RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION ASSAY OF MEGESTROL ACETATE TABLETS USP

A Dissertation submitted to

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

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In partial fulfilment of the award of the degree of

MASTER OF PHARMACY

IN

Branch V – PHARMACEUTICAL ANALYSIS

Submitted by

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APRIL - 2020.

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled, **RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION ASSAY OF MEGESTROL ACETATE TABLETS USP,** submitted by student bearing **Reg. No. 261830203** to **"The Tamil Nadu Dr. M.G.R. Medical University"**, Chennai, for the partial fulfillment of the degree of **MASTER OF PHARMACY in Pharmaceutical Analysis,** was evaluated by us during the examination held on…….

Internal Examiner **External Examiner**

CERTIFICATE

This is to certify that the work embodied in the dissertation entitled, **RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION ASSAY OF MEGESTROL ACETATE TABLETS USP,** submitted to "**The Tamil Nadu Dr. M.G.R. Medical University",** Chennai, was carried out by **T. AMUDHA [Reg.No: 261830203],** for the partial fulfillment of the degree of **MASTER OF PHARMACY** in **PHARMACEUTICAL ANALYSIS**, under direct supervision of **Dr. V. SEKAR, M.Pharm., Ph.D.,** Professor and Head, Department of Pharmaceutical Analysis, J.K.K. Nattraja College of Pharmacy, Komarapalayam, during the academic year 2019-2020.

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DECLARATION

The work presented in this dissertation entitled, **RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION ASSAY OF MEGESTROL ACETATE TABLETS USP,** was carried out by me, under the direct supervision of **Dr. V. SEKAR, M.Pharm., Ph.D.,** Professor and Head, Department of Pharmaceutical Analysis, J.K.K. Nattraja College of Pharmacy, Komarapalayam. I further declare that, this work is original and has not been submitted in part or full for the award of any other degree or diploma in any other university.

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Dedicated to

Almighty

My Beloved Parents,

&

My Family Members

CONTENTS

LIST OF ABBREVIATIONS USED

ICH	-	International conference on Harmonization
USP	-	United states of Pharmacopoeia
λ	-	Lambda
µg/ml	-	Microgram per milliliter
ng /ml	-	Nanogram per milliliter
µl	-	Micro liter
ml	-	Milliliter
mM	-	Milli mole
nm	-	Nanometer
mm	-	Millimeter
%	-	Percentage
%RSD	-	Percentage of Relative standard Deviation
LOD	-	Limit of detection
LOQ	-	Limit of Quantitation
pH	-	Negative Logarithm of Hydrogen Ion
Rt	-	Retention time
S.D	-	Standard Deviation
RP-HPLC	-	Reverse phase –High performance liquid chromatography
min	-	Minute
ml /min	-	Milliliter / minute
v / v	-	Volume /Volume
ml /min	-	Millilitre /Minute

1. INTRODUCTION

Pharmaceutical Analysis is the branch of Chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and quantitative measurements of the substances present in Bulk drug and Pharmaceutical preparations (Sharma B.K, 2000).

Pharmaceutical Analysis simply means analysis of a Pharmaceutical(s). It is generally known that a Pharmaceutical is a chemical entity of Therapeutic interest. A more appropriate term for a Pharmaceutical is Active Pharmaceutical Ingredient (API) or Active Ingredient. Even though the term Active Ingredient is more frequently used, the preferred term is Active Pharmaceutical Ingredient. To distinguish it from the formulated product or drug product, API is also called Drug substance. The drug product is prepared by formulating a drug substance with inert ingredients (excipients) to prepare a drug product that is suitable for administration to patients.

However, it should be recognized that there are situations where a drug substance can be administered after simple dissolution in a solvent such as water. Even in these situations, a suitable Pharmaceutical treatment has to be conducted to assure availability and other safety considerations.

It is well known in the Pharmaceutical industry that Pharmaceutical Analysts in Research and Development (R&D) play a very comprehensive role in new drug development and follow up activities to assure that a new drug product meets the established standards, is stable and continues to meet the purported quality throughout its shelf life. After the drug product is approved by regulatory

authorities, assuring that all batches of drug product are made to the specified standards, utilization of approved ingredients and production methods becomes the responsibility of Pharmaceutical Analysts in the Quality Control (QC) or Quality Assurance (QA) department. The methods are generally developed in an analytical R&D department and transferred to QC or other departments, as needed. At times, they are transferred to other divisions located nationally or abroad or to outsourced companies. By now it should be quite apparent that Pharmaceutical Analysts play a major role in assuring the identity, safety, efficacy, and quality of a Drug product. Safety and efficacy studies require that drug substance and drug product meet two critical requirements:

- Established Identity and Purity.
- Established Bioavailability / Dissolution **(Satinder A & Stephen S, 2001)**

Quality Assurance plays a central role in determining the safety and efficacy of medicines. Highly specific and sensitive analytical techniques hold the key to the design, development, standardization and quality control of medicinal products. They are equally important in pharmacokinetics and in drug metabolism studies, both of which are fundamental to the assessment of bioavailability and the duration of clinical response. However, modern concepts of quality differs and concerned not only with chemical purity, but also with those other characteristics of Pharmaceutical materials which may influence safety, efficacy, formulation and processing of medicines **(Beckett AH & Stenlake JB 1997)**.

1.1 INSTRUMENTAL ANALYSIS

The instrument is only one component of the total analysis. Often, it is necessary to use several instrumental techniques to obtain the information required to solve an analytical problem. Instrumental method may be used by analytical chemists to save time, to avoid chemical separation or to obtain increased accuracy.

Based on Principle Types of Chemical Instrumentation:

A) *Spectrometric Techniques*:

- Atomic Spectrometry (Emission and Absorption)
- Electron Spin Resonance Spectroscopy
- Fluorescence and phosphorescence Spectrophotometry
- Infrared Spectrophotometry
- Nuclear Magnetic Resonance Spectroscopy
- Radiochemical Techniques including activation analysis
- Raman Spectroscopy
- Ultraviolet and visible Spectrophotometry
- X-Ray Spectroscopy

B) Electrochemical techniques

- Potentiometry
- Voltametry
- Stripping techniques
- Amperometric techniques
- Coulometry
- Electrogravimetry
- Conductance techniques.

C) *Chromatographic Techniques*:

- Gas Chromatography
- High performance Liquid Chromatography
- Thin Layer Chromatography

D) *Miscellaneous Techniques*:

- Kinetic Techniques
- Mass Spectrometry
- Thermal Analysis

D) *Hyphenated Techniques*:

- GC-MS (Gas Chromatography - Mass Spectrometry)
- ICP-MS (Inductivity Coupled Plasma - Mass Spectrometry)
- GC-IR (Gas Chromatography - Infrared Spectroscopy)
- MS-MS (Mass Spectrometry - Mass Spectrometry **(Willard H.H. *et al 1986)***

1.1. CHROMATOGRAPHY

Chromatography (from Greek: chroma, color and:"graphein" to write) is essentially a group of techniques for the separation of the compounds of mixtures by their continuous distribution between two phases, one of which is moving fast the other that depends on differential affinities of the solute between two immiscible phases, one of which will be fix with large surface area, while the other is fluid which moves through or over the surface of the fixed phase. **(Beckett AH & Stenlake JB 1997)**

Definitions for Chromatography:

1. Tswett gave the first definition of chromatography. Chromatography is a method in which the compounds of a mixture are separated on an adsorbent column in a flowing system.
2. Chromatography defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases. **(Gurdeep R Chatwal & Sham K.Anand 2002).**
3. IUPAC: chromatography is a physical method of separation in which the compound to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction **(IUPAC, 1993).**

CLASSIFICATION OF CHROMATOGRAPHIC METHODS

(Gurdeep R. Chatwal & Sham K.Ananad2002)

STATIONARY PHASE	MOBILE PHASE	NAME
SOLID	LIQUID	Plane Chromatography
		Paper Chromatography
		Thin layer Chromatography
		Adsorption Column Chromatography
		High Performance Liquid Chromatography
SOLID (Ion exchange resin)	LIQUID	Ion exchange Chromatography
SOLID	GAS	Gas-Solid Chromatography
SOLID MATRIX	LIQUID	Gel permeation Chromatography (Exclusion Chromatography)
LIQUID	GAS	Gas-Liquid Chromatography
LIQUID	LIQUID	Liquid-Liquid Chromatoraphy

1.3. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High Performance Liquid Chromatography (HPLC) was developed in the late 1960s and early 1970s. Today it is widely applied for separations and purifications in a variety of areas including pharmaceuticals, biotechnology, environmental, polymer and food industries.

HPLC has over the past decade become the method of choice for the analysis of a wide variety of compounds. Its main advantage over GC is that the analytes do not have to be volatile, so macromolecules are suitable for HPLC analysis.

PRINCIPLE:

HPLC is accomplished by injection of a small amount of liquid sample into a moving stream of liquid (called the mobile phase) that passes through a column packed with particles of stationary phase. Separation of a mixture into its components depends on different degrees of retention of each component in the column. Since the compounds have different mobilities, they exit the column at different times; i.e., they have different retention times, Rt. The retention time is the time between injection and detection. There are numerous detectors which can be used in liquid chromatography. It is a device that senses the presence of components different from the liquid mobile phase and converts that information to an electrical signal.

Reversed phase HPLC

In this case, the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of

water and an alcohol such as methanol. There will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. There won't be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Polar molecules in the mixture will therefore spend most of their time moving with the solvent. Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of vander Waals dispersion forces. They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules, for example. They therefore spend less time in solution in the solvent and this will slow them down on their way through the column. That means that now it is the polar molecules that will travel through the column more quickly. **(David C. Lee &Michael Webb, 2003)(Synder L.R &Kirkland J.J.,1997)**

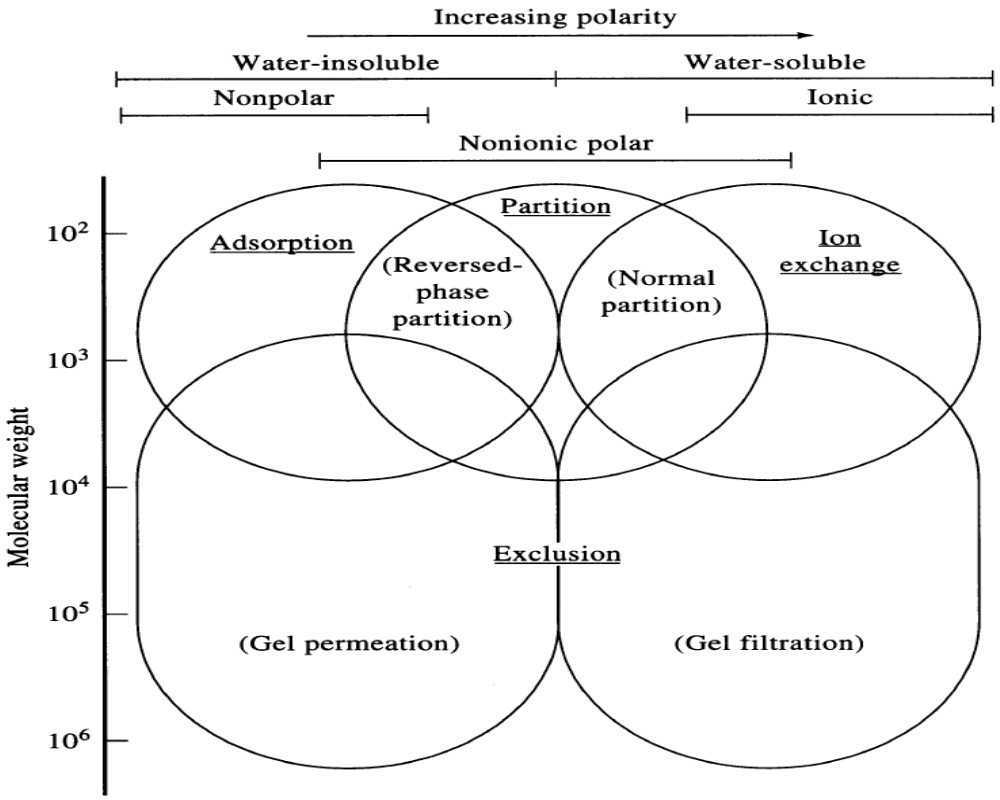
The majority of the HPLC separations are done with Reversed phase separation, probably over 90%. In reversed phase separations organic molecules are separated based on their degree of hydrophobicity. There is a correlation between the degree of lipophylicity and retention in the column.

Types of HPLC techniques

- **Based on Principles of Separations**
  - Partition Chromatography
  - Adsorption (liquid-solid) Chromatography
  - Ion exchange Chromatography
  - Size exclusion Chromatography

- **Based on Modes of Chromatography**
  - Normal Phase Chromatography
  - Reverse Phase Chromatography
- **Based on Elution Techniques**
  - Isocratic Separation
  - Gradient Separation
- **Based on the Scale of Operation**
  - Analytical HPLC
  - Preparative HPLC

Flow Chart: 1. Selection of HPLC methods depending upon Nature of samples

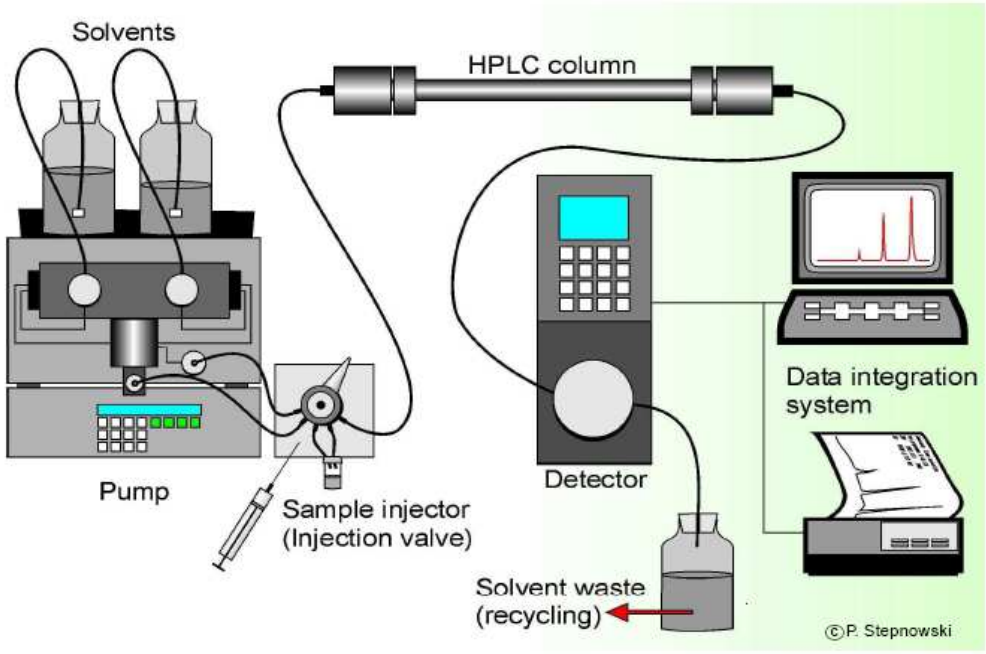


INSTRUMENTATION:

1. COLUMN:

HPLC columns are made of high quality Stainless steel, polished internally to a mirror finish. Standard analytical columns are 4-5 m internal diameter and 10-30 cm in length, shorter column (3-6 cm in length) containing a small particle size packing material (3 or 5 µm). **(Beckett AH& Stenlake J B 1997)**

Figure 1 : Shcematic representation of HPLC



Column packing:

Three forms of column packing material are available based on a rigid structure. These are

i. Microporous supports

ii. Pellicular supports

iii. Bonded phase supports (**Gurdeep R.Chatwal &Sham K.Anand,2002)**

2. MOBILE PHASE RESERVIOR :

The mobile phase reservior can be any clean, inert containers made up of stainless steel and glass. Precaution should be taken to present solvents spills in case of breakage of the reservoir and it should be placed in plastic container. Solvent bottles are available that are coated with a resin material that resist breaking. It usually contain 1 or 2 liter of solvent and it should have a cap that allows the tubing inlet line to pass through it. **(James W. Munson, 2001)**

The choice of mobile to be used in any separation depend on the type of separation to be achieved. Isocratic separation may be made with a single solvent, or two or more solvents mixed in fixed proportion. Alternatively a gradient elution system may be used where the composition of the developing solvent is continuously changed by use of a suitable gradient programmer. All solvents for using HPLC systems must be specially purified since traces of impurities can affect the column and interfere with the detection system. It is also essential that all solvents are degassed before use other wise gassing tends to occur in most pumps. Gassing can alter column resolution and interfere with the continues monitoring of the column effluent. Degassing may be carried out in several way; by warming the solvents, by stirring it vigorously with a magnetic stir, subjecting it to a vacuum, ultrasonic vibrations or by bubbling helium gas through the solvent reservoir. (Gurdeep R.Chatwal & Sham K.Ananad,2002)

The following points should also be considered when choosing a Mobile phase:

1. It is essential to establish that the drug is stable in the Mobile phase for at least the duration of the analysis.

2. Excessive salt concentrations should be avoided. High salt concentrations can result in precipitation, which can damage HPLC equipment.

