Rajveer Bhaskar. *et al.* / *International Journal of Biological & Pharmaceutical Research.* 2015; 6(8): 639-648.

e- ISSN 0976 - 3651
Print ISSN 2229 - 7480

International Journal of Biological & Pharmaceutical Research

Journal homepage: www.ijbpr.com

IJBPR

A REVIEW ON HPLC METHOD FOR THE ASSAY OF NORETHINDRONE ACETATE AND ETHINYL ESTRADIOL IN NORETHINDRONE ACETATE AND ETHINYL ESTRADIOL TABLETS USP

Rajveer Bhaskar*, Monika Ola, P. H. Patil, Sunil Garje

R. C. Patel institute of Pharmaceutical Education and Research Centre, Shirpur, Dhule, Maharashtra, India.

ABSTRACT

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Analytical method validation is the process of demonstrating that an analytical method is reliable and adequate for its intended purpose. Any method that is utilized to determine results during drug substance and formulation development will have to be validated. Validation of HPLC methods focus mainly on the following. Identification Test. Quantitative measurements of the content of related substance. Semi quantitative and limit test for the control of related substances. Quantitative test for the assay of major components (Drug substances and Preservatives) in Samples of drug substance or drug product (assay, content uniformity, dissolution rate, etc.) Analytical method validation is established through documented evidence demonstrating the accuracy, precision, linearity, selectivity, ruggedness, and/or robustness of that particular test method which will be utilized to generate test results for a drug substance or drug product.

Key Words: High Performance Liquid Chromatography, Good Manufacturing Practice, International Conference on Harmonization.

INTRODUCTION

Method Validation

Establish documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes. 1978 Current Good Manufacturing Practices (cGMP), 1987 FDA Validation Guideline, 1994 CDER Reviewer Guidance-Validation of Chromatographic Method, 1995 ICH Validation Definitions, Q2A, Text on Validation of Analytical procedures, 1997 ICH Validation Methodology, Q2B, Validation of Analytical Procedures: Methodology, 1999 Supplement 10 to USP 23 <1225>: Validation of Compendial Methods, 1999 CDER "Bioanalytical Method Validation for Human Studies, 2000 CDER Draft "Analytical Procedures and Method Validation (Analytical Methods, 2006).

Chemistry is a measurement of science consisting of a set of powerful ideas Analytical and methods that are useful in all fields of science and medicine. It seeks ever improved means of measuring the chemical composition of natural and artificial materials. This branch of chemistry, which is both theoretical, and a practical science, is practiced in a large number of laboratories in many diverse ways while analytical method, is a specific application of a technique to solve an analytical problem. Methods of analysis are routinely developed, improved, validated, collaboratively studied and applied. The discipline of analytical chemistry consists of qualitative and quantitative analysis (USFDA, 2006).

Corresponding Author

Rajveer Bhaskar
Email: sunilgarje13@gmail.com

Qualitative analysis- Establishes the chemical identity of the species in the sample.

Quantitative analysis-Determines the relative amounts of these species, or analytes, in numerical terms.

INSTRUMENTAL METHODS OF CHEMICAL ANALYSIS

Chromatographic Techniques

High Performance Liquid Chromatography Gas chromatography

High Performance Thin Layer Chromatography Thin Layer Chromatography

GC- MS (Gas chromatography - Mass Spectroscopy

LC-MS (Liquid Chromatography - Mass Spectroscopy)

High Performance Liquid Chroma Tography

Liquid chromatography (LC) is an analytical chromatographic technique that is useful for separating ions or molecules that are dissolved in a solvent. If the sample solution is in contact with a second solid or liquid phase, to differing degrees due to differences in adsorption, ion exchange, partitioning or size. These differences will allow the mixture components to be separated from each other by using these differences to determine the transit time of the solutes through a column (ICH, 2003).

HPLC SYSTEM

Solvent Reservoirs

This provides storage of sufficient amount of HPLC solvents for continuous operation of the system which can be equipped with an online degassing system and special filters to isolate the solvent from the influence of the environment.

