after oral TU dose yielded efficacy (93%) equivalent to C_{avg}-based titration (87%). Safety profiles were similar in both groups, but oral TU was associated with a mean increase in systolic BP of 3-5 mm Hg.[42]

Pieter Van Renterghem, et al. (2020) validated an ultra-sensitive detection method for steroid esters in plasma for doping analysis using positive chemical ionization GC-MS/MS. The analytical instrumentation used was a GC 7890 GC coupled with a 7000C triple quadrupole (MS/MS) from Agilent mounted with a multipurpose sampler (MPS2) from Gerstel. Splitless injection volume was 2.5 µl in a clean double tapered splitless liner without glass wool at 270°C. It was used as carrier gas at constant flow of 2 ml/min. The GC column was an HP5MS from Agilent (Palo Alto, US) and measured 12 m x 250 µm inner diameter and 0.25 µm film thickness. Temperature program was as follows: initial temperature 110°C (0.45min), ramped with 75°C/min to 235°C, 10°C/min to 300°C and 70°C/min to 315°C, which is kept for 0.5 min. Total run time was 9.3 including a solvent delay of 2.5 min. Transfer line temperature was set at 310°C and the CI source was set at 280°C which operated with ammonia as reagent gas. The triple quadrupole was operated with N_2 collision gas with a flow of 1.5 ml/min and He as quenching gas with a 2.25 ml flow. Tuning of the quadrupoles was performed using methane gas.[43]

Atheer Zgair, et al. (2020) predicted the Intestinal and Hepatic First-Pass Metabolism of Orally Administered Testosterone Undecanoate using human liver microsomes and Caco-2 cell homogenate. The concentrations of TU in microsomal mixtures and Caco-2 cell homogenates were determined by Waters Alliance 2695 HPLC system equipped with Waters 996 photodiode-array detector. Separation of analytes was carried out with an ACE C18 column (100 × 4.6 mm, 5 µm particle size; Hichrom Ltd., Reading, UK), coupled with an ACE C18 3 µm guard column. Samples and column temperatures were maintained at 5 °C and 50 °C, respectively. The mobile phase, consisting of acetonitrile and water (96:04: v/v), was run at a flow rate of 0.5 mL/min for 20 mins. The detector wavelength was set at 240 nm. HPLC data were integrated by Empower TM 2 software.[35]

Amber Bharti, et al. (2019) quantified the potential impurities present in Testosterone Undecanoate active pharmaceutical ingredient by stability indicating HPLC method using UV detector. The chromatographic separation of potential impurities and degradation products were achieved in YMC pack C8 column (150 mm x 4.6 mm, 5 µm) using gradient elution method. For the gradient elution method, mobile phase-A and Mobile phase-B was used. Mobile phase-A was prepared using water and acetonitrile mixture in a ratio of (90:10, v/v) and mobile phase-B was only acetonitrile. Column temperature was kept at 35°C throughout the analysis. Wavelength of 240 nm were selected for the analysis. The current developed method is specific, linear, precise, and accurate. Specificity of the method was confirmed by peak purity analysis using photodiode array detector. Testosterone is the major degradant. The validation study was done as per the current ICH guidelines.[44]

Md Didarul Islam, et al. (2018) developed and validated an analytical method for Testosterone Undecanoate Soft Gelatin Capsule by RP-HPLC. During validation active pharmaceutical ingredient (API) has been separated by C18 (4.6 mm × 250 mm, 5 µm) column, 100% methanol as mobile phase, flow rate of 0.8 ml/min and detection wavelength at 240 nm. The method was validated according to USP and ICH guideline requirements which includes specificity, accuracy, precision, linearity and range and robustness. Linearity of standard spiked sample was observed for each working day and coefficient of determination (r^2) has been found >0.99 each day in concentration ranging from 20-60 ppm. Recovery was found from 98.87-100.02% for 20, 40 and 60 ppm of Testosterone Undecanoate spiked sample. Precision and intermediate precision showed that % RSD of test sample solution were 0.26 and 0.19 respectively and absolute difference between them was 0.52, all of the values were within acceptable limit. The method was also found robust in changing column oven temperature (± 5°C) and flow rate change (± 0.1).[45]

Kishor R More, et al. (2017) identified and characterized Process Related Impurity of Male Hormone Testosterone Undecanoate. An unknown impurity observed in API testosterone undecanote was detected by Liquid chromatography–mass spectrometry (LC-MS) and data revealed that the molecular weight of the impurity is two protons less than the API indicating presence of one additional double bond in the structure. Further the position of the double bond was confirmed by its synthesis. It is found to be the process related impurity. The liquid chromatography-heated electrospray ionization-tandem mass spectrometry (LC-HESI-MS/MS) analysis was carried out on Q Exactive orbitrap mass spectrometer, which was used to achieve high-resolution accurate mass spectral data. The LC unit was consisted of an

Ultimate 3000 quaternary gradient pump with a degasser and auto sampler. A Spherisorb C8 column (250 × 4.6 mm i.d., 5 µm particles) was used for chromatographic separations. The mobile phase composed of 0.77 g ammonium acetate dissolved in 1000 ml of water (A) and acetonitrile (B) in a gradient mode (Tmin/A:B; T0 /94:06; T4 /94:06; T45/70:30; T50/94:06). The flow rate was set to 1.0 ml per minute with UV detector wavelength was fixed at 210 nm. The sample solution (500 ppm) was prepared in mobile phase and 10 µl was injected. Mass parameters were set as; spray voltage was kept at 4.0 kV and capillary temperature at 320°C. Nitrogen was used as both sheath and auxiliary gas. Mass range was kept at m/z 100-1000. MS/MS studies were carried out by maintaining normalized collision energy at about 15% with the mass range m/z 50-750.[46]

Zhou Cheng, et al. (2016) developed a dissolution method for Testosterone Undecanoate Capsule by the method specified in China pharmacopoeia. The dissolution was determined by HPLC. The HPLC column was phenomenex C18 column (250 mm×4.6 mm, 5µm). The mobile phase was 2-propanol-acetonitrile-water (45:45:10,V/V/V) with a flow rate of 1.0 ml/min and the detection wavelength of 240 nm. The column temperature was 40℃ and the injection volume was 20 µl. The average recovery of the method was about 100.55% (n=9). The calibration curves showed good linearity (r^2=0.9999, n=7) within ranges of 1-50 µg/ml. The method is convenient and sensitive in the dissolution determination of Testosterone Undecanoate capsule.[47]

Guro Forsdahl, et al. (2015) detected testosterone esters in blood. In doping control, the detection of an intact ester of testosterone in blood gives unequivocal proof of the administration of exogenous testosterone. Blood was collected throughout a testing period of 60 days. The applied analytical method for blood analysis included liquid-liquid extraction and detected using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The samples were analyzed using a CTC HTS PAL autosampler and an Aria Transcend TLX-1 LC system interfaced to a TSQ Vantage triple quadrupole. A Zorbax Eclipse XDB-C8 precolumn (2.1 × 2.5 mm, particle size 5 µm) was used for sample clean-up and the analytical HPLC column was an X-Bridge C8 (4.6 × 50 mm, particle size 2.5 µm). Column selection was performed by a Maylab Mistra switch column selector. All analytical conditions were set as previously described for the assay. The solvents were 0.2% formic acid in water (A) and methanol containing 0.1% formic acid (B) and the eluent was diverted from the MS/MS to waste for the first 7 min following each injection. Detailed chromatographic conditions have been previously described. The mass spectrometer was equipped with a heated electrospray

ionization (ESI) source and was operated in positive ionization mode with a spray voltage set at 3000 V. The capillary temperature was adjusted to 300 °C. The sheath and auxiliary gas (nitrogen) flow rate was 35 and 15 arbitrary units, respectively. The system was operated in selected ion monitoring (srm) mode with argon as the collision gas at a pressure of 1.5 mTorr.[48]

Sara Odoardi, et al. (2015) determined and quantified anabolic steroids and clenbuterol at trace levels in dietary supplements by liquid chromatography-high-resolution mass spectrometry (LC-HRMS) in atmospheric pressure ionisation (APCI) mode using a single-stage Orbitrap analyser operating at a resolution power of 100,000 full width at half maximum (FWHM). The LC-HRMS system was composed of a Thermo ULTIMATE 3000 system equipped with an analytical column Thermo Acclaim RSLC 120 C18 (2.1 mm × 100 mm, 2.2 μm particle size), coupled to a Thermo single-stage Orbitrap (Exactive) MS system. The whole equipment and the column were provided by Thermo Fisher Scientific. Mobile phase A was a mixture of ultrapure water/methanol 90/10 v/v with 0.1% formic acid; mobile phase B was a solution of methanol with 0.1% formic acid. The analytical column was maintained at 40°C and sample injection volume was 10 μl. The flow rate was set at 400 μl min^{-1}. Mobile phase gradient was as follows: 60% A for 1 min, linear gradient to 100% B in 7 min, held for 5.0 min, column re-equilibration was performed with linear gradient to 60% A in 3.0 min, held for 3.0 min. The APCI source was heated at 400°C. Source current was 6 μA, sheath gas and auxiliary gas (either nitrogen) flow rates were 35 and 15 arbitrary units, respectively; capillary temperature was 290°C. This method was fully validated Limits of detection (LODs) obtained for anabolic androgenic steroids (AASs) varied from 1 to 25 ng g−1 and the limit of quantitation (LOQ) was 50 ng g−1 for all analytes. The calibration was linear for all compounds in the range from the LOQ to 2000 ng g−1 , with correlation coefficients always higher than 0.99. Accuracy (intended as %E) and repeatability (%CV) were always lower than 15%.[49]

Sylvain Lachance, et al. (2015) developed and validated a LC-MS/MS method for measuring Testosterone, Testosterone Undecanoate and Dihydro Testosterone Undecanoate in enzyme-inhibited plasma for oral Testosterone Undecanoate androgen replacement therapy clinical trials. The mobile phase consisted of Milli-Q water/methanol 10/90 ammonium formate 5 mM, formic acid 0.1% and column of Waters BEH Phenyl, 50 × 3 mm, 1.7 μm using the AB SCIEX API 5000 LC-MS/MS. Positive ionization modes using the Turbo Ion Spray were optimized with the mass transitions 457.4→271.3 amu for TU, 476.5→273.3 amu for DHTU,

462.5→276.3 amu for TU-d5 and 497.6→273.3 amu for DHTU-d21. The methods were validated as per the most recent USFDA and EMA validation guidelines. During the method validation, the accuracy, precision, within run, between-run, selectivity, matrix effect as well as the stability (stability in whole blood at 4°C, short term stability in matrix at room temperature, freeze–thaw stability at -20°C/-80°C, long-term stability at -20°C/-80°C) were evaluated.[50]

Mario Thevis, et al. (2014) used Dried Blood Spots (DBS) in doping control analysis of anabolic steroid esters. Dried blood spot (DBS) sampling, a technique for whole blood sampling on a piece of filter paper, has more than 50-years tradition, particularly in the diagnostic analysis of metabolic disorders in neonatal screening. Due to the minimal invasiveness, straight forwardness, robustness against manipulation and fastness, DBS sampling recommends itself as an advantageous technique in doping control analysis. This present approach highlights the development of a screening assay for the analysis of eight anabolic steroid esters including Testosterone Undecanoate in DBS. The detection of the intact esters allows an unequivocal proof of the administration of conjugates of exogenous testosterone and its derivatives. Precise, specific and linear conditions were obtained by means of liquid chromatography high resolution/high accuracy mass spectrometry. LC–MS/MS analysis was performed with a Thermo Dionex Ultimate 3000 liquid chromatograph interfaced to a Q Exactive Plus mass spectrometer, using a heated electrospray ionization (HESI-II) source. The Dionex system was equipped with an Accucore XL C8 analytical column, 3 mm × 100 mm, 4 µm particle size. The eluents consisted of A: formic acid (0.2%), and B: methanol. A gradient program was used, starting with 40% B, raising to 100% B in 11 min, followed by an isocratic step for 2 min and re-equilibration at starting conditions for 3 min. The total run time was 16 mins, applying a flow rate of 600 l/min. Measurements were conducted in positive ion mode. The HESI source temperature was adjusted to 450°C, the temperature of the transfer capillary was set to 300°C and the applied spray voltage was 4 kV. Nitrogen generated by a CMC nitrogen generator was used for collision-induced dissociation experiments in the HCD cell and furthermore as damping gas in the curved linear ion trap. The resolution of the mass spectrometer was set to 17,500 full width at half maximum (FWHM) (at m/z 200), and the precursor isolation window was adjusted to 1.5 Da. The system was operated in targeted MS/MS mode with optimized collision energies.[51]

Sabina Strano-Rossi, et al. (2013) screened exogenous androgen anabolic steroids in human hair by liquid chromatography/orbitrap-high resolution mass spectrometry. Exactive

benchtop Orbitrap mass spectrometer, has been used here and validated. This method involved methanolic incubation of 30 mg of hair and analysis of the relevant extract in the LC–HRMS system composed of Thermo ULTIMATE 3000 system equipped with an analytical column Thermo Acclaim RSLC 120 C18 (2.1 mm × 100 mm, 2.2 m particle size), coupled to a Thermo single-stage Orbitrap (Exactive) MS system, interfaced with an APCI source. Mobile phase A was ultrapure water/methanol 90/10 with 0.1% formic acid, mobile phase B was methanol with 0.1% formic acid. All solvents used were LC–MS grade. The analytical column was maintained at 40°C and sample injection volume was 10 L. The flow rate was set at 400 L min−1. Mobile phase gradient was as follows: 60% A for 1 min, linear gradient to 100% B in 7 min, held for 5.0 min, column re-equilibration was performed with linear gradient to 60% A in 3.0 min, held for 3.0 min. The mass detector, with nominal resolving power of 100,000, operated in full scan mode in APCI under positive ionization mode. Analytes were identified by exact mass, correspondence of isotopic cluster and retention times. The limits of detection obtained varied from 10 to 50 pg mg−1, and limits of quantitation were 0.5 ng mg−1 for all compounds. The method was linear for all analytes in the ranges from the LOQ to 6 ng mg−1, giving correlation coefficients >0.99 for all analytes. Also accuracy (intended as %E) and repeatability (%CV) were always lower than 15%. Specificity was assessed by analysing ten blank samples and fifteen samples from drug abusers. This method was applied to a real-life case, resulting in the identification of Testosterone Undecanoate in the hair of a suspect. The analyte identity was confirmed by the analysis of its in-source fragmentation and comparison to a certified standard.[52]

G Forsdahl, et al. (2013) successfully screened testosterone esters in human plasma. The detection of an intact ester of testosterone in plasma is leading towards unequivocal proof of the administration of exogenous testosterone. In the current study, a sensitive screening method for the detection of nine testosterone esters in human plasma was developed. By preparing oxime derivatives of intact testosterone esters, the sensitivity of the assay was increased. Furthermore, the method included liquid-liquid extraction (LLE) as sample clean-up, as well as online separation of the target analytes from the derivatization solution. The analysis was performed by liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS). The method developed herein is simple and rapid, and was validated according to World Anti-Doping Agency (WADA) guidelines. LC-MS/MS analyses were performed with a TSQ Vantage triple quadrupole coupled to an Aria Transcend TLX-1 LC system and a CTC HTS PAL autosampler. The TLX system was equipped with a Zorbax

Eclipse XDB-C8 pre-column (2.1 × 2.5 mm, particle size 5 μm) for on line sample clean-up and pre-concentration. The analytical column was an X-Bridge C8 (4.6 × 50 mm, particle size 2.5 μm) and column selection was performed by a Maylab Mistraswitch column selector. The temperature of the analytical column was maintained at 20°C. The mass spectrometer was equipped with a heated electrospray ionization (ESI) source and measurements were performed in the positive ion mode. The temperature of the transfer capillary was adjusted to 300°C and the applied ion spray voltage was 3000 V. Nitrogen sheath gas pressure and auxiliary gas pressure were set at 35 and 15 arbitrary units, respectively. The system was operated in selected ion monitoring (srm) mode with argon as the collision gas at a pressure of 1.5 mTorr.[53]

Cornelius E.Uboh, et al. (2011) developed a simultaneous separation and determination method for 16 testosterone and nandrolone esters in equine plasma using ultra high performance liquid chromatography–tandem mass spectrometry for doping control. . Analytes were extracted from equine plasma by liquid–liquid extraction using a mixture of methyl terbutyl ether and ethyl acetate (50:50, v/v) and separated on a sub-2 micron C18 column. Detection of analytes was achieved on a triple-quadrupole mass spectrometer by positive electrospray ionization mode with selected reaction monitoring (SRM). Mobile phase comprised 2 mM ammonium formate and methanol. Deuterium-labeled testosterone enanthate and Testosterone Undecanoate were used as dual-internal standards for quantification. Limits of detection (LOD) and quantification (LOQ) were 25–100 pg/mL and 100–200 pg/mL, respectively. The linear dynamic range of quantification was 100–10,000 pg/mL. For confirmation of the presence of these analytes in equine plasma, matching of the retention time with mass spectrometric ion ratios from MS/MS product ions was used. The limit of confirmation (LOC) was 100–500 pg/mL. The method is sensitive, robust, selective and reliably reproducible.[54]

Oscar J Pozo, et al. (2009) quantified Testosterone Undecanoate in human hair by liquid chromatography– tandem mass spectrometry. The sample procedure consisted of digestion of 200 mg of pulverized hair with tris (2-carboxyethyl) phosphine hydrochloride and liquid–liquid extraction with n-pentane. Several parameters such as the mobile phase, the ionization source and the washing step were optimized. The method was validated at different spiked levels obtaining satisfactory values for accuracy (between 92% and 102%) with relative standard deviations lower than 7% and a limit of detection of 0.2 ng/g. The applicability of

the method was checked by the analysis of three samples from patients using T-C11. A peak for the analyte was detected in all samples with concentrations between 0.4 and 8.4 ng/g.[55]

Peter G Schnabel, et al. (2007) determined the effect of food composition on serum testosterone levels after oral administration of Andriol Testocaps. Testosterone and Dihydrotestosterone Undecanoate concentrations were determined using a validated liquid chromatographic (LC) assay with mass spectrometry (MS) detection after solid-phase extraction. The extracts were quantified by LC-MS using electrospray ionization in multireaction monitoring (MRM) mode. The analysis of testosterone and DHT concentrations in serum were assayed using a validated GC assay with MS detection after solid-phase extraction, derivatization and liquid–liquid extraction with n-hexane. The lower limit of quantification was 0·438 nmol/l for TU, 0·436 nmol/l for DHTU, 0·347 nmol/l for testosterone and 0·344 nmol/l for DHT. The interassay coefficient of variation for the QC samples was in the range 4·8–11·0% for TU, 4·0–30·1% for DHTU, 2·3–5·8% for testosterone and 4·5–11·9% for DHT. The accuracy of the QC samples was in the range 93·9–100·3% for TU, 103·0–114·2% for DHTU, 99·2–102·9% for testosterone and 91·7–102·2% for DHT. Bioanalysis was performed at the Bioanalytics Section of the Department of Metabolism and Kinetics, Organon Development GmbH, Waltrop, Germany, with validated methods and in compliance with GLP principles of the OECD.[56]

David M Shackleford, et al. (2003) developed a study using stable isotope methodology to compare the relative performance of two commercially available Testosterone Undecanoate formulations (Andriol and Andriol Testocaps) with respect to the amount of Testosterone Undecanoate absorbed via the intestinal lymphatics and the resulting systemic exposure of Testosterone after oral administration of the Testosterone Undecanoate formulations. To determine the concentration of labeled and unlabeled TU, T, DHT, and DHTU in serum and lymph, the concentration of TU in the intravenous formulation liquid chromatography coupled to mass spectrometry (LC-MS/MS) has been used. For Serum Concentrations of TU/DHTU, LC system of Hewlett Packard Series 1100 equipped with an LC analytical column (Supelcosil LC-8-DB, 50 mm × 4.6 mm, 5µm particle size) running at a column temperature of 40°C was used. The flow was 0.5 ml/min and an 8-min gradient was used in which the mobile phase composition varied (linearly) from 10% water [0.2% (v/v) acetic acid] in methanol to 100% methanol over 6.9 min. The mass spectrometer was an API 3000 operated at a vaporizer temperature of 200°C and an ionization spray voltage of 5,500 V. In the MS/MS mode the protonated [M H] molecules of TU, [2 H] 3-TU, DHTU, [2 H] 3-

DHTU, and TD were used as the precursor ion and measured as product ions at m/z ratios of 270.75, 274.09, 254.91, 258.15, and 270.86, respectively. The limits of quantitation (LOQ) for TU and DHTU in serum were 0.5 and 1 ng/ml, respectively. For Lymph Concentrations of TU/DHTU, the LC analytical column (Chromolith Speed ROD RP18e, 50 mm × 4.6 mm) was maintained at a column temperature of 40°C, and separations conducted at 1.00 ml/min where an 8-min gradient program was used in which the mobile phase composition remained constant [7% water, 0.2% (v/v) acetic acid in methanol] for 5 min, after which it changed to 100% methanol over 0.1 min where it remained for a further 0.9 min before returning to the starting conditions over a further 0.1 min. The mass spectrometer was an API 3000 (Applied Biosystems), and the operating conditions were identical to those for determination of TU in serum, except that the vaporizer temperature was set at 250°C. In the MS/MS mode, the protonated [M H] molecules of TU, [2 H]3-TU, DHTU, [2 H]3-DHTU, and TD were used as precursor ion and measured as product ions at m/z 271.10, 274.30, 255.20, 258.10, and 271.20, respectively. The LOQ for TU and DHTU (and the corresponding related labeled analytes) in lymph was 2 and 5 ng/ml, respectively. For determination of [2 H]-TU in Intralipid, HPLC system consisting of a Spectra System P4000 pump, Spectra System AS3000 autosampler, and Spectra System UV2000 UV detector equipped with an LC analytical column (Kromasil C18, 100 mm × 4.6 mm, 5-m particle size; Varian Medical Systems) operated at a column temperature of 30°C was used. The mobile phase was 100% methanol flowing at 1.0 ml/min, the injection volume was 10 l, and the detection wavelength was 254 nm.[57]

Wilma M Bagchus, et al. (2003) studied the Effect of Food on the Bioavailability of Testosterone Undecanoate, testosterone and 5a-dihydrotestosterone (DHT) after administration of a new oral Testosterone Undecanoate formulation, Andriol Testocaps. Serum concentrations of Testosterone Undecanoate were assayed by liquid chromatography with mass spectrometry detection. Testosterone and DHT were assayed by gas chromatography with mass spectrometry detection. Serum concentrations of testosterone, Testosterone Undecanoate, and DHT were low to negligible when Testosterone Undecanoate was administered to subjects in a fasting state; these values were significantly higher when the test drug was co-administered with food. For testosterone, the maximum serum concentration and area under the plasma concentration–time curve were 0.67 ng/ml and 5.37 ng hr/ml, respectively, in the fasting state, versus 10.7 ng/ml and 56.4 ng hr/ml, respectively, in the fed state. The same parameters were also significantly higher for Testosterone

Undecanoate and DHT in the fed versus fasting subjects. From this it can be concluded that, food increases the bioavailability of Testosterone Undecanoate, testosterone, and DHT. For proper absorption, Andriol Testocaps must be taken with meals.[58]

Jordi Segura, et al. (2002) determined Orally administered Testosterone Undecanoate (TU), an anabolic, androgenic steroid, which have been potentially abused by athletes. Indirect evidence for detecting oral TU intake could be deduced from the changes in steroid profile post-administration. Direct evidence could be obtained by detection of unchanged TU in plasma. To this end, both urinary and plasma steroid profiles of six healthy male subjects given a single oral dose of 120 mg of TU were studied by gas chromatography/mass spectrometry (GC/MS) and gas chromatography/tandem mass spectrometry (GC/MS/MS). For GC-MS, a Hewlett-Packard 6890 II GC model fitted with a HP 7673A autosampler was connected to a HP 5973 mass selective detector has been used. The separation was carried out by a methyl silicone fused-silica capillary column (HP Ultra-1; 17 m× 0.2 mm (i.d.), film thickness 0.11 m) with the following oven temperature program: an initial temperature of 181°C increased to 230°C at a rate of 3.0°C/min, then to 310°C at a rate of 40°C/min and held for 3 min. Helium was used as carrier gas with a flow rate of 0.8 ml/min (measured at 180°C). The injector (operated in 10:1 split mode) and the interface were both maintained at 280°C. The mass spectrometer was operated in selected ion monitoring acquisition mode with one or more ions selected for the TMS derivative of each substance. For GC-MS/MS, a Finnigan GCQ ion trap mass spectrometer was connected with a GCQ gas chromatograph equipped with a model A200S autosampler has been used. A HP Ultra-1 capillary column (19 m × 0.2 mm (i.d.), film thickness 0.11 m) was used for the separation with the following oven temperature program: an initial temperature of 180°C (maintained for 0.5 min) increased to 210°C at a rate of 30°C/min, then to 230°C at a rate of 4.0°C/min, and finally to 315°C at a rate of 40°C/min and held for 7 min. The injector (in 15:1 split mode) and the interface were maintained at 290°C and 300°C, respectively. Helium was used as carrier gas with a flow rate of 0.8 ml/min (measured at 180°C). The ion source temperature was maintained at 200°C. [59]

J Segura, et al. (2000) detected anabolic steroids in hair samples. A sensitive and specific assay has been developed for the simultaneous determination of testosterone, nandrolone and some of their esters in hair. The analytes were extracted from finely cut hair with methanol–trifluoroacetic acid overnight. After the incubation, the mixture was evaporated to dryness, redissolved and extracted with hexane. The dried organic layer was silanised and analysed by GC–MS and GC–MS–MS. A sensitivity of at least 20 pg injected was obtained for all the

analytes. GC–MS analysis was performed using a Hewlett Packard 6890 gas chromatograph equipped with 7683A autosampler and MSD 5973. Separation was carried out using a 5% phenylmethylsilicone fused-silica capillary column (HP5 Ultra 2; 12 m 30.2 mm I.D., film thickness 0.33 mm). The injector (splitless mode) and the interface were both maintained at 280℃, and helium was the carrier gas at a flow-rate of 0.8 ml/min. The oven temperature was programmed from 190℃ (held for 0.5 min.), to 280℃ at a rate of 30 ℃/min, then to 310℃ at a rate of 40 ℃/min, and maintained at 310℃ for 3.5 min. The mass spectrometer was operated in selected ion monitoring (SIM) acquisition mode. For quantitative determinations, the following ions were used: m/z 418 for N-bis–trimetnylsilane (N-bis–TMS), m/z 432 for testosterone-bis–TMS, m/z 416 for testosterone 17b-propionate–TMS, m/z 472 for testosterone 17b-enanthate–TMS, m/z 500 for nandrolone 17b-decanoate–TMS and m/z 402 for testosterone 17b-acetate–TMS. GC–MS–MS identification analysis of hair samples was performed using a Finnigan Mat GC 8000 TOP gas chromatograph equipped with A 200 autosampler and a GCQ ion-trap mass spectrometer. Separation was carried out using methyl silicone fused-silica capillary column (HP1; 15 m 30.2 mm I.D., film thickness 0.11 mm). The injector (splitless mode) and the interface were maintained at 280℃ and 300℃, respectively and helium was the carrier gas at a flow-rate of 1 ml/min. The oven temperature was programmed from 180℃ (held for 0.5 min), to 210℃ at a rate of 15 ℃/min, then to 240℃ at a rate of 10 ℃/min and finally to 310℃ at a rate of 30 ℃/min, and maintained at 310℃ for 3 mins. The mass spectrometer was operated in multiple reaction monitoring daughter scan acquisition mode using an excitation energy in the range 1.10–1.25 V, an isolation time of 8 ms and an excitation time of 15 ms.[60]

Yvan Gaillard, et al. (1999) determined anabolic steroids and their esters in hair as a application in doping control and meat quality control by using Gas chromatographic–tandem mass spectrometry. The gas chromatograph was a 5890 series II plus from Hewlett-Packard equipped with an A200S autosampler from Finnigan Mat distributed by Thermo Quest. The GC system was interfaced to a triple quadrupole mass spectrometer TSQ 7000 from Finnigan Mat. The analytical column was a CPSIL 8 CB, 30 m 30.25 mm I.D., 0.25 mm film thickness from Chrompack. Helium was used as the carrier gas at a flow-rate of 1.1 ml/min in the constant flow mode (i.e., 56 kPa at 80℃). Pulsed splitless injection was done at 290℃ and 180 kPa during 0.75 min. Injection volume was 2 ml. MS temperatures were: interface=300℃, ionsource=160℃, quadrupole=70℃. The initial oven temperature was 80℃ for 2 min and was increased to 310℃ at 15℃/min and held for 14 min. The chromatographic

run time was 31 min. The MS instrument was operated in the electronic impact ionization mode. The collision gas was argon at 1.7mTorr (1 Torr =133.322 Pa). Dwell time per parent ion was set at 200 ms.[61]