3. The Mobile phase should have a pH between 2.5 to pH 7.0 to maximize the lifetime of the column.

4. Reduce cost and toxicity of the Mobile phase by using methanol instead of acetonitrile when possible.

5. Minimize the absorbance of buffer. Since trifluroacetic acid or formic acid absorb at shorter wavelengths, they may prevent detection of products with out chromophores above 220 nm. Carboxylic acid modifiers can be frequently replaced by phosphoric acid, which does not absorb above 200 nm.

6. Use volatile Mobile phases when possible to facilitate collection of products and LC - MS analysis. Volatile Mobile phases include ammonium acetate, ammonium phosphate, and formic acid, acetic acid and trifluroacetic acid. Some caution is needed as these buffers absorb below 220 nm.

3. INJECTORS:

Injection ports are of two basic types

a. Those in which the sample is injected directly into the column

b. Those in which the sample is deposited before the column inlet and then swept by a vying action into the column by the mobile phase.

On –column injection involves the injection of the sample by means of a syringe through a septum into the center of the packing material. The column and the capacity of the packing material is typically 5-25µl for analytical column.

High- pressure syringes that can be used at pressure up to 650 atmospheres allow the injection of the sample while the mobile phase is flowing. While using Low- pressure syringes the flow must be stopped.

Modern injectors are based on injection valves which allow the sample at atmospheric pressure to be transferred to the high-pressure mobile phase immediately before the column inlet. With the injection in LOAD position, the sample is injected from a syringe through a needle port into the loop.

The valve lever is then turned through 60^{o} to the inject position and the sample is swept into the flowing mobile phase. An excess of sample is flushed through the loop in the LOAD position, the volume injected is the volume of the loop, which is typically 10-20µl for analytical separation. **(Beckett AH & Stenlake J B 1997)**

4. PUMPS:

The pumping system is one of the most important features of a HPLC system. There is a high resistance to solvent due to the narrow columns packed in small particles, high pressure are required to achieve satisfactory flow rate.

The requirements for an HPLC pumping system are several; They include

a. The generation of pressures of up to 6000 psi (lbs/in^2)
b. Pulse- free output
c. Flow rates ranging from 0.1 to 10ml/min
d. Good flow control capacity
e. All materials in the pump should be chemically resistance to all solvents

These pumping system available which operate on the principle of constant pressure or constant displacement.

Constant pressure pumps produce a pulse less flow through the column, but any decrease in the permeability of the column will result in lower flow rates for which the pumps will not compensate. These pumps operate by the introduction of high pressure gas into the pump, and the gas in turn forces the solvent from the pump chamber in to the column. The intermediate solvent between the gas and the eluting solvent reduce the chances of dissolved gas directly enter in the eluting solvent and causing problems during the analysis.

Constant displacement pump maintain a constant flow rate through the column irrespective of changing conditions within the column. One form of constant displacement pump is a motor-driven syringe type pump where a fixed volume of solvent is forced from the pump to the column by a piston driven by a motor. Such pumps, as well as providing uniform solvents flow rates, also yields a pulse less solvent flow which is important as certain in detectors are sensitive to change in solvent flow rate.

The reciprocating pump is most commonly used form of constant displacement pump. The piston is moved by a motorized crank and entry of solvent from the reservoir to the pump chamber and exit of solvent to the column is regulated by check valves. On the compression stroke solvent is forced from the pump chamber in to the column. During the return stroke the exit check valve closes and solvent is drawn in via entry valve to t e pump chamber, ready to be pumped on to the column on the next compression stroke. **(Gurdeep R. Chatwal & Sham K. Anand, 2002)**

5. DETECTORS:

The detector for an HPLC is the compound that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. It is positioned immediately posterior to the stationary phase on order to detect the compounds as they elute from the column. The bandwidth and height of the peaks may usually be adjusted using the coarse and fine tuning controls, and the detection and sensitivity parameters may also be controlled (in most cases). There are many types of detectors that can be used with HPLC.

Types of Detectors

1. Solute specific detectors (UV, visible, fluorescence, electrochemical, infra-red, radioactivity).
2. Bulk property detectors (refractive index, viscometer, conductivity).
3. Desolvation detectors (flame ionization etc.).
4. LC-MS detectors.
5. Reaction detectors.

Absorbance Detectors

Absorbance detectors is a typical, Z- shaped, flow through cell for absorbance measurements on eluent from chromatographic column. Volumes are limited to 1 to 10 µl and cell lengths to 2 to 10 mm, and the pressure not greater than 600 psi. Many Absorbance detectors are double-beam devices in which one beam passes through the eluent cell and the other through a filter to reduce the intensity.

Ultraviolet Absorbance Detectors

UV Absorbance Detectors are available in two types, UV Absorbance Detectors with Filter and with monochromators. Most HPLC manufactures offer detectors that consist of a scanning spectrophotometer with grating optics. Some are limited to UV radiation; others encompass both UV and Visible radiation. The most powerful UV Spectrophotometric detectors are diode - array instruments.

Refractive Index Detectors

RI Detectors are also called as Universal analyte detector. RI detectors have the significant advantage of responding to nearly all solutes. That is they are general detectors analogous to flame detectors in gas chromatography. In addition they are reliable and unaffected by flow rate. They are highly temperature sensitive and must be maintained at a constant temperature to a few thousands of a degree centigrade. They are not as sensitive as most other type of detectors and generally cannot be used with gradient elution.

Fluorescence Detectors

Excitation wavelength generates fluorescence emission. Analytes must contain a Flurophore group it reacts with the same group of the reagent. The inherent advantage of fluorescence methods is their high sensitivity. Results are dependent up on the separation condition. **(Gennaro A.R. Remigton, 2000)**

1.4. STEPS FOR ANALYTICAL DEVELOPMENT

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are

developed to reduce the cost and time for better precision and ruggedness. Trial runs are conducted, method is optimized and validated.

1. Analyte standard characterization:

a) All information about the analyte i.e., physical and chemical properties, toxicity,

b) The standard analyte (100% purity) is obtained. Made an arrangement for the proper storage (refrigerator, desiccators and freezer).

c) When multiple components are to be analyzed in the sample matrix, the number of components is noted, data is assembled and the availability of standards for each one is determined.

d) Only those methods (MS, GC, HPLC etc.,) that are compatible with sample stability are considered.

2. Method requirements:

The goals of the analytical method that need to be developed are considered. The detection limits, selectivity, linearity, range, accuracy and precision are defined.

3. Literature search and prior methodology:

The information related to the analyte is surveyed for synthesis, physical and chemical Properties, solubility and relevant analytical methods. Books, periodicals and USP / NF, and publications are reviewed. Chemical Abstracts Service (CAS) automated computerized literature searches are convenient.

4. Choosing a method:

a) Using the information in the literatures, methodology is adapted. The methods are modified wherever necessary. Sometimes it is necessary to acquire

additional instrumentation to reproduce, modify, improve or validate existing methods for in-house analytes and samples.

b) If there are no prior methods for the analyte in the literature, from analogy, the compounds that are similar in structure and chemical properties are investigated and are worked out. There is usually one compound for which analytical method already exist that is similar to the analyte of interest.

5. Instrumental setup and initial studies:

a) The required instrumentation is setup Installation, operational and performance qualifications of instrumentation verified by using laboratory Standard Operating Procedures (SOP's).

b) Always new solvents, filters are used, for example, method development is never started, on a HPLC column that has been used earlier.

c) The analyte standard in a suitable injection / introduction solution and in known concentrations and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (e.g., Bulk drug), then it is possible to start work with the actual sample.

d) Analysis is done using analytical conditions described in the existing literature.

6. Optimization:

During optimization one parameter is changed at a time, and set of conditions are isolated, rather than using a trial and error approach. Work has been

done from an organized methodical plan, and every step is documented (in a lab notebook) in case of dead ends.

7. Documentation of analytical figures of merit:

The originally determined analytical figures of merit Limit of Quantitation (LOQ), Limit of Detection (LOD), linearity, time per analysis, cost, sample preparation etc., are documented.

8. Evaluation of method development with actual samples:

The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.

9. Determination of percent recovery of actual sample and demonstration of quantitative sample analysis:

a) Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery (average +/- standard deviation) from sample to sample and whether recovery has been optimized is determined. It is not necessary to obtain 100% recovery as long as the results are reproducible and known with a high degree of certainty.

b) The validity of analytical method can be verified only by laboratory studies. Therefore documentation of the successful completion of such studies is a basic requirement for determining whether a method is suitable for its intended applications. (Michael E & Schartz IS)

1.5. OPTIMIZATION OF CHROMATOGRAPHIC CONDITION

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that all the compounds are detected by more or less symmetrical peaks on the chromatogram. By a slight change of the mobile phase composition, the shifting of the peaks can be expected. From a few experimental measurements, the position of the peaks can be predicted within the range of investigated changes. An optimized chromatogram is the one in which all the peaks are symmetrical and are well separated in less run time. **(Munson J.W, 1994)**

The peak resolution can be increased by using a more efficient column with higher theoretical plate number, N.

The parameters that are affected by the changes in chromatographic conditions are,

- Resolution (R_s),
- Capacity factor (k'),
- Selectivity (α),
- Column efficiency (N) and
- Peak asymmetry factor (As).

i) Resolution (R_s)

The resolution, R_s, of two neighboring peaks is defined by the ratio of the distance between the two peak maxima. It is the difference between the retention

times of two solutes divided by their average peak width. For baseline separation, the ideal value of R_s is 1.5. It is calculated by using the formula,

$$R_s = \frac{Rt_2 - Rt_1}{0.5(W_1 + W_2)}$$

Where, Rt_1 and Rt_2 are the retention times of components 1 and 2 and

W_1 and W_2 are peak widths of components 1 and 2.

ii) Capacity factor (k')

Capacity factor, k', is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. Capacity factor is a measure of how well the sample molecule is retained by a column or TLC plate during an isocratic separation. The ideal value of k' ranges from 2-10. Capacity factor can be determined by using the formula,

$$k' = \frac{V_1 - V_0}{V_0} \times S$$

Where, V_1 = retention volume at the apex of the peak (solute) and

V_0 = void volume of the system.

The values of k'of individual band increase or decrease with changes in solvent strength. In reverse phase HPLC, solvent strength increases with the increase in the volume of organic phase in the water / organic mobile phase. Typically an increase in percentage of the organic phase by 10 % by volume will decrease k' of the bands by a factor of 2-3.

iii) Selectivity (α)

The selectivity (or separation factor), α, is a measure of relative retention of two components in a mixture. The ideal value of selectivity is 2. It can be calculated by using the formula,

$$\alpha = \frac{V_2 - V_0}{V_1 - V_0}$$

Where, V_0 is the void volume of the column and V_2 and V_1 are the retention volumes of the second and the first peak respectively.

iv) Column efficiency (N)

Efficiency, N, of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Smaller the band spread, higher is the number of theoretical plates, indicating good column and system performance. Columns with N ranging from 2000 - 100,000 plates/meter are ideal for a good system. Efficiency is calculated by using the formula,

$$N = 16\frac{Rt^2}{W^2},$$

Where, Rt is the retention time and W is the peak width.

v) Peak asymmetry factor (As)

Peak asymmetry factor, can be used as a criterion of column performance. The peak half width, b, of a peak at 10 % of the peak height, divided by the corresponding front half width, a, gives the asymmetry factor.

$$As = \frac{b}{a}$$

For a well packed column, an asymmetry factor of 0.9 to 1.1 should be achievable. **(Jeffery G.H *et al., 2003)***

1.6. VALIDATION

The word "validation" means "Assessment" of validity or action of validity or action of providing effectiveness'. Validation is, of course, a basic requirement to ensure quality and reliability of the results for all analytical applications. However, in comparison with Analytical Chemistry, in Pharmaceutical Analysis, some special aspects and conditions exist that need to be taken into consideration. Validation of an analytical method is the process by which it is established by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications.

Definitions:

Validation is a systematic approach to gathering and analyzing sufficient data which will give reasonable assurance (documented evidence), based upon scientific judgment, that a process, when operating within specified parameters, will consistently produce results within predetermined specifications.

Validation is defined as follows by different agencies:

European Committee (EC):

Action of providing in accordance with the principles of Good Manufacturing Practice (GMP) that any procedure, process, equipment, material, activity or system actually leads to the expected results. In brief validation is a key process for effective Quality Assurance.

Food and Drug Administration (FDA):

Provides a high degree of assurance that specific process will consistently produce a product meeting its predetermined specification and quality attributes.

World Health Organization (WHO):

Action of providing that any procedure, process, equipment, material, activity, or system actually leads to the expected results.

History:

Since the mid-1970s validation has become an increasingly dominant influence in the manufacturer and quality assurance of pharmaceutical products. In 1976 the FDA proposed a whole set of current GMP regulations which were revised several times.

Objective of the Validation

There are two important reasons for validating assays in the Pharmaceutical Industry.

- The first, and by for the most important, is that assay validation is an integral part of the quality control system.
- The second is that current Good Manufacturing Practice (GMP) regulation requires assay validation. In industry it would be difficult to confirm that the product being manufactured is uniform and that meet the standards set to assure fitness for use. The varying nature of the differences between the analytical development laboratory and quality control laboratory is a good reason for validation program.(USP 1985) (Joachim Ermer & Miller2005)

ANALYTICAL METHOD VALIDATION

Method Validation is the process of proving that an analytical method is acceptable for its intended purpose. Methods need to be validated or revalidated- Before their introduction into routine use, whenever the conditions change for which the method has been validated, whenever the method is changed and the change is outside the original scope of the method.

- United States Pharmacopoeia (USP).
- Food and Drug Administration (FDA).
- World Health Organization (WHO).
- International Conference on Harmonization (ICH).

These guidelines provide a framework for performing Validation. In general, methods for routine analysis, standardization or regulatory submission must include studies on specificity, linearity, accuracy, precision, range, limit of detection, limit of Quantitation and robustness.

In the early stages of drug development, it is usually not necessary to perform all of the various validation studies. Many researchers focus on specificity, linearity, accuracy, and precision studies for drugs in the preclinical through Phase II (preliminary efficacy) stages. The remaining studies are performed when the drug reaches the Phase III (efficacy) stage of development and has a higher probability of becoming a marketed product. The process of validating a method cannot be separated from the actual development of the method conditions, because the developer will not know whether the method conditions are acceptable until validation studies are performed. The development and validation of a new

analytical method may therefore be an iterative process. Results of validation studies may indicate that a change in the procedure is necessary, which may then require revalidation.

During each validation study, key method parameters are determined and then used for all subsequent validation steps. To minimize repetitious studies and ensure that the validation data they are generated under conditions equivalent to the final procedure. **(Mark JG.)**

Benefits of Method Validation:

A fully validated process may require less in-process control and end product testing. It deepens the understanding of processes, decrease the risks of processing problems, and thus assure the smooth running of the process**.**(WHO 1999)

Validation Parameters of Analytical Method:

According to ICH guidelines, typical analytical performance characteristics that should be considered in the validation of the types of methods are

Typical Validation Characteristics which should be considered are:

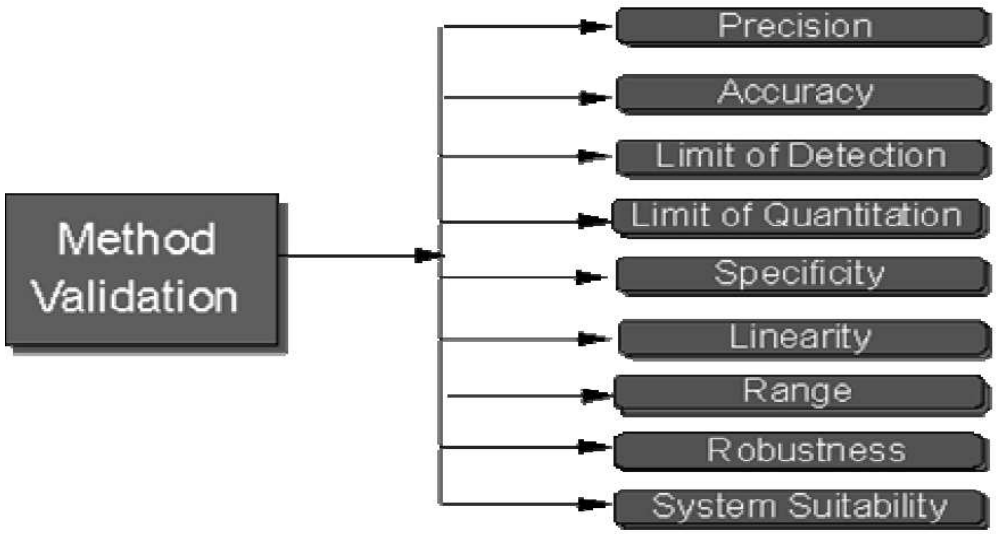


Figure 5: The USP and ICH Method Validation Parameter

1. Accuracy:

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found.