Pump: This provides the constant and continuous flow of the mobile phase through the system; most modern pumps allow controlled mixing of different solvents from different reservoirs.

Injector: This allows an introduction (injection) of the analytes mixture into the stream of the mobile phase before it enters the column; most modern injectors are auto samplers, which allow programmed injections of different volumes of samples that are withdrawn from the vials in the auto sampler tray.

Column: This is the heart of HPLC system; it actually produces a separation of the analytes in the mixture. A column is the place where the mobile phase is in contact with the stationary phase, forming an interface with enormous surface. Most of the chromatography development in recent years went toward the design of many different ways to enhance this interfacial contact (Skoog DA *et al.,* 2004).

Detector: This is a device for continuous registration of specific physical (sometimes chemical) properties of the column effluent. The most common detector used in pharmaceutical analysis is UV (ultraviolet), which allows monitoring and continuous registration of the UV absorbance at a selected wavelength or over a span of wavelengths (diode array detection). Appearance of the analyte in the detector flow cell causes the change of the absorbance. If the analyte absorbs greater than the background (mobile phase), a positive signal is obtained. UV detector does not respond to mass but on the basis of absorbance at the monitoring wavelength, accurate quantification of individual impurity levels requires knowledge of the response factor relative to that of the parent. Determining response factors can be a time consuming and expensive undertaking (Murali Krishna P *et al*., 2011).

Data Acquisition and Control System: Computer-based system that controls all parameters of HPLC instrument (eluent composition (mixing of different solvents); temperature, injection sequence, etc.) and acquires data from the detector and monitors system performance (continuous monitoring of the mobile-phase composition, temperature, backpressure, etc.) (Lloyd R *et al*., 2012).

Reverse Phase HPLC (RP HPLC)

Reversed-phase chromatography employs mainly dispersive forces (hydrophobic or van der Waals interactions). The polarities of mobile and stationary phases are reversed, such that the surface of the stationary phase in RP HPLC is hydrophobic and mobile phase is polar, where mainly water-based solutions are employed. Reversed-phase HPLC is by far the most popular mode of chromatography. Almost 90% of all analyses of low molecular-weight samples are carried out using RP HPLC. One of the main drivers for its enormous popularity is the ability to discriminate very closely related compounds and the ease of variation of retention and selectivity.

Chromatography in the Pharmaceutical World

In the modern pharmaceutical industry, high-performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development, and production. The development of new chemical entities (NCEs) is comprised of two major activities: drug discovery and drug development. Throughout the drug discovery and drug development paradigm, rugged analytical HPLC separation methods are developed and are tailored by each development group (i.e., early drug discovery, drug metabolism, pharmacokinetics, process research, preformulation, and formulation). At each phase of development the analyses of a myriad of samples are performed to adequately control and monitor the quality of the prospective drug candidates, excipients, and final products. Effective and fast method development is of paramount importance throughout the drug development life cycle.

Method Development

Prior to the initiation of method development, all the known information about the analyte such as its

Rajveer Bhaskar. *et al.* / *International Journal of Biological & Pharmaceutical Research.* 2015; 6(8): 639-648.

structure, physical and chemical properties, toxicity, purity, hygroscopitiy, solubility, and stability should be determined. The goals or requirements of the HPLC method that needs to be developed should be known as well as the analytical figures of merit, which include the required detection limits, selectivity, linearity, range, and accuracy and precision.

METHOD DEVELOPMENT CONSIDERATION

Sample Properties

Analyte Structure and pKa

If the target analyte is ionizable, the pKa of the analyte should be determined or obtained. The optimal pH, to commence method development, is at a pH that is at least 1–2 units from the analyte p*Ka* in the particular hydro-organic mixture that is employed.

Solubility of Components

Solubility of the analyte is also very important. The analyte must be soluble in the diluent and must not react with any of the diluent components. The diluent should match to the starting eluent composition of the assay to ensure that no peak distortion will occur, especially for early eluting components (Kailas Bansal *et al*., 1997).