Cedric H.L. Shackleton, et al. (1997) identified 17β-fatty acid esters of testosterone in blood plasma by using HPLC-MS. These drugs are therapeutic but are increasingly misused by athletes in an attempt to improve sports performance. The mass spectral properties of testosterone esters under electrospray ionization are described. These esters (testosterone acetate, propionate, isocaproate, benzoate, enanthate, cypionate, phenylpropionate, decanoate, and undecanoate) essentially give only a protonated molecular ion (MH+) under "optimum sensitivity" mass spectrometric conditions but could be induced to fragment in the source or collision cell of a triple quadrupole mass spectrometer. The underivatized steroid esters were analyzed by direct infusion because development of solvent systems compatible with high-performance liquid chromatography (HPLC) was not achieved for these nonpolar compounds. HPLC/MS (mass spectrometry) was possible when the steroids were converted to polar, water soluble, Girard hydrazones, and almost all compounds were separated by microbore C4 HPLC using water, acetonitrile, TFA gradient. The mass spectra under optimal ionization conditions essentially comprised only a molecular ion (M+), but source fragmentation gave major ions at M - 59 and M – 87 for all compounds. The molecular ion and these fragment ions were monitored in a selected-ion-recording (SIR) method developed for detecting the steroids in plasma. Using this methodology, testosterone enanthate and undecanoate could be detected after intramuscular injection or oral administration of the drugs. Further development of the technique could form the basis of a protocol for confirming the misuse of testosterone in sport, especially if sensitivity could be improved.[62]

Xavier de la Torre, et al. (1995) detected Testosterone Esters in Human Plasma. Liquid-liquid extraction of human blood plasma and appropriate derivatization of nine testosterone esters are described. The Tandem mass spectrometric analysis was successful in the detection and determination of intramuscularly administered testosterone propionate and testosterone enanthate and orally ingested testosterone undecanoate. For GC-MS analysis, a Model 5890 Series II gas chromatograph fitted with a Model 7673A autosampler was connected to a Model 5971A mass-selective detector (HP) has been used. Separation was carried out using a 5% phenylmethylsilicone fused-silica capillary column (HP5 Ultra 2; 12 m x 0.2 mm i.d., film thickness 0.33 pm). The injector, operated in the splitless mode (0.3 min), and the interface were both maintained at 280°C. The oven temperature programmes were as follows:

(a) program A1: initial temperature 190°C (held for 0.5 min), rate 1 30°C/min to 280°C, rate 2 40°C/min to 310°C, and maintained at 310°C for 3.5 min; (b) program A2: as for programme A1 except that the final temperature was 315°C the analytes eluting - 1 min before. Helium was used as the carrier gas at a flow-rate of 0.8 ml/min. For GC-MS/MS analysis, a Finnigan TSQ 700 triple quadrupole mass spectrometer equipped with a Varian Model 3400 gas chromatograph has been used. The fused-silica capillary column (HP5 Ultra 2; 12 m x 0.2 mm i.d., film thickness 0.33 pm) was programmed (temperature programme B) from 200°C (held for 2 min) to 315°C at 40°C/min (7 min isothermal at 315°C). The injector (split less, 2 min) was maintained at 290°C and the interface at 300°C. Helium was used as the carrier gas at a flow-rate of 0.8 ml/min. [63]

C.Gooijer, et al. (1992) demonstrated Impurity Profiling of Testosterone Undecanoate using LC interfaced with FT-IR. Reversed-phase liquid chromatography (RP-LC) was coupled to Fourier transform infrared spectrometry (FT-IR) with the use of a spray jet assembly to eliminate the solvent and to deposit the effluent on zinc selenide. The usefulness of the LC/FT-IR system in impurity profiling is demonstrated for the steroid drug testosterone undecanoate (TU). FTIR transmission spectra of components of the steroid sample separated by LC are identified directly by library search or are interpreted to yield structural information. The study also shows the occasional similarity of the spectra of testosterone enanthate (TE) and testosterone decanoate (TD), two possible impurities of TU. This phenomenon, which can hinder identification, is attributed to the fact that TE and TD can both be either in a crystalline or in an amorphous state. An LDC/Milton Roy microbore pump or a Perkin-Elmer 250 pump were used with a home-made injection valve having an internal loop of 1.9 µL. Separations were done on a 170 × 1.1mm-i.d. column packed with 5 µm Rosil C18 or a 150 × 1.0mm-i.d. column packed with 3 µm Spherisorb ODS-2 at a flow rate of 20 µL/rain with methanol/water (95:5 v/v) as mobile phase. The LC effluent was led to the spray jet assembly through a fused-silica capillary, which was connected to a stainless-steel needle. A heated nitrogen flow around the needle, which partially protruded through a nozzle, ensured deposition of the effluent and provided removal of the mobile phase. During deposition the zinc selenide substrate was moved at constant speed by a computer-controlled Bruker microscope X-Y stepper table, which was also used for the IR-scanning of chromatograms. The interface conditions applied in this study were: needle protrusion distance, 0.5 mm; needle-to-substrate distance, 0.5 mm; nozzle i.d., 600 mm; nitrogen gas pressure, 7 bar; nitrogen gas temperature, 120°C; table speed, 1 mm/min.[64]

4. AIM & OBJECTIVE

Testosterone Undecanoate is an unsaturated, aliphatic, fatty acid ester of Testosterone which is formulated as a Soft Gelatin capsule dosage form to treat Hypogonadal patients. Testosterone Undecanoate is not officially listed in Indian Pharmacopoeia (IP), British Pharmacopoeia (BP), and United States Pharmacopoeia (USP). So there is a need for a novel routine method of analysis.

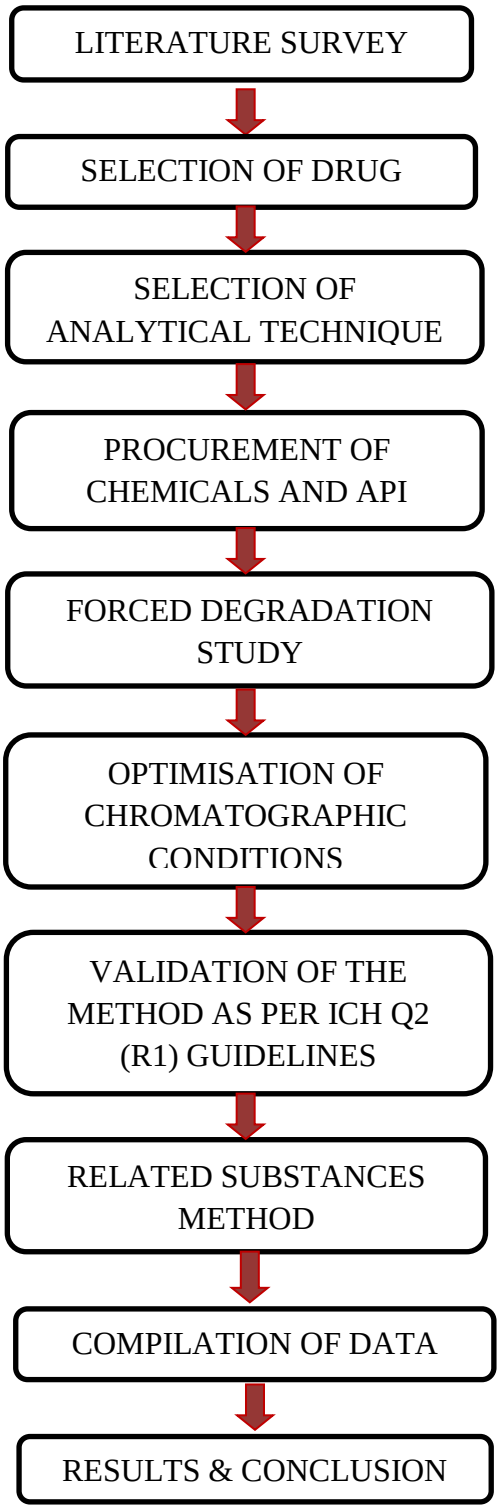
4.1. AIM

The main aim of this study is to develop a simple, precise, and accurate Stability indicating Related substances method for the estimation of Testosterone undecanoate & its related degradation impurities in the drug product of Testosterone undecanoate Soft Gelatin Capsules 40mg by RP-HPLC method.

4.2. OBJECTIVE

- To develop a novel stability-indicating RP-HPLC method for the estimation of Testosterone Undecanoate & Related substances in the capsule dosage form.
- To conduct various trials for estimation of Testosterone Undecanoate & Related substances in bulk and capsule dosage form by changing chromatographic conditions.
- To optimize the chromatographic conditions for quantification of Testosterone Undecanoate & Related substances using RP-HPLC.
- To validate the developed method as per ICH Q2 (R1) guidelines for its accuracy, precision, linearity, selectivity, robustness, etc.
- To perform the forced degradation studies for the Testosterone Undecanoate & Related substances in the capsule dosage form.
- To ensure that the developed method can be utilized for routine analysis.

5. PLAN OF WORK



Reviewing of literature for selection of the drug molecule.

Selection of analytical technique, study about physio-chemical properties of the drug, defining HPLC parameters.

Procuring the chemicals needed for mobile phase, standard API and sample (with placebo).

Executing the degradation studies like acid, alkali, photolytic, thermal, oxidative degradation.

Improved the developed method to gain greater confidence on the generation of analytical data.

The developed method was validated for its Accuracy, Linearity, Precision, Recovery, Sensitivity, Robustness, etc as per ICH guidelines.

Separation and Quantitation of impurities from stress studies.

Data's obtained from method development & validation are compiled properly.

Information/ data gained during the study are discussed, documented and concluded.

6. MATERIALS AND METHODS

6.1. Chemicals and Reagents

Table 6: List of Chemicals and Reagents used

S.No.	Name of the Material	Grade	Make
1	Acetonitrile	HPLC	Rankem
2	Methanol	HPLC	MERCK
3	Water	HPLC	Milli pore
4	Sodium Hydroxide	AR	Rankem
5	Hydrochloric acid	AR	Rankem
6	Hydrogen Peroxide	AR	Rankem

6.2. Working Standard and Impurities

Table 7: List of Working Standard and Impurities used

S.No.	Name of the components	Purity (% w/w)	Make
1	Testosterone Undecanoate	99.8	Symbiotec Pvt. Ltd.
2	Testosterone	99.84	Symbiotec Pvt. Ltd.
3	Testosterone Decanoate	97.77	Symbiotec Pvt. Ltd.

6.3. Sample and Placebo

Table 8: List of Sample and Placebo used

S.No	Name of the Sample	Make
1	Testosterone Undecanoate capsule 40mg	Steril-Gene Life Sciences
2	Testosterone Undecanoate capsule 40mg -Placebo	Steril-Gene Life Sciences

6.4. Equipment

Table 9: List of Equipment used

S.No.	Name of the Instrument	Make
1	HPLC	Agilent
2	Analytical Balance	LC/GC
3	Sonicator	Pci Analytics
4	Centrifuge	Remi R-24
5	Hot air oven	Technico
6	Photo stability chamber	Remi

6.5. Column and Filters

Table 10: List of Column and Filters used

S.No.	Name	Make
1	Inertsil-ODS-3, 4.6 mm x 250 mm, 5µm	Inertsil
2	0.45 µm Nylon filter & 0.45 µm PVDF filter	Axiva

7. METHOD DEVELOPMENT

7.1. Selection of Optimal method conditions

Trial 1

The initial method was referred to from the API methodology of Testosterone Undecanoate, which was provided by the manufacturer – Symbiotec Pharmalab Pvt. Limited, Pithampur.

Chromatographic conditions:

Column description : Inertsil-ODS-3, 4.6 mm x 250 mm, 5µm

Flow rate : 1.5 mL/ minute

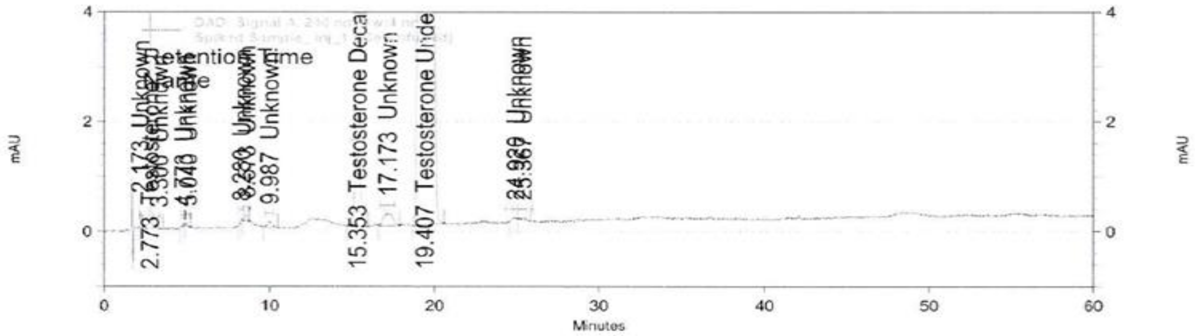
Injection volume : 20 µL

Column temperature : 25°C

Detector : 240 nm

Run time : 60 minutes

Mobile phase : Acetonitrile and Water (95:5)



Name	Ret. Time	Area	RRT
Unknown	2.17	3141	0.112
Testosterone	2.77	84578	0.143
Unknown	3.30	1087	0.170
Unknown	4.77	801	0.246
Unknown	5.04	1164	0.260
Unknown	8.28	1560	0.427
Unknown	8.57	2348	0.442
Unknown	9.99	4892	0.515
Testosterone Decanoate	15.35	64516	0.791
Unknown	17.17	18830	0.885
Testosterone Undecanoate	19.41	20621302	1.000
Unknown	24.92	1668	1.284
Unknown	25.37	2384	1.307

Observation:

Based on the above study the known and unknown impurities are well separated from each other. However, the testosterone impurity peak was found to be eluting along with the void volume peak. To make it separate from void volume peak further trials are executed.

Trial 2

Based on the above statement, to separate Testosterone impurity from void volume peak, the isocratic method was changed to the gradient method. The detailed mobile phase composition, diluent and chromatographic condition details are given below,

Chromatographic conditions:

Column description : Inertsil-ODS-3 C18, 4.6 mm x 250 mm, 5µm.

Flow rate : 1.5 mL/ minute.

Injection volume : 20 µL.

Column temperature : 25°C

Sample thermostat : Ambient

Detector : 240 nm

Run time : 50 minutes

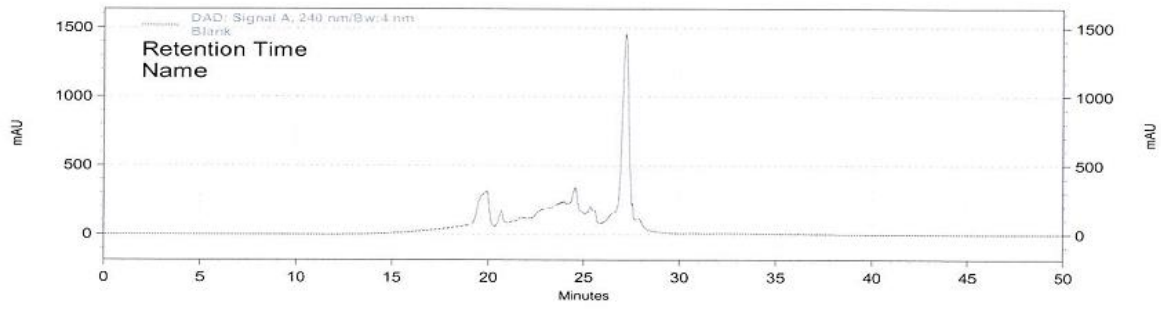
Mobile phase : A - Water (100%), B - ACN (100%)

Diluent : Acetonitrile and Water (95:5)

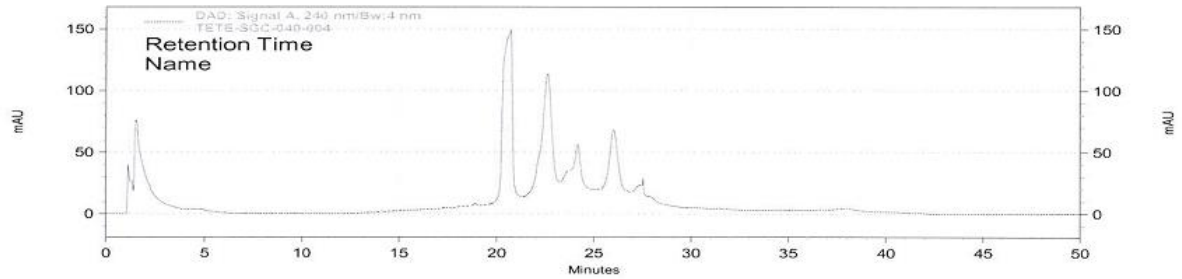
Gradient Programme :

Time (min)	Mobile phase A (%v/v)	Mobile Phase B (% v/v)
0.0	60	40
10.0	60	40
20.0	0	100
25.0	0	100
30.0	30	70
35.0	30	70
40.0	60	40
50.0	60	40

Blank Chromatogram



Sample Chromatogram



Observation:

No peaks were observed in this chromatographic condition. Hence the gradient program was altered and started with fresh preparation.

Trial 3

Chromatographic conditions:

Column description : Inertsil-ODS-3 C18, 4.6 mm x 250 mm, 5µm.

Flow rate : 1.5 mL/ minute.

Injection volume : 20 µL.

Column temperature : 25°C

Sample thermostat : Ambient

Detector : 240 nm

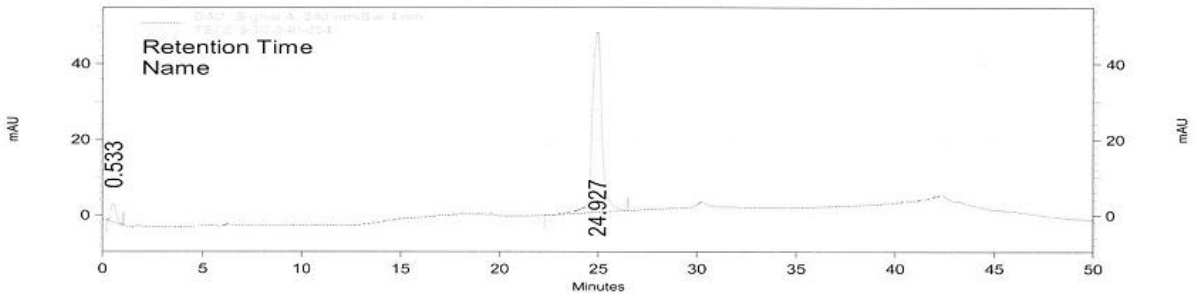
Run time : 50 minutes

Mobile phase : A - Water (100%), B - ACN (100%)

Diluent : Acetonitrile and Water (95:5)

Gradient Programme :

Time (min)	Mobile phase A (%v/v)	Mobile Phase B (% v/v)
0.0	60	40
10.0	60	40
15.0	30	70
20.0	30	70
40.0	5	95
55.0	60	40
60.0	60	40



Name	Retention Time	Area	Asymmetry	Theoretical Plates
Unknown	0.533	184315	1.3	22
Unknown	24.927	3395247	1.2	15839

Observation:

No peaks were observed in this chromatographic condition. Hence the gradient program was altered and started with fresh preparation.

Trial 4

Chromatographic conditions:

Column description : Inertsil-ODS-3 C18, 4.6 mm x 250 mm, 5µm.

Flow rate : 1.5 mL/ minute.

Injection volume : 20 µL.

Column temperature : 25°C

Sample thermostat : Ambient

Detector : 240 nm

Run time : 60 minutes

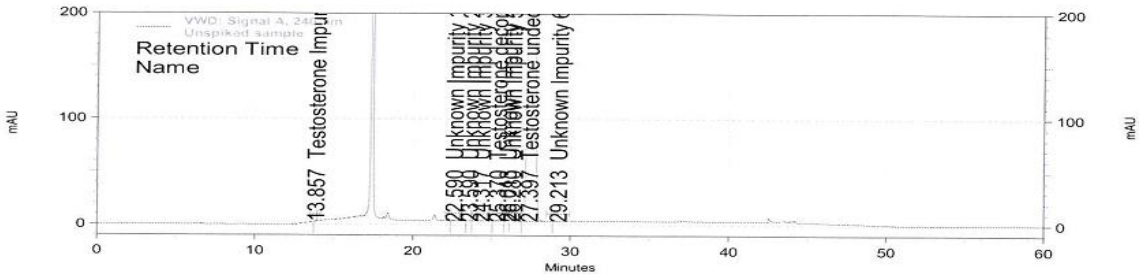
Retention time : About 27 minutes

Mobile phase : A - Water, B - Acetonitrile

Diluent : Acetonitrile and Water (95:5)

Gradient Programme :

Time (min)	Mobile phase A (%v/v)	Mobile Phase B (% v/v)
0.0	60	40
10.0	60	40
15	0	100
40	0	100
50	60	40
60	60	40



Name	Retention Time	Area
Testosterone Impurity	13.857	44002
Unknown 1	22.590	98346
Unknown 2	23.590	5512
Unknown 3	24.317	114028
Testosterone Decanoate Impurity	25.370	203868
Unknown 4	26.013	80149
Unknown 5	26.280	115814
Testosterone Undecanoate	27.397	593140573
Unknown 6	29.213	60320

Observation:

Based on the above trials, trial number 4 is having a very good response, and all the peaks are well separated from each other. There was no interference from blank and known impurities at the retention time of Testosterone Undecanoate observed. The known and unknown impurity's peak purity meets the acceptance criteria requirements. So this method was taken as an optimized method and selected for quantitative estimation.

7.2. Quantitative Estimation

Optimized Chromatographic Conditions:

Column description : Inertsil-ODS-3 C18, 4.6 mm x 250 mm, 5µm

Flow rate : 1.5 mL/ minute.

Injection volume : 20 µL

Column temperature : 25°C

Sample thermostat : Ambient

Detector : 240 nm

Run time : 60 minutes

Retention time : About 27 minutes

Mobile phase : A - Water, B - Acetonitrile

Diluent : Acetonitrile and Water (95:5)

Gradient Programme :

Time (min)	Mobile phase A (%v/v)	Mobile Phase B (%v/v)
0.0	60	40
10.0	60	40
15	0	100
40	0	100
50	60	40
60	60	40

7.2.1. SYSTEM SUITABILITY

Preparation of Standard solution:

25 mg of Testosterone Undecanoate Working standard was weighed accurately in a 100 mL volumetric flask. 30 ml of Acetonitrile was added to it and sonicated for 10 minutes. Then the volume was made up with Acetonitrile. Further diluted 2 ml of the above solution to 100 ml with diluent and mixed well (5 ppm).

Procedure:

System suitability was performed by injecting the standard solution in six replicates and the chromatograms were recorded.

Observation:

Table 11: Summary of System Suitability results

System Suitability	Peak Area	Theoretical plates	Tailing factor
Standard Inj - 1	243157	59261	1.12
Standard Inj - 2	243515	59715	1.11
Standard Inj - 3	244354	58815	1.11
Standard Inj - 4	246399	58953	1.12
Standard Inj - 5	246597	58577	1.11
Standard Inj - 6	245536	58207	1.12
Average	244926	58921	1.11
SD	1468.44	526.58	0.005
% RSD	0.60	0.89	0.49

Acceptance Criteria:

- The percentage relative standard deviation of peak areas of Testosterone Undecanoate from six replicate injections of standard preparation should be NMT 5.0
- The Tailing factor of Testosterone Undecanoate peak standard preparation should be NMT 2.0
- The Theoretical plates of Testosterone Undecanoate peak standard preparation should be NLT 2000.

Conclusion:

System suitability parameters met the acceptance criteria. Hence the system and method found suitable for Testosterone Undecanoate.

7.2.2. SPECIFICITY

The specificity of the method was demonstrated by injecting Blank (diluent), standard solution, Placebo solution, sample solution, and sample solution spiked with known impurities at specification limit and injected individual known impurities at Specification Limit. Evaluated the interference of matrix and blank peaks at the retention time of Testosterone Undecanoate. The results are summarized in below Table 12.

Standard preparation:

25 mg of Testosterone Undecanoate Working standard was weighed accurately in a 100 mL volumetric flask. 30 ml of Acetonitrile was added to it and sonicated for 10 minutes. Then the

volume was made up with Acetonitrile. Further diluted 2 ml of the above solution to 100 ml with diluent and mixed well. (5 ppm).

Placebo preparation:

Weighed accurately 212 mg of placebo (50 mg equivalent of Testosterone Undecanoate) into 50 ml volumetric flask. 10 ml of water was added to it & sonicated for 10 minutes and then 25 mL of Acetonitrile was added & sonicated for 15 minutes with intermediate shaking. Volume was made up with diluent. Finally, the placebo solution was centrifuged for about 10 minutes at 2500 rpm and injected into the chromatographic condition.

Unspiked sample preparation:

20 capsules were taken and weighed to calculate the net content of the capsules. Then weighed accurately 262 mg of sample (50mg equivalent of Testosterone Undecanoate) into a 50 ml volumetric flask with 10 ml of water and sonicated for 10 minutes. 25 mL of Acetonitrile was added to it and sonicated for 15minutes with intermediate shaking. Volume was made up with the diluent. Finally, the solution was centrifuged for about 10 minutes at 2500 rpm and injected into the chromatographic condition.

Testosterone impurity stock preparation:

Weighed about 2 mg of Testosterone and transferred into a 20 ml volumetric flask, dissolved and diluted to the volume with diluent.

Testosterone impurity preparation:

Pipetted out 1 ml of Testosterone impurity stock solution into a 50 ml volumetric flask, diluted the volume with diluent.

Testosterone Decanoate impurity stock preparation:

Weighed about 2 mg of Testosterone Decanoate and transferred into a 20 ml volumetric flask, dissolved and diluted to the volume with diluent.

Testosterone Decanoate impurity preparation :

Pipetted out 1 ml of Testosterone Decanoate impurity stock solution into a 50 ml volumetric flask, diluted the volume with diluent.

Spiked sample preparation:

Weighed 20 capsules and calculated the net content of the capsules. Weighed and transferred 50 mg equivalent of Testosterone Undecanoate sample into 50 ml volumetric flask. Added 10 ml of water and sonicated for 10minutes. Then added 25 mL of Acetonitrile and sonicated for 15 minutes with intermediate shaking. Finally transferred 1 mL each of the Testosterone stock solution and Testosterone Decanoate stock solution into the flask and made up the volume with diluent. Centrifuged the sample solution for 10 minutes at 2500 rpm.

Observation:

Table 12: Specificity results

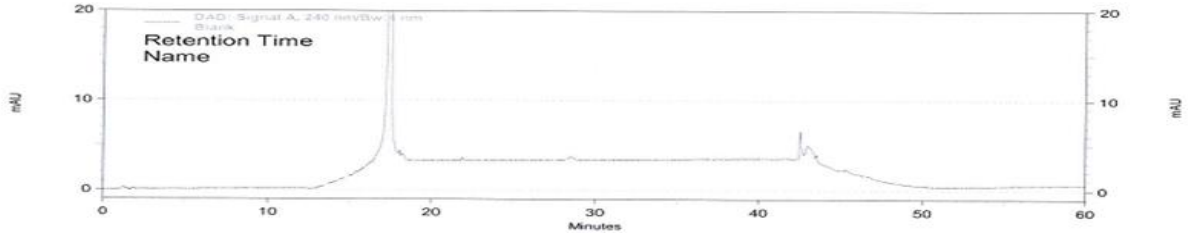
Sample	Interference			
	RT	RRT	Peak Purity	Result
Blank	NAP	NAP	NAP	No interference
Placebo	NAP	NAP	NAP	No interference
Standard solution	27.707	1.00	1.0	Nil
Spiked Sample Solution				
Testosterone Decanoate Impurity	25.413	0.91	1.0	Nil
Testosterone Impurity	13.707	0.50	0.9	Nil
Testosterone Undecanoate	27.507	1.00	1.0	Nil
Unspiked Sample Solution				
Testosterone Decanoate Impurity	25.380	0.91	1.0	Nil
Testosterone Impurity	13.713	0.50	1.0	Nil
Testosterone Undecanoate	27.507	1.00	1.0	Nil
Individual Impurity				
Testosterone Decanoate Impurity	25.427	0.91	1.0	Nil
Testosterone Impurity	13.720	0.50	1.0	Nil

RT-Retention time, RRT-Relative Retention Time, NAP-not Applicable

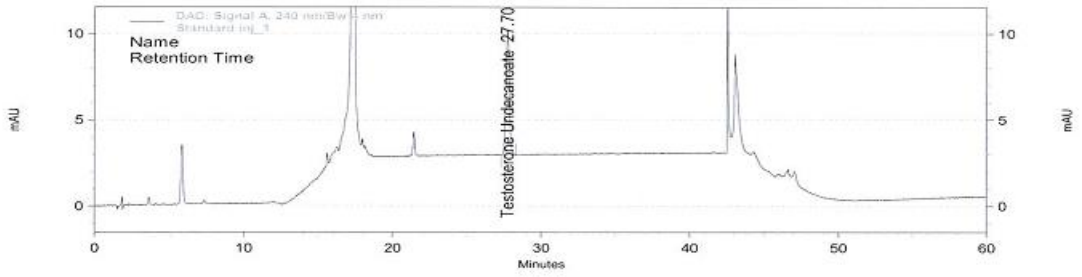
Acceptance Criteria:

- There should not be any interference of blank and placebo peaks at the Retention Time of the main analyte and its impurities.
- The Peak Purity should be NLT 0.9 in Open lab software.