The ICH documents recommended that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentrations levels the specified range (i.e., three concentrations and three replicates of each concentration). Accuracy was tested (% Recovery and % RSD of individual measurements) by analyzing samples at least in triplicate, at each level (80,100 and 120 % of label claim) is recommended. For each determination fresh samples were prepared and assay value is calculated. Recovery was calculated from following regression equation obtained in linearity study.

The % recovery was calculated using the formula,

$$\%\,\mathrm{Recovery} = \frac{(a+b)-a}{bX100}$$

Where,

a – Amount of drug present in sample

b – Amount of standard added to the sample

2. Precision:

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

Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. The ICH documents recommend the repeatability should be assessed using a minimum of nine determinations covering specified range of procedure.

***2.1) Repeatability*:**

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

***2.2) Intermediate Precision*:**

Intermediate precision expresses with in laboratories variations: different days, different analyst and different equipment.

***2.3) Reproducibility*:**

When the procedure is carried out by different analyst in different laboratories using different equipment, regents and laboratories setting reproducibility was determined by measuring repeatability and intermediate precision. Reproducibility is assessed by means of an inter-laboratory trial.

3. Specificity:

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

of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

The definition has the following implications:

Identification test:

To ensure identity of an analyte.

Purity test:

To ensure that all the analytical procedures performed allow an accurate statement of the content of impurity of the content of impurity of an analyte i.e. related substances test, heavy metals, residual solvents etc.

Assay:

To provide an exact result, this allows an accurate statement on the content or potency of the analyte in a sample.

5. Linearity:

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

The linearity is determined from 60% of the ICH reporting level to 140 % of the proposed shelf life specifications of the related substance as a minimum.

6. Range:

The range of an analytical procedure is the interval between the upper and lower of analyte, which is studied.

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

7. Ruggedness:

Degree of reproducibility of test results obtained by the analysis of the same samples under a variety of condition such as different laboratories, different analysts, different instruments etc, normally expressed as the lack of influence on test results of operational and environmental variable of the analytical method. Ruggedness is a measurement of reproducibility of test results under the variation in condition normally expected from laboratory to laboratory and from analyst to analyst. Degree of representative of test results is then determined as a function of the assay variable.

8. Robustness:

Robustness of an analytical method is measure of its capacity to remain unaffected small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. (www.waters.com, USP specification) (ICH Guidelines 1996)

Table C: Acceptance criteria of validation for HPLC

S. No.	Characteristics	Acceptance criteria
1.	Accuracy	Recovery 98-102% with 80,90,100,120 spiked sample
2.	Precision	RSD < 2
a)	Repeatability	RSD < 2
b)	Intermediate precision	RSD < 2
3.	Specificity / Selectivity	No interference
4.	Detection limit	S/N > 2 or 3
5.	Quantitation limit	S/N > 10
6.	Linearity	$r^2 > 0.999$
7.	Range	80 - 120%
8.	Stability	>24hr or < 8hr

2. LITERATURE REVIEW

Systematic literature survey is the main basis for the planning of any scientific work and due to the same reasons here the review of literature regarding estimation of *Megestrol acetate Tablets 40mg* formulation **Mahesh.**

1. **Gaver RC, Movahhed HS, Farmen RH, Pittman KA**. The Megestrol acetate belong to a group of Anti-cancer drugs A simple, sensitive, and reproducible high-performance liquid chromatographic (HPLC) procedure was developed for the quantitative analysis of megestrol acetate in human plasma. An internal standard, 2,3-diphenyl-1-indenone, was added to 0.5 mL of plasma followed by extraction with hexane. The residue remaining after evaporation of hexane was reconstituted in methanol and injected onto a mu-Bondapak C18 column. The column was eluted with acetonitrile : methanol : water : acetic acid (41:23:36:1), and the eluant was monitored at 280 nm. Megestrol acetate and the internal standard eluted at 6-7 and 12-14 min, respectively. The peak height ratio (megestrol acetate/internal standard) versus plasma concentration was linear over a range of 10-600 ng of megestrol acetate/mL of plasma, and the limit of detection was 5 ng/mL. The mean intra- and interassay accuracies were within 3% of the actual values. The mean intra- and interassay precision, as estimated by RSD, were 4 and 6%, respectively. Constituents in human plasma and megestrol, a possible degradation product, did not interfere in the assay. The procedure was applied to the analysis of plasma samples from subjects receiving 40 mg of Megace q.i.d.

2. **Jankana Burana-osot, Sooksri Ungboriboonpisal, Lawan Sriphong.** A stability-indicating HPLC assay method has been developed and validated for medroxyprogesterone acetate (MPA) in bulk drug and injectable suspension. An

isocratic RP-HPLC was achieved on a Hichrom C(18) column (150 mm x 4.6mm i.d., 5 microm) utilizing a mobile phase of methanol 0.020 M acetate buffer pH 5 (65:35, v/v) and a photodiode array detector at 245 nm. The stress testing of MPA was carried out under acidic and alkaline hydrolysis, and oxidation conditions. MPA was well resolved from its degradation products, a main related substance (megestrol acetate) and two preservatives (methyl paraben and propyl paraben) with the resolution >or=2. The proposed method was validated for selectivity, linearity, accuracy, precision and solution stability. The method was found to be suitable for the quality control of MPA in bulk drug and injections as well as the stability-indicating studies.

3. AIM AND OBJECTIVE OF WORK

The drug analysis plays an important role in the development of drugs, their manufacture and the therapeutic use. Pharmaceutical industries rely upon quantitative chemical analysis to ensure that the raw materials used and the final product obtained meets the required specification. The number of drugs and drug formulations introduced in to the market has been increasing at an alarming rate. These drugs or formulation may be either in the new entities in the market or partial structural modification of the existing drugs or novel dosage forms or multi component dosage forms.

The multi component dosage form proves to be effective due to the combined mode of action on the body. The complexity of dosage forms including the presence of multiple drug entities posses considerable challenge to the analytical chemist during the development of assay procedure. The estimation of individual drugs in these multi component dosage forms becomes difficult due to cumbersome extraction or isolation procedures.

For the present study of *Megestrol acetate* was selected. The extensive literature survey carried out and revealed that there is one method reported for the simultaneous estimation of these drugs. Hence an attempt was made to develop a specific, precise, accurate, linear, simple, rapid, validated and cost effective RP-HPLC method for the simultaneous estimation of these drugs in combined dosage forms.

4. PLAN OF WORK

To develop and validate an effective RP – HPLC method for the estimation of *Megestrol acetate* in bulk and its pharmaceutical dosage forms.

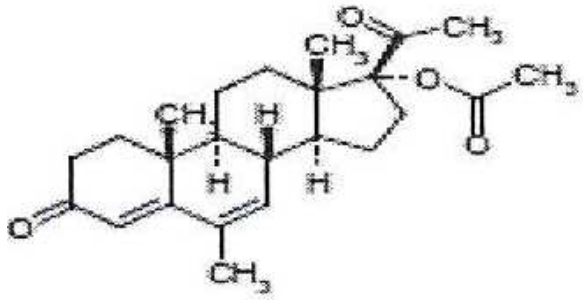
So ,the plan of work for the designed study was as follows:

- Gathering physical chemical properties of drug
- From the UV- analysis ,selection of λ max
- Selection of chromatographic condition
    - Selection of stationary phase
    - Selection of mobile phase
    - Selection of flow rate
    - Selection of Initial separation condition
- Optimization of chromatographic condition
- Validation of proposed method
- Applying developed method to the marketed formulation.
- Summarize methodology, finalize documentation.

5. DRUG PROFILE

Megestrol Acetate

Structure :



Chemical name : 6-Methyl-3,20-dioxopregna-4,6-dien-17-yl acetate

Description : White to creamy white, tasteless and essentially odorless crystalline power

Molecular formula : $C_{24}H_{32}O_4$

Molecular mass : 384.51 g/mol

pKa : Strong Acidic(17.3) and Strong Base(-4.8)

BCS Classification : Class II (Low Soluble high permeability)

Melting point : In between 213°C and 220°C

Solubility : Very soluble in chloroform; soluble in acetone; sparingly soluble in ethanol and slightly soluble in ether in fixed iols; insoluble in water.

Pregnancy Category : US: X (Contraindicated)

Elimination Half life : Mean:34hours

Range:13-105hours

M.O.A: The precise mechanism by which megestrol acetate produces effects in anorexia and cachexia is unknown at the present time, but its progestin antitumour activity may involve suppression of luteinizing hormone by inhibition of pituitary function. Studies also suggest that the megestrol's weight gain effect is related to its appetite-stimulant or metabolic effects rather than its glucocorticoid-like effects or the production of edema. It has also been suggested that megestrol may alter metabolic pathyways via interferences with the production or action of mediators such as Cachectin, a hormone that inhibits adipocyte lipogenic enzymes.

Adverse reaction

- Weight gain
- Vaginal bleeding
- Nausea
- Edema
- Hypogonadism
- Secondary sexual characteristics
- Sexual dysfunction
- Osteoporosis
- Reversible infertility in men
- Premenopausal women
- Cushing syndrome

Dose : 40mg

Pharmacokinetics

The oral bioavailability of MGA is approximately 100% After a single low oral dose of 4 mg MGA, peak serum concentrations of MGA were about 7 ng/dL(18 nmol/L) and occurred after 3 hours. Following a single high oral dose of 160 mg micronized MGA in men, peak circulating levels of MGA were 125 ng/mL (325 nmol/L) and occurred after 6.3 hours. This study found that micronized MGA at this dose showed considerably improved absorption relative to its conventional tablet form. In terms of plasma protein binding, MGA is bound mostly to albumin (82.4%) and is not bound to sex hormone-binding globulin or to corticosteroid-binding globulin. MGA metabolized in the liver mainly by hydroxylation of the C21, C2α, and C6 positions, as well as by reduction and conjugation. Its elimination half-life is 34 hours on average, with a range of 13 to 105 hours. MGA is excreted 57 to 78% in urine and 8 to 30% in feces.

At high doses, MGA appears to have far greater bioavailability and potency than medroxyprogesterone acetate, regardless of whether the route of administration of the latter is oral or parenteral. Following oral administration of 80 to 160 mg MGA or 500 to 1,000 mg medroxyprogesterone acetate, circulating levels of MGA were 2- to 10-fold higher than those of medroxyprogesterone acetate. Similar findings have been found for oral MGA relative to medroxyprogesterone acetate administered via intramuscular injection. MGA also reaches steady-state levels more quickly than medroxyprogesterone acetate. The improved potency of MGA compared to medroxyprogesterone acetate may be due to increased resistance to metabolism of MGA afforded by its C6(7) double bond (medroxyprogesterone acetate being identical to MGA in structure except lacking this feature).

The pharmacokinetics of MGA have been reviewed.

Pharmacodynamics

MGA has progestogenic activity, antigonadotropic effects, weak partial androgenic activity, and weak glucocorticoid activity.

Relative affinities (%) of Megestrol acetate show

- v
- t
- e

Relative affinities (%) of antiandrogens at steroid-hormone receptors show

6. MATERIALS AND INSTRUMENTS

Instruments used:

- System : HPLC(Agilent)
- Pump : I80 (LC – 10 AT Vp series)
- Detector : UV/Visible E2469
- Column : Intertsil ODS-3 C18 (300mm x 3.9 mm, 5 µ i.d.)
- Semi-Micro balance : LC/GC
- Analytical balance : Sartorious
- Hot air oven : Technico
- Mechanical shaker : Remi RS-24 Plus
- Sonicator : PCI Analytics

Reagents and Chemicals

- Acetonitrile : HPLC grade
- Water : HPLC grade
- Hydrogen peroxide : AR
- Sodium hydroxide : AR grade
- Hydrochloric acid : EMPARTA

Reference Standards

1.	**Megestrol acetate**	**:**	Purity 98.7 %
2.	**Propyl paraben**	**:**	Purity 100%
	Brand Used	**:**	Megace
	Label claim	**:**	Megestrol Acetate Tablets USP(40mg)

7. METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

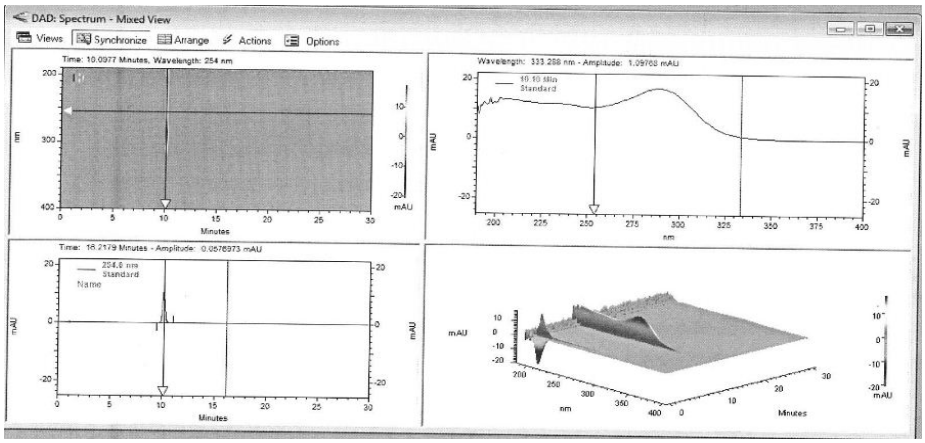
SOLUBILITY

According to literature, Very soluble in chloroform; soluble in acetone; sparingly soluble in ethanol and slightly soluble in ether in fixed iols; insoluble in water.

SELECTION OF CHROMATOGRAPHIC CONDITION

Proper selection of the method depends upon the nature of the sample (ionic / ionisable / neutral molecule), its molecular weight and solubility. The drugs selected in the present study are polar in nature and hence reversed phase or ion-pair or ion exchange chromatography method may be used. The reversed phase HPLC was selected for the separation because of its simplicity and suitability.

SELECTION OF WAVELENGTH (λ max)



In setting up the conditions for the development of the Assay method, the choice of detection wavelength was based on the scanned absorption for *Megestrol Acetate*. The spectrum was scanned over the range of 190 – 400nm and was obtained by measuring the absorption of 1.0 mg/ml solution of Megestrol Acetate in

Acetonitrile and water prepared from stock solution. The spectrum was obtained by using HPLC. λ_{max} of Megestrol Acetate was 280. Hence for estimation 280 nm was selected. **UV – spectrum of Megestrol Acetate**

7.1. METHOD DEVELOPMENT TRIALS

Trial –1

Preparation of Buffer Mobile Phase A:

0.20 M Acetate Buffer and adjusted at pH 5.0.

Preparation of Mobile phase B: Methanol.

Diluent : Methanol and water

Mobile phase Composition : Mobile phase A : Mobile phase B

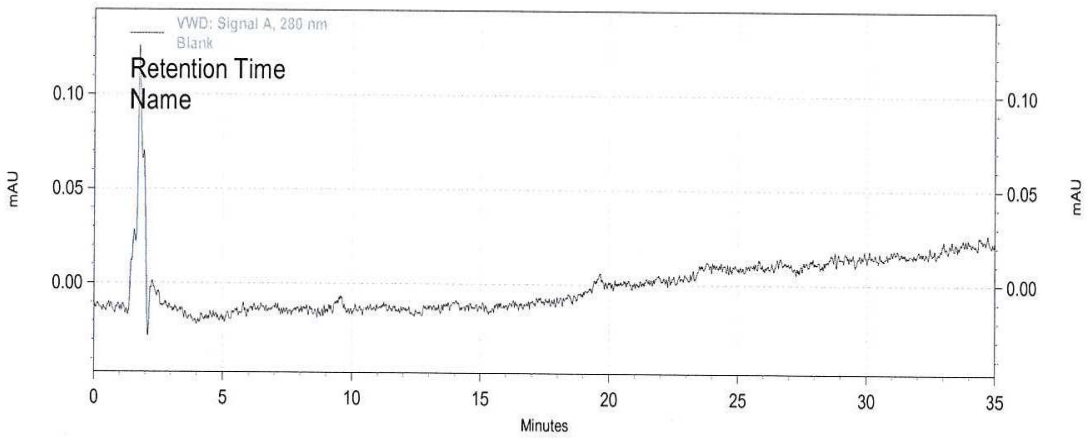
35 35 : 65

The trail 1 was performed in the mobile phase of mobile phase A and mobile phase B in the ratio of 35 : 65 with the flow rate of 1.0 ml/min by using the C_{18} Hichrom 150mm x4.6mm , 5µm column.

Result:

While injecting the above chromatographic condition, the peaks are not eluted properly and going to another method.