Detector Considerations

The choice of the proper detection scheme is dependent on the properties of the analyte. Different types of detectors are available such as ultraviolet (UV), fluorescence, electrochemical, light scattering, refractive index (RI), flame ionization detection (FID), evaporative light scattering detection (ELSD), corona aerosol detection (CAD), mass spectrometric (MS), NMR, and others. However, the majority of reversed-phase and normal-phase HPLC method development in the pharmaceutical industry is carried out with UV detection. A wavelength for UV detection must be chosen so that an accurate mass balance may be determined. Therefore, if area% normalization is to be used, then all the impurities and the active pharmaceutical ingredient must have similar relative response factors (area response/weight). The UV spectra of target analyte and impurities must be taken and overlaid with each other, and the spectra should be normalized due to different amounts present in the mixture. A wavelength must be chosen such that adequate response is obtained for the active and that at least a 0.05 v/v% solution of the active at target concentration could be quantified (S/N greater than 10). The wavelength chosen should not be on a distinct slope of the spectrum, and the relative difference in the absorbance at a certain wavelength is not significantly different from the impurities/degradation products present (ICH Guidelines, 1997).

Mobile phase parameters

Organic Solvent Strength and Selectivity

Solvent strength or % organic solvent content in the mobile phase controls the retention time of the analyte and that different organic solvents (MeOH, ACN, THF) can have a dramatic effect on selectivity. In the development of reversed-phase separation methods the organic part of the eluent is considered the strong solvent. Organic solvent increases the solvent strength and allows for elution of the species in a mixture, resulting in smaller analyte retention factors or retention volumes. Increasing the concentration of the organic modifier generally leads to an exponential decrease in the analyte retention volume. The general rule of thumb is that for every 10 v/v% increase in organic modifier there is a two- to threefold decrease in the analyte retention factors for analytes with molecular weights of less than 1000Da.Increasing the fraction of the Mobile-phase strength depends not only on the concentration of the organic modifier, but also on the type of organic modifier used. The solvent strength of the most common organic eluents used at the same volume percentage (v/v %) in reversed-phase chromatography would be: methanol < acetonitrile < tetrahydrofuran (USP, 2009).

Buffers

Many drugs have either acidic or basic functional groups and can exist in solutions in ionized or non-ionized forms. The ionic state and degree of ionization greatly affect their chromatographic retention in RPLC. Typically, the ionic form does not partition well into the hydrophobic stationary phase and therefore has significantly lower k' than the neutral, un-ionized form. Buffers are commonly used to control the pH of the mobile phase for the separation of acidic or basic analytes. Volatile acids and their ammonium salts are used for the development of mass spectrometer (MS) compatible HPLC methods. Since a buffer is only effective within 1-2 pH unit from its pKa, judicious selection of the proper buffer within its buffering range is paramount.

Acidic Mobile Phase

A mobile phase at acidic pH of 2.5-3 is a good starting point for most pharmaceutical applications because the low pH suppresses the ionization of most acidic analytes resulting in their higher retention. Common acids used for mobile-phase preparations are phosphoric acid, formic acid, and acetic acid. Low pH also minimizes the interaction of basic analytes with surface silanols on the silica packing (because silanols do not ionize at acidic pH). Also, the lifetime of most silica-based columns is excellent in the pH range of 2-8. However, basic analytes are ionized at low pH and might not be retained unless ion-pairing reagents are used.

Ion-pairing Reagent

Ion-pairing reagents are detergent-like molecules added to the mobile phase to provide additional retention

or selectivity for the analytes with opposite charge. Long-chain alkyl sulfonates are commonly used for the separation of water-soluble basic analytes. Amine modifiers such as triethylamine (TEA) are often added in the mobile phase to reduce peak tailing caused by the strong interaction of basic analytes with acidic surface silanols. For acidic analytes, ion-pairing reagents such as tetraalkylammonium salts are often used.