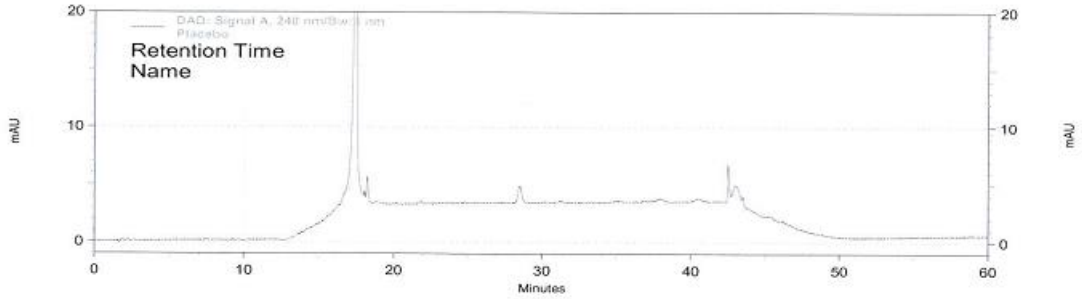
Blank Chromatogram



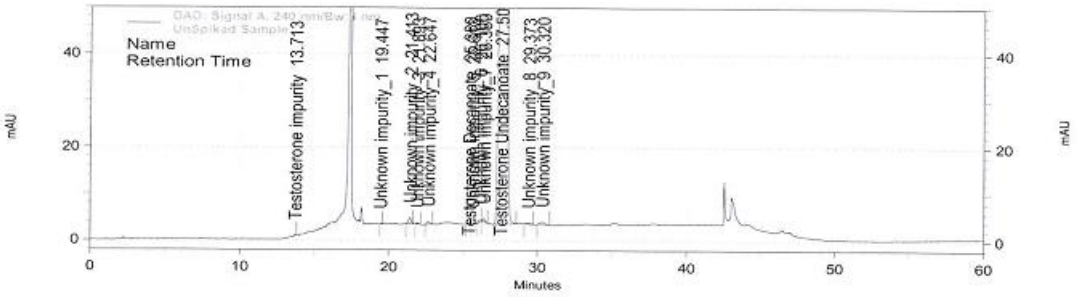
Standard Chromatogram



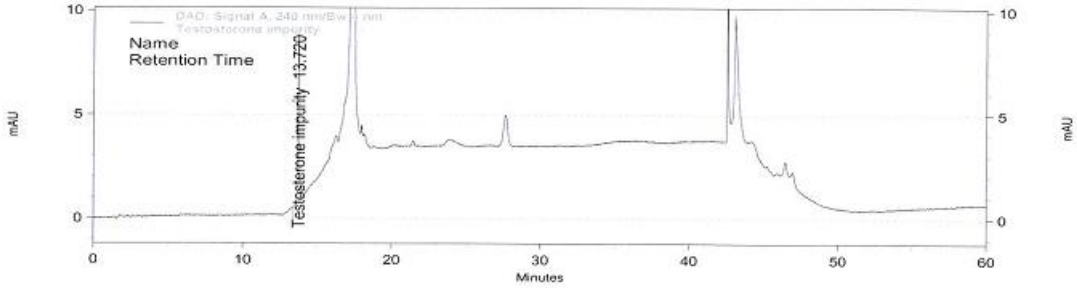
Placebo Chromatogram



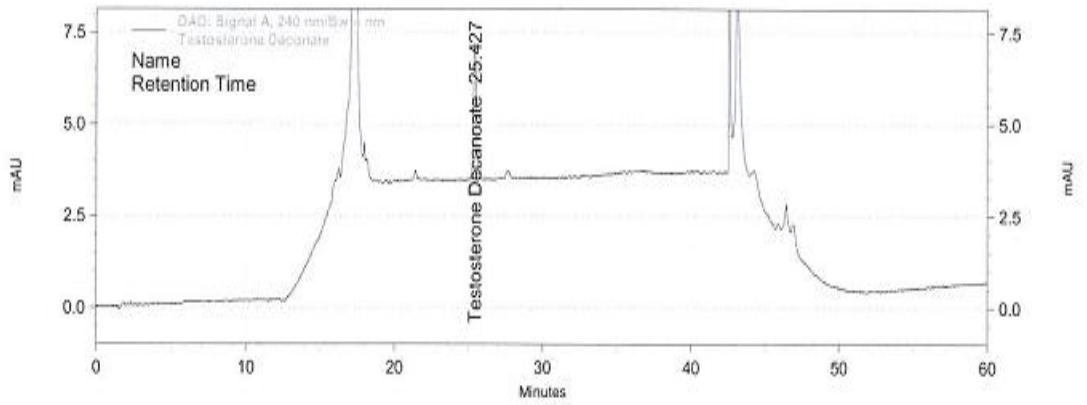
Unspiked Sample Chromatogram



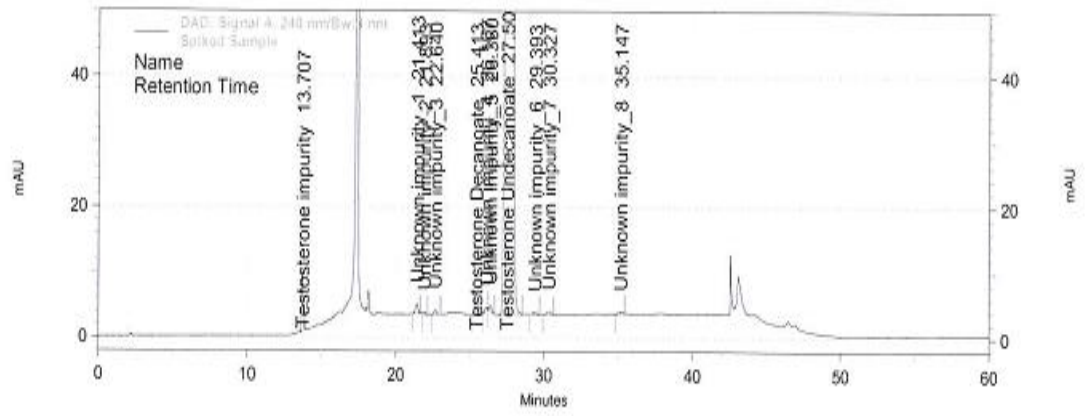
Testosterone Impurity Chromatogram



Testosterone Decanoate Impurity Chromatogram



Spiked Sample Chromatogram



7.2.2.1. FORCED DEGRADATION STUDY

A study was conducted to demonstrate the effective separation of Degradation from Testosterone Undecanoate Soft Gelatin Capsules 40mg of related substances method. Drug product, Placebo, and blank were exposed to the following stress conditions to induce degradation.

Stressed samples of the drug product and Placebo were injected separately into the HPLC system equipped with PDA (Photo Diode Array) detector by using test method conditions. The results are summarized in Tables 13 & 14.

Observation:

Table 13: Peak purity for Forced Degradation samples

For Stress Condition	Peak Purity			% Degradation
	A	B	C	
As such Sample	0.9	1.0	1.0	0.0877
Added 5mL of 0.5N Hydrochloric acid solution kept in the water bath at 30minutes and neutralized with 5mL of 0.5N Sodium Hydroxide solution.	1.0	1.0	1.0	4.6224
Added 5mL of 0.5N Sodium Hydroxide solution kept in the water bath at 30minutes and neutralized with 5mL of 0.5N Hydrochloric acid solution.	1.0	1.0	1.0	1.1641
Added 2mL of 30% hydrogen peroxide solution kept in the water bath at 30 min.	1.0	1.0	1.0	0.1137

A- Testosterone Undecanoate, B- Testosterone impurity, C- Testosterone Decanoate impurity

Table 14: Mass Balance for Forced Degradation samples

Mass Balance			
Stressed condition	**% Degradation**	**Assay (%w/w)**	**Mass Balance**
Unstressed Sample	0.0877	101.0	-
Acid Stressed Sample	4.6224	91.7	95.3
Base stressed Sample	1.1641	103.1	103.2
Oxidation Stressed Sample	0.1137	104.5	103.5

Acceptance Criteria:

- There should not be any interference of blank and placebo peaks at the RT of the main analyte and its impurities.
- The Peak Purity should be NLT 0.9 in Open lab software.
- Mass Balance for all stressed conditions should be achieved between 95.0 to 105.0%.
- The degradation of the drug substance should be not more than 20%.

Conclusion:

There was no interference observed from blank, placebo peak at the retention time of Testosterone undecanoate, Testosterone Decanoate, and Testosterone impurities. The Peak Purity for standard, Spiked & Unspiked sample impurities of Testosterone Undecanoate met the acceptance criteria. Testosterone Undecanoate impurities are well separated from co-eluents and the main peak. Hence, the method found specific for Testosterone Undecanoate SG capsules 40mg. Forced degradation study acid stressed sample getting degradation about 4.5 % and mass balance also meeting the acceptance limit. Hence, the method is stability-indicating.

8. ANALYTICAL METHOD VALIDATION

Preparation of Testosterone Decanoate impurity stock solution:

Weighed accurately 2 mg of Testosterone Decanoate impurity into 100 mL volumetric flask with 30 mL of diluent and sonicated to dissolve the content. Made up the volume with diluent and mixed well.

Resolution solution preparation:

Weighed accurately 50 mg of Testosterone Undecanoate working standard into 50 mL volumetric flask. Then 5 mL of Testosterone Decanoate impurity stock solution and 30 mL of diluent were added. Finally, the content was sonicated and made up the volume with diluent.

Standard preparation:

25 mg of Testosterone Undecanoate Working standard was weighed accurately in a 100 mL volumetric flask. 30 ml of Acetonitrile was added to it and sonicated for 10 minutes. Then the volume was made up with Acetonitrile. Further diluted 2 ml of the above solution to 100 ml with diluent and mixed well.

Placebo preparation:

Weighed accurately 50 mg equivalent of Testosterone Undecanoate placebo into 50 ml volumetric flask. 10 ml of water was added to it & sonicated for 10minutes and then 25mL of Acetonitrile was added & sonicated for 15minutes with intermediate shaking. Volume was made up with diluent. Finally, the placebo solution was centrifuged for about 10 minutes at 2500 rpm and injected into the chromatographic condition.

Sample preparation:

20 capsules were taken and weighed to calculate the net content of the capsules. Then weighed 50mg equivalent of Testosterone Undecanoate sample into 50 ml volumetric flask with 10 ml of water and sonicated for 10minutes. Then 25mL of Acetonitrile was added to it and sonicated for 15minutes with intermediate shaking. Volume was made up with the diluent. Finally, the solution was centrifuged for about 10 minutes at 2500 rpm and injected into the chromatographic condition.

8.1. SYSTEM SUITABILITY

Blank, resolution solution, and standard solution prepared as per test procedure and made six replicate injections of standard solution. The system suitability parameters were evaluated as per the test procedure and the results are summarized in below Table 15.

Table 15: Summary results of System Suitability

Parameters	%RSD Peak Area	Tailing factor	Theoretical plates	Resolution
System Precision	0.2	1.0	65394	5.51
Method Precision	0.2	1.0	65394	5.51
Intermediate Precision	0.1	1.0	60422	5.41
Specificity	0.3	1.0	61207	5.77
Forced degradation-1	0.5	1.0	55242	5.59
Forced degradation-2	0.3	1.2	21733	3.84
Forced degradation-3	0.3	0.8	42039	5.18
LOD and LOQ precision	0.6	1.0	60111	5.82
Linearity	2.8	1.0	63622	5.43
Accuracy	0.2	1.0	69907	5.85
Solution stability (Initial)	0.1	0.9	60103	5.43
Solution Stability (Day-1)	1.9	0.9	47101	4.98
Solution Stability (Day-2)	0.1	1.0	55927	5.20
Mobile phase stability (Initial)	0.8	1.0	64356	5.91
Mobile phase stability (Day-1)	0.4	1.0	62603	5.79
Mobile phase stability (Day-2)	0.2	1.0	63444	5.86
Low Flow Variation (Test condition)	0.3	1.0	61207	5.77
High Flow Variation (Test condition)	0.3	1.0	61207	5.77
High Column Temperature variation	0.6	1.0	60111	5.82
Wavelength variation (Test condition)	0.3	1.0	61207	5.77
Gradient variation-10% (Test condition)	0.2	1.0	60342	5.70
Gradient variation-3% (Test condition)	0.4	1.0	60020	5.77
Filter validation-1	0.2	1.0	65394	5.51
Filter validation-2	0.1	0.9	60103	5.43

Acceptance Criteria:

- The percentage relative standard deviation for peak areas of Testosterone Undecanoate obtained from six replicate injections of standard solution should be not more than 5.0.

- The Tailing factor of the Testosterone Undecanoate peak obtained from the standard solution should be not more than 2.0.
- The Theoretical plates of the Testosterone Undecanoate peak obtained from the standard solution should be not less than 2000.
- The Resolution between Testosterone Decanoate and Testosterone Undecanoate peaks obtained from the resolution solution should be not less than 2.0.

Conclusion:

System suitability Parameters met the acceptance criteria for all the validation parameters. Hence the method found suitable for Testosterone Undecanoate Capsules 40mg.

8.2. SPECIFICITY

Specificity is the ability to check unequivocally the analyte in the presence of components that may be expected to be present. Typically, these might include impurities, degradants, and matrices.

8.2.1. Blank and placebo interference

The specificity of the method was demonstrated by injecting Blank, Placebo solution, standard solution, sample solution, and sample solution spiked with known impurities at specification level concentration and injected individual known impurities at specification level concentration. Interference of matrix, blank and placebo peaks are evaluated at the retention time of Testosterone Undecanoate and its known impurities. The results are summarized in below Table 16.

Testosterone impurity stock preparation:

Weighed about 2 mg of Testosterone and transferred into a 20 mL volumetric flask, dissolved and diluted to the volume with diluent.

Testosterone impurity preparation:

Pipetted out 1 mL of Testosterone impurity stock solution into a 50mL volumetric flask and diluted to the volume with diluent.

Testosterone Decanoate impurity stock preparation:

Weighed about 2 mg of Testosterone Decanoate and transferred into a 20 mL volumetric flask, dissolved and diluted to the volume with diluent.

Testosterone Decanoate impurity preparation:

Pipetted out 1 mL of Testosterone Decanoate stock solution into a 50 mL volumetric flask and diluted to the volume with diluent.

Placebo preparation:

Weighed and transferred the placebo equivalent to 50 mg of Testosterone Undecanoate into a 50 ml volumetric flask. Added 10 ml of water and sonicated for 10 minutes. Then added 25 mL of Acetonitrile and sonicated for 15 minutes with intermediate shaking. Finally made up the volume with diluent and centrifuged the solution for 10 minutes at 2500 rpm.

Unspiked sample preparation:

Weighed 20 capsules and calculated the net content of the capsules. Weighed and transferred 50 mg equivalent of Testosterone Undecanoate sample into 50 ml volumetric flask. Added 10 ml of water and sonicated for 10minutes. Then added 25 mL of Acetonitrile and sonicated for 15 minutes with intermediate shaking. Finally made up the volume with diluent and centrifuged the sample solution for 10 minutes at 2500 rpm.

Spiked sample preparation:

Weighed 20 capsules and calculated the net content of the capsules. Weighed and transferred 50 mg equivalent of Testosterone Undecanoate sample into 50 ml volumetric flask. Added 10 ml of water and sonicated for 10 minutes. Then added 25 mL of Acetonitrile and sonicated for 15 minutes with intermediate shaking. Finally transferred 1 mL each of the Testosterone stock solution and Testosterone Decanoate stock solution into the flask and made up the volume with diluent. Centrifuged the sample solution for 10 minutes at 2500 rpm.

Table 16: Summary results of Specificity (Blank and Placebo Interference)

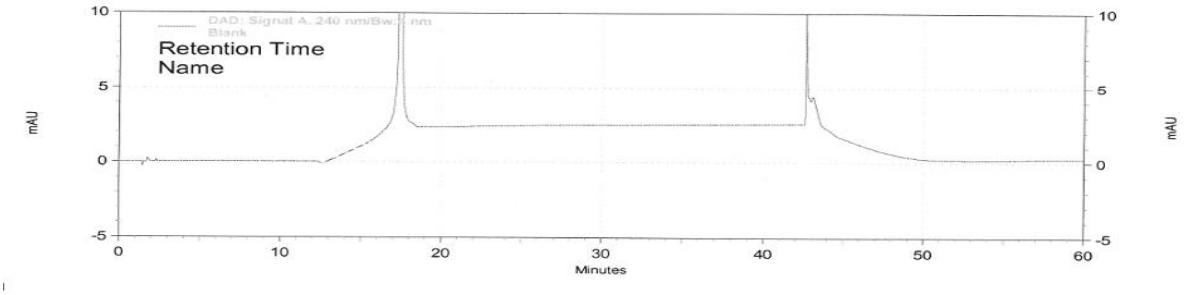
Sample ID	Interference results			
	RT	RRT	Peak Purity	Result
Blank	NAP	NAP	NAP	No interference
Placebo	NAP	NAP	NAP	No interference
Testosterone Undecanoate standard	29.093	1.00	1.0	Nil
Testosterone Decanoate – Resolution solution	26.487	0.91	1.0	Nil
Testosterone Undecanoate – Resolution solution	28.993	1.00	1.0	Nil
Spiked Sample Solution				
Testosterone	13.360	0.46	1.0	Nil
Testosterone Decanoate	26.493	0.91	1.0	Nil
Testosterone Undecanoate	28.993	1.00	1.0	Nil
Unspiked Sample Solution				
Testosterone	13.360	0.46	1.0	Nil
Testosterone Decanoate	26.487	0.91	1.0	Nil
Testosterone Undecanoate	28.993	1.00	1.0	Nil
Individual Impurity				
Testosterone	13.347	NAP	1.0	Nil
Testosterone Decanoate	26.487	NAP	1.0	Nil

RT-Retention time, RRT-Relative Retention Time, NAP-not Applicable

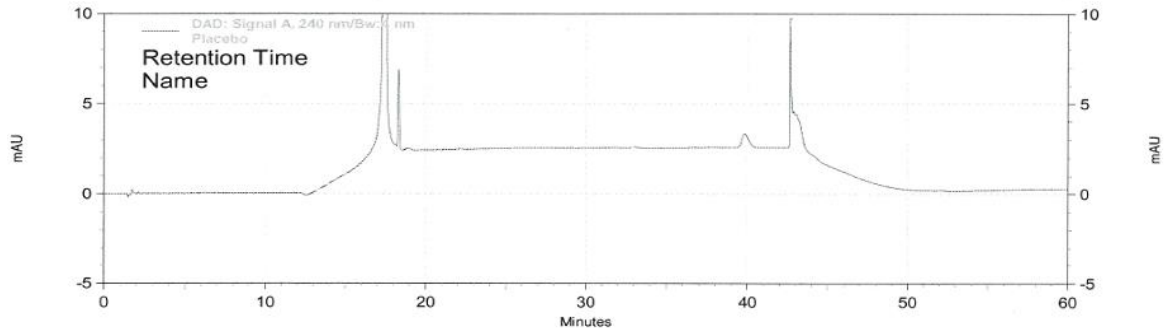
Acceptance Criteria:

- There should not be any interference of blank and placebo peaks at the Retention Time of the main analyte and its impurities.
- The Peak Purity should be NLT 0.9 in open lab software.

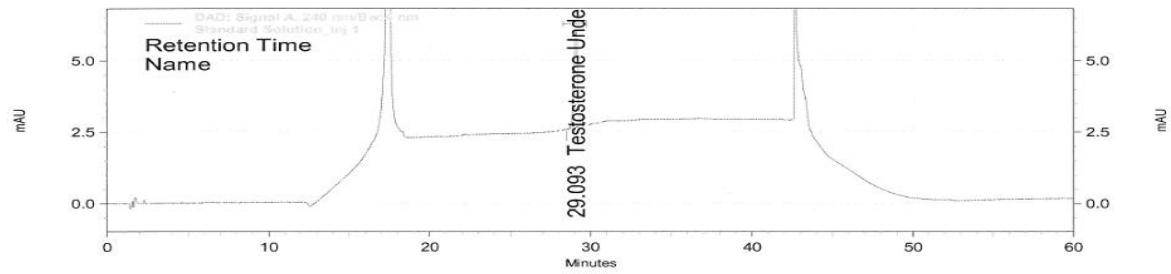
Blank Chromatogram



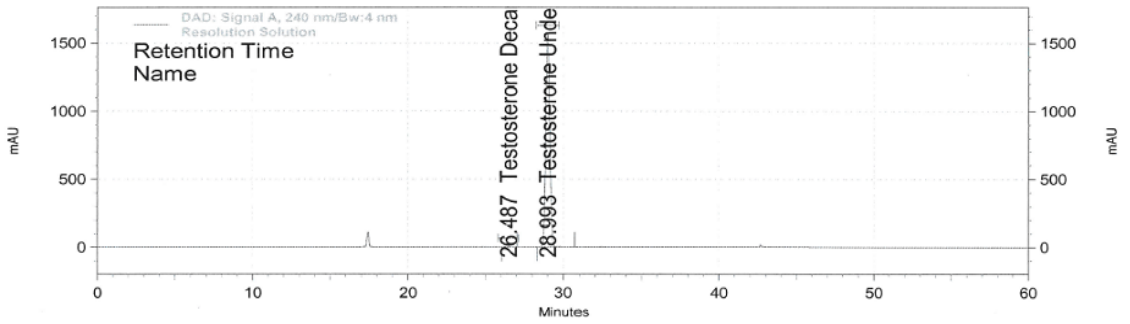
Placebo Chromatogram



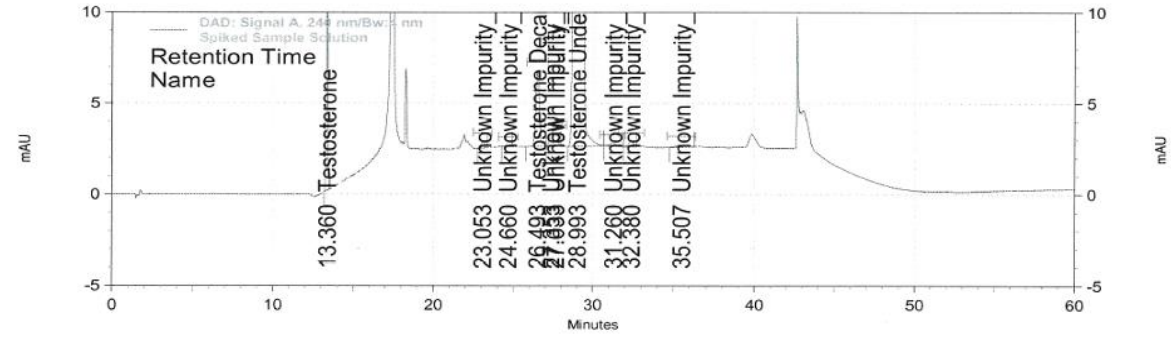
Standard Chromatogram



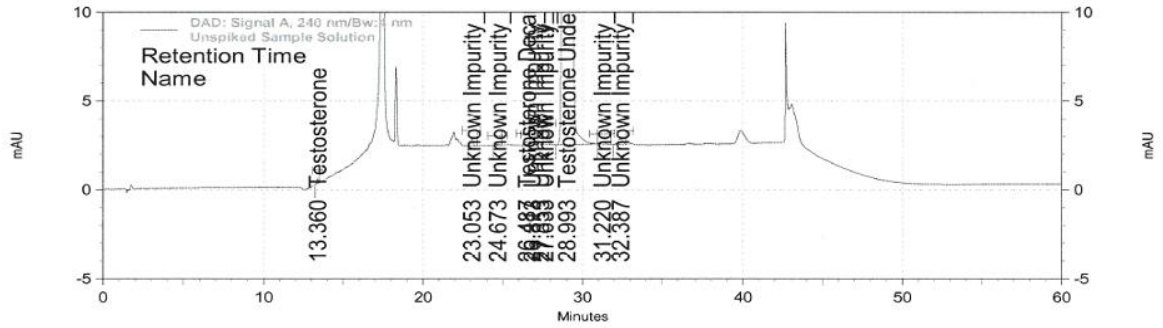
Resolution Solution Chromatogram



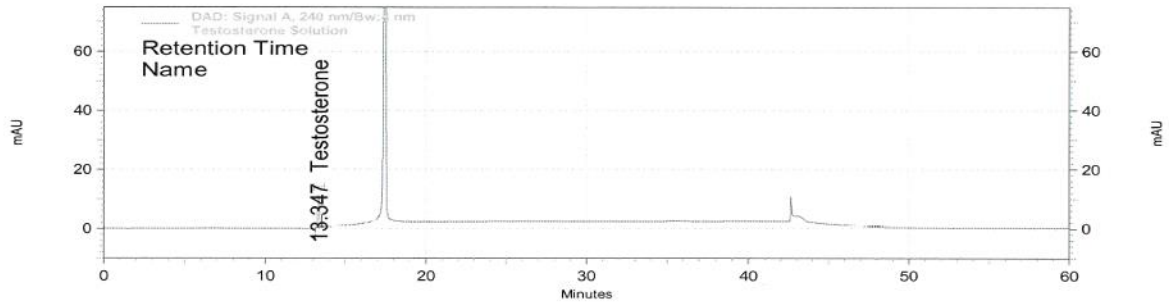
Spiked Sample Chromatogram



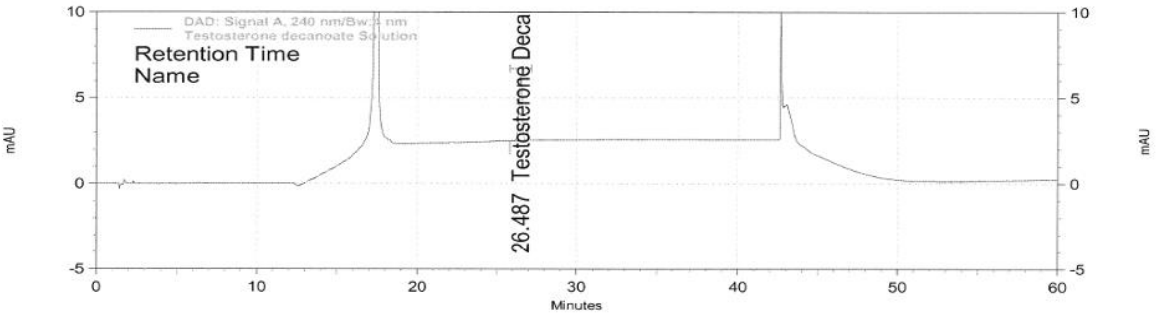
Unspiked Sample Chromatogram



Testosterone Impurity Chromatogram



Testosterone Decanoate Impurity Chromatogram



Conclusion:

There was no interference observed from blank, placebo peak at the retention time of Testosterone, Testosterone Decanoate, and Testosterone Undecanoate. The Peak Purity for standard, Unspiked samples, and spiked samples of Testosterone Undecanoate & its known impurities met the acceptance criteria. Testosterone Undecanoate impurities are well separated from co-eluents and the main peak. Hence, the method found specific for Testosterone Undecanoate Capsules 40mg.