Chromatogram 1



Trial –2

Preparation of Mobile Phase A: Water

Preparation of Mobile phase B: 100% Acetonitrile

Diluent : Acetonitrile and water

Mobile phase Composition : Mobile phase A : Mobile phase B

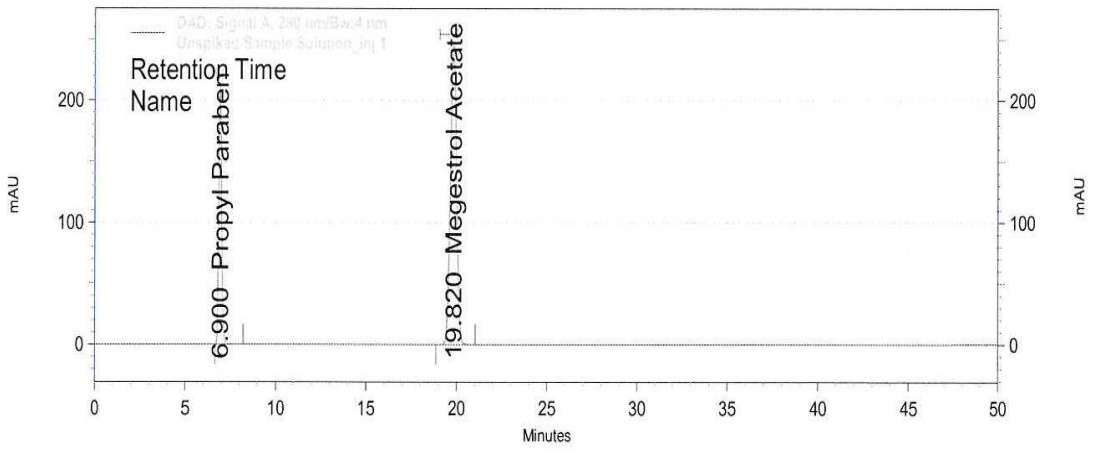
35 60 : 40

The trail 2 was performed in the mobile phase of Mobile phase A and Mobile phase B in the ratio of 60 : 40 with the flow rate 1.0 ml/min C_{18} Intertsil ODS-3 300x3.9, 5µm column.

Result:

While injecting the above chromatographic condition, the impurities was merged with analyte peak, Internal Standard also detected.

Chromatogram 2



Trial –3

Preparation of Mobile Phase A: Water

Preparation of Mobile phase B: 100% Acetonitrile

Diluent : Acetonitrile and water

Mobile phase Composition : Mobile phase A : Mobile phase B

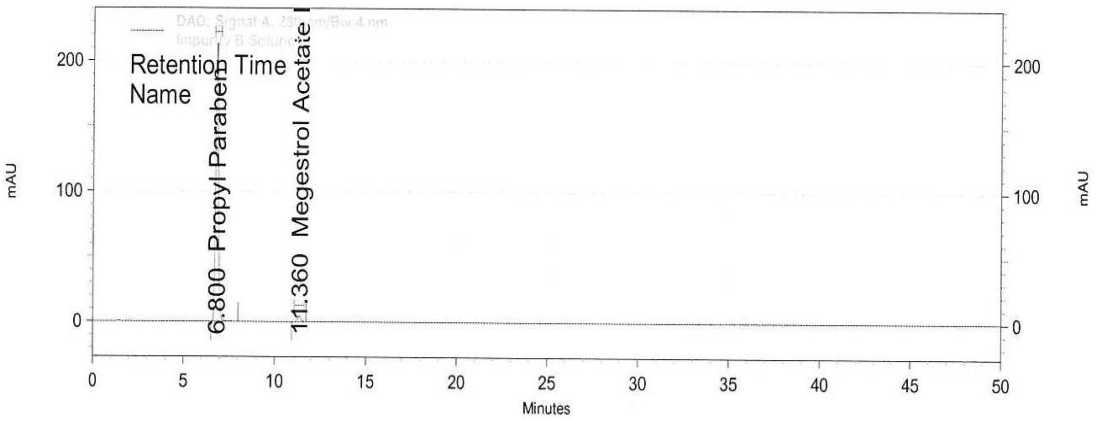
35 55 : 45

The trail 3 was performed in the mobile phase of Mobile phase A and Mobile phase B in the ratio of 55 : 45 with the flow rate 1.0 ml/min C_{18} Intertsil ODS-3 300x3.9, 5µm column.

Result:

While injecting the above chromatographic condition, the impurities was separated and main peak eluted later, Internal Standard also detected.

Chromatograms 3 System suitability



Trial –4

Preparation of Mobile Phase A: Water

Preparation of Mobile phase B: 100% Acetonitrile

Diluent : Acetonitrile and water

Mobile phase Composition : Mobile phase A : Mobile phase B

35 50 : 50

The trail 4 was performed in the mobile phase of Mobile phase A and Mobile phase B in the ratio of 50 : 50 with the flow rate 1.0 ml/min C_{18} Intertsil ODS-3 300x3.9, 5µm column.

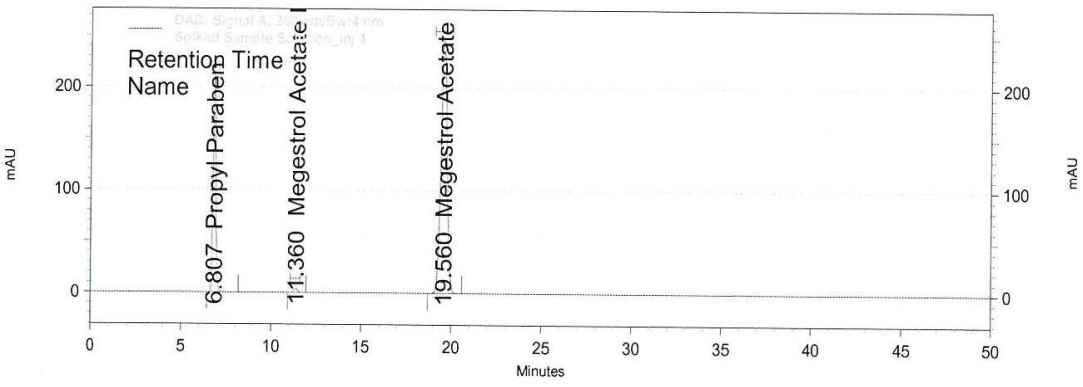
Result:

While injecting the above chromatographic condition, main peak eluted in RT 19.56 and Internal Standard are detected.

The resolution between Megestrol acetate and Impurity-B peak was obtained 15.96.

As per pharmacopoeia recommended resolution between two peaks is not less than 8.0 Still, the method should be developed.

Chromatograms 4 System suitability



Trial –5

Preparation of Mobile Phase A: Water

Preparation of Mobile phase B: 100% Acetonitrile

Diluent : Acetonitrile and water

Mobile phase Composition : Mobile phase A : Mobile phase B

35 45 : 55

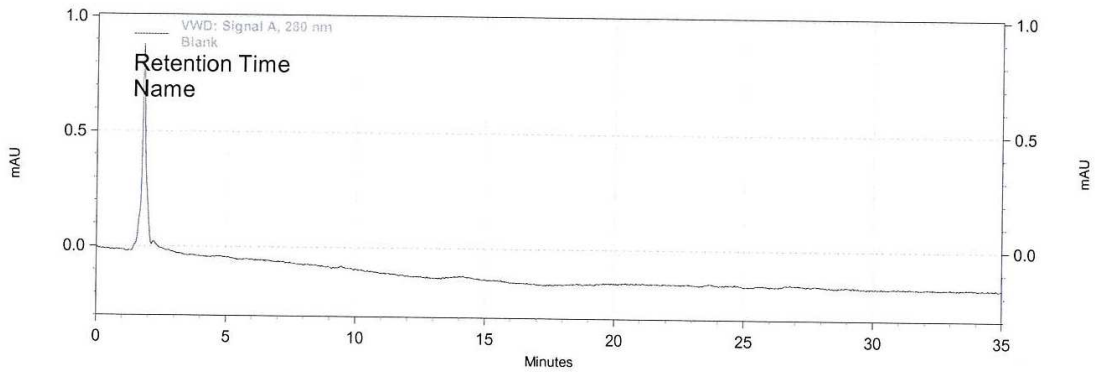
The trail 5 was performed in the mobile phase of Mobile phase A and Mobile phase B in the ratio of 45 : 55 with the flow rate 1.0 ml/min C_{18} Intertsil ODS-3 300x3.9, 5µm column.

Result:

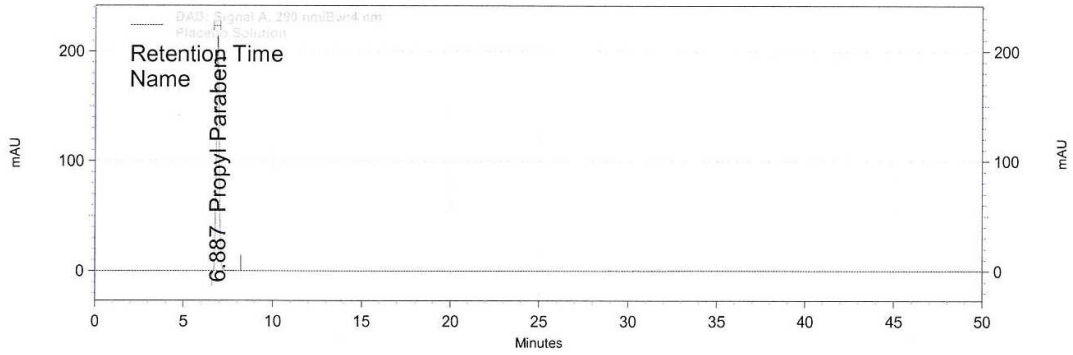
The above mentioned method, Megestrol Acetate RT and all placebo RT were separated from main peak and peak purity will be passes. The isocratic method base line is set properly. The resolution between Megestrol acetate and Propylparaben peak was obtained 26.0.

As per pharmacopoeia recommended resolution between two peaks is not less than 8.0 and the method should be developed.

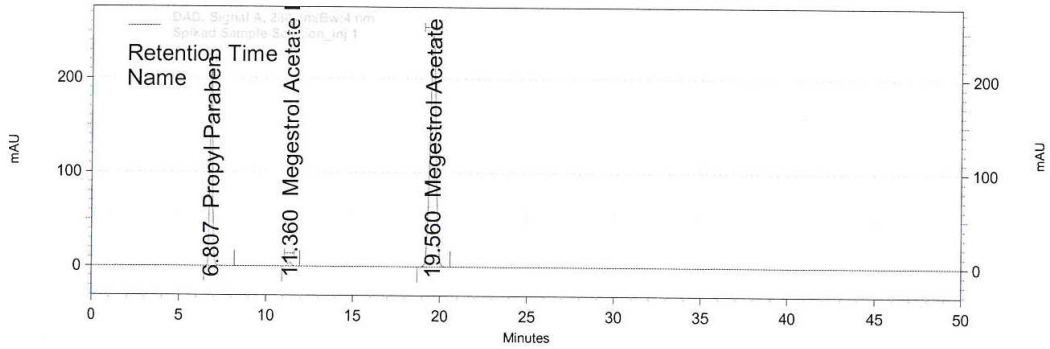
Chromatogram-5 Blank



Chromatogram-6 Placebo with internal Standard



Chromatogram-7 System suitability



DAD: Signal A, 280 nm/Bw:4 nm Results

Name	RT	Area	Theoretical plates (USP)	Asymmetry	Integration Codes	Resolution (USP)	Peak purity
Propyl Paraben	6.800	4759710	8886	1.3	vv	0.00	1.000
Megestrol Acetate	11.353	130222	13973	1.1	vv	13.54	0.976
Impurity B Megestrol Acetate	19.553	12318155	14742	1.0	vv	15.95	1.000
Totals		17208087					

Chromatograph conditions

Column : Intertsil ODS-3 C18, 300 X 3.9 mm, 5µm

Flow rate : 1.0 ml / minute

Injection volume : 25 µl

Detector Wave length : 280 nm

Column temperature : 25°C

Run time : 35 min

Preparation of mobile phase:

Mix well Water and Acetonitrile in the ratio of 45:55 v/v and sonicate to degas.

Preparation of Diluent:

Mix well Acetonitrile and water in the ratio of 40:60 v/v and sonicate to degas.

Preparation of internal Standard stock solution:

Weigh accurately and transfer about 80.0 mg of Propylparaben working standard into a 100 mL volumetric, add 25ml of acetonitrile, sonicate for 10mins make upto volume with acetonitrile.

Preparation of standard stock solution:

Weigh accurately and transfer about 50.0 mg of Megestrol acetate working standard into a 50 mL volumetric flask, add 25ml of acetonitrile, sonicate for 10mins and make upto volume with Acetonitrile.

Preparation of standard solution:

Pipette out 4 mL of standard stock solution and 5ml of internal standard solution into a 50 mL volumetric flask and dilute to volume with diluent (Concentration: 80 µg/ml of Megestrol acetate and Propylparaben).

Note: Prepare duplicate preparation of standard solution.

Sample solution:

Weigh and powdered 20 Tablets. Weighed accurately powdered sample equivalent to 80mg of Megestrol Acetate (aboutv280mg of tablet powder) into 100ml volumetric flask, and add 10ml of water and shake for 10mins. Then add

75ml of acetonitrile and shake for 30mins at 200rpm and make up to volume with acetonitrile. Centrifuge the above solution for 10mins at 3000rpm. Transfer 5ml of supernatant solution and 5ml of internal standard stock solution into 50ml volumetric flask and dilute to the volume with diluents.(Concentration: 80 µg/ml each of Megestrol acetate and Propylparaben).

Procedure

Inject 25 µl of diluent as blank, System suitability solution**,** Standard solution and sample solution into the chromatograph, record the chromatogram and measure the peak response. The related sequence as mentioned below table.

Name of the Solution	Number of Injection
Blank (Diluent)	1
System suitability solution	1
Standard Solution-1	1
Standard Solution-2	5
Sample Solution	1
Standard Solution (Bracketing standard)	1

Note: Inject bracketing standard after every six injections of the test preparation or end of the sequence. The area difference between each bracketing standard and average area of standard preparation should be with in ±2.0%.

System suitability:

1. The %RSD for peak ratio of Megestrol acetate to Propylparaben five replicate injection of Standard solution-2 should be NMT 2.0.

2. Resolution between Megestrol acetate and Propylparaben in standard solution-2 should be NLT 8.0.

3. Similarity factor between standard preparation-1 and first injection of standard preparation-2 for Megestrol acetate peak should be within 0.98 to 1.02.

Calculation for similarity factor

$$\frac{AS1}{AS2} \times \frac{WS2}{WS1}$$

Where,

AS1= Peak response ratio of Megestrol Acetate obtained from the standard solution-1

AS2= Peak response ratio of Megestrol Acetate obtained from first injection of standard solution-2

WS1= Weight of the standard solution-1 in mg

WS2= Weight of the standard solution-2 in mg

Calculation for Peak Response Ratio:

$$\textbf{Peak Response Ratio} = \frac{\text{Megestrol peak Area}}{\text{Propyl paraben Peak area}}$$

Calculate the % Assay of Megestrol Acetate in the portion of Tablets taken:

$$\frac{RU}{RS} \times \frac{Wstd}{100} \times \frac{4}{50} \times \frac{100}{\frac{Wsp}{1}} \times \frac{50}{5} \times \frac{P}{100} \times \frac{Avg.Wt}{L.C} \times 100$$

RU = Peak response ration of Megestrol Acetate to Propylparaben from the sample solution

RS = Average Peak response ratio of Megestrol acetate to Propylparaben from the standard solution

Wstd = Weight of Megestrol acetate Standard taken, in mg

Wspl = Weight of sample taken(mg)

Avg. Wt = Average Weight(mg)

P = Purity of Megestrol Acetate(%). (on as such basis)

L.C = Labeled claim(mg)

8. VALIDATION OF RP-HPLC METHOD

After development of HPLC method for the estimation of the Single component dosage forms validation of the method was carried out. This section describes the procedure followed for the validation of the developed method.

SYSTEM SUITABILITY STUDIES

System suitability studies were carried out as specified in the United States Pharmacopoeia (USP). These parameters include column efficiency, resolution, tailing factor and RSD were calculated in present study.

Prepared Standard Preparations as per test procedure and made six replicate injections. Evaluated system suitability parameters as per the test procedure and tabulated the results in the table given below.

Table No: 1 System suitability parameters

System Suitability Parameters	Observed value	Acceptance criteria
Resolution between Megestrol acetate and propylparaben in standard solution-2	26.06	NLT 8.0
The relative standard deviation obtained from six replicate injections of standard solution in megestrol acetate	0.2	NMT 2.0
The relative standard deviation obtained from six replicate injections of standard solution in propylparaben	0.1	NMT 2.0

No of injection	Megestrol acetate area ratio	Megestrol acetate RT	Propylparaben RT
Standard injection-01	2.729	19.587	6.700
Standard injection-02	2.734	19.577	6.697
Standard injection-03	2.737	19.570	6.693
Standard injection04	2.724	19.567	6.690
Standard injection-05	2.733	19.570	6.693
Standard injection06	2.729	19.567	6.693
Mean	2.731	19.573	6.694
Stdev.	0.005	0.008	0.004
% RSD	0.2	0.04	0.1

Acceptance criteria:

1. The resolution between Megestrol acetate and propylparaben peaks from system suitability solution is not less than 8.0.
2. % RSD for six replicate injections of standard solution should not be more than 2.0.