High pH Mobile Phase

The advent of silica-based columns stable under high-pH conditions (e.g., pH 1-12) offers an important alternate approach for the separation of basic analytes. The application of this approach is increasing for assay and impurity testing of many water-soluble basic drugs. The advantages of high-pH separation as opposed to ion-pairing are mass spectrometry compatibility, better sensitivity and unique selectivity.

Isocratic vs. Gradient analysis

Traditionally, most pharmaceutical assays are isocratic analysis employing the same mobile phase throughout the elution of the sample. Isocratic analyses are particularly common in quality control applications since they use simpler HPLC equipment and premixed mobile phases. Notable disadvantages of isocratic analysis are limited peak capacity (the maximum number of peaks that can be accommodated in the chromatogram), and problems with samples containing analytes of diverse polarities. Also, late eluters (such as dimers) are particularly difficult to quantitate in isocratic analysis due to excessive band broadening with long retention times.

In contrast, gradient analysis in which the strength of the mobile phase is increased with time during sample elution, is suited for complex samples and those containing analytes of wide polarities. Gradient chromatography is amenable for high-throughput screening applications and for impurity testing. The disadvantages of gradient analysis are the requirements for more complex instrumentation and greater skills in method development, and difficulties in method transfer.

Stationary Phases for reverse phase HPLC

Silica-based packing materials dominate in applications for RP separations in the pharmaceutical industry. Hydrophobic surface of these packing's typically are made by covalent bonding of organosilanes on the silica surface. This modification involves the reaction of monofunctional alkyldimethylchlorosilanes with the surface silanol groups. Octadecylsilane was the first commercially available silica-based bonded phase and is still the most commonly utilized. Also, alkyl-type ligands of different number of carbon atoms (C1, C4, C8, and C12) are often used as well as phases with phenyl functionality. Polar embedded phases provide an additional avenue for potential modification of the chromatographic selectivity, and some of these phases offer an enhancement of retention of polar analytes. These phases can be used with high aqueous mobile phases, even 100% aqueous, without loss of analyte retention that sometimes could be observed for more hydrophobic phases. Most silica-based reversed-phase packing materials have a relatively narrow applicable pH range

Screening several different types of stationary phases during method development for a particular separation is often useful because different columns usually have different selectivity for components in a sample.

Parameters for the method validation

Specificity/Selectivity

The term specific generally refers to a method that produces a response for a single analyte only while the term selectivity refers to a method, which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective.

Precision and Reproducibility

The precision of a method is the extent to which individual test results of multiple injections of a series of standards agree the measured standard deviation can be subdivided into three categories:

Repeatability

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

Intermediate precision

Intermediate precision expresses within laboratories variations: different day, different analyst, different equipment etc.

Reproducibility

Reproducibility expresses the precision between laboratories collaborative studies, usually supplied to standardization of methodology.

Ruggedness

Ruggedness is the precision obtained when the assay is performed by multiple analysts, using multiple instruments, on multiple days, in one laboratory, different sources of reagents and multiple lots of columns should also be included in this study.

Accuracy

The test for accuracy is intended to demonstrate the closeness of agreement between the value found and the value that is accepted either as a conventional true value or as an accepted reference value. Thus the accuracy of a method is the closeness of the measured value to the

true value for the sample. The accuracy can also be demonstrated by recovery of the impurity spiked to a drug substance or into a placebo with drug substance. The percentage recovery with the certain acceptance criteria at each defined level is reported. Accuracy should be assessed using a minimum of nine determinations at a minimum of three concentration levels covering the specified range.

Linearity

The purpose of the test for linearity is to demonstrate that the entire analytical system (including detector and data acquisition) exhibits a linear response and is directly proportional over the relevant concentration range for the target concentration of the analyte. It is recommended to perform the linearity of the API and related substances independently; and once linearity has been demonstrated, linearity could be performed containing both API and specific related substance if necessary. At least five concentrations within the range specified above for the linearity test should be used.

Limit of detection

LOD is defined as lowest concentration of analyte that can be detected, but not necessarily quantified, by the analytical method.