8.2.2. Forced Degradation Study

Forced degradation testing studies are those undertaken to degrade the sample deliberately. These studies are used to evaluate the overall sensitivity of the material / Drug product for method validation purposes and/or degradation pathway elucidation.

Stressed samples of drug product and API were injected separately into the HPLC system equipped with a PDA detector by using test method conditions. The results are summarized in Tables 17-20.

Acid hydrolysis stress study:

Weighed and transferred 50 mg equivalent of Testosterone Undecanoate sample into 50 ml volumetric flask. Added 5 mL of 4N Hydrochloric acid solution to it and kept in the water bath at 80°C for 30minutes and neutralized with 5mL of 4N Sodium Hydroxide solution. Added 10 ml of water and sonicated for 10minutes. Then added 25mL of Acetonitrile and sonicated for 15minutes with intermediate shaking. Finally made up the volume with diluent and centrifuged the sample solution for 10 minutes at 2500rpm.

Base hydrolysis stress study:

Weighed and transferred 50 mg equivalent of Testosterone Undecanoate sample into 50 ml volumetric flask. Added 2 mL of 0.1N Sodium Hydroxide solution to it and kept in the water bath at 80°C for 30 minutes and neutralized with 2 mL of 0.1N Hydrochloric acid solution. Added 10 ml of water and sonicated for 10minutes. Then added 25 mL of Acetonitrile and sonicated for 15 minutes with intermediate shaking. Finally made up the volume with diluent and centrifuged the sample solution for 10 minutes at 2500 rpm.

Peroxide oxidation stress study:

Weighed and transferred 50 mg equivalent of Testosterone Undecanoate sample into 50 ml volumetric flask. Added 2mL of 3% Hydrogen peroxide solution to it and kept in the water bath at 80°C for 30 minutes. Added 10 ml of water and sonicated for 10minutes. Then added 25 mL of Acetonitrile and sonicated for 15minutes with intermediate shaking. Finally made up the volume with diluent and centrifuged the sample solution for 10 minutes at 2500 rpm.

Water degradation stress study:

Weighed and transferred 50 mg equivalent of Testosterone Undecanoate sample into 50 ml volumetric flask. Added 2 mL of water to it and kept in the water bath at 80°C for 30minutes. Added 10 ml of water and sonicated for 10minutes. Then added 25 mL of Acetonitrile and sonicated for 15 minutes with intermediate shaking. Finally made up the volume with diluent and centrifuged the sample solution for 10 minutes at 2500 rpm.

Heat stress study:

Kept the sample in a hot air oven at 50°C for 5 hours. Weighed and transferred 50 mg equivalent of Testosterone Undecanoate sample into 50 ml volumetric flask. Added 10 ml of water and sonicated for 10minutes. Then added 25 mL of Acetonitrile and sonicated for 15 minutes with intermediate shaking. Finally made up the volume with diluent and centrifuged the sample solution for 10 minutes at 2500rpm. Repeat the same procedure for samples stressed at 24 hours and 48 hours respectively.

Photo stability stress study:

Kept the sample in a photo stability chamber with and without a closed container for overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 Watt-hours / square meter. Weighed and transferred 50 mg equivalent of Testosterone Undecanoate sample into 50 ml volumetric flask. Added 10 ml of water and sonicated for 10minutes. Then added 25 mL of Acetonitrile and sonicated for 15 minutes with intermediate shaking. Finally made up the volume with diluent and centrifuged the sample solution for 10 minutes at 2500 rpm.

Note: Blank, API, and Placebo preparations follow the same procedure of sample preparation above for each specified condition.

% Known Impurity determination:

$$= \frac{A_K}{As} \times \frac{Ws}{100} \times \frac{2}{100} \times \frac{50}{W_{SP}} \times \frac{\text{Avg fill wt}}{LC} \times \frac{P}{100} \times 100 \times \frac{1}{RRF}$$

% Unknown impurity determination:

$$= \frac{A_{UN}}{As} \times \frac{Ws}{100} \times \frac{2}{100} \times \frac{50}{W_{SP}} \times \frac{\text{Avg fill wt}}{LC} \times \frac{P}{100} \times 100$$

% of Total Impurities = Sum of known impurities + Unknown impurities.

Where,

A_K = Area of Known impurity peak obtained with sample preparation.

A_{UN} = Area of Unknown impurity peak obtained with sample preparation.

As = Average area Testosterone Undecanoate peak obtained with standard preparation.

W_S = Weight of Testosterone Undecanoate working standard in mg.

W_{SP} = Weight of Sample in mg.

P = Purity of Testosterone Undecanoate working standard in percentage (on as basis).

LC = Label claim of Testosterone Undecanoate in mg/capsule

Avg fill wt = Average net content of Capsule

RRF = Relative response factor

Mass Balance determination:

The following general formula shall be used for Mass balance calculation.

%Total content of stressed sample =% Assay of stressed sample+%Total impurities

%Total content of unstressed sample =% Assay of unstressed sample+%Total impurities

$$\% \text{ Mass Balance} = \frac{\%\text{Total content of the stressed sample}}{\%\text{Total content of the unstressed sample}} \times 100$$

Table 17: Peak Purity for Forced Degradation Samples

For Stress Condition	Peak Purity			% Degradation
	A	B	C	
Unstressed Sample	1.0	1.0	1.0	0.2508
Added 2mL of 0.1N Sodium Hydroxide solution kept in the water bath at 80°C for 30minutes and neutralized with 2mL of 0.1N Hydrochloric acid solution.	1.0	1.0	1.0	0.0859
Added 2mL of 3% hydrogen peroxide solution kept in the water bath at 80°C for 30minutes.	1.0	1.0	1.0	0.0931
Added 2mL of water, kept in the water bath at 80°C for 30minutes.	1.0	1.0	1.0	0.0878
Photo stressed sample (open condition)	1.0	1.0	1.0	0.0919

Photo stressed sample (closed condition)	1.0	1.0	1.0	0.0941
Heat the sample kept in a hot air oven at 50ºC for 5 hours.	1.0	1.0	1.0	0.0757
Heat the sample kept in a hot air oven at 50ºC for 24 hours (with blister).	1.0	1.0	1.0	0.5894
Heat the sample kept in a hot air oven at 50ºC for 48 hours (with blister).	1.0	1.0	1.0	0.1236
Added 5mL of 4N Hydrochloric acid solution in the water bath at 80°C for 30minutes and neutralized with 5mL of 4N Sodium Hydroxide solution.	1.0	1.0	1.0	9.3671

A- Testosterone; B- Testosterone Decanoate; C- Testosterone Undecanoate

Table 18: Mass Balance for Forced Degradation Samples

Mass Balance			
Stressed condition	**% Degradation**	**Assay (%w/w)**	**Mass Balance**
Unstressed Sample	0.2508	99.6	-
Base stressed Sample	0.0859	98.4	98.6
Oxidation Stressed Sample	0.0931	98.5	98.7
Water Stressed Sample	0.0878	100.9	101.1
Photo stressed sample (open condition)	0.0919	95.5	95.7
Photo stressed sample (closed condition)	0.0941	98.6	98.8
Thermal stressed sample (50°C, 5 hours)	0.0757	99.4	99.6
Thermal stressed sample (50°C, 24 hours, with blister)	0.5894	99.2	100.6
Thermal stressed sample (50°C, 48 hours, with blister)	0.1236	100.2	99.7
Acid Stressed Sample	9.3671	90.6	100.7

Table 19: Peak purity for Forced Degradation samples - API

For Stress Condition	**Peak Purity**			**% Degradation**
	A	**B**	**C**	
Unstressed Sample	1.0	1.0	1.0	0.4814
Added 2mL of 0.1N Sodium Hydroxide solution, kept in the water bath at 80°C for 30minutes, and neutralized with 2mL of 0.1N Hydrochloric acid solution.	1.0	1.0	1.0	0.5529
Added 2mL of 3% hydrogen peroxide solution kept in the water bath at 80°C for 30minutes.	1.0	1.0	1.0	0.4619
Added 2mL of water, kept in the water bath at 80°C for 30minutes.	1.0	1.0	1.0	0.4730
Photo stressed sample (open condition)	1.0	1.0	1.0	0.5758
Photo stressed sample (closed condition)	1.0	1.0	1.0	0.5358

Heat the sample kept in a hot air oven at 50ºC for 5 hours.	1.0	1.0	1.0	0.4902
Added 5mL of 4N Hydrochloric acid solution in the water bath at 80°C for 30minutes and neutralized with 5mL of 4N Sodium Hydroxide solution.	1.0	1.0	1.0	5.5034

A- Testosterone; B- Testosterone Decanoate; C- Testosterone Undecanoate

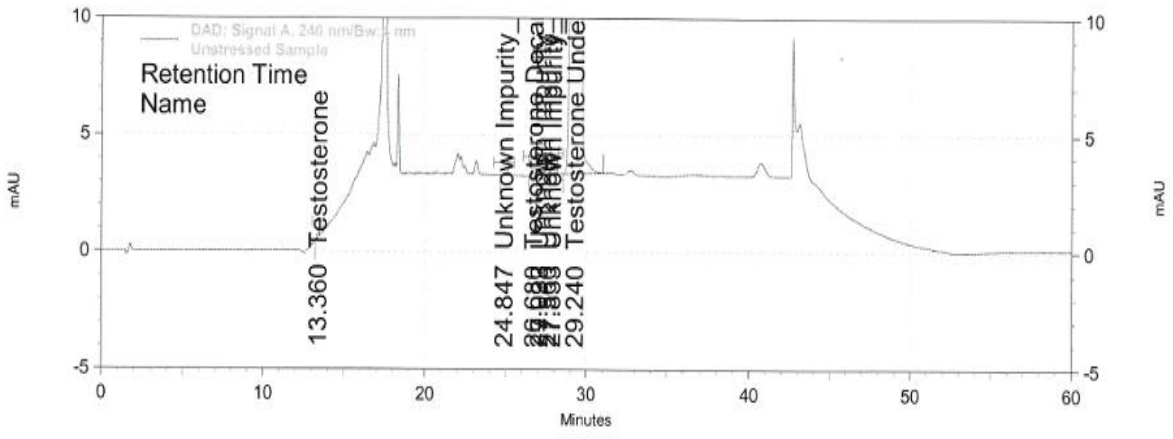
Table 20: Mass Balance for Forced Degradation samples - API

Mass Balance			
Stressed condition	**% Degradation**	**Assay (%w/w)**	**Mass Balance**
Unstressed Sample	0.4814	99.4	-
Base stressed Sample	0.5529	100.5	101.2
Oxidation Stressed Sample	0.4619	100.5	101.1
Water Stressed Sample	0.4730	100.9	101.5
Photo stressed sample (open condition)	0.5758	100.8	101.5
Photo stressed sample (closed condition)	0.5358	100.5	101.4
Thermal stressed sample	0.4902	100.5	101.1
Acid Stressed Sample	5.5034	90.2	102.4

Acceptance Criteria:

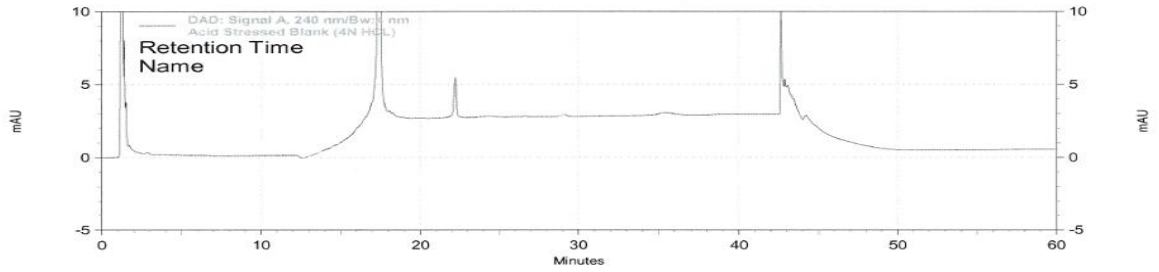
- There should not be any interference of blank and placebo peaks at the RT of the main analyte and its impurities.
- The Peak Purity should be NLT 0.9 in Open lab software.
- Mass Balance for all stressed conditions should be achieved between 95.0 to 105.0%.
- The degradation of the drug substance should be not more than 20%.

Unstressed Sample Chromatogram

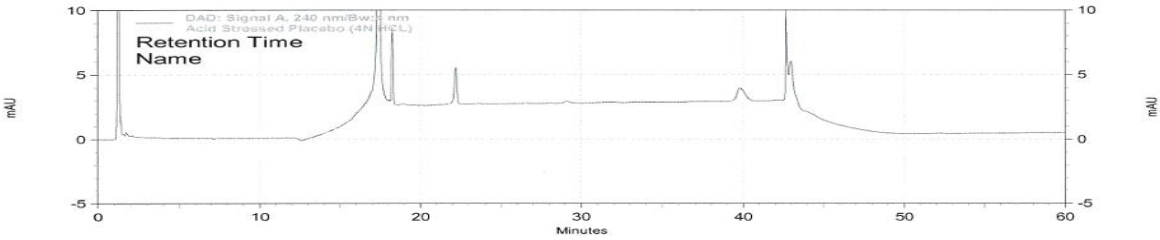


Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.360	2918	0.46	1.00
Unknown Impurity - 1	24.847	1855	0.85	1.00
Testosterone Decanoate	26.680	7793	0.91	1.00
Unknown Impurity – 2	27.033	2446	0.92	1.00
Unknown Impurity - 3	27.560	19655	0.94	1.00
Unknown Impurity – 4	27.833	12524	0.95	1.00
Testosterone Undecanoate	29.240	53011192	1.00	1.00

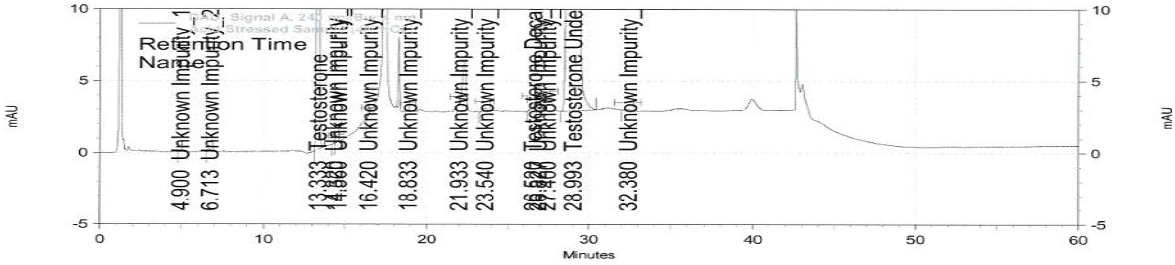
Acid stressed Blank Chromatogram



Acid stressed Placebo Chromatogram



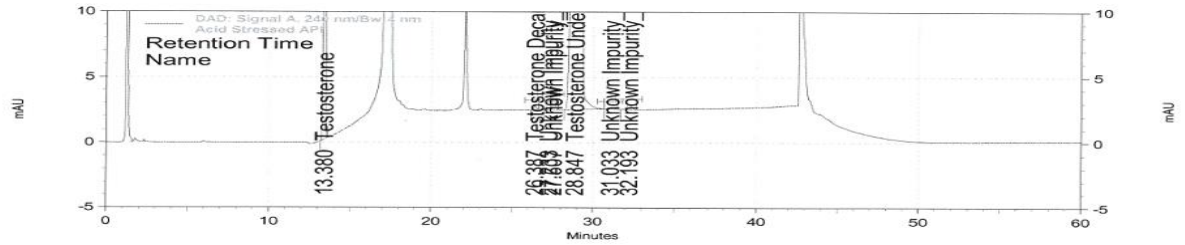
Acid stressed Sample Chromatogram



Name	Retention Time	Area	Relative Retention Time	Peak Purity
Unknown Impurity - 1	4.900	811	0.17	1.0
Unknown Impurity - 2	6.713	4251	0.23	1.0
Testosterone	13.333	7945046	0.46	1.0
Unknown Impurity – 3	14.320	974	0.49	1.0

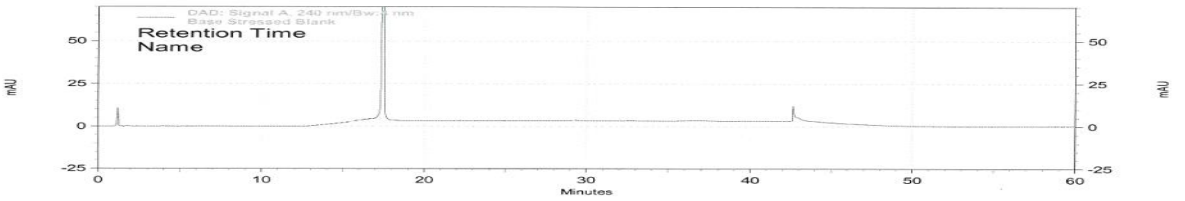
Unknown Impurity – 4	14.560	1120	0.50	1.0
Unknown Impurity – 5	16.420	904	0.57	1.0
Unknown Impurity – 6	18.833	1351	0.65	1.0
Unknown Impurity – 7	21.933	1445	0.76	1.0
Unknown Impurity – 8	23.540	9397	0.81	1.0
Testosterone Decanoate	26.520	12951	0.91	1.0
Unknown Impurity – 9	26.827	1921	0.93	1.0
Unknown Impurity – 10	27.400	16671	0.95	1.0
Testosterone Undecanoate	28.993	85961437	1.00	1.0
Unknown Impurity – 11	32.380	4820	1.12	1.0

Acid stressed API Chromatogram

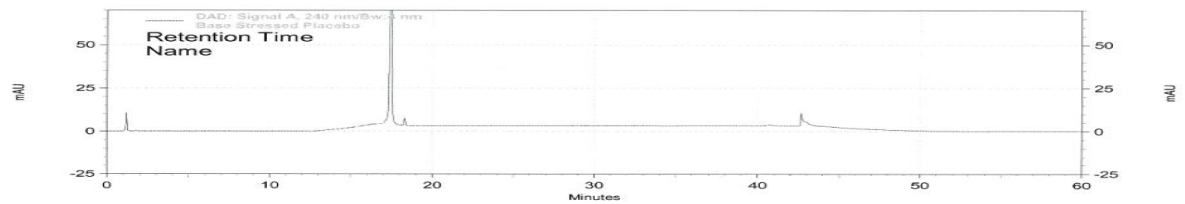


Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.380	774457	0.46	1.0
Testosterone Decanoate	26.387	6364	0.91	1.0
Unknown Impurity - 1	27.233	22397	0.94	1.0
Unknown Impurity - 2	27.507	23447	0.95	1.0
Testosterone Undecanoate	28.847	57658498	1.00	1.0
Unknown Impurity - 3	31.033	5215	1.08	1.0
Unknown Impurity - 4	32.193	15330	1.12	1.0

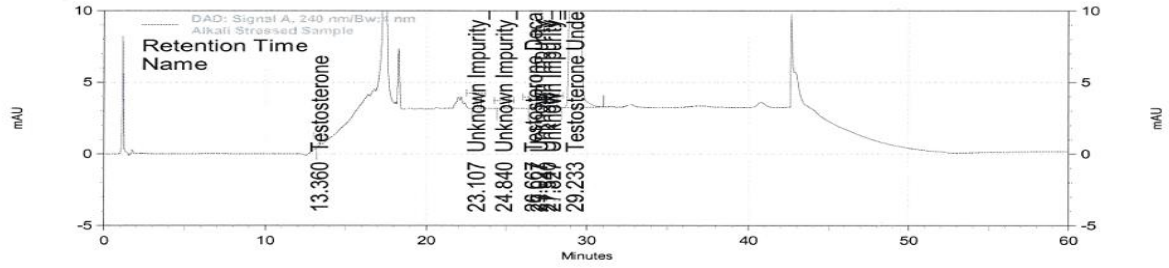
Base stressed Blank Chromatogram



Base stressed Placebo Chromatogram

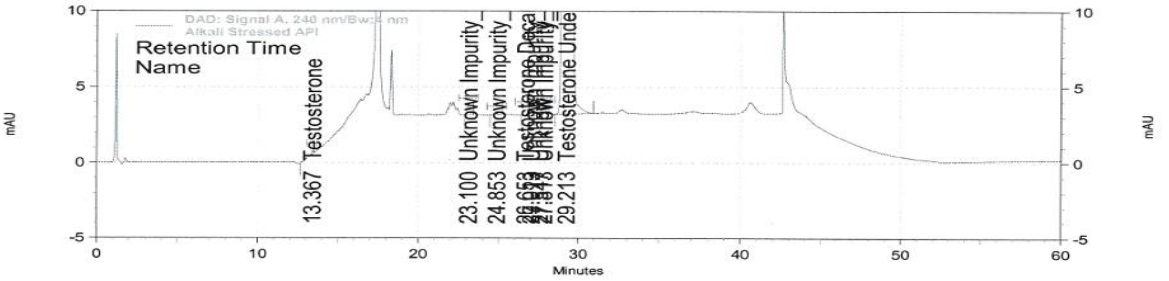


Alkali stressed Sample Chromatogram



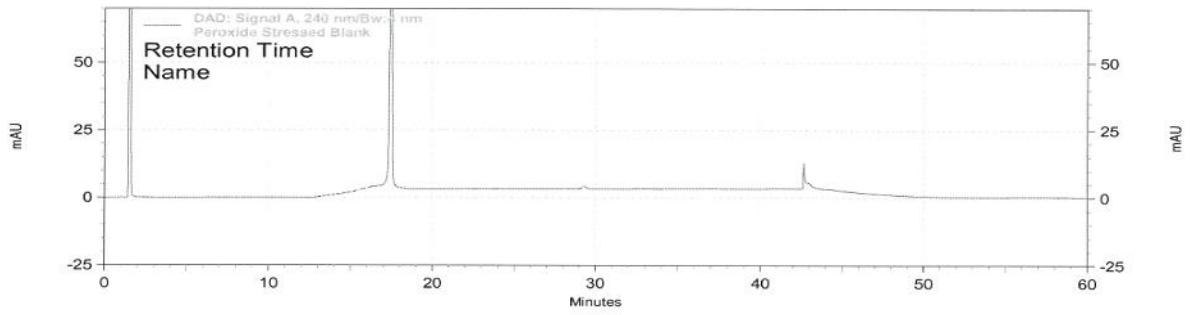
Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.360	3452	0.46	1.0
Unknown Impurity - 1	23.107	14181	0.79	1.0
Unknown Impurity - 2	24.840	2810	0.85	1.0
Testosterone Decanoate	26.667	6478	0.91	1.0
Unknown Impurity – 3	27.027	2067	0.92	1.0
Unknown Impurity – 4	27.540	16422	0.94	1.0
Unknown Impurity - 5	27.827	11032	0.95	1.0
Testosterone Undecanoate	29.233	43365737	1.00	1.0

Alkali stressed API Chromatogram

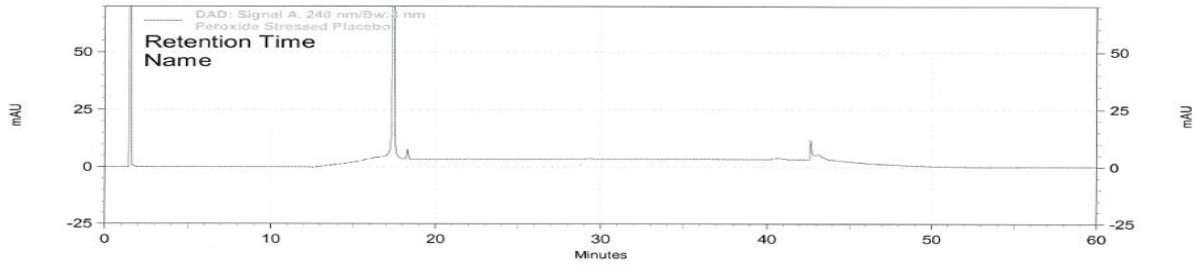


Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.367	4063	0.46	1.0
Unknown Impurity - 1	23.100	15336	0.79	1.0
Unknown Impurity – 2	24.853	3095	0.85	1.0
Testosterone Decanoate	26.653	8149	0.91	1.0
Unknown Impurity – 3	27.013	2805	0.92	1.0
Unknown Impurity – 4	27.547	20624	0.94	1.0
Unknown Impurity - 5	27.813	13444	0.95	1.0
Testosterone Undecanoate	29.213	56037727	1.00	1.0

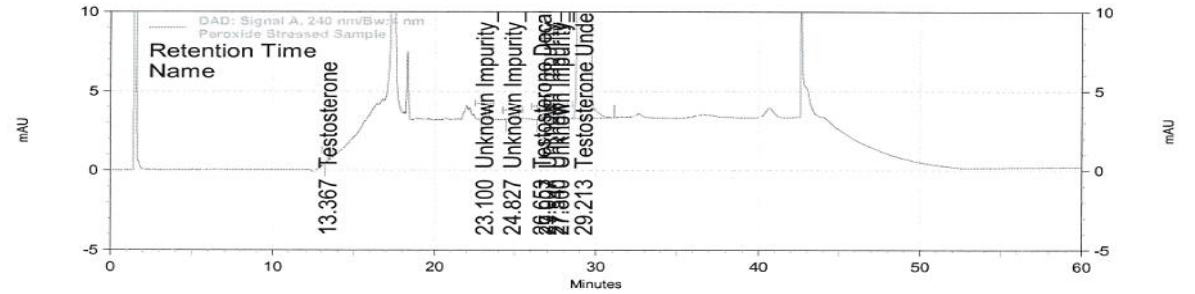
Peroxide stressed Blank Chromatogram



Peroxide stressed Placebo Chromatogram

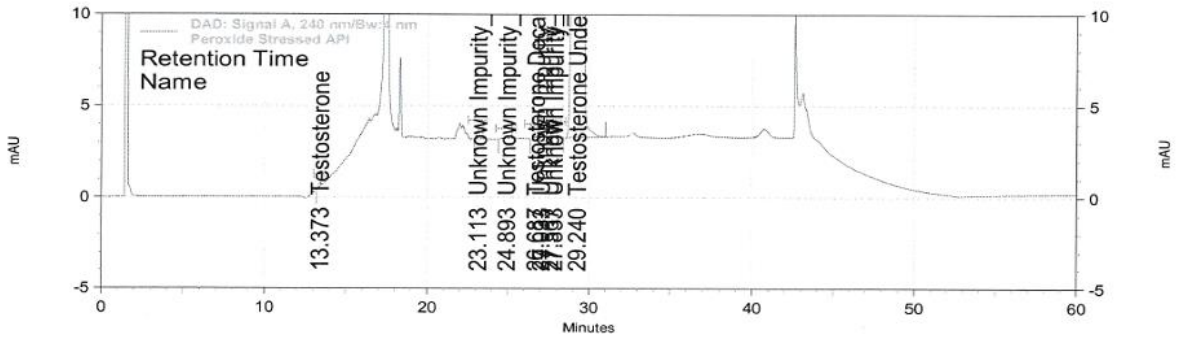


Peroxide stressed Sample Chromatogram



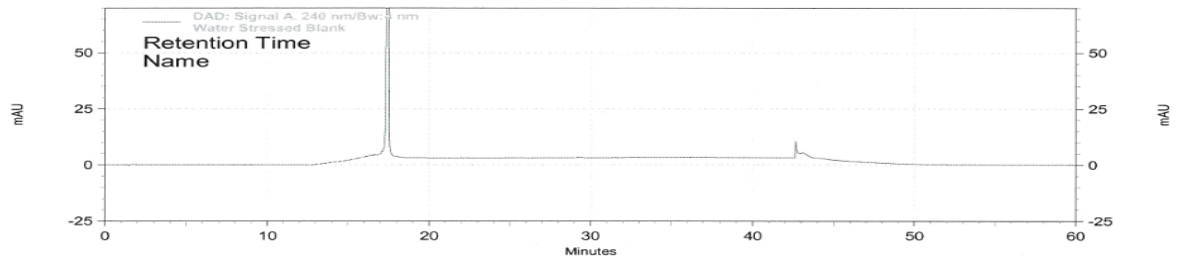
Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.367	3164	0.46	1.0
Unknown Impurity - 1	23.100	14058	0.79	1.0
Unknown Impurity – 2	24.827	3512	0.85	1.0
Testosterone Decanoate	26.653	7478	0.91	1.0
Unknown Impurity – 3	27.007	2243	0.92	1.0
Unknown Impurity – 4	27.540	19291	0.94	1.0
Unknown Impurity - 5	27.800	11152	0.95	1.0
Testosterone Undecanoate	29.213	52615776	1.00	1.0