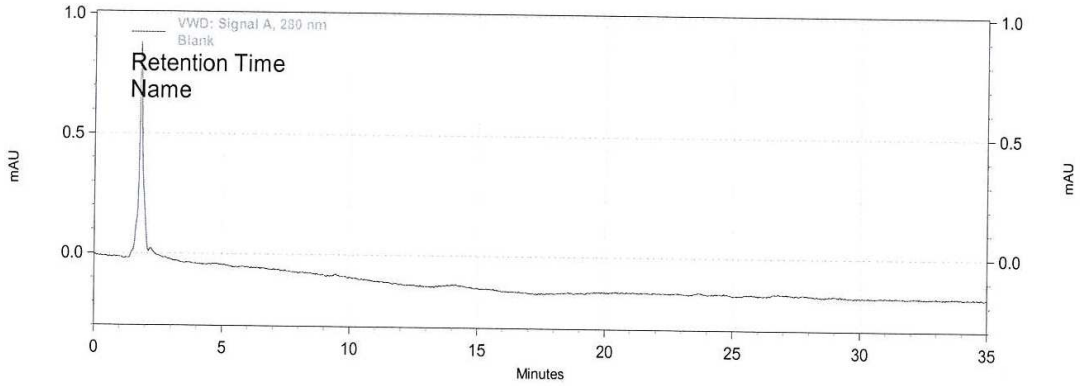
Conclusion: The System suitability parameters are within the limit.

SPECIFICITY

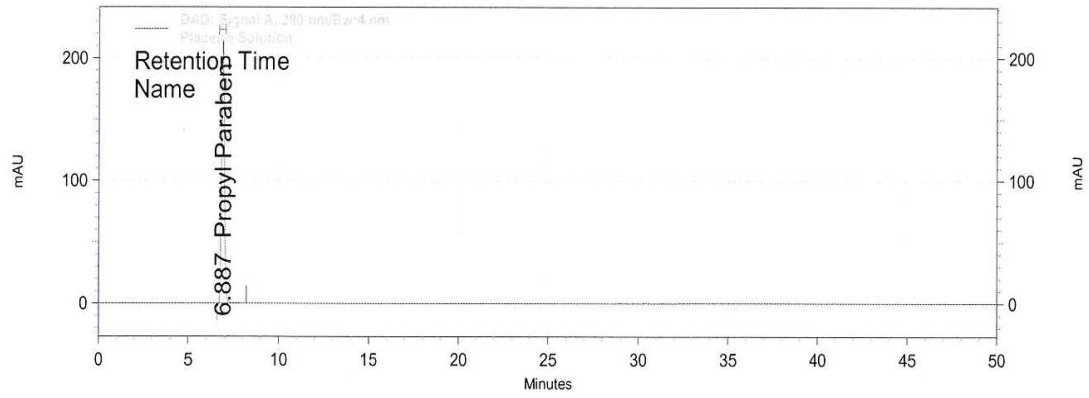
The following methods were employed for demonstrating specificity for HPLC method. In the first method, the conditions of HPLC method developed, namely, percentage of the organic solvent in mobile phase, pH of the mobile phase, flow rate, etc. were changed in HPLC and the presence of additional peaks, if any, was observed. The second method involves the peak purity test method using diode array detector. The diode array derivative spectrums and derivative chromatograms

of the standard and sample drug peaks were recorded and compared. The third method was based on measurement of the absorbance ratio of the drug peaks at different wavelengths.

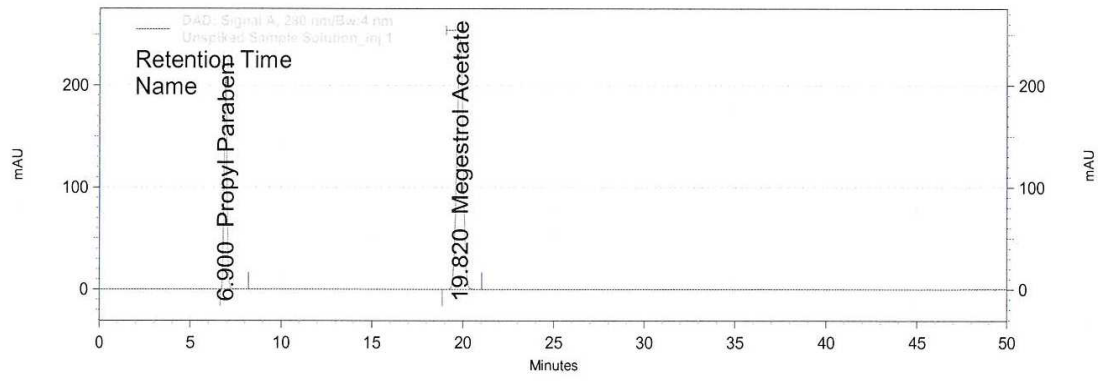
Chromatogram Blank



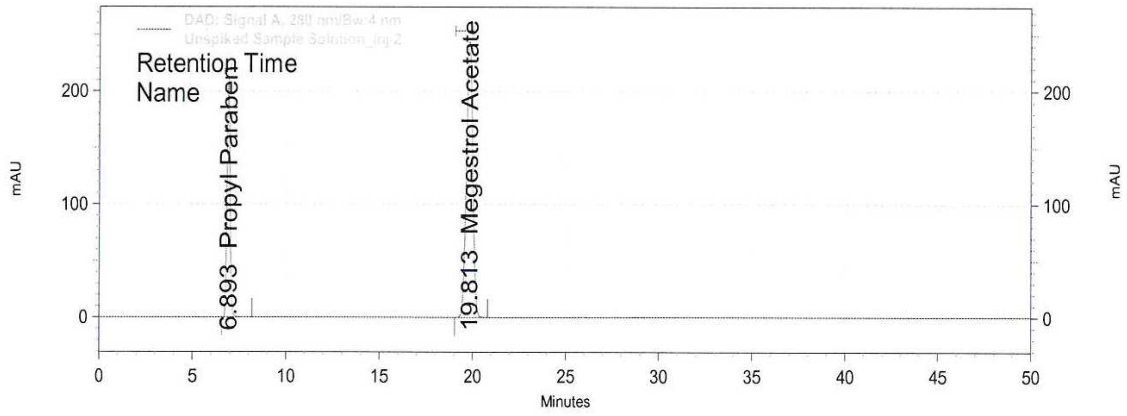
Chromatogram Placebo



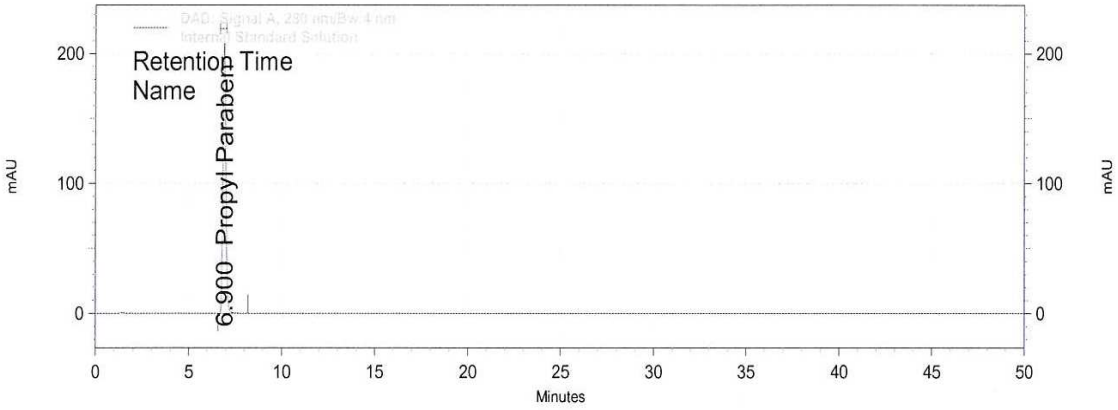
Chromatogram Unspiked sample solution 1



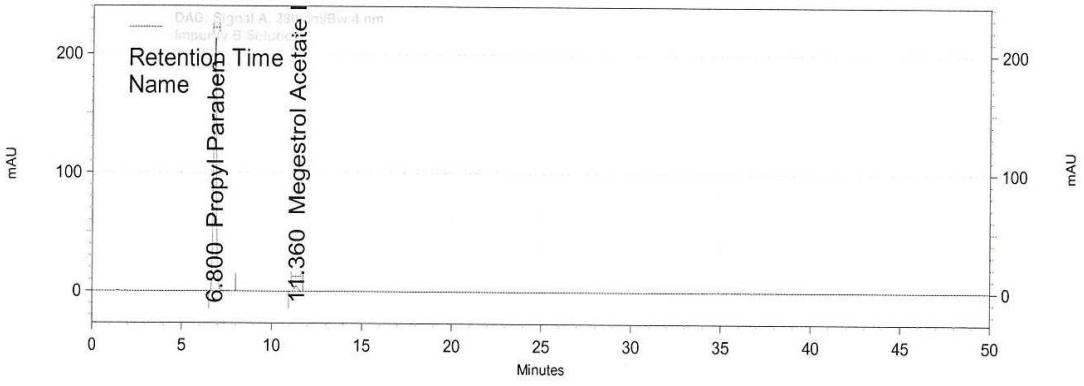
Chromatogram Unspiked sample solution 2



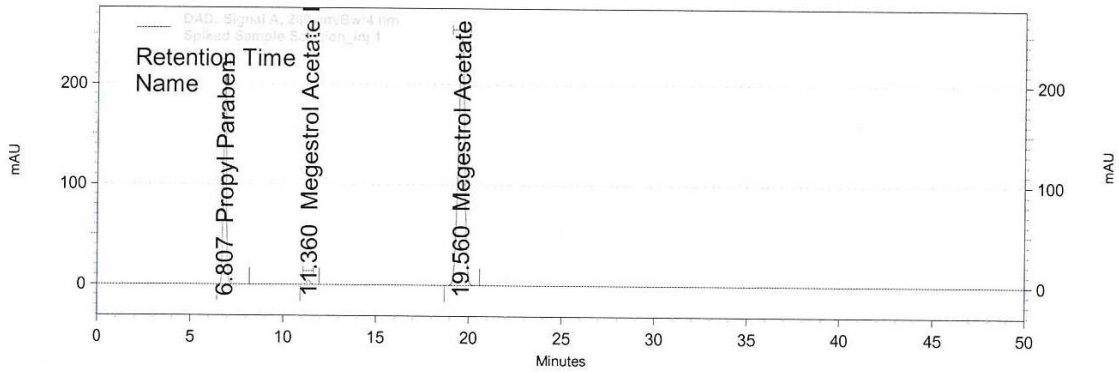
Internal Standard solution



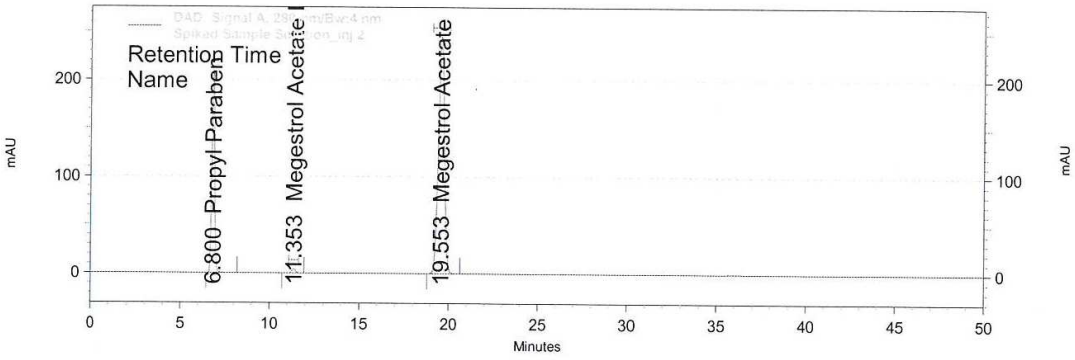
Impurity B Solution



Spiked sample solution 1



Spiked sample solution 2



Placebo interference

Specificity is the ability of the method to measure the analyte in the presence of matrix components. The Specificity will be demonstrated by injecting the solutions of blank, placebo mixture, standard, internal Standard solution, impurity B Solution, Unspiked sample solution and Spiked sample solution. The interference with placebo mixture is checked.

Table No: 2 Summarized the results in the table given below.

Sample ID	Interference (Megestrol acetate)	
	RT (min)	**Peak purity**
Blank	Nil	NA
propylparaben with placebo solution	6.887	1.0
Unspiked sample solution 1(Megestrol acetate)	19.820	1.0
Unspiked sample solution 1(Megestrol acetate)	19.813	1.0
Internal Standard Solution	6.900	1.0
Impurity B solution	11.36	1.0
Spiked sample solution 1(Megestrol acetate)	19.560	1.0
Spiked sample solution 1(Megestrol acetate)	19.553	1.0

Acceptance Criteria

1. There should not be any interference of blank, placebo peaks at the Retention Time (RT) of main peak and known impurity peaks.
2. The Peak Purity should be not less than 0.9 in open lab software / purity angle should less than purity threshold for Empower Software.

Conclusion

The above observation reveals that no interference of any of the blank and placebo was observed at the retention time (RT) of main peak and known impurity peaks.

Interference from Degradation products

A study was conducted to demonstrate the effective separation of degradants from Megestrol acetate tablets USP 40 mg of assay method. Drug product, Placebo and Blank were exposed to the following stress conditions to induce degradation.

Table No: 3 Degradation of the product in the table given below

Stress Condition	Megestrol acetate		
	RT (min)	**% degradation**	**Peak purity**
Kept in water bath at 80°c with 2 mL of 0.5M HCl for 25 minutes (Acid Hydrolysis).	19.753	19.1	1.00
Kept in water bath at 80°c with 2 mL of 0.5M NaOH for 30minutes (Base Hydrolysis).	19.753	11.0	1.00
Kept water bath at 80°c with 2.5 mL of 30% Hydrogen peroxide solution for 30 minutes (Oxidation).	19.547	4.23	1.00
Kept water bath at 80°c with 2 mL of water for 30 minutes (water hydrolysis).	19.553	0.52	1.00
Exposed to Dry heat at 105° C for about 24 hours.	19.542	0.08	1.00
Exposed to humidity at 95% RH for about 24 hours.	19.57	0.06	1.00
Exposed to 1.2million lux hours and at 200 watt hour/square meter near ultraviolet energy(Photolytic).	19.572	5.82	1.00

Acceptance Criteria

1. There should not be any interference of degradants at the Retention Time (RT) of main peak and known impurity peaks.
2. The Peak Purity should be not less than 0.9 in open lab software / purity angle should less than purity threshold for Empower Software.

Conclusion

The above observation reveals that no interference of degradants was observed on the area of Megestrol acetate. This demonstrates that the method is specific for assay of Megestrol acetate tablets USP 40 mg.

ACCURACY

Accuracy of the method was determined by recovery experiments. To the formulation, the reference standards of the respective drugs were added at the level of 100 %. These were further diluted by procedure as followed in estimation of formulation. The resulting sample solutions were analyzed by HPLC. The amount of the each drug present, percentage recovery, percentage relative standard deviation (% RSD) was calculated. The percentage recovery was calculated using the formula,

$$\text{Percentage recovery} = \frac{[a+b]-a}{b} x\,100$$

Accuracy is the closeness of the test results obtained by the method to the true value. Accuracy may often be expressed as percent recovery by the assay of known, added amounts of analyte. Accuracy is a measure of the exactness of analytical method. Injected triplicate preparations by spiking Megestrol acetate on placebo from 50%, 100% and six preparation for 25% , and 150% with respect to

target concentration. Calculated the % Recovery for Megestrol acetate. Summarized the results in the table given below.

Table No: 4 Accuracy of the product in the table given below

Series	No of Sample	Added in ppm	Found in ppm	Recovery in %	Average in %
25%	**01**	20.1	20.1	99.8	**99.2**
	02	20.0	19.9	99.5	
	03	20.2	19.9	98.9	
	04	20.0	19.9	99.2	
	05	20.1	19.9	98.8	
	06	20.2	19.9	98.8	
50%	**01**	40.1	39.9	99.7	**99.6**
	02	40.0	39.8	99.5	
	03	40.0	39.7	99.4	
100%	**01**	79.1	78.6	99.4	**99.8**
	02	79.5	79.6	100.2	
	03	79.1	79.0	99.8	
150%	**01**	119.1	119.0	99.9	**99.8**
	02	118.7	118.8	100.0	
	03	118.6	118.5	100.0	
	04	119.1	118.5	99.5	
	05	118.9	118.3	99.5	
	06	118.8	118.4	99.7	
			Mean	99.5	
			Stdev	0.40	
			% RSD	0.4	

Acceptance Criteria

1. The % Recovery at 25%, 50 %, 100% to 150% level should not be less than 98.0% and not more than 102.0%.
2. The % RSD for % recovery at each level should be NMT 2.0.
3. The overall % RSD for all accuracy determination should be NMT 2.0.