Based on visual evaluation

The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Based on signal to noise

A signal to noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

Based on standard deviation of the response and slope

The limit of detection (LOD) may be expressed as

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

Where σ = the standard deviation of the response, S = slope of calibration curve of analyte.

Limit of quantification (LOQ)

LOQ is defined as the lowest concentration of analyte that can be determined with acceptable accuracy and precision by the analytical method.

Based on visual evaluation

The quantitation limit is determined by the analysis of samples with known concentrations of analyte.

Based on signal to noise ratio

Signal to noise ratio between 10:1 is generally considered.

Based on standard deviation of the response and slope

The limit of quantitation (LOQ) may be expressed as

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

Where σ = the standard deviation of the response.

S = slope of calibration curve of analyte.

Robustness

Robustness tests examine the effect operational parameters have on the analysis results.

Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample.

System Suitability Parameters

The purpose of the system suitability test is to ensure that the complete testing system (including instrument, reagents, columns, analysts) is suitable for the intended application.

After the method has been validated an overall system suitability test should be routinely run to determine if the operating system is performing properly.

Commonly used system suitability parameters are as follows:

Retention Time (RT)

Retention Time is the time of elution of peak of maximum after injection of compound.

Theoretical Plates (N)

It is also called as column efficiency. A column can be considered as being made of large number of theoretical plates where distribution of sample between liquid-liquid or solid-solid phase occurs. The number of theoretical plates in column is given by the relationship

$$\mathbf{N = 16\ (t/w)^2}$$

Where t is the retention time and w is the width at the base of the peak.

L = length of column

HETP =L/N

Theoretical Plates should be more than 2000.

Resolution

It is a function of Column efficiency and is specified to ensure that closely eluting compounds are resolved from each other to establish the general resolving power of the system. For the separation of two components in mixture the resolution is determined by equation.

$$\mathbf{R = 2\ (t_2 - t_1) / (W_1 + W_2)}$$

Where t_2 and t_1 is the retention time of second and first compounds respectively, where as W_2 and W_1 are the

corresponding widths at the bases of peak obtained by extrapolating straight sides of the peaks to baselines.

R should be more than 2 between peak of interest and the closest eluted potential interferences (impurities, excipients, degradation products or internal standard).

Tailing factor

It is the measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing become more pronounced.

$$R = W0.05 / 2F$$

Where, $W_{0.05}$ is the width of peak at 5 % height and F is the distance from the peak maximum to the leading edge of the peak height from the baseline.
Tailing factor should be less than 2.

Capacity Factor (K')

It is calculated by the formula

$$K' = t / t_a - 1$$

Where t is the retention time of drug t_a is the retention time of non retarded component, air with thermal conductivity detection.

Selectivity (α)

Also known as separation factor, it is a measure of peak spacing and expressed as

$$\alpha = k / k'_1$$

Pharmaceutical Development

Our pharmaceutical development process for this product involved the following sequential steps:

- An analysis of the reference product identified a target product profile thatincluded rapid dissolution and other aspects of product quality and equivalence.
- Preformulation characterization of the drug substance (2.3.P.2.1.1) identified particle sizeand polymorphic form as mechanistic factors critical to product performance.
- A list of mechanistic factors that are needed to reach the target product profilewas identified.
- The need for content uniformity of the low dose API and its poor flow properties led usto choose wet granulation as the manufacturing process.
- Since the amount of active ingredient is less than 1%, we selected an established set ofexcipients known to provide pharmaceutically acceptable tablets by wet granulation.
- We evaluated the compatibility of these excipients with the active and found no evidence of incompatibility.
- During process development the manufacturing steps and critical process parameters thatcontrolled each of the mechanistic factors were identified.