Peroxide stressed API Chromatogram

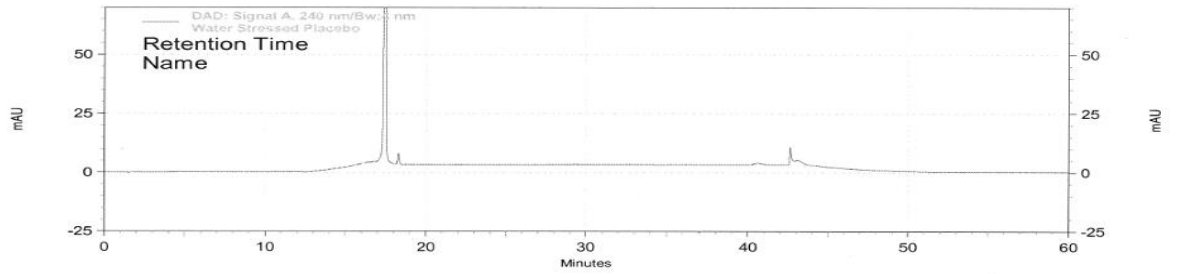


Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.373	3286	0.46	1.0
Unknown Impurity - 1	23.113	13658	0.79	1.0
Unknown Impurity – 2	24.893	3052	0.85	1.0
Testosterone Decanoate	26.687	6875	0.91	1.0
Unknown Impurity – 3	27.003	1812	0.92	1.0
Unknown Impurity – 4	27.567	17945	0.94	1.0
Unknown Impurity - 5	27.833	9905	0.95	1.0
Testosterone Undecanoate	29.240	49572353	1.00	1.0

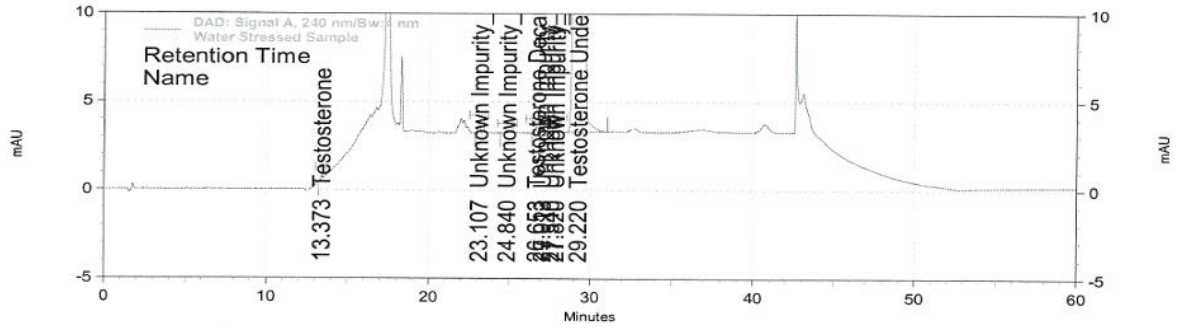
Water Stressed Blank Chromatogram



Water stressed Placebo Chromatogram

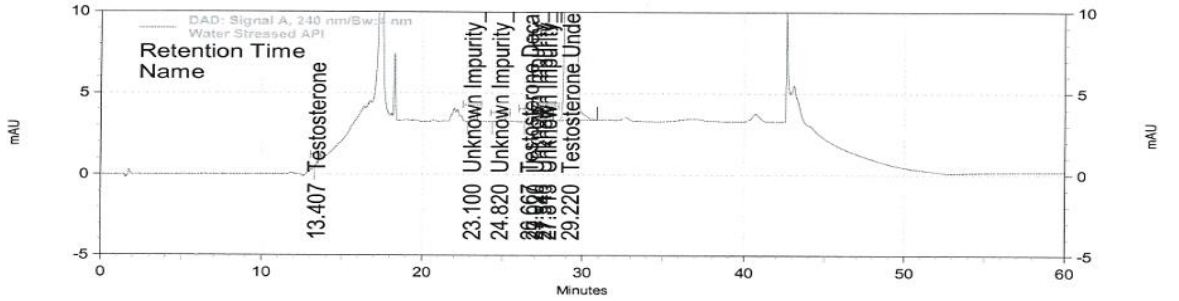


Water stressed Sample Chromatogram



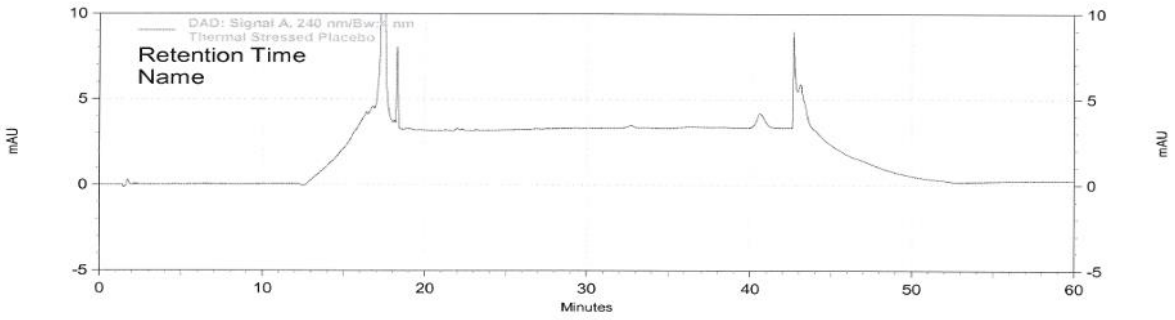
Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.373	2982	0.46	1.0
Unknown Impurity - 1	23.107	13047	0.79	1.0
Unknown Impurity – 2	24.840	2730	0.85	1.0
Testosterone Decanoate	26.653	7128	0.91	1.0
Unknown Impurity – 3	27.013	1909	0.92	1.0
Unknown Impurity – 4	27.540	17586	0.94	1.0
Unknown Impurity - 5	27.820	10776	0.95	1.0
Testosterone Undecanoate	29.220	48158598	1.00	1.0

Water stressed API Chromatogram

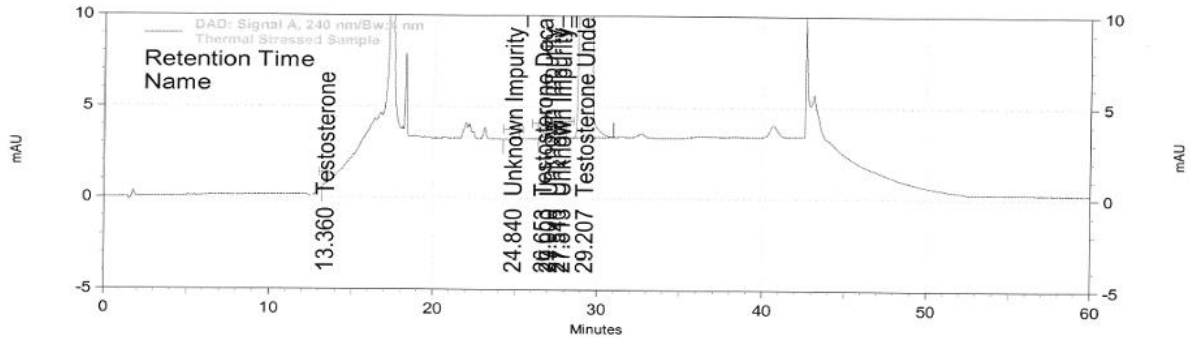


Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.407	2150	0.46	1.0
Unknown Impurity - 1	23.100	13598	0.79	1.0
Unknown Impurity – 2	24.820	2715	0.85	1.0
Testosterone Decanoate	26.667	7026	0.91	1.0
Unknown Impurity – 3	27.020	2255	0.92	1.0
Unknown Impurity – 4	27.540	18610	0.94	1.0
Unknown Impurity - 5	27.813	11285	0.95	1.0
Testosterone Undecanoate	29.220	48517023	1.00	1.0

Thermal stressed Placebo Chromatogram

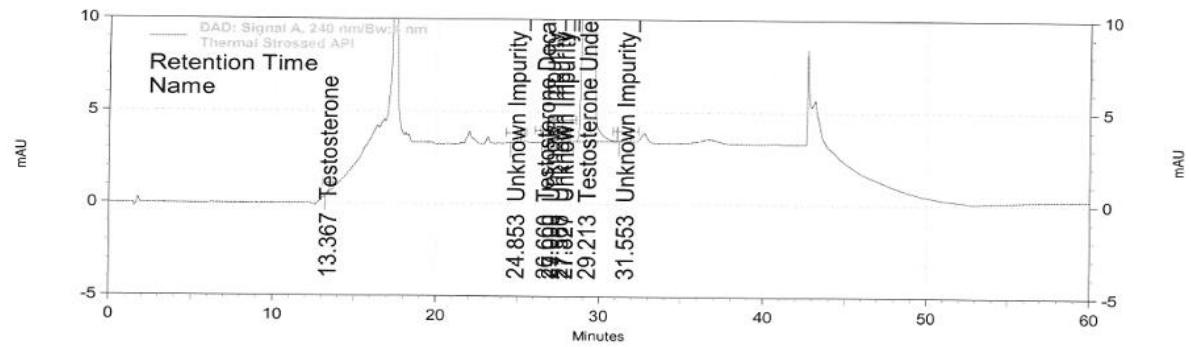


Thermal stressed Sample Chromatogram



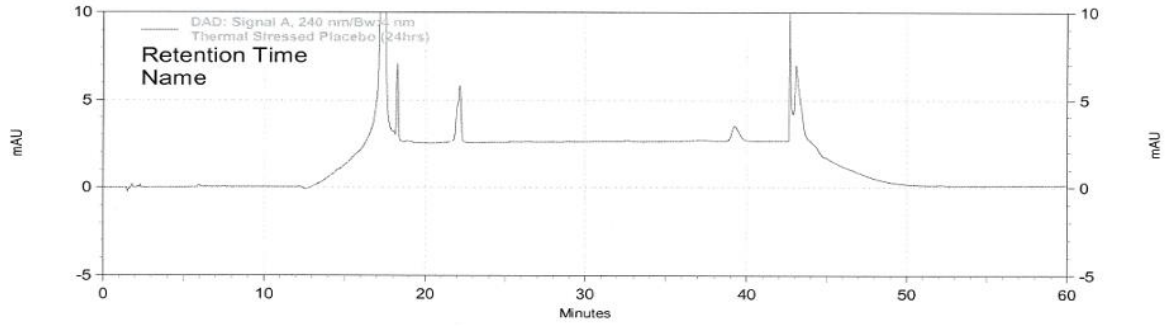
Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.360	3212	0.46	1.0
Unknown Impurity - 1	24.840	2575	0.85	1.0
Testosterone Decanoate	26.653	7958	0.91	1.0
Unknown Impurity – 2	27.000	2386	0.92	1.0
Unknown Impurity – 3	27.540	20972	0.94	1.0
Unknown Impurity - 4	27.813	12391	0.95	1.0
Testosterone Undecanoate	29.207	55970640	1.00	1.0

Thermal stressed API Chromatogram

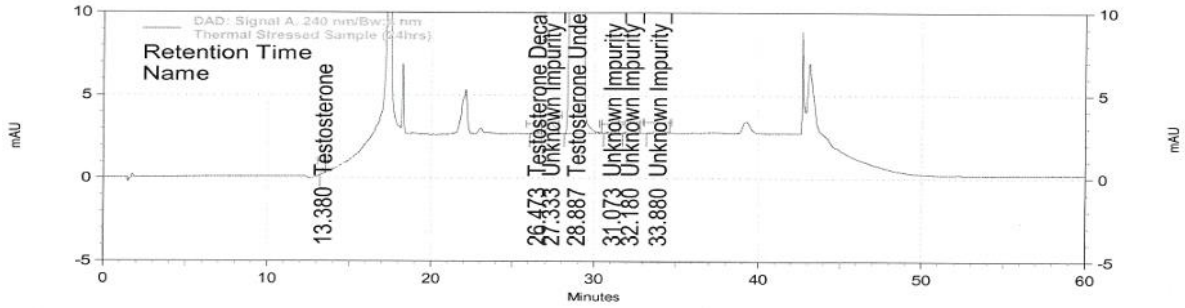


Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.367	1532	0.46	1.0
Unknown Impurity - 1	24.853	2575	0.85	1.0
Testosterone Decanoate	26.660	3609	0.91	1.0
Unknown Impurity – 2	27.000	1207	0.92	1.0
Unknown Impurity – 3	27.560	23090	0.94	1.0
Unknown Impurity - 4	27.827	22166	0.95	1.0
Testosterone Undecanoate	29.213	58914712	1.00	1.0
Unknown Impurity - 5	31.553	5328	1.08	1.0

Thermal stressed Placebo (24Hrs, 50°C) Chromatogram

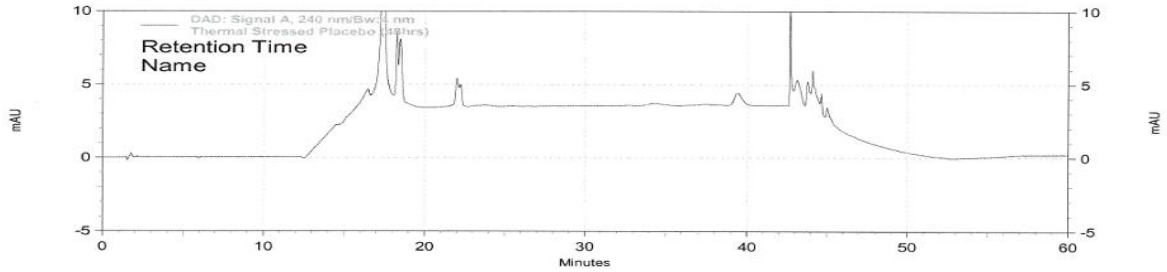


Thermal stressed sample (24Hrs, 50°C) Chromatogram

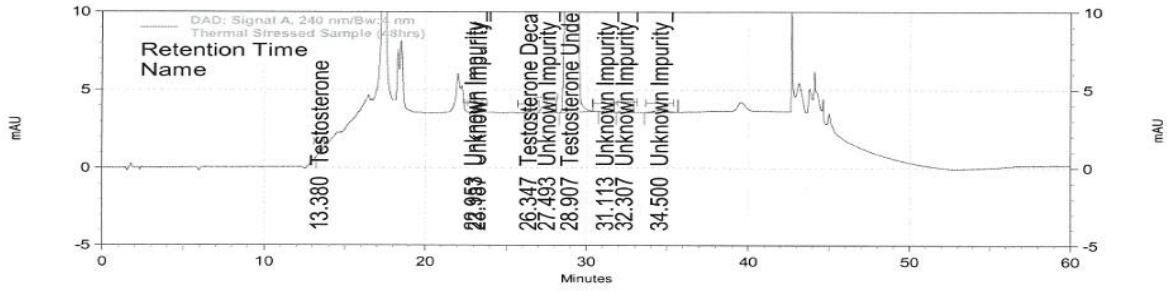


Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.380	5367	0.46	1.0
Testosterone Decanoate	26.473	5754	0.92	1.0
Unknown Impurity – 1	27.333	38952	0.95	1.0
Testosterone Undecanoate	28.887	54289316	1.00	1.0
Unknown Impurity – 2	31.073	5968	1.08	1.0
Unknown Impurity - 3	32.180	13407	1.11	1.0
Unknown Impurity - 4	33.880	15541	1.17	1.0

Thermal stressed Placebo (48Hrs, 50°C) Chromatogram



Thermal stressed Sample (48Hrs, 50°C) Chromatogram



Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.380	4407	0.46	1.0
Unknown Impurity – 1	22.953	1361	0.79	1.0
Unknown Impurity – 2	23.187	1274	0.80	1.0
Testosterone Decanoate	26.347	1042	0.91	1.0
Unknown Impurity - 3	27.493	35647	0.95	1.0
Testosterone Undecanoate	28.907	45556981	1.00	1.0
Unknown Impurity - 4	31.113	4343	1.08	1.0
Unknown Impurity – 5	32.307	12317	1.12	1.0
Unknown Impurity - 6	34.500	16034	1.19	1.0

Photo stressed Placebo (Open condition) Chromatogram

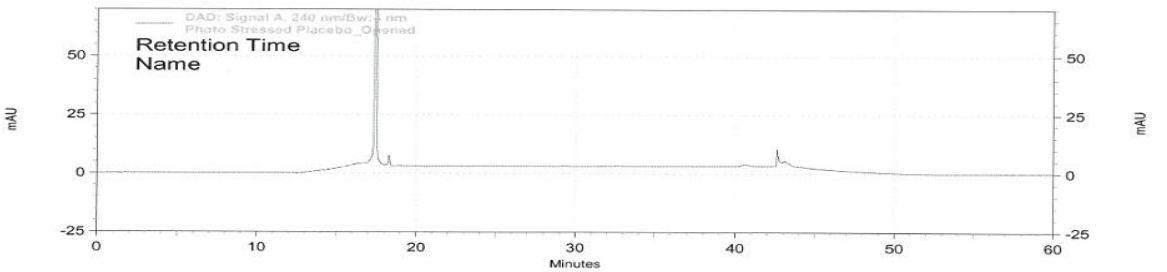
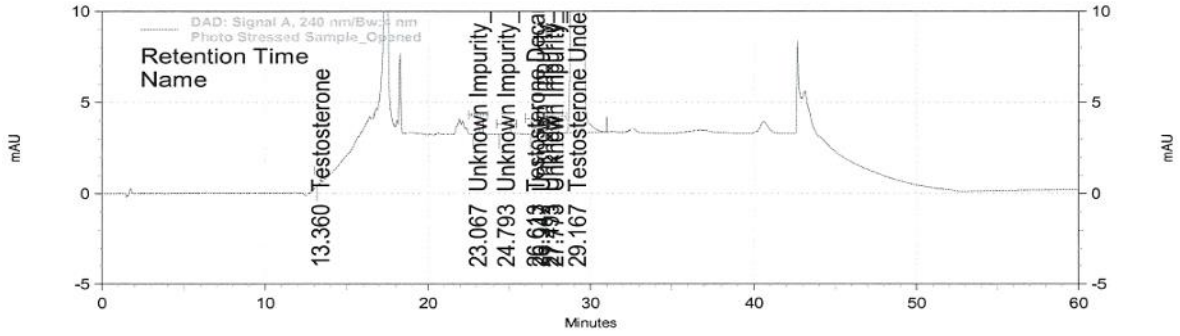
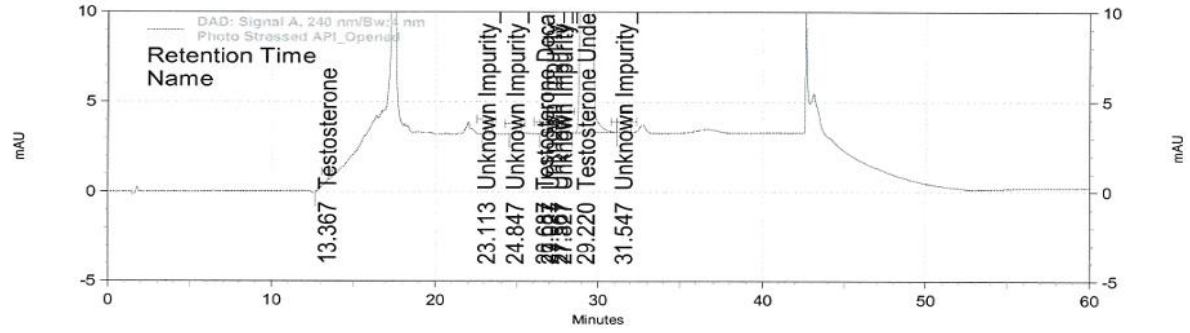


Photo stressed Sample (Open condition) Chromatogram



Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.360	3075	0.46	1.0
Unknown Impurity – 1	23.067	14133	0.79	1.0
Unknown Impurity – 2	24.793	2490	0.85	1.0
Testosterone Decanoate	26.613	7791	0.91	1.0
Unknown Impurity - 3	26.947	2350	0.92	1.0
Unknown Impurity - 4	27.493	19765	0.94	1.0
Unknown Impurity – 5	27.773	12312	0.95	1.0
Testosterone Undecanoate	29.167	53739182	1.00	1.0

Photo stressed API (Open condition) Chromatogram



Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.367	1834	0.46	1.0
Unknown Impurity – 1	23.113	8680	0.79	1.0
Unknown Impurity – 2	24.847	2816	0.85	1.0
Testosterone Decanoate	26.687	3564	0.91	1.0
Unknown Impurity - 3	27.027	1164	0.92	1.0
Unknown Impurity - 4	27.567	23345	0.94	1.0
Unknown Impurity – 5	27.827	21748	0.95	1.0
Testosterone Undecanoate	29.220	58328039	1.00	1.0
Unknown Impurity – 6	31.547	5535	1.08	1.0

Photo stressed Placebo (Closed condition) Chromatogram

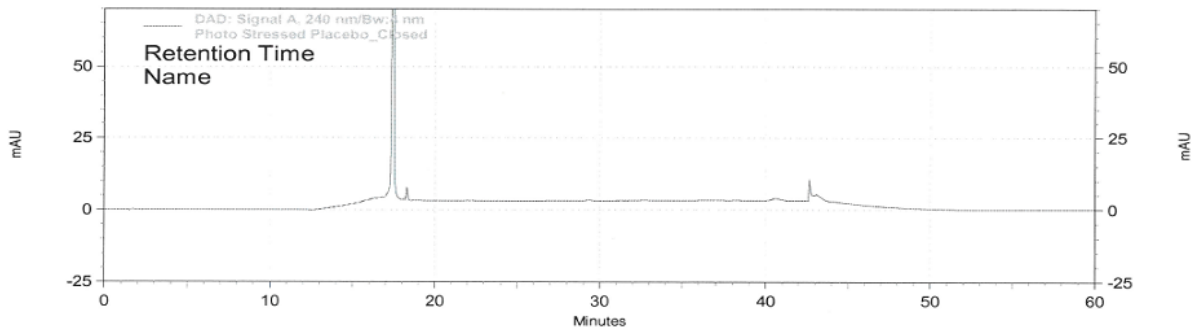
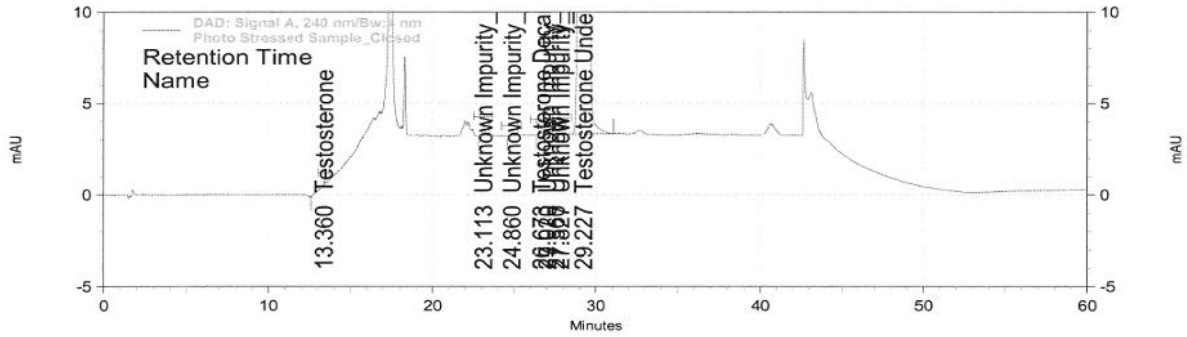
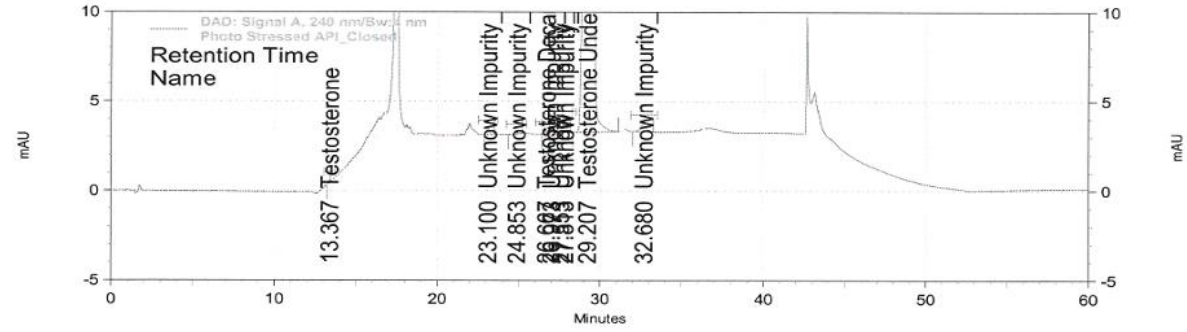


Photo stressed Sample (Closed condition) Chromatogram



Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.360	3798	0.46	1.0
Unknown Impurity – 1	23.113	14304	0.79	1.0
Unknown Impurity – 2	24.860	2279	0.85	1.0
Testosterone Decanoate	26.673	7468	0.91	1.0
Unknown Impurity - 3	27.020	2191	0.92	1.0
Unknown Impurity - 4	27.560	19964	0.94	1.0
Unknown Impurity – 5	27.827	11943	0.95	1.0
Testosterone Undecanoate	29.227	53353079	1.00	1.0

Photo stressed API (Closed condition) Chromatogram



Name	Retention Time	Area	Relative Retention Time	Peak Purity
Testosterone	13.367	1553	0.46	1.0
Unknown Impurity – 1	23.100	8492	0.79	1.0
Unknown Impurity – 2	24.853	3027	0.85	1.0
Testosterone Decanoate	26.667	3480	0.91	1.0
Unknown Impurity - 3	26.973	1075	0.92	1.0
Unknown Impurity - 4	27.553	22709	0.94	1.0
Unknown Impurity – 5	27.813	22581	0.95	1.0
Testosterone Undecanoate	29.207	58291698	1.00	1.0
Unknown Impurity – 6	32.680	25497	1.12	1.0

Conclusion:

All impurities were well separated from each other. The peak purity and mass balance met the acceptance criteria as per the protocol. These results show that the method is stability-indicating and intended to be used for Testosterone Undecanoate Capsules 40mg.

8.3. PRECISION

The precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of a homogenous sample. The precision of the analytical method is usually expressed as the standard deviation and relative standard deviation.

8.3.1. System Precision

The system precision was checked to ensure that the analytical system is working properly. The retention time and area response for Testosterone Undecanoate from six standard replicate injections were measured and calculated the percentage of relative standard deviation. The results are summarized in below Table 21.

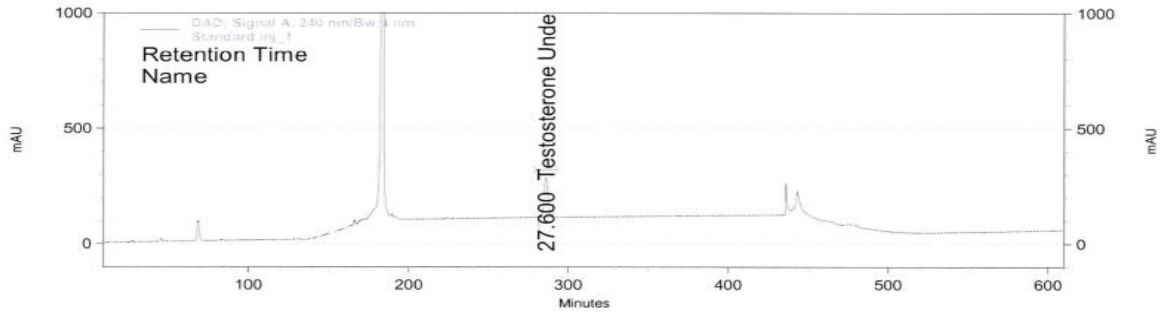
Table 21: Results of System Precision

No of injection	Area	Retention time
	Testosterone Undecanoate	Testosterone Undecanoate
Standard injection 1	2420828	27.600
Standard injection 2	2414432	27.627
Standard injection 3	2418284	27.653
Standard injection 4	2422922	27.660
Standard injection 5	2427131	27.680
Standard injection 6	2415976	27.680
Average	2419929	27.65
% RSD	0.2	0.1

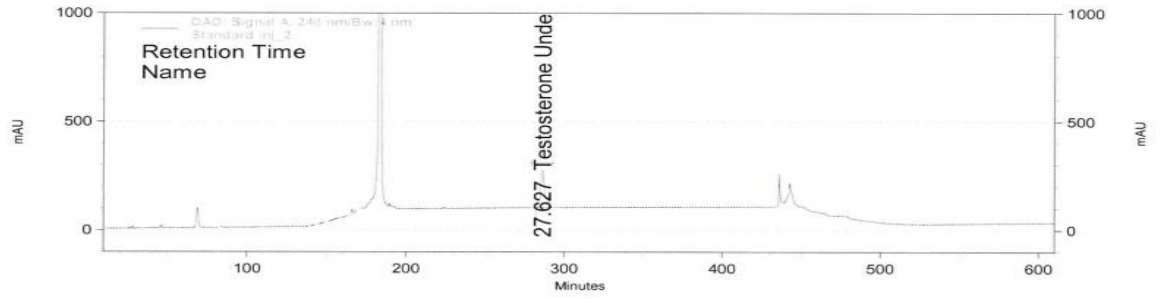
Acceptance Criteria:

- % RSD for Area response for Testosterone Undecanoate obtained from six replicate injections of standard solution should be NMT 5.0.
- % RSD for retention time of Testosterone Undecanoate obtained from six replicate injections of standard solution should be NMT 1.0.