Conclusion:

The % Recovery for Megestrol acetate were found within the limits. Comparison of above results meeting the accuracy acceptance criteria.

PRECISION

- **Method Precision (Repeatability)**

To demonstrate the method precision of the related substances method by analyzing six replicates of sample preparation. Calculated the mean value, the standard deviation and the relative standard deviation for assay of Megestrol acetate. Summarized the results in the table given below.

Table No: 5 Repeatability of the product results in the table given below

No. of Sample	% Assay
	Megestrol acetate Tablets USP 40mg
01	99.1
02	100.1
03	99.7
04	99.5
05	99.4
06	99.8
Mean	99.6
SD	0.346
% RSD	0.4

Acceptance Criteria

1. % Assay of individual sample preparation and the mean % assay obtained should be between 95% and 105%.
2. The % RSD for % assay of Six replicate sample preparations should be NMT 2.0.

Conclusion

The % RSD for % assay of 6 replicate preparations are meeting the acceptance criteria as per protocol. Hence, the method found precise for Megestrol acetate Tablets USP 40mg.

Intermediate Precision

Performed the procedure as detailed in the method precision study on a different day, by a different analyst, preferably using a different instrument and with freshly prepared mobile phase, sample and standard preparation. Prepared the test solution in replicate (Six Preparations) using the same batch, which is taken for method precision, study.

Calculated the mean value, the standard deviation, the relative standard deviation for Megestrol acetate. Summarized the results in the table given below

Table No: 6 Intermediate precision of the product results in the table given below

No. of Sample	% Assay
	Megestrol acetate Tablets USP 40mg
01	99.6
02	99.2
03	99.4
04	99.3
05	99.2
06	99.6
Mean	99.4
SD	0.183
% RSD	0.2

Table No: 7 Comparison of method precision and intermediate precision results:

No. of Sample		Overall % RSD
Method precision	**01**	99.1
	02	100.1
	03	99.7
	04	99.5
	05	99.4
	06	99.8
Intermediate precision	**07**	99.6
	08	99.2
	09	99.4
	10	99.3
	11	99.2
	12	99.6
Overall Mean (n=12)		99.5
SD		0.287
% RSD		0.3

Acceptance Criteria

1. Overall cumulative % RSD for Method precision and Intermediate precision (12 samples) should be NMT 2.0.
2. The Difference between mean values from Method precision and Intermediate precision should be NMT 2.0%.

Conclusion:

The % RSD of % assay obtained from six replicate of sample solution were found within the limits. Comparison of the results obtained by two different days with different analysts and different instruments, shows that the Assay method was meeting the Intermediate precision acceptance criteria.

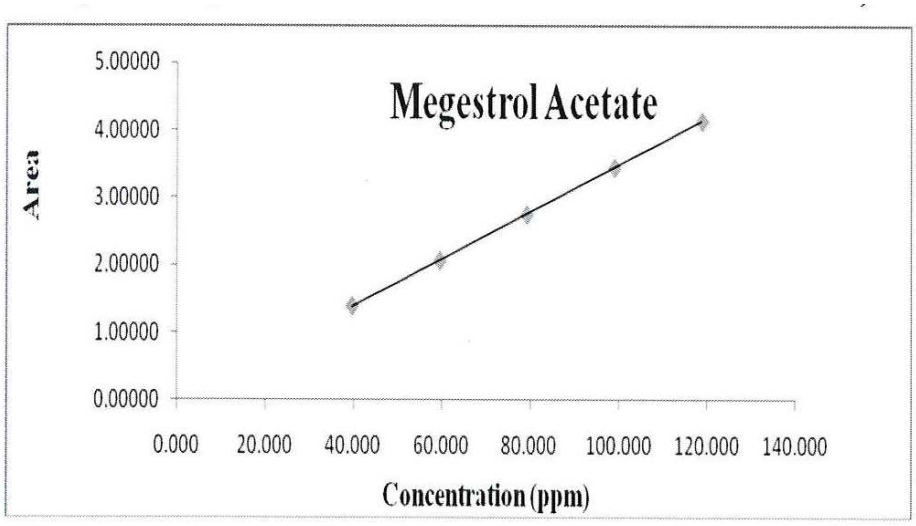
LINEARITY & RANGE

Ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Performed the linearity in the concentrations at 25%, 50%, 75%, 100%, 125% and 150% of specification limit. Recorded the area for each level and calculate slope, y- intercept & coefficient of correlation and coefficient of regression. Plotted the graph of Megestrol acetate concentration on X- axis and area response on Y-axis. Summarized the results in the below table.

Table No: 8 Linearity of the sample calculation given below

Sr. No.	% Level	Concentration in µg/ml (ppm)	Peak Response ratio of Megestrol acetate
01	25	19.814	0.68897
02	50	39.628	1.39333
03	75	59.442	2.06840
04	100	79.256	2.75253
05	125	99.070	3.44970
06	150	118.884	4.14058
		Slope	**0.0348**
		Y intercept	**0.004**
		Coefficient of correlation r	**1.0000**
		Coefficient of regression(r^2)	**1.0000**
		%Y- intercept	**0.16**

Linearity Graph



(fig:01)

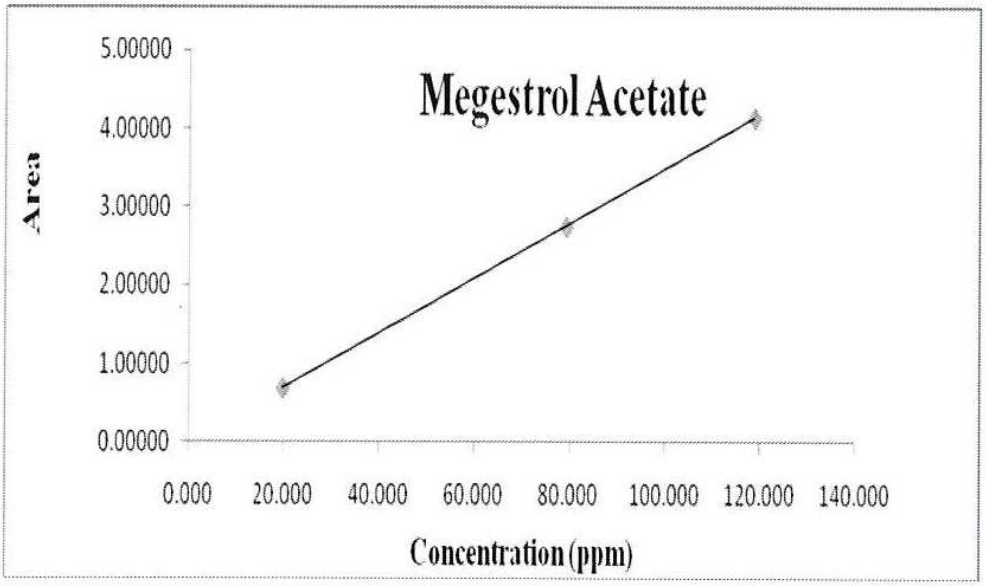
Table No: 9 Linearity lower and higher precision calculation given below

S.No	Sample name	Lower Precision Area	Higher Precision Area
1	Injection 1	0.68804	4.15806
2	Injection 2	0.68769	4.14063
3	Injection 3	0.68726	4.13755
4	Injection 4	0.69041	4.14047
5	Injection 5	0.69044	4.12838
6	Injection 6	0.69000	4.13837
Average		0.68897	4.14058
%RSD		0.2	0.2

Table No: 10 Range for Megestrol acetate calculation given below

S. No.	% Level	Concentration in µg/mL	Peak Response of Megestrol Acetate
1	25	19.814	0.68897
2	100	79.256	2.75253
3	150	118.884	4.14058
Slope			0.0348
Intercept			-0.003
Coefficient of correlation r			1.0000
Coefficient of regression (r^2)			1.0000

Linearity Range Graph



Acceptance Criteria:

1. The Coefficient of correlation(r)value should be not less than 0.999.
2. The Y intercept should be ± 2.0% of the active response at 100% concentration.
3. The % RSD for peak area due to Megestrol acetate at lower and higher precision should be NMT 2.0.

Conclusion

Form the statistical treatment of the linearity data Megestrol Acetate, it is clear that the response of Megestrol Acetate is linear 25% to 150% of the assay method working concentration for Megestrol acetate Tablets USP 40mg. The correlation and regression are more than 0.999. in addition, the value of the Y-intercept is within the ±2 % of the area response at 100% level. Hence, the method was found linear and within the range for Megestrol acetate Tablets USP 40mg.

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 provides an indication of its reliability during normal usage.

In order to demonstrate the robustness of the method, the following optimized conditions were slightly varied.

1. Change in organic variation in Mobile phase ± 10%
2. Change in flow variation ± 0.1ml
3. Change Column Oven Temperature ± 3°C

The separation factor, retention times and peak symmetry were then calculated.

Tablet No: 11 Robustness

Acceptance criteria	%RSD	Resolution	Similarity factor
Low flow variation (0.9mL/Minute)	0.05	25.11	1.00
High flow variation (1.1mL/Minute)	0.2	22.54	1.00
Low column temperature Variation (22°C)	0.1	25.53	1.00
High column temperature Variation (28°C)	0.1	25.21	1.00
Low organic Variation ACN: Water (495mL:505 v/v)	0.1	29.30	1.00
High organic Variation ACN: Water (605:395 v/v)	0.1	21.04	1.00

Acceptance criteria	The % difference in mean assay values obtained for each robustness parameter and mean % Assay

Table No:12 compare from Method precision

	from method precision
Low flow variation (0.9mL/Minute)	1.1
High flow variation (1.1mL/Minute)	0.3
Low column temperature Variation (22°C)	0.2
High column temperature Variation (28°C)	0.6
Low organic Variation ACN: Water (495mL:505 v/v)	0.4
High organic Variation ACN: Water (605:395 v/v)	0.6

Acceptance criteria

1. The % RSD for peak ratio of Megestrol Acetate to Propylparaben five replicate injections of standard solution-2 should NMT 2.0.
2. Resolution between Megestrol acetate and Propylparaben in standard solution-2 should be NLT 8.0.

3. Similarity factor between standard preparation-1 and first injection of standard preparation-2 for Megestrol acetate peak should be 0.98 to 1.02
4. The % Assay of sample preparation obtained for each robustness parameter should be between 95.0% and 105.0%.
5. The difference in mean assay values obtained for each robustness parameter and mean % Assay from method precision should NMT 2.0%.

Conclusion

The robustness parameter was performed as per protocol with chromatographic conditions and mobile phase slight variation namely flow variation(low and high flow), Column temperature variation(low and high column temperature) and organic variation(low and high organic) all the robustness condition was meeting the acceptance criteria. Hence, the method found robust for flow variation (low and high flow), column temperature variation (low and high column temperature), organic variation (low and high organic) for Megestrol acetate Tablets USP 40mg.

SOLUTION STABILITY

Evaluated the stability of analytical solution by injecting the standard and sample solution at 48 hours. The results are summarized in the table(13-14) for standard and sample solutions

Table No: 13 Solution stability for Standard

Time in hours	% Assay	% Difference
Initial	100.0	-
48 Hours	99.8	0.2

Table No: 14 Solution stability for Sample

Time in hours	% of Assay for 40mg	% Diff for Sample
Initial	98.2	-
48 Hours	97.9	0.3

Acceptance Criteria

1. The % difference for % assay of standard and sample preparation between initial and different time intervals should be within ± 2.0.

Conclusion

The % of assay difference between the initial and respective time points of standard and sample solutions meet the acceptance limit for 48Hours at room temperature(25°C). From the above results, it is concluded that the standard solution and sample solution are stable for 48hours at room temperature(25°C).

MOBILE PHASE STABILITY

To evaluated stability of mobile phase, the suitability criteria were established at initial, Day1 and Day2 using same mobile phase with freshly prepared standard solutions. The mobile phase stability was established for two days, where the system suitability criteria meeting the acceptance criteria and not observed any physical change in the appearance of mobile phase. The results are summarized below table 15.

Table No: 15 Mobile phase Stability

Time Interval	% RSD	Resolution	Similarity Factor	Physical Appearance
Initial	0.2	26.06	1.00	Clear
Day-1	0.1	24.97	1.00	Clear
Day-2	1.4	24.51	0.98	Clear

Acceptance criteria

1. The percentage relative standard deviation for peak ratio of Megestrol acetate to Propylparaben five replicate injections of standard solution-2 should be not more than 2.0.
2. Resolution between Megestrol acetate and Propylparaben in Standard solution-2 should be not less than 8.0.
3. Similarity factor between standard preparation-1 and first injection of Standard preparation-2 for Megestrol acetate peak should be within 0.98 to 1.02.

Conclusion

The system suitability parameter met the acceptance criteria for 2Days mobile phase and appearance was found clear. Hence, the mobile phase is stable for 2 days at room temperature with closed container for Megestrol acetate Tablets USP 40mg.

FILTER VALIDATION

Sample solutions are filtered through 0.45µm PVDF filter and 0.45µm Nylon filter and are summarized in the below. The results are summarized table 16.

Table No: 16 Filter interference study

Sample Name	%Assay	% difference
Centrifuged Sample	100.2	-
0.45µm PVDF Filter 2mL Discarded	100.3	-0.1
0.45µm PVDF Filter 4mL Discarded	100.0	0.2
0.45µm PVDF Filter 6mL Discarded	100.4	-0.2
0.45µm NYLON Filter 2mL Discarded	100.8	-0.6
0.45µm NYLON Filter 4mL Discarded	100.2	0.0
0.45µm NYLON Filter 6mL Discarded	99.6	0.6

Conclusion

The % assay difference between centrifuged sample and 0.45µm Nylon filtered and 0.45µm PVDF filtered sample met the acceptance criteria. Hence, it is concluded that 0.45µm Nylon and 0.45µm PVDF filters are suitable for filtering the sample solution of Megestrol acetate Tablets USP 40mg.

ASSAY OF PROPOSED METHOD:

Procedure:

Separately inject both the standard and sample preparations into liquid chromatogram and record the peak area responses. The % RSD is not more than 2.0.

Calculate of similarity factor following formula

$$\frac{AS1}{AS2} \times \frac{WS2}{WS1}$$

Where,

AS1= Peak response ratio of Megestrol Acetate obtained from the standard solution-1

AS2= Peak response ratio of Megestrol Acetate obtained from first injection of standard solution-2

WS1= Weight of the standard solution-1 in mg

WS2= Weight of the standard solution-2 in mg

Calculation for Peak Response Ratio:

$$\textbf{Peak Response Ratio} = \frac{\text{Megestrol peak Area}}{\text{Propyl paraben Peak area}}$$

Calculate the % Assay of Megestrol Acetate in the portion of Tablets taken:

$$\frac{RU}{RS} \times \frac{Wstd}{100} \times \frac{4}{50} \times \frac{100}{\frac{Wsp}{1}} \times \frac{50}{5} \times \frac{P}{100} \times \frac{Avg.Wt}{L.C} \times 100$$

RU = Peak response ration of Megestrol Acetate to Propylparaben from the sample solution

RS = Average Peak response ratio of Megestrol acetate to Propylparaben from the standard solution

Wstd = Weight of Megestrol acetate Standard taken, in mg

Wspl = Weight of sample taken(mg)

Avg. Wt = Average Weight(mg)

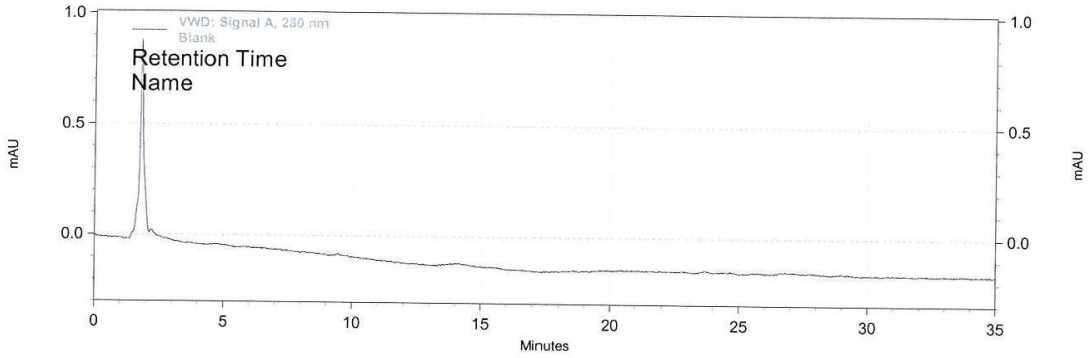
P = Purity of Megestrol Acetate(%). (on as such basis)

L.C = Labeled claim(mg)