INSTRUMENTS AND REAGENTS

HPLC SYSTEMS - AR/VAL/HPLC- 31, 73
Waters 2695 separation module
Waters 2996 PDA detector
Waters 2487 dual wavelength detector
Waters Empower Software
Balance (AR/VAL/BALN-11)
pH meter (AR/LAB-II/PHMT-11)
Columns: C8/G/34, C8/G/345\

Standard

Norethindrone Acetate standard: Use the standard as such and use % potency on as is basis for calculations. Keep the container tightly closed.
Batch No. : GHWS0091302 Potency: 99.5 % on as is basis

Ethinyl Estradiol standard: Use the standard as such and use % potency on as is basis for calculations. Keep the container tightly closed.
Batch No. : GHWS0051301 Potency: 99.8 % on as is basis

Reagents

Acetonitrile (HPLC grade)
Water (Milli Q or equivalent)

Nonbinding Recommendations

Guidance on Ethinyl Estradiol, Norethindrone Acetate. This Following guidance, once finalized, will represent the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA. The use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations.
Active ingredient: Ethinyl Estradiol; Norethindrone Acetate
Form/Route: Tablets/Oral
Recommended studies: 2 studies

Type of study: FastingDesign

Single-dose, two-way, crossover in-vivo
Strength: 0.030 mg/1.5 mg (28 day packet)
Subjects: Healthy nonpregnant females, general population
Additional Comments: Subjects should not be taking hormonal contraceptives

Type of study: Fed Design

Single-dose, two-way, crossover in-vivo
Strength: 0.030 mg/1.5 mg (28 day packet)
Subjects: Healthy nonpregnant females, general population
Analytes to measure: Ethinyl Estradiol; Norethindrone in plasma
Bioequivalence based on (90% CI): Ethinyl Estradiol, Norethindrone

Waiver request of in-vivo testing: 0.02 mg/1 mg (28 day), 0.02/1 mg (24 day) and 0.02 mg/1 mg (21 day) based on (i) acceptable bioequivalence studies on the 0.030 mg/1.5 mg (28 day packet) strength, (ii) acceptable dissolution testing across all strengths, and (iii)

proportional similarity in the formulations across all strengths.

Cross-referencing of in-vivo testing: For the same strength, 0.030 mg/1.5 mg (21 day packet), as that used in the bioequivalence studies but submitted in a separate ANDA, based on (i) acceptable bioequivalence studies of this strength in the separate ANDA, (ii) acceptable in- vitro dissolution testing of the formulations of the same strength, and (iii) sameness (except for the colorants) of the formulations of the same strength.

If only the lower strength, 0.02 mg/1 mg is to be marketed first, then the fasting and fed studies should be conducted on this lower strength, comparing it with the equal strength of the RLD. However, if the higher strength, 0.03 mg/1.5 mg, is to be marketed following the in-vivo studies of the lower strength, then an additional fasting study will be requested for the higher strength.

A stability indicating HPLC method is official in USP for the determination for the Assay of Norethindrone Acetate and Ethinyl Estradiol in Norethindrone Acetate and Ethinyl Estradiol Tablets USP. This Report is intended for the performed on the Assay method for Norethindrone Acetate and Ethinyl Estradiol in Norethindrone Acetate and Ethinyl Estradiol Tablets USP.

METHODOLOGY

Evaluation of System Suitability

Inject five replicate injections of standard solution into the chromatograph and record the chromatograms. The relative standard deviation of five replicate injections should not be more than 2.0% for Ethinyl Estradiol and Norethindrone Acetate peaks. The theoretical plates should not be less than 2000 for Norethindrone acetate and Ethinyl Estradiol peaks.

Procedure

Separately inject equal volumes of Blank and Sample solution (in duplicate) into the chromatograph and record the chromatograms. Measure the area counts for the Ethinyl estradiol and Norethindrone acetate peaks.

Calculation

For Norethindrone acetate

Calculate the percentage of Norethindrone acetate in tablet using following formula:

$$= \frac{AT}{AS} \times \frac{\text{Std wt. (mg)}}{50\ mL} \times \frac{3\ ml}{20\ ml} \times \frac{200\ ml}{10\ tablet} \times \frac{P}{100} \times \frac{100}{LC}$$

Where,

AT = Area count of Norethindrone acetate in the chromatogram of sample solution. AS = Average area count of Norethindreone acetate in the chromatogram of standard solution.