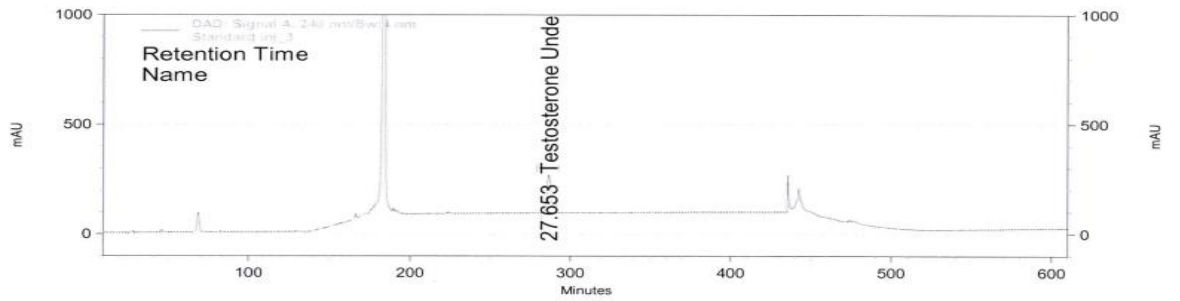
Standard Injection – 1 Chromatogram



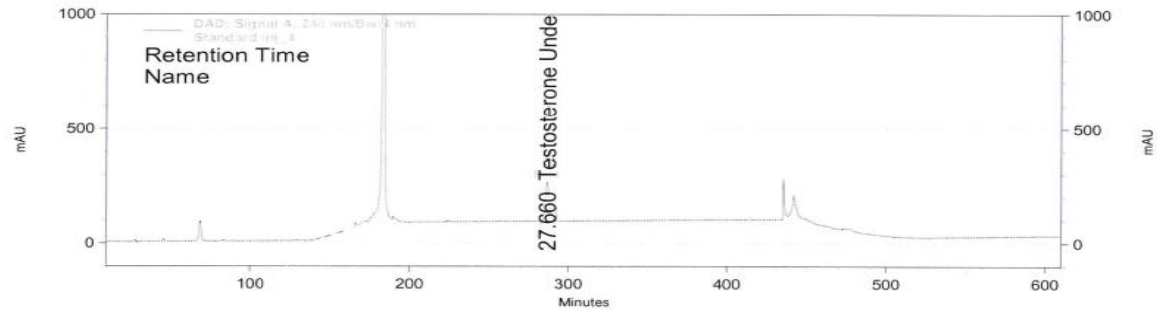
Standard Injection – 2 Chromatogram



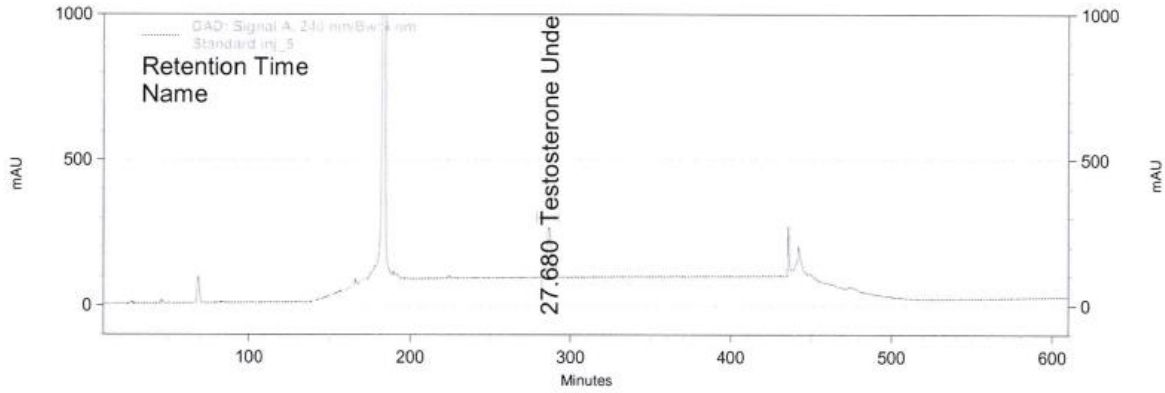
Standard Injection – 3 Chromatogram



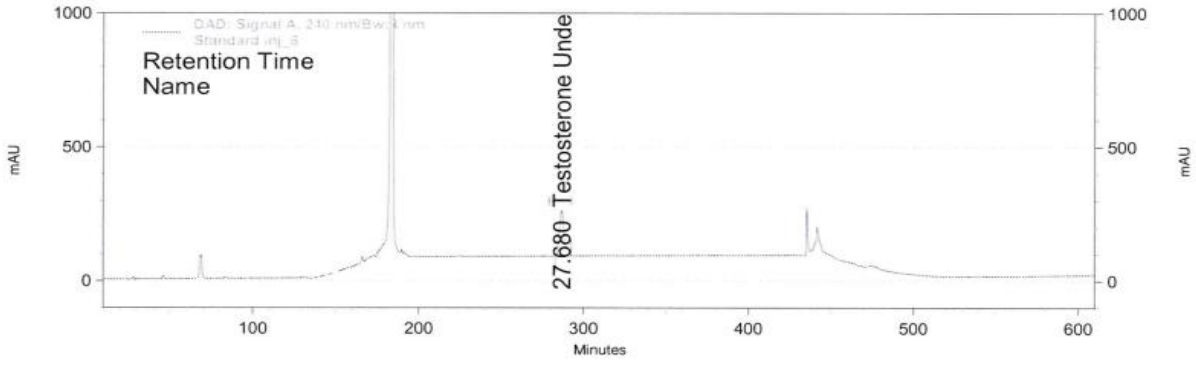
Standard Injection – 4 Chromatogram



Standard Injection – 5 Chromatogram



Standard Injection – 6 Chromatogram



Conclusion:

From the above results, the retention time and area response are consistent as evidenced by relative standard deviation and the results met the acceptance criteria. Hence, the system was found to be precise.

8.3.2. Method Precision

To determine the precision of the method, six test solutions of Testosterone Undecanoate Capsules 40mg were prepared and treated according to the method of analysis.

The sample solution was prepared six replicates by spiking known impurity at specification level and calculated the % of known impurity. Six replicates of unspiked sample solutions were prepared and calculated the % of unknown impurity with respect to standard preparation. Total impurities were calculated from both spiked and unspiked samples. The results obtained are given in below table 22.

Preparation of Impurity stock solution:

Weighed about 2 mg each of Testosterone and Testosterone Decanoate. Transferred it into a 20 mL volumetric flask, dissolved, and diluted to the volume with diluent.

Preparation of Placebo solution:

Weighed and transferred the placebo equivalent to 50 mg of Testosterone Undecanoate into a 50 ml volumetric flask. Added 10 ml of water and sonicated for 10 minutes. Then added 25 mL of Acetonitrile and sonicated for 15 minutes with intermediate shaking. Finally made up the volume with diluent and centrifuged the solution for 10 minutes at 2500 rpm.

Unspiked Sample Preparation:

Weighed 20 capsules and calculated the net content of the capsules. Weighed and transferred 50 mg equivalent of Testosterone Undecanoate sample into 50 ml volumetric flask. Added 10 ml of water and sonicated for 10 minutes. Then added 25 mL of Acetonitrile and sonicated for 15 minutes with intermediate shaking. Finally made up the volume with diluent and centrifuged the sample solution for 10 minutes at 2500 rpm.

Spiked Sample Preparation:

Weighed 20 capsules and calculated the net content of the capsules. Weighed and transferred 50 mg equivalent of Testosterone Undecanoate sample into 50 ml volumetric flask. Added 10 ml of water and sonicated for 10 minutes. Then added 25 mL of Acetonitrile and sonicated for 15 minutes with intermediate shaking. Finally transferred 1 mL of impurity stock solution into the flask and made up the volume with diluent. Centrifuged the sample solution for 10 minutes at 2500 rpm.

Table 22: Results of Method Precision

Sample Name	Testosterone (%w/w)	Testosterone Decanoate (%w/w)	Highest Unknown impurity (% w/w)	Total Impurities (% w/w)
Sample1	1.0024	0.9405	0.0875	2.1608
Sample 2	1.0254	0.9535	0.0881	2.1958
Sample 3	0.9975	0.9332	0.0866	2.1441
Sample 4	1.0599	1.0339	0.0883	2.3121
Sample 5	1.0495	0.9750	0.0871	2.2383
Sample 6	1.0046	0.9363	0.0876	2.1540
Average	1.0232	0.9620	0.0875	2.2009
STD. Dev	0.0264	0.0384	0.0006	0.06455
% RSD	2.6	4.0	0.7	2.9

Acceptance Criteria:

- All the results should be within the specification limit.
- The Percentage RSD of Known, Unknown, and Total impurities should be NMT 10.0. If the unknown impurities are below 0.1%, it should be NMT 15.0, and are above 0.1% it should be NMT 10.0.

Conclusion:

The percentage Relative Standard Deviation for % of Known, Unknown, and Total impurities from six replicate sample preparations met the acceptance criteria. The result shows that the method is acceptable with respect to Method precision for Testosterone Undecanoate Capsules 40mg.

8.3.3. Intermediate Precision

The intermediate precision was carried out to ensure that the analytical results remain unaffected due to changes in the environmental conditions like change in the instrument, analyst, column, and day.

Repeated the method precision study using different analysts, different instruments, different columns, and different days. The results obtained are given in Tables 23 & 24.

Table 23: Results of Intermediate Precision

Sample Name	Testosterone (%w/w)	Testosterone Decanoate (%w/w)	Highest Unknown impurity (% w/w)	Total Impurities (% w/w)
Sample1	1.0268	0.9423	0.1034	2.1932
Sample 2	1.0252	0.9412	0.1022	2.1906
Sample 3	1.0290	0.9490	0.1023	2.2028
Sample 4	1.0398	0.9617	0.1044	2.1872
Sample 5	1.0233	0.9475	0.1031	2.1966
Sample 6	1.0218	0.9416	0.1038	2.1884
Average	1.0277	0.9472	0.1032	2.1931
STD. Dev	0.0065	0.0078	0.0009	0.00582
% RSD	0.6	0.8	0.8	0.3

Table 24: Comparison between Method and Intermediate Precision

Sample Name	Testosterone (%w/w)		Testosterone Decanoate (%w/w)		Highest Unknown impurity (% w/w)		Total Impurities (% w/w)	
	I	II	I	II	I	II	I	II
Sample 1	1.0024	1.0268	0.9405	0.9423	0.0875	0.1034	2.1608	2.1932
Sample 2	1.0254	1.0252	0.9535	0.9412	0.0881	0.1022	2.1958	2.1906
Sample 3	0.9975	1.0290	0.9332	0.9490	0.0866	0.1023	2.1441	2.2028
Sample 4	1.0599	1.0398	1.0339	0.9617	0.0883	0.1044	2.3121	2.1872
Sample	1.0495	1.0233	0.9750	0.9475	0.0871	0.1031	2.2383	2.1966
Sample	1.0046	1.0218	0.9363	0.9416	0.0876	0.1038	2.1540	2.1884
Average	1.0254		0.9546		0.0954		2.1970	
%RSD	1.8		2.9		8.6		2.0	

I-Analyst 1 and II- Analyst 2

Acceptance Criteria:

- All the results should be within the specification limit.
- The Percentage RSD of Known, Unknown, and Total impurities should be NMT 10.0. If the unknown impurities are below 0.1% it should be NMT 15.0 and for above 0.1% it should be NMT 10.0.
- Overall Percentage RSD of Known, Unknown, and Total impurities between method precision and Intermediate precision (12 samples) should be NMT 10.0. If the Unknown impurities are below 0.1% it should be NMT 15.0 and for above 0.1% it should be NMT 10.0.

Conclusion:

The percentage Relative Standard Deviation for Known impurities, Unknown impurities, and Total impurities from six replicated preparation met the acceptance criteria. The percentage Relative Standard Deviation for Testosterone, Testosterone Decanoate, Unknown impurities, and Total impurities from 12 sample preparations met the acceptance limit. It shows that the related substances method met the repeatability acceptance criteria for Known impurities, Unknown impurities, and Total impurities. Hence, the method for related substances of Testosterone Undecanoate Capsules 40mg was found rugged.

8.4. LIMIT OF DETECTION AND LIMIT OF QUANTITATION

Limit of Detection

It is the lowest amount of analyte in the sample that can be detected but not necessarily quantitated, under the stated experimental conditions.

Limit of Quantitation

It is the lowest amount of analyte in the sample that can be quantitated with acceptable accuracy and precision, under the stated experimental conditions.

Determination of LOD and LOQ:

Calculated the LOD and LOQ by using peak slope method, formulae are followed.

$$LOD = \frac{3.3 \times \sigma}{Slope}$$

$$LOQ = \frac{10 \times \sigma}{Slope}$$

Where

σ = Residual standard deviation

Slope = Slope of the linear curve

Procedure:

Prepared and injected Testosterone Undecanoate, Testosterone and Testosterone Decanoate from 10% to 100% at specification level concentration (0.02% to 2.0% with respect to test concentration) and determined the LOD, LOQ by slope method.

Preparation of Prediction LOD/LOQ Stock solution:

Weighed accurately about each 2 mg of Testosterone Undecanoate working standard, Testosterone impurity, and Testosterone Decanoate impurity into 100 mL volumetric flask with 70 mL of diluent added to it and sonicated to dissolve the content. Finally made up the volume with diluent and mixed well.

Preparation of Prediction LOD and LOQ solution 1:

Pipetted out 1mL of above solution into 100mL with diluent and mixed well. (0.2ppm)

Preparation of Prediction LOD and LOQ solution 2:

Pipetted out 1mL of above solution into 50mL with diluent and mixed well. (0.4ppm)

Preparation of Prediction LOD and LOQ solution 3:

Pipetted out 1mL of above solution into 20mL with diluent and mixed well. (1.0ppm)

Preparation of Prediction LOD and LOQ solution 4:

Pipetted out 1.5mL of above solution into 20mL with diluent and mixed well. (1.5ppm)

Preparation of Prediction LOD and LOQ solution 5:

Pipetted out 2mL of above solution into 20mL with diluent and mixed well. (2.0ppm)

Table 25: Testosterone – LOD/LOQ Establishment

S. No.	% Level	Concentration in µg/mL	Peak Response of Testosterone
1	0.02	0.220	141318
2	0.04	0.440	288335
3	0.10	1.100	724364
4	0.15	1.650	1092599
5	0.20	2.199	1442158
Slope			658773.5418
Intercept			-1213
Coefficient of correlation (r)			1.0000
Coefficient of regression (r^2)			0.9999
Standard Deviation			5428
LOQ (ppm)			0.082
LOD (ppm)			0.027

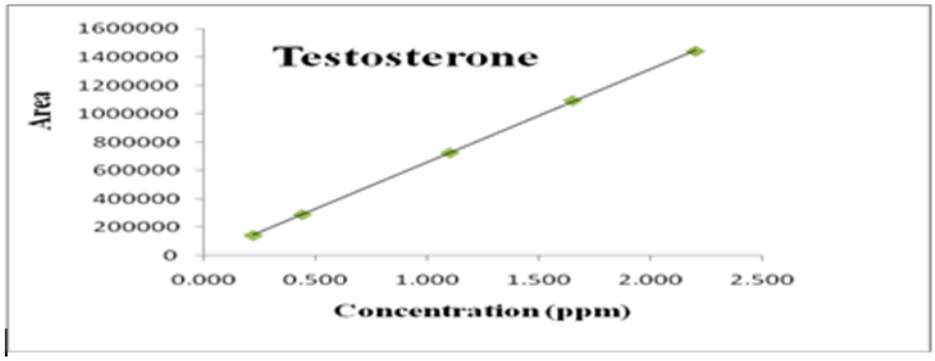


Figure 9: Testosterone – LOD/LOQ Establishment Graph

Table 26: Testosterone Decanoate – LOD/LOQ Establishment

S. No.	% Level	Concentration in µg/mL	Peak Response of Testosterone Decanoate
1	0.02	0.217	126317
2	0.04	0.433	241159
3	0.10	1.083	585308
4	0.15	1.625	875209
5	0.20	2.167	1148757
Slope			526058.111
Intercept			14078
Coefficient of correlation (r)			1.0000
Coefficient of regression (r^2)			0.9999
Standard Deviation			4873
LOQ (ppm)			0.093
LOD (ppm)			0.031

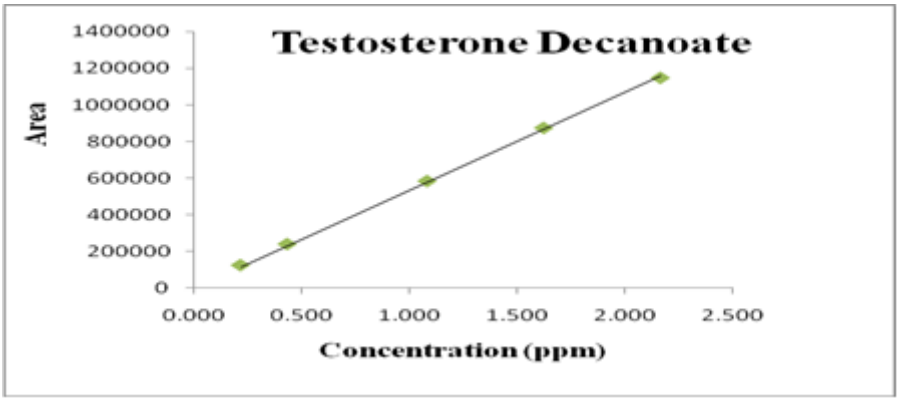


Figure 10: Testosterone Decanoate – LOD/LOQ Establishment Graph

Table 27: Testosterone Undecanoate – LOD/LOQ Establishment

S. No.	% Level	Concentration in µg/mL	Peak Response of Testosterone Undecanoate
1	0.02	0.227	133735
2	0.04	0.454	222178
3	0.10	1.135	546248
4	0.15	1.703	820319
5	0.20	2.270	1086661
Slope			470356.4103
Intercept			17189
Coefficient of correlation (r)			0.9998
Coefficient of regression (r^2)			0.9997
Standard Deviation			8170
LOQ (ppm)			0.174
LOD (ppm)			0.057

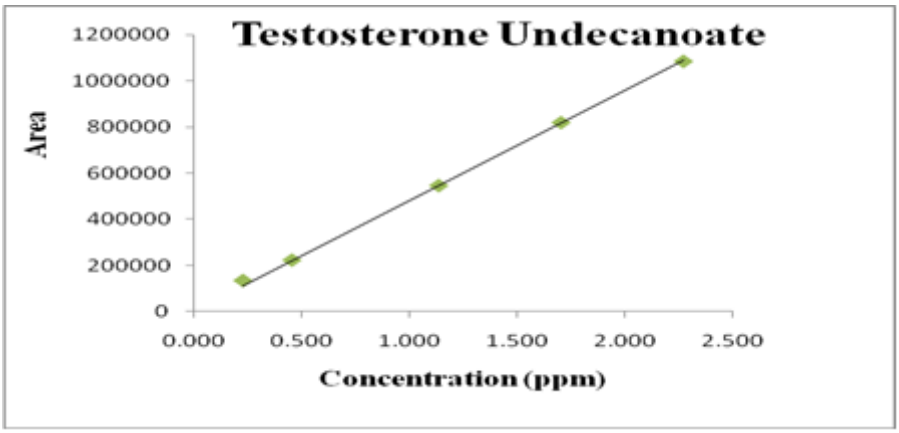


Figure 11: Testosterone Undecanoate – LOD/LOQ Establishment Graph

Table 28: LOD and LOQ

Peak Name	Level	Concentration in ppm	% w/w Impurity
Testosterone	LOQ	0.1033	0.0103
	LOD	0.0341	0.0034
Testosterone Decanoate	LOQ	0.1003	0.0100
	LOD	0.0331	0.0033
Testosterone Undecanoate	LOQ	0.2063	0.0206
	LOD	0.0681	0.0068

8.4.1. Precision at LOD and LOQ Level

Table 29: Precision at LOD Level for Testosterone, Testosterone Decanoate, and Testosterone Undecanoate

Injection number	LOD Level for Testosterone	LOD Level for Testosterone Decanoate	LOD Level for Testosterone Undecanoate
1	67191	57040	104460
2	66660	56303	103231
3	67700	55446	101815
4	66825	59037	103566
5	67866	56476	104182
6	69448	56896	103645
Mean	67615	56866	103483
STD.DEV	1014	1202	930
% RSD	1.5	2.1	0.9

Table 30: Precision at LOQ Level for Testosterone, Testosterone Decanoate, and Testosterone Undecanoate

Injection number	LOQ Level for Testosterone	LOQ Level for Testosterone Decanoate	LOQ Level for Testosterone Undecanoate
1	63710	58921	109155
2	63014	57990	107067
3	63554	59787	106987
4	63892	60112	106794
5	63160	58795	106601
6	63825	59429	107660
Mean	63526	59172	107377
STD.DEV	361.6896	765.5230	941.4497
% RSD	0.6	1.3	0.9

Acceptance Criteria:

- Report the LOD and LOQ
- The % RSD for peak area due to Testosterone Undecanoate, Testosterone, and Testosterone Decanoate at LOD Level should be NMT 30.0.
- The % RSD for peak area due to Testosterone Undecanoate, Testosterone, and Testosterone Decanoate at LOQ Level should be NMT 10.0.

Conclusion:

The precision at LOD and LOQ level met the acceptance criteria for Testosterone Undecanoate, Testosterone, and Testosterone Decanoate. This shows that the method has repeatability at LOD and LOQ concentration level.

8.5. LINEARITY AND RANGE

The Linearity of the analytical method is its 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.

The linearity was performed with Testosterone Undecanoate, Testosterone, and Testosterone Decanoate in the range of LOQ to 200% of the Specification limit. Precision was performed at a higher level.

Recorded the area response at each level and calculated slope, intercept, correlation coefficient, and regression coefficient (R square). A graph plotted of concentration (ppm) on X-axis and area response under the curve on the y-axis. The results are summarized in Tables 31-36.

Linearity stock preparation:

Weighed accurately and transferred about 2.5 mg each of Testosterone Undecanoate, Testosterone, and Testosterone Decanoate into a 50mL volumetric flask, dissolved and diluted to the volume with diluent.

Preparation of **LOQ Level solution:**

Based on the slope method LOQ determination was injected.

Preparation of 50.0% solution:

Pipetted out 1.0mL of linearity stock solution into a 50mL volumetric flask and made up the volume with diluent and mixed well.

Preparation of 100.0% solution:

Pipetted out 2mL of linearity stock solution into a 50mL volumetric flask and made up the volume with diluent and mixed well.

Preparation of 150.0% solution:

Pipetted out 3mL of linearity stock solution into a 50mL volumetric flask and made up the volume with diluent and mixed well.

Preparation of 200.0% solution:

Pipetted out 4mL of linearity stock solution into a 50mL volumetric flask and made up the volume with diluent and mixed well.

Table 31: Testosterone Linearity

S. No.	% Level	Concentration in µg/mL	Peak Response of Testosterone
1	LOQ	0.1003	67615
2	50	5.1487	3504331
3	75	7.7231	5285743
4	100	10.2975	7013181
5	125	12.8719	8620399
6	150	15.4462	10592850
7	200	20.5950	13779847
Slope			671712
Intercept			53992
Coefficient of correlation (r)			0.9998
Coefficient of regression (r^2)			0.9996
% Y-Intercept			0.77
Standard Deviation			106544
LOQ			1.586
LOD			0.523
RRF			1.37

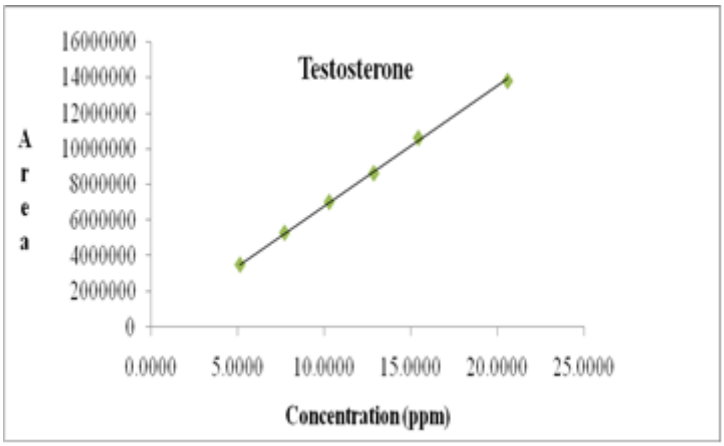


Figure 12: Testosterone Linearity Graph

Table 32: Testosterone - Range

S. No.	% Level	Concentration in µg/mL	Peak Response of Testosterone
1	LOQ	0.1003	67615
2	100	10.2975	7013181
3	200	20.5950	13779847
Slope			669043
Intercept			41718
Coefficient of correlation (r)			0.9999
Coefficient of regression (r^2)			0.9999

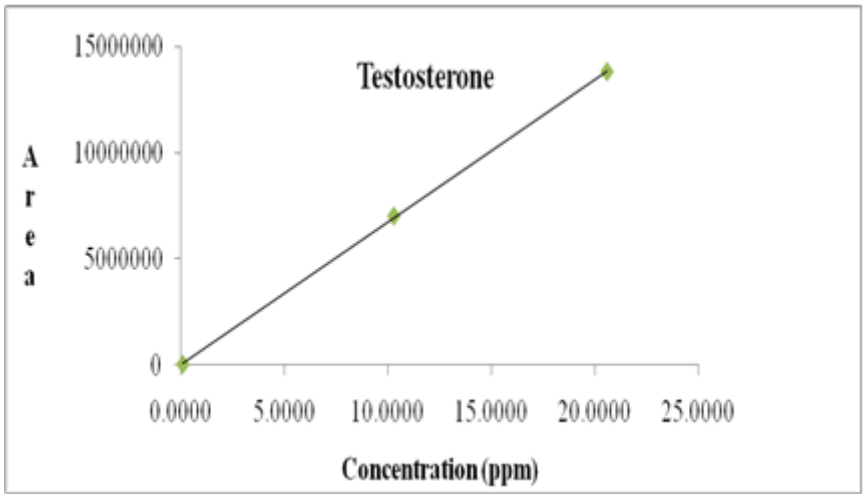


Figure 13: Testosterone Range Graph

Table 33: Testosterone Decanoate - Linearity

S. No.	% Level	Concentration in µg/mL	Peak Response of Testosterone Decanoate
1	0.01	0.0983	56866
2	50	5.1036	2651009
3	75	7.6554	4000145
4	100	10.2072	5252200
5	125	12.7590	6605015
6	150	15.3108	7954793
7	200	20.4144	10603809
Slope			518817
Intercept			459
Coefficient of correlation (r)			1.0000
Coefficient of regression (r^2)			1.0000
% Y-Intercept			0.01
Standard Deviation			28165
LOQ			0.543
LOD			0.179
RRF			1.06