9. CHROMATOGRAMS

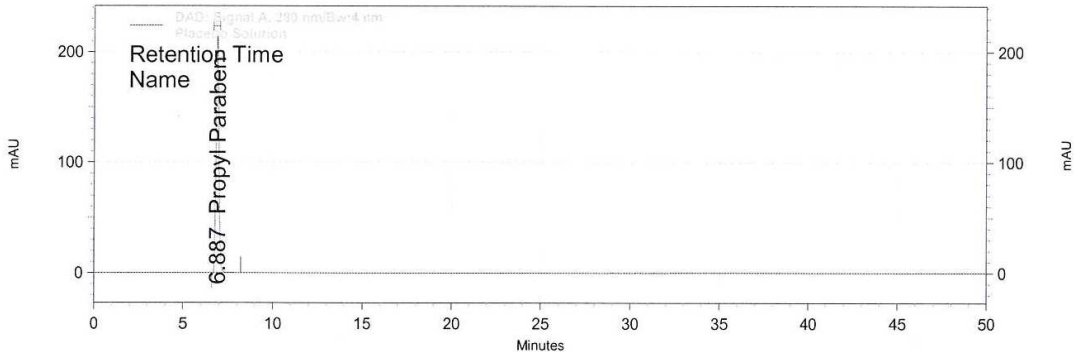
Chromatogram No`:1

A Representative chromatogram of Blank



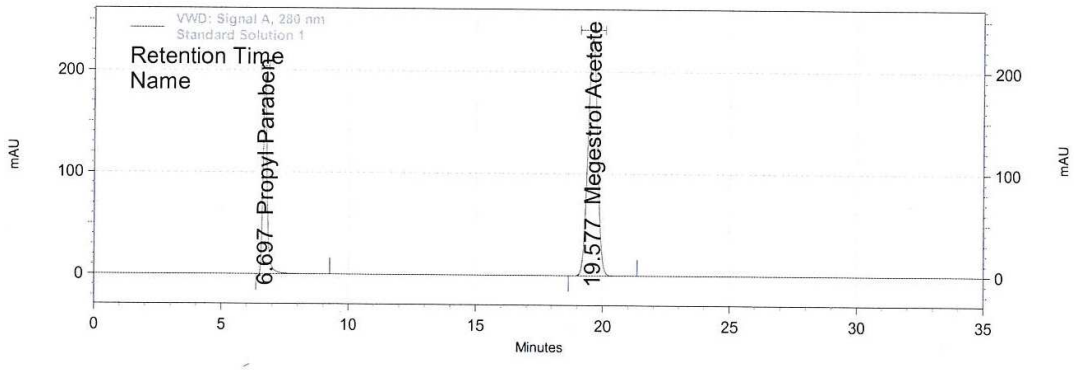
Chromatogram No: 2

A Representative chromatogram of placebo



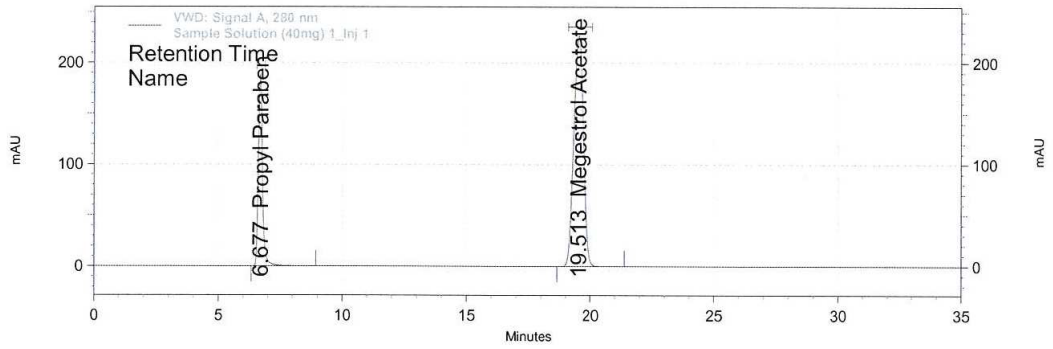
Chromatogram No: 3

System suitability



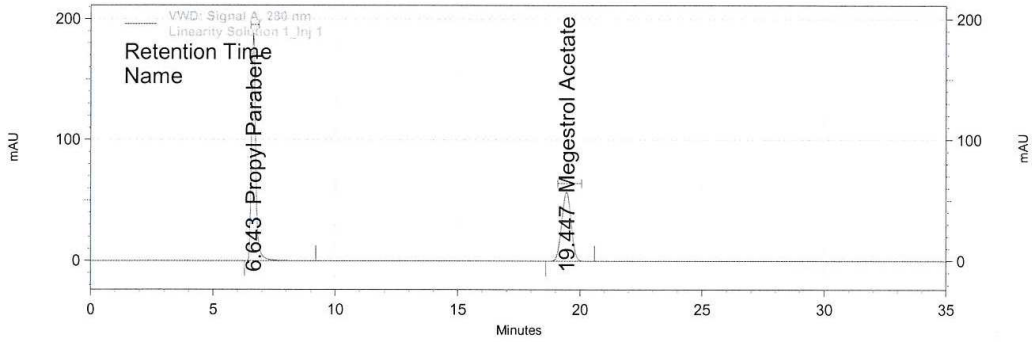
Chromatogram No: 4

A Representative chromatogram of Sample



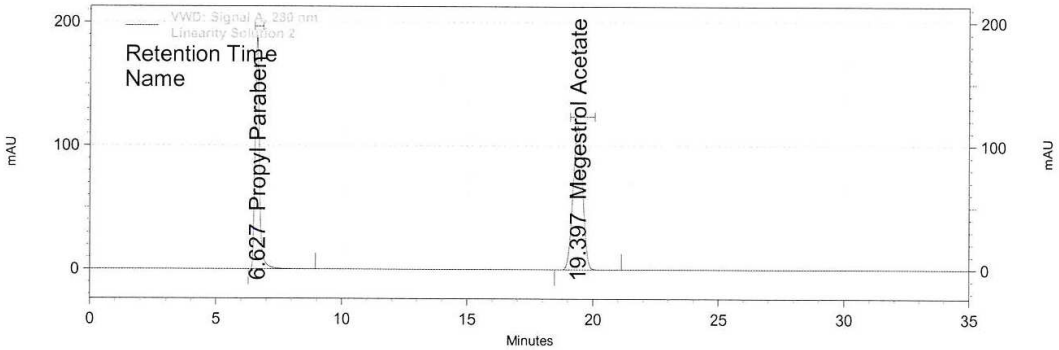
Chromatogram No: 5

Representative chromatogram of the linearity solution 1(25% solution)



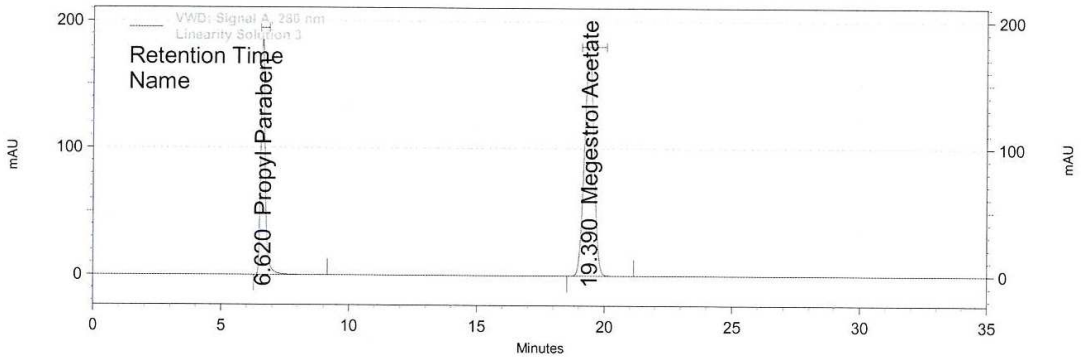
Chromatogram No: 6

A Representative chromatogram of the linearity solution 2 (50% solution)



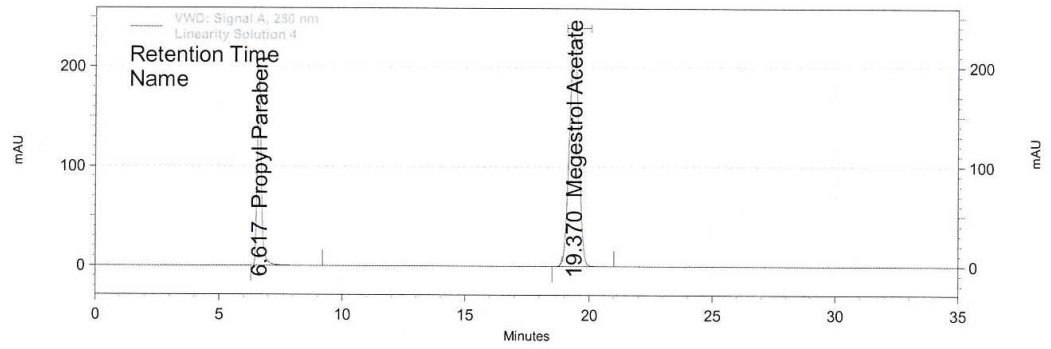
Chromatogram No: 7

A Representative chromatogram of the linearity solution 3 (75% solution)



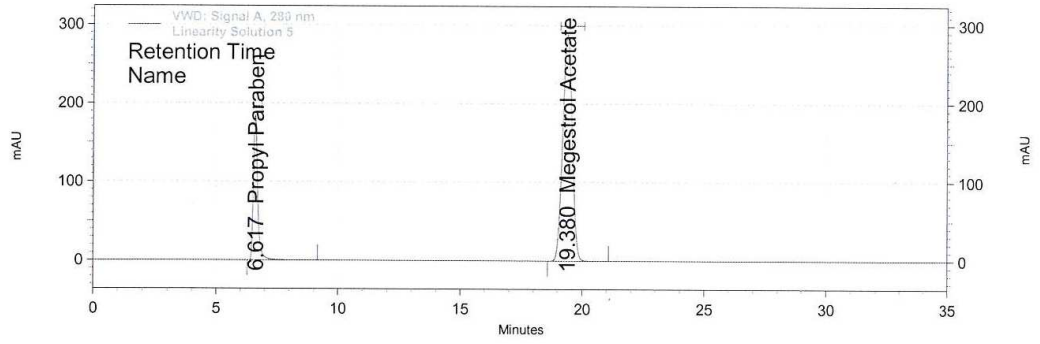
Chromatogram No: 8

A Representative chromatogram of the linearity solution 4 (100% solution)



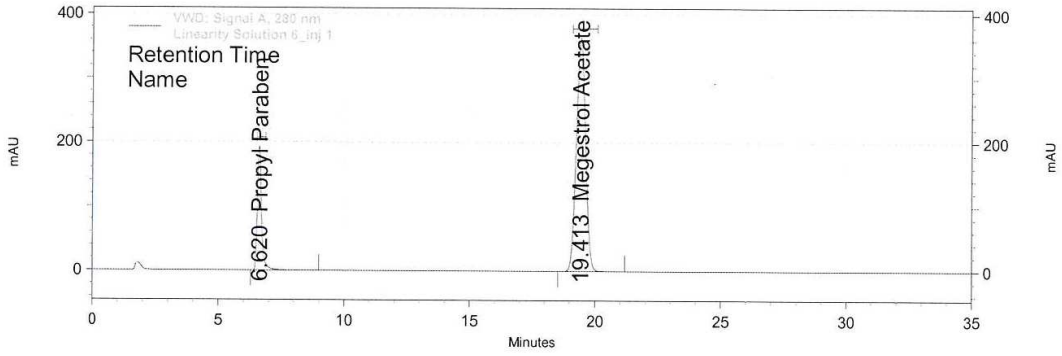
Chromatogram No: 9

Representative chromatogram of the linearity solution 5 (125% solution)



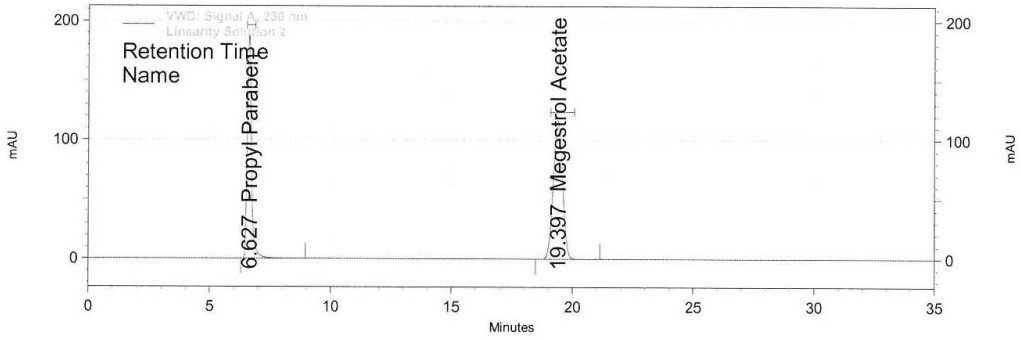
Chromatogram No: 10

Representative chromatogram of the linearity solution 6 (150% solution)