P = Percentage potency of Norethindreone acetate working standard on as is basis. LC = Label claim in mg for Norethindreone acetate.

For Ethinyl estradiol

Calculate the percentage of Ethinyl estradiol in tablet using following formula:

$$\frac{AT_1}{AS_1} \times \frac{\text{Std wt. (mg)}}{100\ mL} \times \frac{5\ ml}{200\ ml} \times \frac{4ml}{20ml} \times \frac{200\ mL}{10\ tablet} \times \frac{P_1}{100} \times \frac{100}{LC_1}$$

Where,

AT_1 = Area count of Ethinyl estradiol peak in the chromatogram of sample solution. AS_1 = Average area count of Ethinyl estradiol peak in the chromatogram of standard solution. P_1 = Percentage potency of Ethinyl estradiol working standard, on as is basis. LC_1 = Label claim in mg for Ethinyl estradiol.

System Suitability

Standard solution

Record the % RSD of five replicate injections for both Norethindrone acetate and Ethinyl Estradiol. Record the theoretical plates for both the peaks.

Acceptance Criteria

Standard solution

The relative standard deviation of five replicate injections should not be more than 2.0% for both Ethinyl Estradiol and Norethindrone Acetate .The theoretical plates should not be less than 2000 for both the peaks.

Table 1. System suitability Parameter and Their values

Parameter	Limit
Capacity factor	K'>2
Injection precision	RSD<1% for n≥5
Resolution	Rs>2
Tailing factor	T≤2
Theoretical plate	N>2000

Table 2. Samples

Strength	Sample Batch no.	Placebo Batch no.
1.5/0.03 mg	473/1.5+0.03/09.14/016	468/0+0/01-14/076PL

Rajveer Bhaskar. *et al.* / *International Journal of Biological & Pharmaceutical Research.* 2015; 6(8): 639-648.

Table 3. Table for System Suitability

Parameters	%RSD of Standard preparation		USP tangent	
	Norethindrone Acetate	Ethinyl estradiol	Norethindrone acetate	Ethinyl estradiol
Precision	NMT- 2%	NMT- 2%	NLT-2000	NLT-2000
Linearity	NMT- 2%	NMT- 2%	NLT-2000	NLT-2000
Accuracy	NMT- 2%	NMT- 2%	NLT-2000	NLT-2000
Ruggedness	NMT- 2%	NMT- 2%	NLT-2000	NLT-2000
Specificity	NMT- 2%	NMT- 2%	NLT-2000	NLT-2000

Table 4. Table for Forced Degradation Studies – Norethindrone Acetate

Sr. No.	Experiment	Degradation Condition
1	Control	--
2	Acid Degradation	5N HCl/ 24 hours/RT (Without Neutrilisation)
3	Base Degradation	0.1N NaOH/30 mins/RT
4	Peroxide Degradation	50% H_2O_2/24 hours/RT