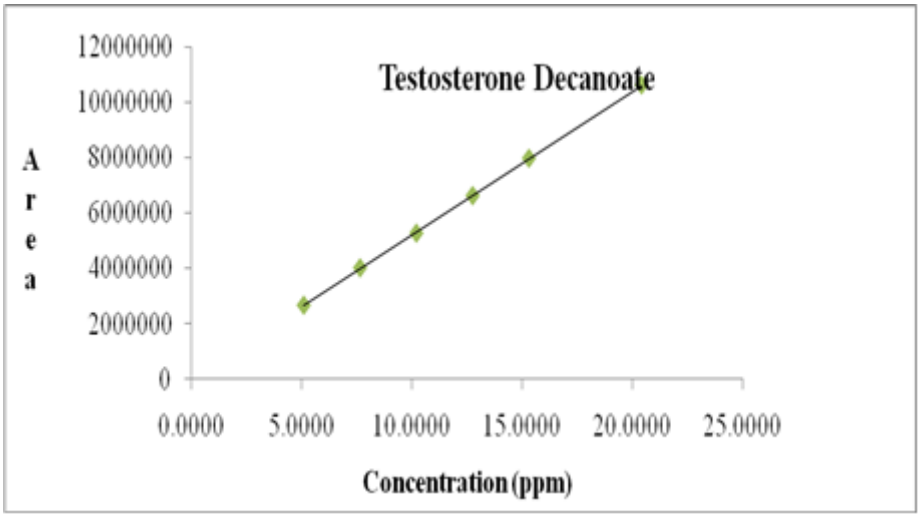


Figure 14: Testosterone Decanoate Linearity Graph

Table 34: Testosterone Decanoate - Range

S. No.	% Level	Concentration in µg/mL	Peak Response of Testosterone Decanoate
1	LOQ	0.0983	56866
2	100	10.2072	5252200
3	200	20.4144	10603809
Slope			519151
Intercept			-11795
Coefficient of correlation (r)			1.0000
Coefficient of regression (r^2)			1.0000

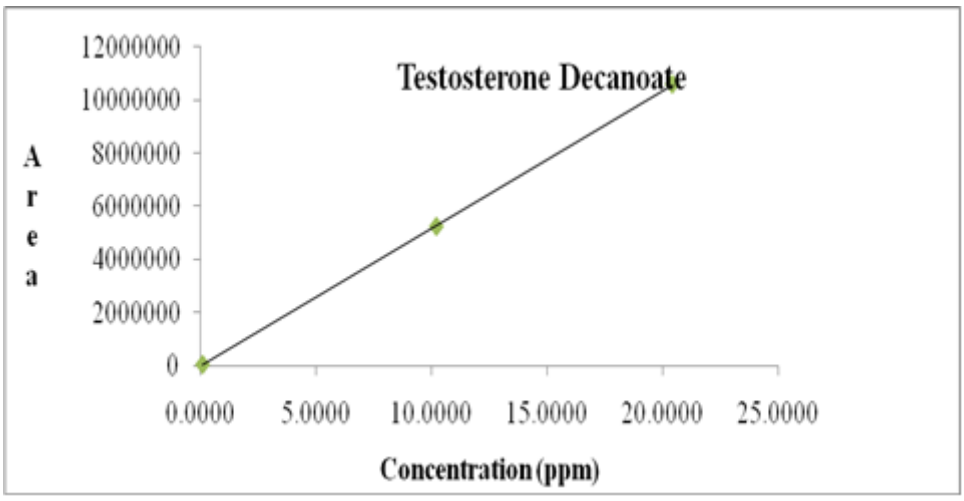


Figure 15: Testosterone Decanoate Range Graph

Table 35: Testosterone Undecanoate - Linearity

S. No.	% Level	Concentration in µg/mL	Peak Response of Testosterone Undecanoate
1	LOQ	0.1990	103483
2	50	2.5160	1240621
3	75	3.7739	1866658
4	100	5.0319	2454394
5	125	6.2899	3084768
6	150	7.5479	3720589
7	200	10.0638	4929617
Slope			489674
Intercept			7817
Coefficient of correlation (r)			1.0000
Coefficient of regression (r^2)			1.0000
% Y-Intercept			0.32
Standard Deviation			13671
LOQ			0.279
LOD			0.092

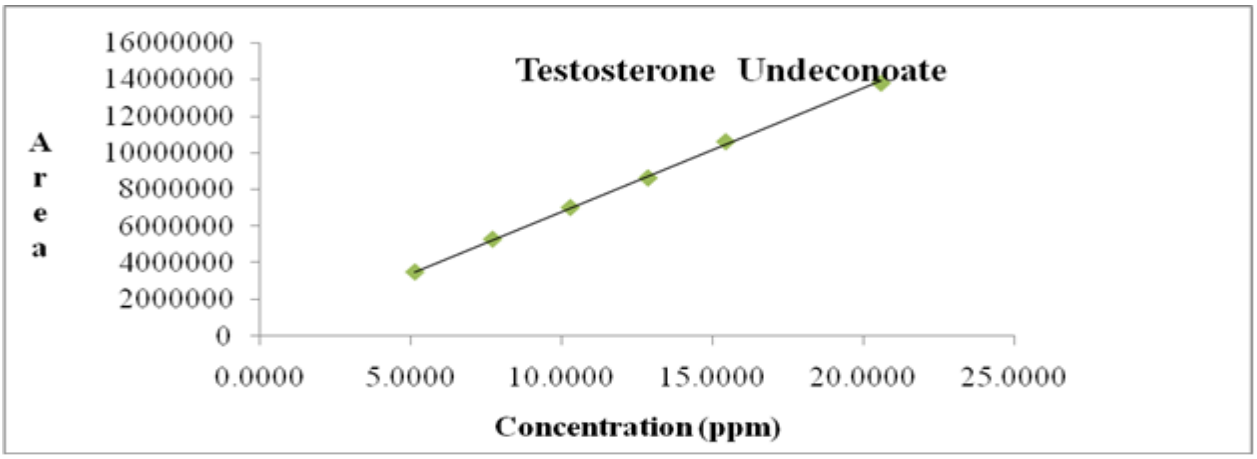


Figure 16: Testosterone Undecanoate Linearity Graph

Table 36: Testosterone Undecanoate - Range

S. No.	% Level	Concentration in µg/mL	Peak Response of Testosterone Undecanoate
1	LOQ	0.1990	103483
2	100	5.0319	2454394
3	200	10.0638	4929617
Slope			489242
Intercept			1561
Coefficient of correlation (r)			1.0000
Coefficient of regression (r^2)			1.0000

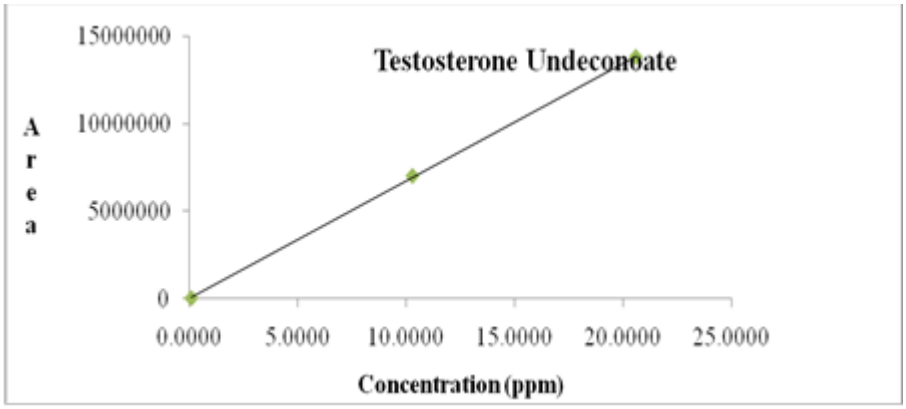
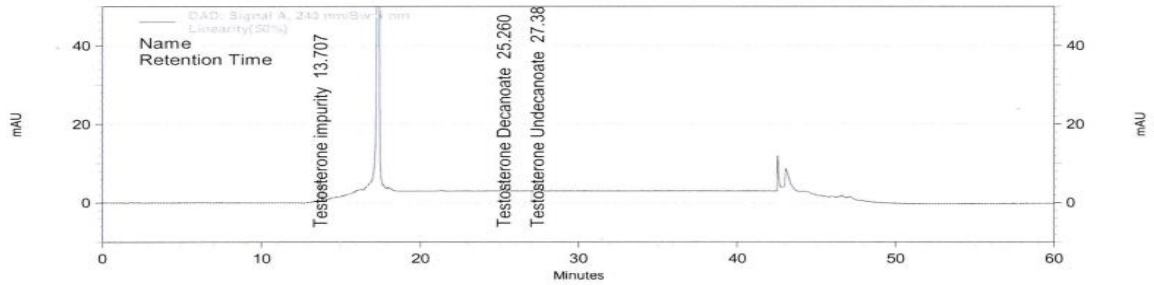


Figure 17: Testosterone Undecanoate Range Graph

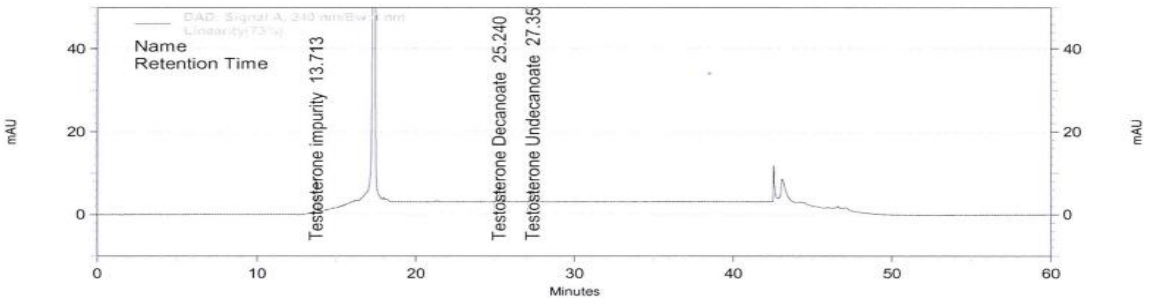
Acceptance Criteria:

- The correlation coefficient and regression coefficient shall be NLT 0.995 and 0.990 respectively.
- The Y-intercept should be ± 5.0% of the active response at 100% concentration.
- The % RSD for peak area due to Testosterone Undecanoate, Testosterone, and Testosterone Decanoate at Linearity Higher precision should be NMT 5.0.

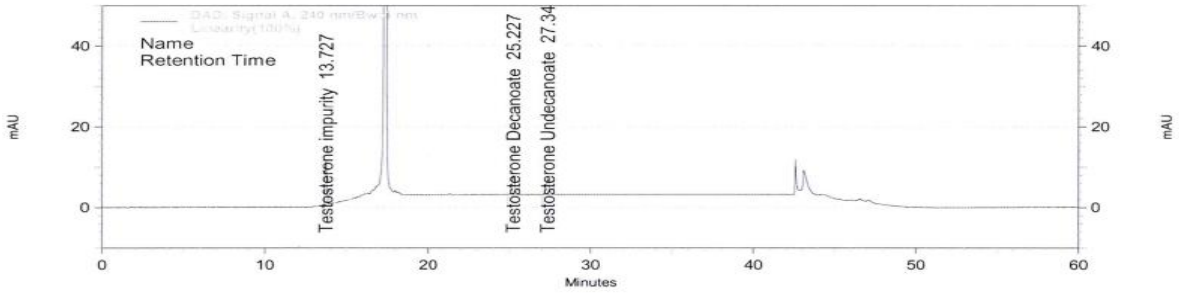
Linearity 50% Chromatogram



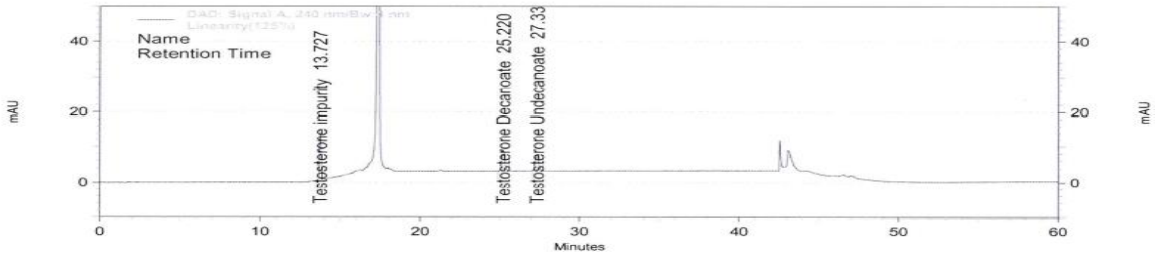
Linearity 75% Chromatogram



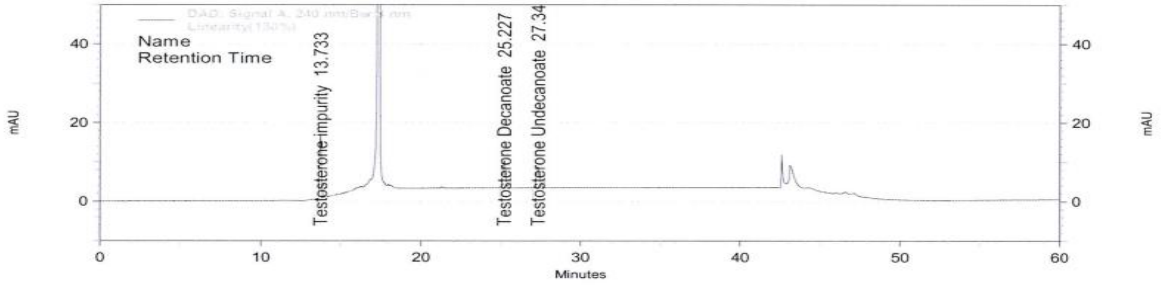
Linearity 100% Chromatogram



Linearity 125% Chromatogram



Linearity 150% Chromatogram



Linearity 200% Chromatogram

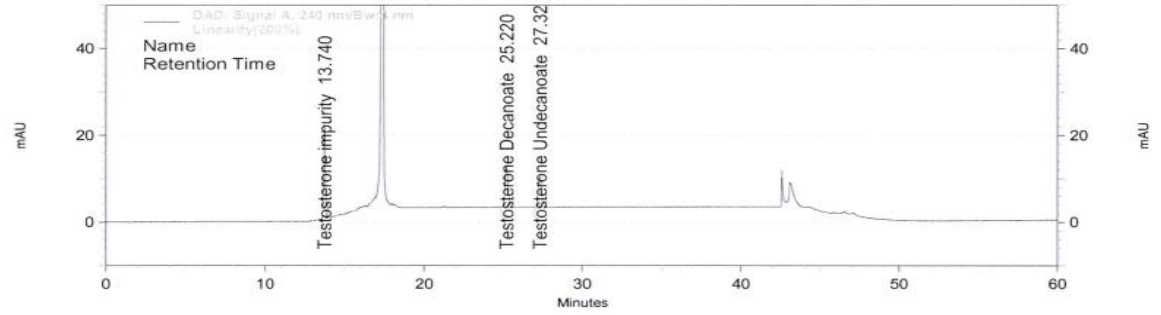


Table 37: Precision at a higher level for Testosterone, Testosterone Decanoate, Testosterone Undecanoate

Number of Injection	Linearity Higher Level Precision for Testosterone	Linearity Higher Level Precision for Testosterone Decanoate	Linearity Higher Level Precision for Testosterone Undecanoate
Injection 1	13775732	11245533	4934790
Injection 2	13799609	10482514	4939232
Injection 3	13812212	10491322	5014536
Injection 4	13803510	10458325	4889532
Injection 5	13744697	10467287	4899118
Injection 6	13743319	10477870	4900493
Average	13779847	10603809	4929617
STD.DEV	30281	314592	46294
%RSD	0.2	3.0	0.9

Conclusion:

From the statistical treatment of the linearity data from Testosterone, Testosterone Decanoate, and Testosterone Undecanoate it is clear that the response of Testosterone, Testosterone Undecanoate, and Testosterone Decanoate was linear between LOQ to 200% of the related substances method specification level for Testosterone Undecanoate Capsules 40mg. The correlation coefficient for Testosterone found 0.9998, the correlation coefficient for Testosterone Decanoate found 1.0000, and correlation coefficient for Testosterone Undecanoate found 1.0000. In addition, the value of the intercept is within the ± 5 % of the area response at 100% level. Hence, the method found Linear and within the range for Testosterone, Testosterone Decanoate, and Testosterone Undecanoate.

8.6. ACCURACY

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value, and the found value. Added known quantity of Testosterone Undecanoate standard, Testosterone impurity, and Testosterone Decanoate impurity at LOQ, 50%, 100%, 150%, and 200% of related substances specification limit concentration into the placebo. Analysed LOQ and 200% samples in six preparations and 50%, 100%, and 150% samples in triplicate for each level. From the results calculated the % recovery. The results are summarized in below tables 38-40.

Accuracy Stock preparation:

Weighed accurately and transferred about each 5 mg of Testosterone Undecanoate, Testosterone impurity, and Testosterone Decanoate impurity into a 100mL volumetric flask, dissolved and diluted to the volume with diluent.

Accuracy at LOQ Level preparation:

Based on the slope method LOQ determination was injected.

Preparation of 50.0 % Level solution:

Weighed and transferred the placebo equivalent to 50 mg of Testosterone Undecanoate into a 50 ml volumetric flask. Pipetted out 1.0 mL of accuracy stock solution into the flask, added 10 ml of water, and sonicated for 10 minutes. Then added 25 mL of Acetonitrile and sonicated for 15 minutes with intermediate shaking. Finally made up the volume with diluent and centrifuged the solution for 10 minutes at 2500 rpm.

Preparation of 100.0 % Level solution:

Weighed and transferred the placebo equivalent to 50 mg of Testosterone Undecanoate into a 50 ml volumetric flask. Pipetted out 2.0 mL of accuracy stock solution into the flask, added 10 ml of water, and sonicated for 10 minutes. Then added 25 mL of Acetonitrile and sonicated for 15 minutes with intermediate shaking. Finally made up the volume with diluent and centrifuged the solution for 10 minutes at 2500 rpm.

Preparation of 150.0 % Level solution:

Weighed and transferred the placebo equivalent to 50 mg of Testosterone Undecanoate into a 50 ml volumetric flask. Pipetted out 3.0 mL of accuracy stock solution into the flask, added 10 ml of water, and sonicated for 10 minutes. Then added 25 mL of Acetonitrile and sonicated for 15 minutes with intermediate shaking. Finally made up the volume with diluent and centrifuged the solution for 10 minutes at 2500 rpm.

Preparation of 200.0 % Level solution:

Weighed and transferred the placebo equivalent to 50 mg of Testosterone Undecanoate into a 50 ml volumetric flask. Pipetted out 4.0 mL of accuracy stock solution into the flask, added 10 ml of water, and sonicated for 10minutes. Then added 25 mL of Acetonitrile and sonicated

for 15 minutes with intermediate shaking. Finally made up the volume with diluent and centrifuged the solution for 10 minutes at 2500 rpm.

Table 38: Accuracy for Testosterone

Levels	No of Sample	Added in ppm	Found in ppm	% Recovery	Average % recovery	%RSD
LOQ	1	0.0997	0.1025	102.7	103.4	0.9
	2	0.0997	0.1024	102.7		
	3	0.0997	0.1049	105.1		
	4	0.0997	0.1026	102.9		
	5	0.0997	0.1030	103.3		
	6	0.0997	0.1034	103.7		
50%	1	5.0144	5.0474	100.7	101.0	0.4
	2	5.0144	5.0847	101.4		
	3	5.0144	5.0685	101.1		
100%	1	10.0287	10.0879	100.6	100.6	0.0
	2	10.0287	10.0961	100.7		
	3	10.0287	10.0903	100.6		
150%	1	15.0431	15.1565	100.8	101.5	1.3
	2	15.0431	15.1457	100.7		
	3	15.0431	15.5000	103.0		
200%	1	20.0575	20.0911	100.2	100.8	0.8
	2	20.0575	20.2383	100.9		
	3	20.0575	20.1490	100.5		
	4	20.0575	20.5313	102.4		
	5	20.0575	20.1521	100.5		
	6	20.0575	20.1603	100.5		
			Average	101.7		
			%RSD	1.4		

Table 39: Accuracy for Testosterone Decanoate

Levels	No of Sample	Added in ppm	Found in ppm	% Recovery	Average % recovery	%RSD
LOQ	1	0.1025	0.1010	98.6	95.4	2.9
	2	0.1025	0.0989	96.5		
	3	0.1025	0.0994	97.0		
	4	0.1025	0.0967	94.4		
	5	0.1025	0.0979	95.5		
	6	0.1025	0.0928	90.6		
50%	1	4.9120	4.9157	100.1	100.0	0.3
	2	4.9120	4.9218	100.2		
	3	4.9120	4.8944	99.6		

100%	1	9.8239	9.8196	100.0	100.1	0.1
	2	9.8239	9.8401	100.2		
	3	9.8239	9.8412	100.2		
150%	1	14.7359	14.7853	100.3	100.3	0.2
	2	14.7359	14.7574	100.1		
	3	14.7359	14.8038	100.5		
200%	1	19.6479	19.6063	99.8	100.0	0.2
	2	19.6479	19.6258	99.9		
	3	19.6479	19.6909	100.2		
	4	19.6479	19.6523	100.0		
	5	19.6479	19.6402	100.0		
	6	19.6479	19.6673	100.1		
			Averag	98.7		
			%RSD	2.6		

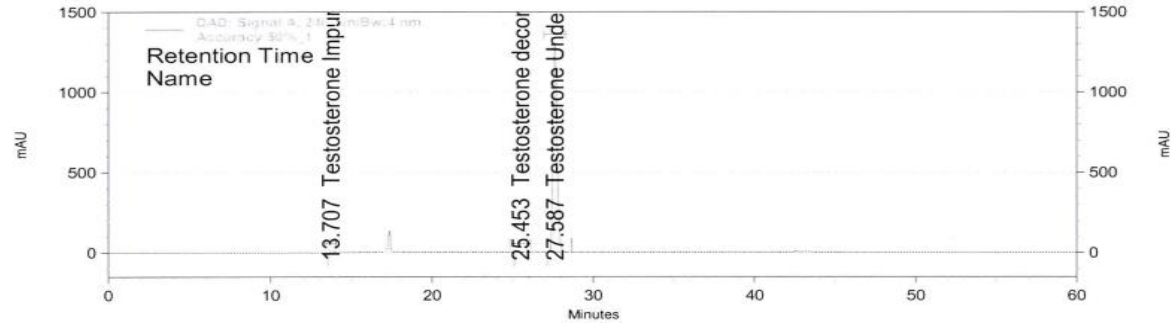
Table 40: Accuracy for Testosterone Undecanoate

Levels	No of Sample	Added in ppm	Found in ppm	% Recovery	Average % recovery	%RSD
LOQ	1	0.2000	0.2290	114.5	112.1	4.4
	2	0.2000	0.2292	114.6		
	3	0.2000	0.2270	113.5		
	4	0.2000	0.2295	114.7		
	5	0.2000	0.2265	113.2		
	6	0.2000	0.2041	102.0		
50%	1	2.4934	2.4928	100.0	100.0	0.7
	2	2.4934	2.5127	100.8		
	3	2.4934	2.4766	99.3		
100%	1	4.9868	4.9825	99.9	100.1	0.2
	2	4.9868	5.0002	100.3		
	3	4.9868	4.9992	100.2		
150%	1	7.4802	7.4982	100.2	100.2	0.3
	2	7.4802	7.4696	99.9		
	3	7.4802	7.5096	100.4		
200%	1	9.9736	9.9235	99.5	99.8	0.2
	2	9.9736	9.9400	99.7		
	3	9.9736	9.9690	100.0		
	4	9.9736	9.9581	99.8		
	5	9.9736	9.9536	99.8		
	6	9.9736	9.9740	100.0		
			Averag	103.4		
			%RSD	5.9		

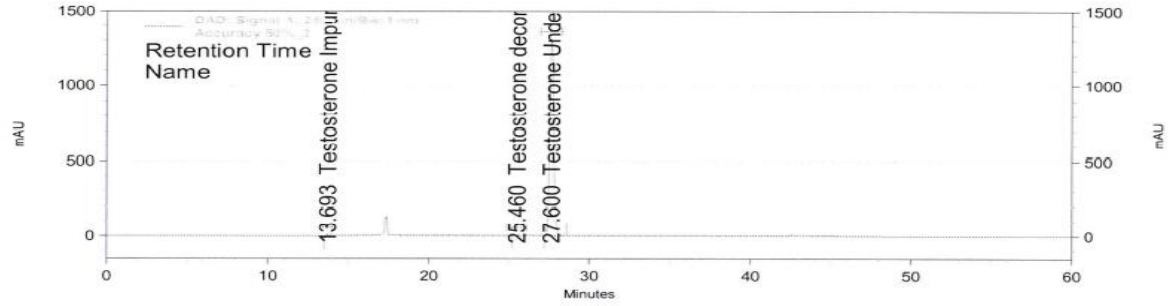
Acceptance Criteria:

- The Percentage Recovery at 50 % to 200% level should be NLT 90.0 and NMT 110.0.
- The Percentage Recovery at the LOQ level should be NLT 85.0 and NMT 115.0.
- The percentage RSD of LOQ level recovery for known and unknown impurities should be NMT 10.0.
- The overall Percentage RSD of all recovery levels should be NMT 10.0.
- The percentage RSD of each level recovery for Known and Unknown impurities should be NMT 5.0.