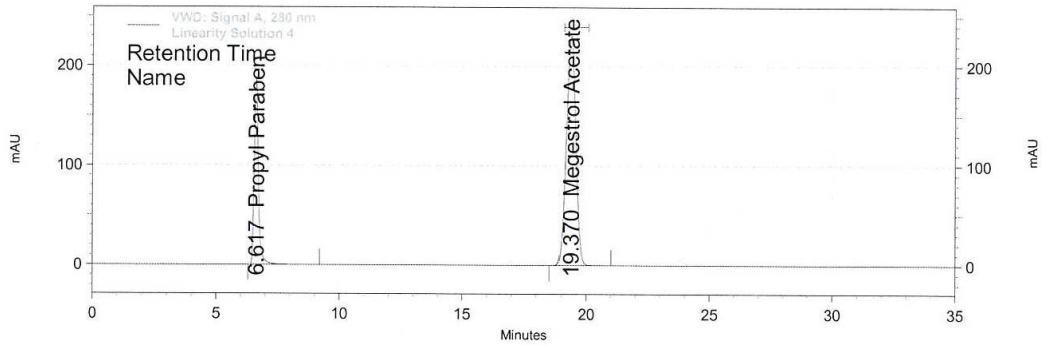
Chromatogram No: 11

A Representative chromatogram of Accuracy sample 50 %



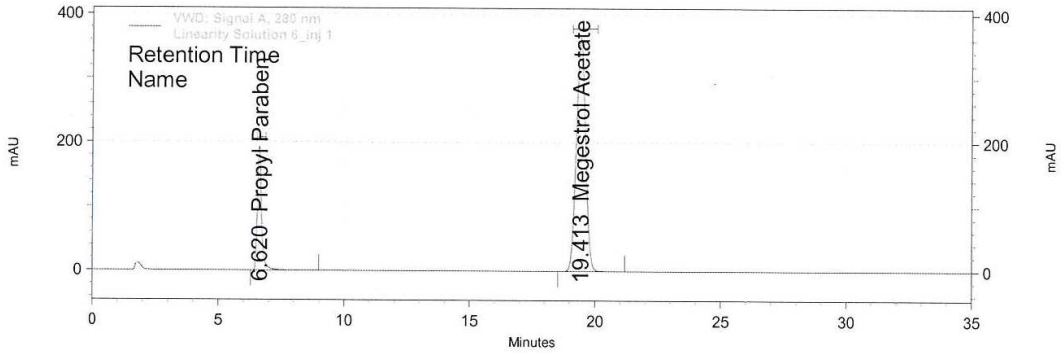
Chromatogram No: 12

A Representative chromatogram of Accuracy sample 100 %



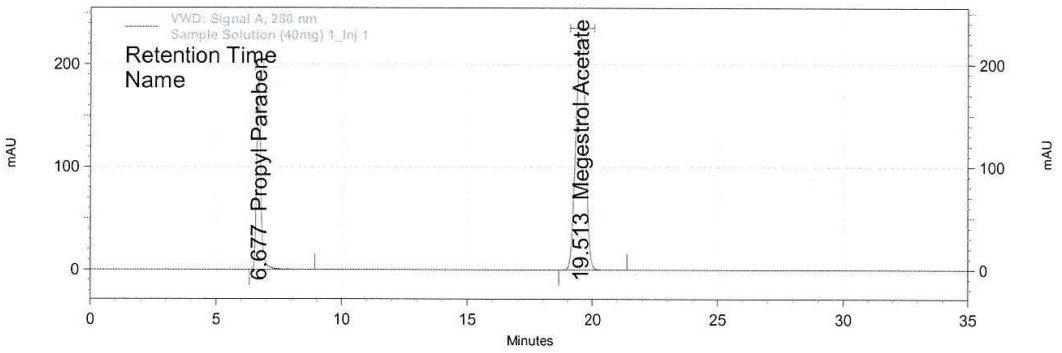
Chromatogram No: 13

A Representative chromatogram of Accuracy sample 150 %



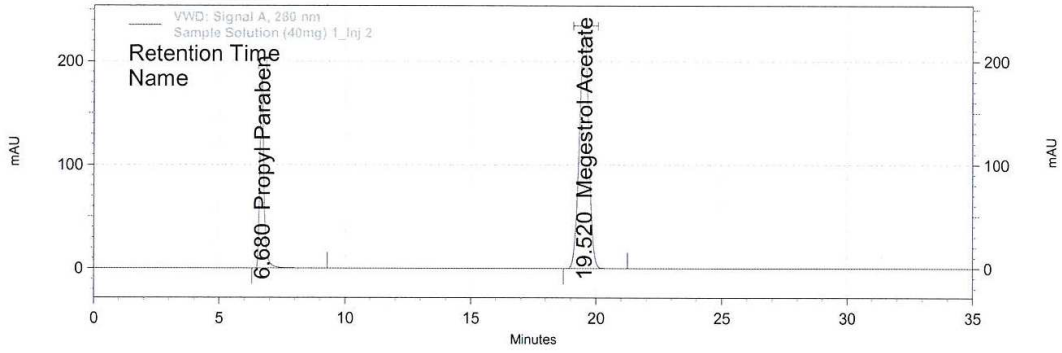
Chromatogram No: 14

A Representative chromatogram of Robustness low organic sample



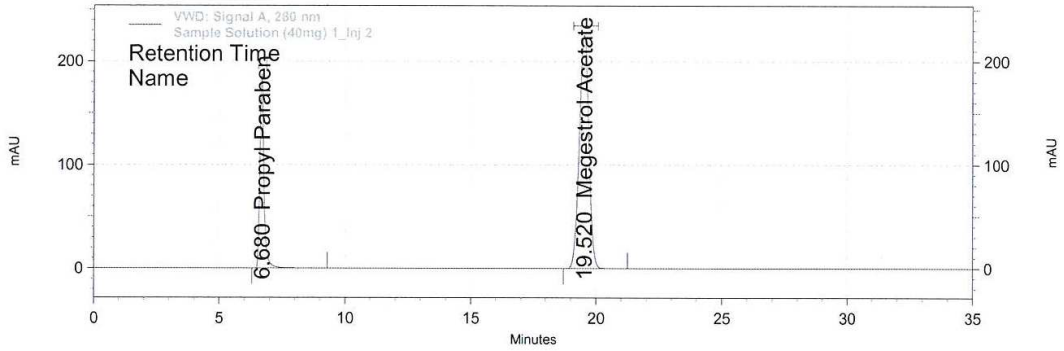
Chromatogram No: 15

A Representative chromatogram of Robustness High organic sample



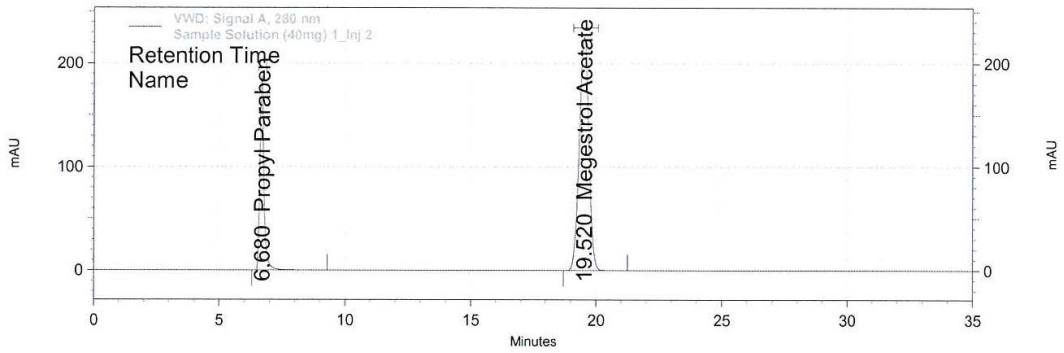
Chromatogram No: 16

A Representative chromatogram of Robustness low column temperature sample



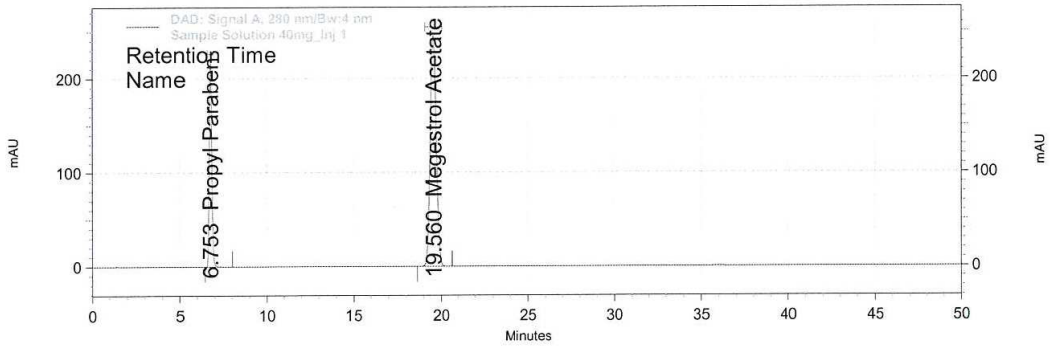
Chromatogram No: 17

A Representative chromatogram of Robustness High column temperature sample



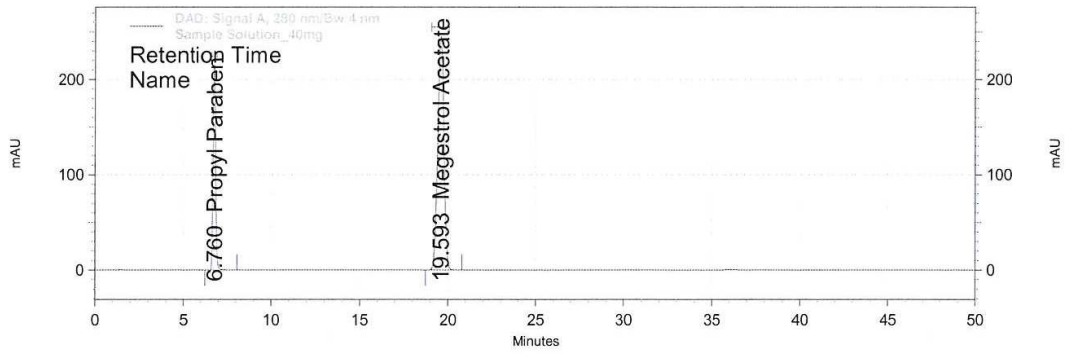
Chromatogram No: 18

A Representative chromatogram of solution stability Sample



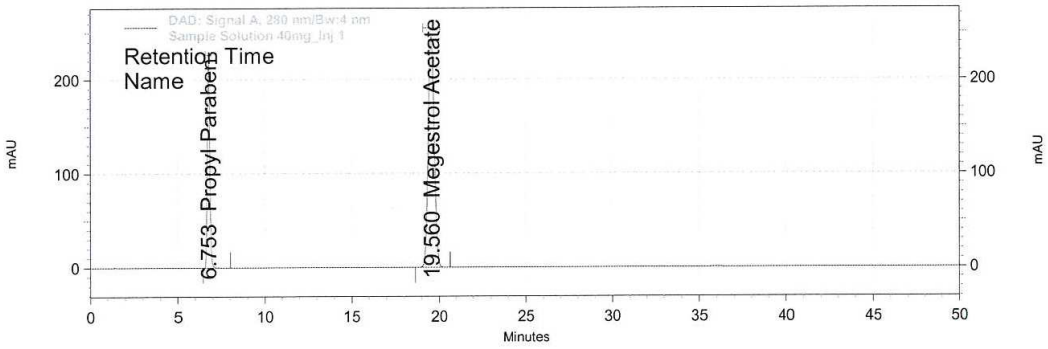
Chromatogram No: 19

Representative chromatogram of Acid stress



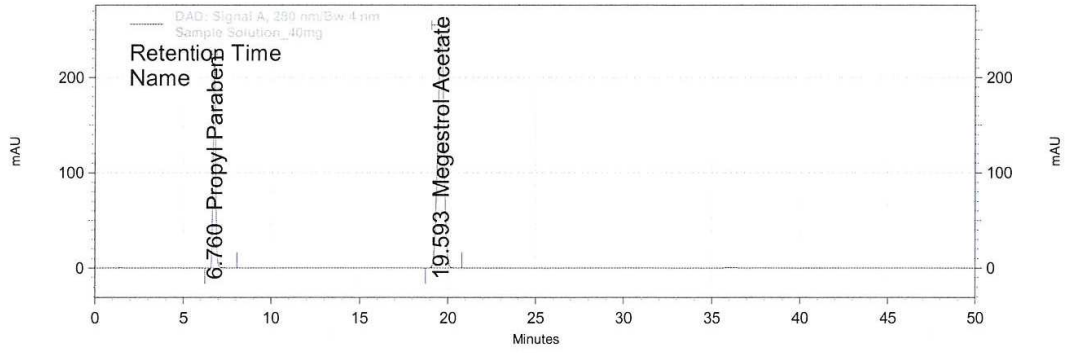
Chromatogram No: 20

Representative chromatogram of Base stress



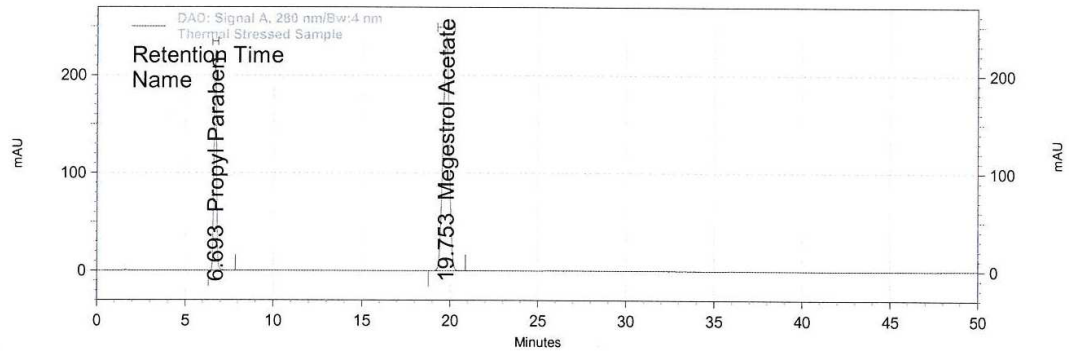
Chromatogram No: 21

Representative chromatogram of peroxide stress



Chromatogram No: 22

Representative chromatogram of thermal stress



10. RESULT AND DISCUSSION

VALIDATION OF THE METHOD

The suitability of the system was studied by the values obtained for Theoretical plate, Resolution and tailing factor, %RSD of the chromatogram of standard drugs and presented in the table(1). The selectivity of the method was revealed by the repeated injection of mobile phase and no interference was found and presented in Table (2) and degradation of stress study of the product calculation presented in Table (3)

The accuracy of the method was determined by recovery experiments. The recovery studies were carried out by preparing 4 individual samples with same procedure from the formulation and injecting. The percentage recovery and percentage relative standard deviation of the percentage recovery was calculated and presented in Tables (4). From the data obtained, added of standard drugs were found to be accurate.

The precision of the method was demonstrated by system and method precision. and intermediate precision of all solutions were injected into the chromatographic system. The peak area and percentage relative standard deviation were calculated and presented in tables (5) & (6) ,The comparision of precision an intermediate precision presented in table (7)

The linearity of proposed method were performed by using the concentration range of 25 to 200% of standard concentration i.e 0.02 µg/ml to 0.120 µg/ml of *Megestrol acetate* was presented in Table (8). The response factor, slope, intercept and correlation co-efficient were calculated. The slope, intercept, correlation co-

efficient were found to be within the limit for *Megestrol acetate.* The calibration curves were plotted using response factor (Vs) concentration of standard solutions (fig: 01). The calibration graph shows that linear response was obtained over the range of concentration used in the procedure. These data demonstrates that the method have adequate sensitivity to the analytes. The range demonstrate that the method is linear outside the limits of expected use.

The robustness of the method was studied by carrying out experiments by changing conditions discussed earlier. The response factors for these changed chromatographic parameters were almost same as that of the fixed chromatographic parameters (table 11,12) and hence developed method is said to be robust and ruggedness performed by analyst 1 and analyst 2 .

The stability studies were carried out at zero hour and after 48 hour, results were tabulated in table (13 & 14).

VALIDATION REPORT SUMMARY

Parameter	Experiment	Observation	Acceptance criteria
System suitability	1. % RSD	0.2%	1.The % RSD for area ratio of Megestrol Acetate peak response ratio obtained from six replicate injections of standard solution should be NMT 2.0.
	2. Retention time	0.04%	2.The % RSD for Retention time of Megestrol Aetate and Propylparaben peak obtained from six replicate injections of standard solution should be NMT 1.0.
	3. Resolution	26.06	3.Resolution between Megestrol acetate and Propylparaben in Standard solution-2 should be NLT 8.0.
Specificity	Placebo and Blank, Impurity interference and Interference from Degradation products	Complies	1.There should not be any interference of blank, placebo peaks at the Retention Time (RT) of main peak and known impurity peaks.
		1.0	2.The Peak Purity should be not less than 0.9 in open lab software / purity angle should less than purity threshold for Empower Software.
Linearity and Range	Coefficient of correlation (r)	1.0000	1.The Coefficient of correlation should not be less than 0.995.
		0.16 %	2.The Y- intercept shall be ± 5.0% of the active response at 100% concentration.

Accuracy	% Recovery	Complies Complies Complies	1.The % of Recovery at 50 % to 200% level should not be less than 98.0% and not more than 102.0%. 2.The % RSD for % recovery at each level should be NMT 2.0. 3.The Overall % RSD for all accuracy determination should be NMT 2.0.
Precision	Method Precision	99.6% 0.4 %	1.The Assay of individual sample preparation and the mean % Assay obtained should be between 95.0% and 105.0%. 2.The % RSD for assay of 6 replicate sample preparations should be NMT 2.0.
	Intermediate Precision	0.3 % 0. 5%	3.The Overall cumulative % RSD for Method precision and Intermediate precision(12 samples) should be NMT 2.0. 4.The % Difference between mean from Method precision and Intermediate precision should be NMT 2.0%.
Solution stability	Bench top stability of standard solution	48 hours	The difference between initial and bench top stability sample for % of Relative standard deviation for assay
	Bench top stability of Test solution	48 hours	

Robustness	1.Variation in column oven temperature 2.Variation in flow rate 3.OrganicVariation in mobile phase	Complies Complies Complies Complies	1.The % RSD for peak ratio of Megestrol acetate to Propylparaben five replicate injections of Standard solution-2 should be NMT 2.0. 2.Resolution factor between Megestrol acetate and Propylparaben in Standard solution-2 should be NLT 8.0. 3.The % Assay of Sample preparation obtained for each robustness parameter should be between 95.0% and 105.0%. 4.The difference in mean assay values obtained for each robustness parameter and mean % Assay from method precision should NMT 2.0%.
Degradation Study	Acid stress Base stress Peroxide stress Thermal stress Humidity stress	Complies 1.0	% Assay should be ±10% The Peak Purity should be not less than 0.9 in open lab software / purity angle should less than purity threshold for Empower Software.

11. SUMMARY AND CONCLUSION

From the reported literature, there were few methods established for the determination of *Megestrol Acetate* in individual and in combination with other drug.

It was concluded that there was two method reported for the simultaneous estimation of the above selected multi component dosage form, which promote to pursue the present work. The scope and object of the present work is to develop and validate a new simple HPLC method for estimation of *Megestrol Acetate Tablets* in dosage form.

In RP-HPLC method development, the estimation was carried out by using the Intertsil ODS-3 C_{18} column (300mm X 3.9mm) with 5-micron particle size. Injection volume of 25µl is injected and eluted with the mobile phase Water, Acetonitrile with the isocratic program , which is pumped at the flow rate of 1.0 ml / min. Detection was carried out at 280 nm. Quantitation was done by calibration curve method with the above mentioned optimized chromatographic condition. This system produced symmetric peak shape, good resolution and reasonable retention times of *Megestrol acetate and Propylparaben* were found to be resolution is 8.0 and retension time is 19.82 and 6.9 minutes respectively.

The 20.0 µg/ml to 120.0 µg/ml of *Megestrol Acetate* respectively. The slope intercept and correlation coefficient(s) were found to be, within the limit for which indicates excellent correlation factor Vs concentration of standard solutions.

Precision of the developed methods was studied under system precision, method precision. The %RSD values for precision was found to be within the

acceptable limit, which revealed that the developed method was precise. The developed method was found to be robust. The %RSD values for recovery percentage of *Megestrol Acetate* was found to be within the acceptable criteria. The result indicates satisfactory accuracy of method for estimation of the above mentioned drugs.

Hence, the chromatographic method developed for *Megestrol Acetate* are rapid, simple, specific, sensitive, precise, Accurate. The RP-HPLC was simple and does not suffer from common excipients in pharmaceutical preparation and highly useful in the analysis of drugs in pharmaceutical formulation.

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