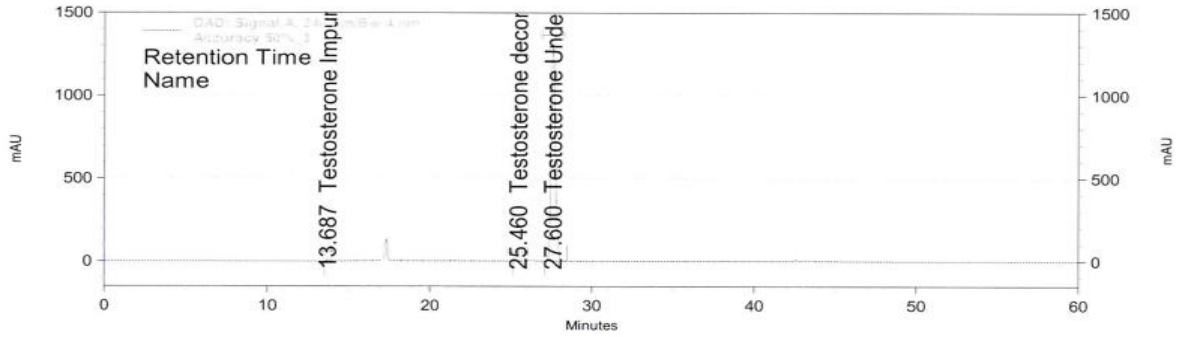
Accuracy 50% - 1 Chromatogram



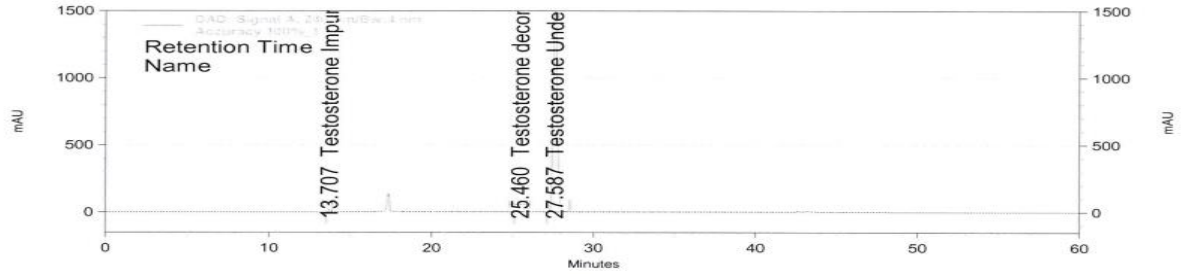
Accuracy 50% - 2 Chromatogram



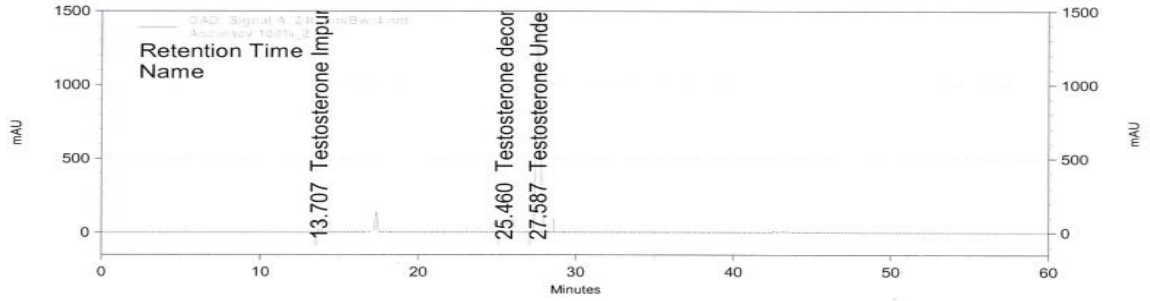
Accuracy 50% - 3 Chromatogram



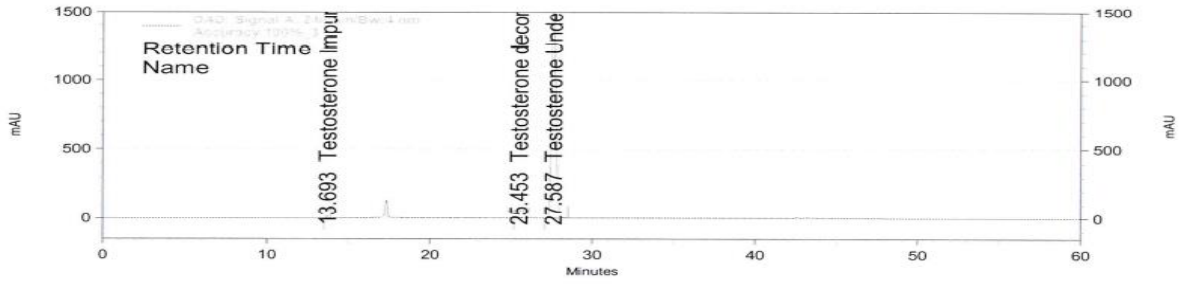
Accuracy 100% - 1 Chromatogram



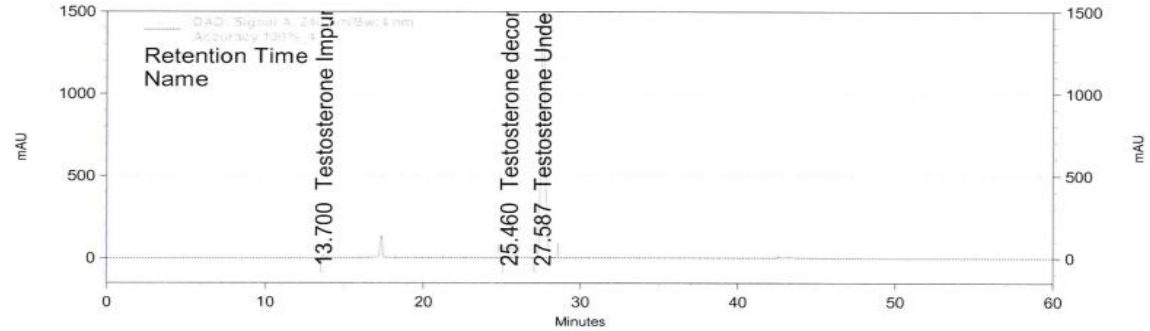
Accuracy 100% - 2 Chromatogram



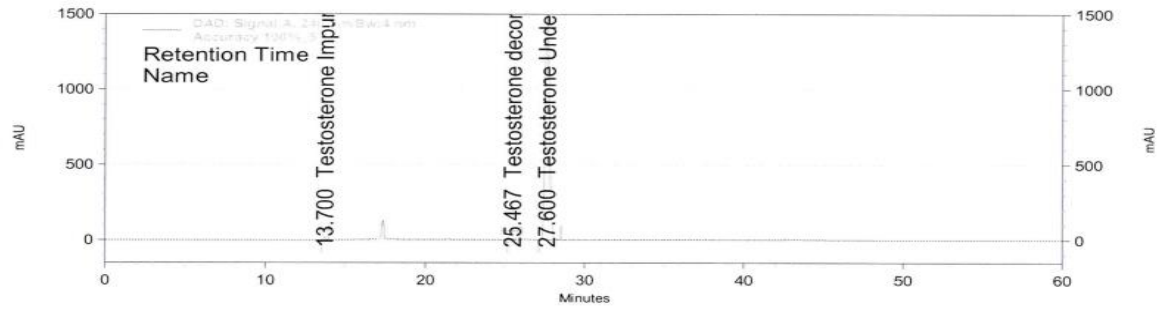
Accuracy 100% - 3 Chromatogram



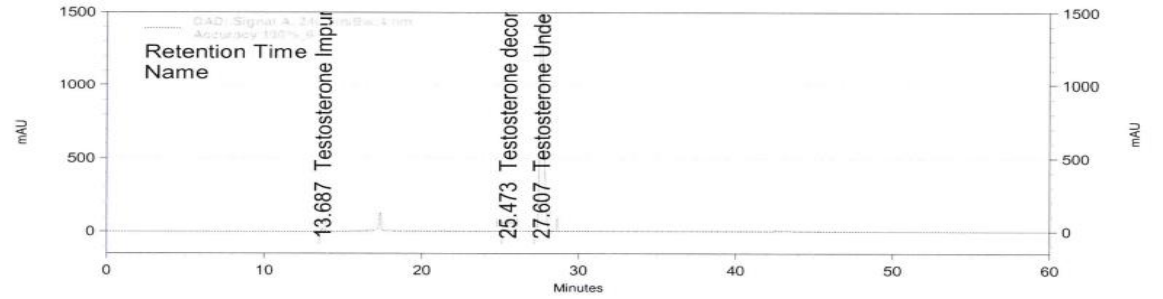
Accuracy 100% - 4 Chromatogram



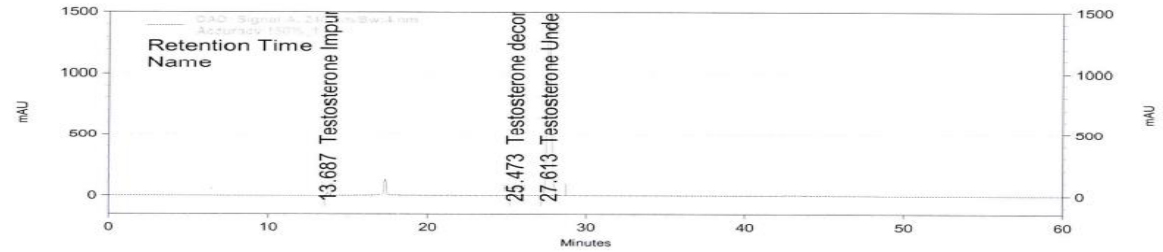
Accuracy 100% - 5 Chromatogram



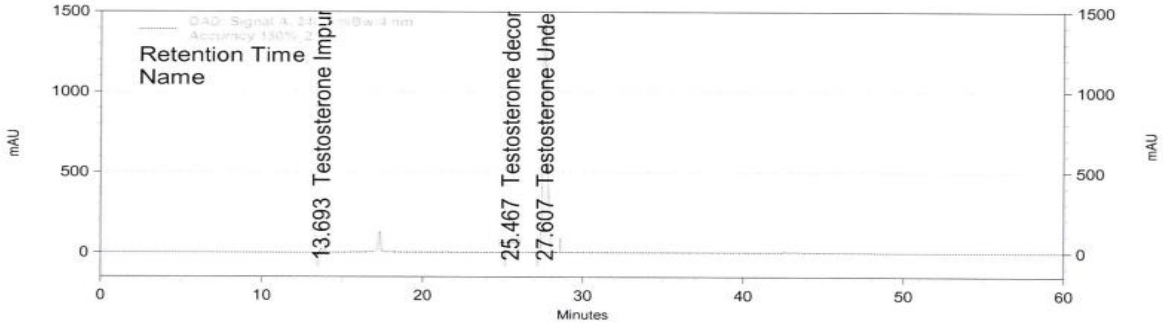
Accuracy 100% - 6 Chromatogram



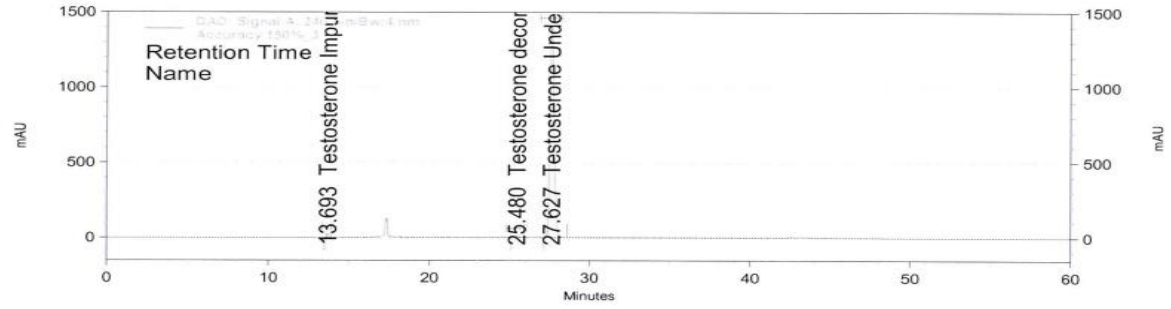
Accuracy 150% - 1 Chromatogram



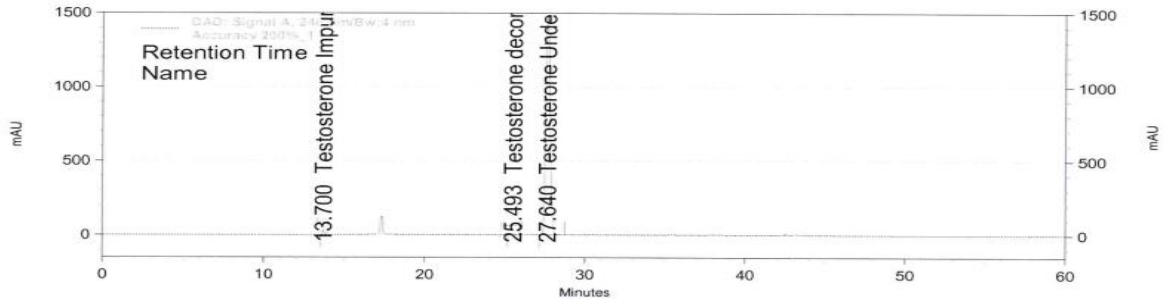
Accuracy 150% - 2 Chromatogram



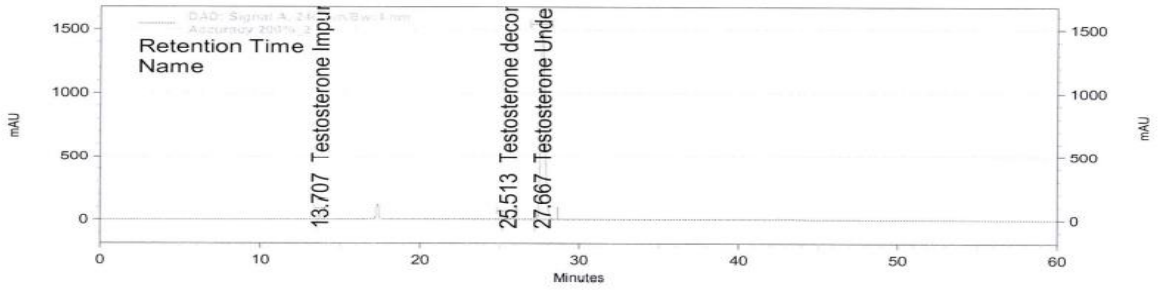
Accuracy 150% - 3 Chromatogram



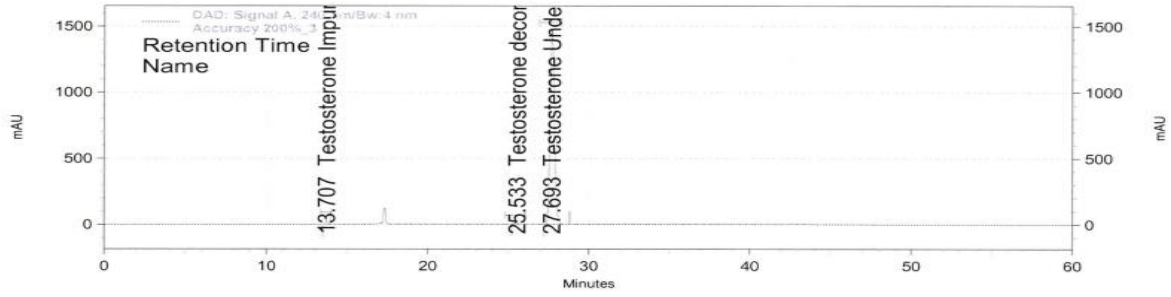
Accuracy 200% - 1 Chromatogram



Accuracy 200% - 2 Chromatogram



Accuracy 200% - 3 Chromatogram



Conclusion:

The results met the acceptance criteria for all Accuracy levels. The result shows that the method is Accurate for Testosterone Undecanoate Capsules 40mg.

8.7. SOLUTION STABILITY

Stability of the analytical solution evaluated by using the following procedure, injected standard and sample solution at the interval of initial, 24hrs and 48hrs. The results are summarized in the tables 41 & 42 for standard and sample solutions.

Table 41: Solution Stability for Standard

Time in hours	Initial	24hrs	48hrs
% of Testosterone Undecanoate standard	100	99.2	100.1
% Diff for Testosterone Undecanoate standard	-	0.8	-0.1

Table 42: Solution Stability for Sample

Time in hours/Name	Initial	24hrs		48hrs	
	% Impurity (%w/w)	% Impurity (%w/w)	% Difference	% Impurity (%w/w)	% Difference
Testosterone (%w/w)	0.9716	0.9607	1.1	0.9878	-1.7
Testosterone Decanoate (%w/w)	0.9308	0.9160	1.5	0.9470	-1.7
Highest unknown impurity (%w/w)	0.0294	0.0303	-3.0	0.0300	-2.0
Total impurities (% w/w)	1.9318	1.9069	1.3	1.9929	-3.2

Acceptance Criteria:

- The % difference in area response of standard solution between initial and after specified period should be NMT ± 15.0.
- The % difference in % Known, Unknown and Total impurities of sample solution between initial and after specified period should be NMT ± 15.0.

Conclusion:

The standard and sample solution met the acceptance criteria for 48 hours at Room Temperature (25°C). Hence, the standard and sample solution was found to be stable for 48 hours at Room Temperature (25°C).

8.8. MOBILE PHASE STABILITY

The mobile phase solution stability was established by preparing fresh standards every day and evaluating the system suitability parameters using the same mobile phase prepared on the initial day. The mobile phase stability was established for initial, day-1, and day-2 where the system suitability criteria met the acceptance criteria and not observed any physical change in the appearance of the mobile phase. The results are summarized in below table 43.

Table 43: Mobile Phase Stability

Time Interval	% RSD	Tailing factor	Theoretical plates	Resolution	Physical Appearance
Initial	0.8	1.0	64356	5.91	Clear
Day-1	0.4	1.0	62603	5.79	Clear
Day-2	0.2	1.0	63444	5.86	Clear

Acceptance Criteria:

- The percentage relative standard deviation for peak areas of Testosterone Undecanoate obtained from six replicate injections of standard solution should be not more than 5.0
- The Tailing factor of Testosterone Undecanoate peak obtained from standard solution should be not more than 2.0
- The Theoretical plates of the Testosterone Undecanoate peak obtained from standard solution should be not less than 2000.
- The Resolution between Testosterone Decanoate and Testosterone Undecanoate peaks obtained from the resolution solution should be not less than 2.0.

Conclusion:

The system suitability parameters met the acceptance criteria with 2 days old mobile phases, no haziness was found. Hence, the Mobile phase was found to be stable for 2 days at room temperature with closed container for Testosterone Undecanoate Capsules 40mg.

8.9. FILTER VALIDATION

The filter compatibility study was evaluated for the spiked sample preparation solution by using centrifuged sample at 2500rpm for 10minutes and filtered sample by using 0.45µm PTFE and 0.45-µm Nylon Filter. The results are summarized in tables 44 & 45.

Table 44: Filter-1 Validation Study

Impurity Name	Centrifuged Sample	Filter-1 (0.45-µm PVDF) 2mL Discarded		Filter-1 (0.45-µm PVDF) 4mL Discarded	
	% Impurity (%w/w)	% Impurity (%w/w)	% Difference	% Impurity (%w/w)	% Difference
Testosterone (%w/w)	0.9721	0.9759	-0.4	0.9740	-0.2
Testosterone Decanoate (%w/w)	0.9296	0.9315	0.2	1.0320	11.0
Highest unknown impurity (%w/w)	0.0813	0.0821	-0.9	0.0818	-0.5
Total impurities (% w/w)	2.1477	2.2078	-2.8	2.2983	-7.0

Table 45: Filter-2 Validation study

Impurity Name	Centrifuged Sample	Filter-2 (0.45-µm Nylon) 2mL Discarded		Filter-2 (0.45-µm Nylon) 4mL Discarded	
	% Impurity (%w/w)	% Impurity (%w/w)	% Difference	% Impurity (%w/w)	% Difference
Testosterone (%w/w)	0.9721	0.9706	0.2	0.9364	3.7
Testosterone Decanoate (%w/w)	0.9296	0.8708	-6.3	0.9288	-0.1
Highest unknown impurity (%w/w)	0.0813	0.0813	0.02	0.0820	-0.9
Total impurities (% w/w)	2.1477	2.0869	2.8	2.1103	1.7

Acceptance Criteria:

The % difference between centrifuged samples and filtered samples for Known, Unknown Impurities and Total impurities should be ±15.0.

Conclusion:

From the above data, it is concluded that the 0.45µm PVDF filtered and 0.45µm Nylon filtered sample solution met the acceptance criteria. Hence, the 0.45µm PVDF filters and 0.45µm Nylon filters was found to be suitable for the filtration of sample solution preparation for the related substances method of Testosterone Undecanoate Capsules 40mg.

8.10. ROBUSTNESS

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

Robustness Parameters:

1. Change in wavelength variation ± 2nm
2. Change in flow variation ± 0.2mL
3. Change column oven temperature +2°C
4. Change in gradient variation ± 10%
5. Change in gradient variation ± 3%

Effect of variation in wavelength:

Prepared and injected the blank, standard, spiked sample and bracketing standard solution with deliberate change in the wavelength variation as follows, for low wavelength with 238 nm and high Wavelength 242 nm and calculated the percentage RSD for Testosterone Undecanoate area in standard solution at each condition.

Effect of variation in Flow Rate:

Prepared and injected the blank, standard, spiked sample and bracketing standard solution with deliberate change in the flow variation as follows, for low flow variation with 1.3 mL/min and high flow variation 1.7 mL/min and calculated the percentage RSD for Testosterone Undecanoate area in standard solution at each condition.

Effect of variation in Column Oven Temperature:

Prepared and injected the blank, standard, Spiked Sample solution and bracketing standard solution with deliberate change in the Column oven temperature variation as follows for high column oven temperature variation (27°C) and calculated the percentage RSD for Testosterone Undecanoate area in standard solution at each condition.

Effect of variation in Gradient:

Prepared and injected the blank, standard, Spiked Sample solution and bracketing standard solution with deliberate change in the gradient (low and high gradient-3% variation) and calculated the percentage RSD for Testosterone Undecanoate area in standard solution at each condition.

Table 46: Robustness

Acceptance criteria	**The percentage relative standard deviation for peak areas of Testosterone Undecanoate obtained from six replicate injections of standard solution should be not more than 5.0**
Test Condition	0.6
Low flow variation (1.3mL/Minute)	0.2
High flow variation (1.7mL/Minute)	0.3
High column temperature Variation	0.4
Low wavelength variation (238nm)	0.3
High wavelength variation (242nm)	0.3
Low gradient (3%)	0.3
High gradient (3%)	0.9
Acceptance criteria	**The Tailing factor of Testosterone Undecanoate peak obtained from standard solution should be not more than 2.0**
Test Condition	1.0
Low flow variation (1.3mL/Minute)	1.0
High flow variation (1.7mL/Minute)	1.0
High column temperature Variation	1.0
Low wavelength variation (238nm)	1.0
High wavelength variation (242nm)	1.0
Low gradient (3%)	0.9
High gradient (3%)	1.0
Acceptance criteria	**The Theoretical plates of Testosterone Undecanoate peak obtained from standard solution should be not less than 2000.**
Test Condition	60111

Low flow variation (1.3mL/Minute)	58116
High flow variation (1.7mL/Minute)	64228
High column temperature Variation	59151
Low wavelength variation (238nm)	61276
High wavelength variation (242nm)	61133
Low gradient (3%)	56748
High gradient (3%)	45684
Acceptance criteria	**The Resolution between Testosterone Decanoate and Testosterone Undecanoate peaks obtained from the resolution solution should be not less than 2.0.**
Test Condition	5.82
Low flow variation (1.3mL/Minute)	5.94
High flow variation (1.7mL/Minute)	5.50
High column temperature Variation	5.47
Low wavelength variation (238nm)	5.77
High wavelength variation (242nm)	5.77
Low gradient (3%)	5.67
High gradient (3%)	6.42

Note:

- Since the peak elution pattern is not matching with as such conditions, the method is not robust with a high gradient variation of 10 %.
- Since RRT of testosterone peak is not matching with as such conditions, the method is not robust with a low gradient variation of 10%.

Hence robustness parameter of gradient variation has been performed with 3% and the results are reported.

Conclusion:

The robustness parameters were performed as per protocol with chromatographic conditions slight variation namely flow variation (Low and high flow), Column temperature variation (High column temperature), Wavelength variation (Low and High wavelength variation), and gradient variation (Low and high gradient), all the robustness condition was meeting the acceptance criteria. Hence, the method was found to be robust for flow variation (Low and high flow), Column temperature variation (High column temperature), Wavelength variation (Low and High wavelength variation), and gradient variation (Low and high gradient) of Testosterone Undecanoate Capsules 40mg.

9. SUMMARY AND CONCLUSION

In this study, the stability-indicating RP-HPLC based Related substances method was developed for the estimation of Testosterone Undecanoate and its related degradation impurities in capsule dosage form and validated according to ICH guidelines.

There are no official compendial methods available for the estimation of Testosterone Undecanoate in both bulk and pharmaceutical dosage forms.

This developed gradient RP-HPLC method is found useful in achieving the complete separation and quantification of all possible impurities that could be present after degradation with suitable resolution criteria between the analyte of interest and related impurities.

The Optimized chromatographic conditions, summary results of Validation, Degradation results of Sample and API are furnished in Tables 47, 48, 49 & 50 respectively.

Table 47: Summary of Optimized Chromatographic Conditions

Stationary Phase	Inertsil-ODS-3, 4.6 mm × 250 mm, 5 µm		
Flow rate	1.5 ml/min		
Detection wavelength	240 nm		
Column Temperature	25°c		
Mobile Phase Flow Composition	Time (min)	A – Water (100%)	B – Acetonitrile (100%)
	0.00	60.0	40.0
	10.00	60.0	40.0
	15.00	00.0	100.0
	40.00	00.0	100.0
	50.00	60.0	40.0
	60.00	60.0	40.0
Injection volume	20µL		
Run time	60 minutes		
Retention time	Testosterone Undecanoate – About 27 minutes		
Diluent	Acetonitrile and Water (95:5)		

Table 48: Validation summary of the proposed method

S.No	Parameters	Observation		Acceptance Criteria
1	**Specificity**	No Interference		There should not be any interference of blank and placebo peaks at the Retention Time of the main analyte and its impurities.
		1.0		Peak Purity – NLT 0.9 in Open Lab Software
		Complies		% Degradation – NMT 20%
		Complies		Mass Balance – 95% - 105%
2	**System Precision**	0.2		% RSD for Standard Peak Area – NMT 5.0
		0.1		% RSD for Standard Retention Time – NMT 1.0
3	**Method Precision**			
	Testosterone	2.6		The Percentage RSD of Known, Unknown, and Total impurity % W/W should be NMT 10.0.
	Testosterone Decanoate	4.0		
	Highest Unknown Impurity	0.7		
	Total Impurity	2.9		
4	**Intermediate Precision**			
	Testosterone	0.6		The Percentage RSD of Known, Unknown, and Total impurity % W/W should be NMT 10.0.
	Testosterone Decanoate	0.8		
	Highest Unknown Impurity	0.8		
	Total Impurity	0.3		
5	**Limit of Detection**	PPM	%RSD	The % RSD for peak area due to Testosterone Undecanoate, Testosterone, and Testosterone Decanoate at LOD Level should be NMT 30.0.
	Testosterone	0.0341	1.5	
	Testosterone Decanoate	0.0331	2.1	
	Testosterone Undecanoate	0.0681	0.9	
6	**Limit of Quantification**	PPM	%RSD	The % RSD for peak area due to Testosterone Undecanoate, Testosterone, and Testosterone Decanoate at LOQ Level should be NMT 10.0.
	Testosterone	0.1033	0.6	
	Testosterone Decanoate	0.1003	1.3	
	Testosterone Undecanoate	0.2063	0.9	
7	**Linearity**			
	Testosterone	0.9996		The correlation coefficient should be NLT 0.995
	Testosterone Decanoate	1.0000		
	Testosterone Undecanoate	1.0000		
8	**Range**			
	Testosterone	0.9999		

	Testosterone Decanoate	1.0000		The correlation coefficient should be NLT 0.995
	Testosterone Undecanoate	1.0000		
9	**Accuracy**			
	Testosterone	103.4		The Percentage Recovery at the LOQ level should be NLT 85.0 and NMT 115.0.
	Testosterone Decanoate	95.4		
	Testosterone Undecanoate	112.1		
10	**Solution Stability**	24 Hrs	48 Hrs	The standard and sample solution was found to be stable up to 48 hrs at Room Temperature (25°C).
	Testosterone Undecanoate	0.8	-0.1	The % difference in % Known, Unknown and Total impurities of sample solution between initial and after specified period should be NMT ± 15.0.
	Testosterone	1.1	-1.7	
	Testosterone Decanoate	1.5	-1.7	
	Highest Unknown Impurity	-3.0	-2.0	
	Total Impurity	1.3	-3.2	
11	**Mobile Phase Stability**	Day 1	Day 2	The mobile phase was found to be stable up to 2 days at Room Temperature with no Haziness.
		0.4	0.2	The percentage Relative Standard Deviation for peak areas of Testosterone Undecanoate obtained from six replicate injections of standard solution should be not more than 5.0.
12	**Filter Validation**	Filter-1	Filter-2	Filter 1 & 2 was found to be suitable for the filtration of sample solution preparation.
	Testosterone	-0.2	3.7	The % difference between centrifuged samples and filtered samples for Known, Unknown Impurities and Total impurities %W/W should be ±15.0.
	Testosterone Decanoate	11.0	-0.1	
	Highest Unknown Impurity	-0.5	-0.9	
	Total Impurity	-7.0	1.7	
13	**Robustness**	No deliberate change in Peak area with a slight change in Wavelength, Flowrate, Column Temp. & Gradient variation		The percentage Relative Standard Deviation for peak areas of Testosterone Undecanoate obtained from six replicate injections of standard solution should be not more than 5.0

Table 49: Summary of Degradation Study - Sample

Stressed condition	% Degradation	Mass Balance
Unstressed Sample	0.2508	-
Base stressed Sample	0.0859	98.6
Oxidation Stressed Sample	0.0931	98.7
Water Stressed Sample	0.0878	101.1
Photo stressed sample (open condition)	0.0919	95.7
Photo stressed sample (closed condition)	0.0941	98.8
Thermal stressed sample (50°C, 5 hours)	0.0757	99.6
Thermal stressed sample (50°C, 24 hours, with blister)	0.5894	100.6
Thermal stressed sample (50°C, 48 hours, with blister)	0.1236	99.7
Acid Stressed Sample	9.3671	100.7
Acceptance Criteria	**NMT 20%**	**95%-105%**

Table 50: Summary of Degradation Study - API

Stressed condition	% Degradation	Mass Balance
Unstressed API	0.4814	-
Base stressed API	0.5529	101.2
Oxidation Stressed API	0.4619	101.1
Water Stressed API	0.4730	101.5
Photo stressed API (open condition)	0.5758	101.5
Photo stressed API (closed condition)	0.5358	101.4
Thermal stressed API	0.4902	101.1
Acid Stressed API	5.5034	102.4
Acceptance Criteria	**NMT 20%**	**95%-105%**

The Validation and Degradation results were found to be complying with the acceptable limits. Hence the developed method was found to be stability-indicating for the estimation of Testosterone Undecanoate and its related degradation impurities in Capsule dosage form.

CONCLUSION

The developed stability-indicating Related Substances method for the determination of Testosterone Undecanoate and its degradation impurities using the RP-HPLC gradient method was found to be simple, accurate, precise, robust, rugged, and specific. Hence this method can be used for routine quality control and stability analysis. Identification and characterization of the impurities present may be taken up as further research in the study.

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