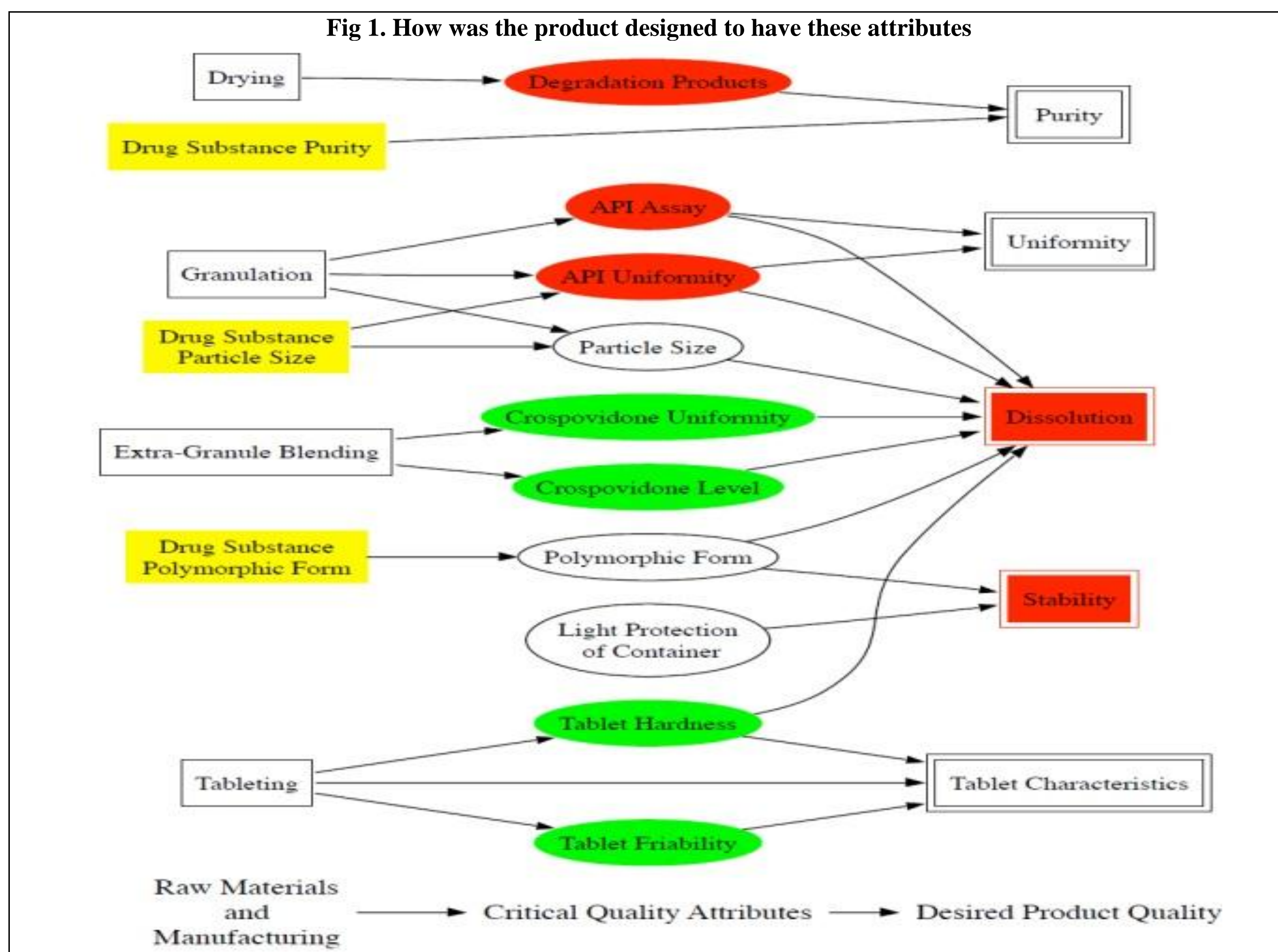
Table 5. Table for Forced Degradation Studies - Ethinyl Estradiol

Sr. No.	Experiment	Degradation Condition
1	Control	--
2	Acid Degradation	5N HCl/ 24 hours/RT (Without Neutrilisation)
3	Base Degradation	0.1N NaOH/30 mins/RT
4	Peroxide Degradation	50% H_2O_2/24 hours/RT

Table 6. Validation Parameters

Sr. No.	Validation Parameter	Acceptance Criteria
1.	SPECIFICITY	
1.1	Identification	Results should be comparable with respect to Retention time.
1.2	Placebo Interference	Blank and Placebo should not show any peak at the retention time of Norethindrone Acetate and Ethinyl Estradiol Peaks. Peak purity should pass for Norethindrone Acetate and Ethinyl Estradiol Peaks in standard and sample.
1.3	Known impurity interference	Difference in mean of spiked and un-spiked (control) sample Assay should not be more than 1%. Peak purity should pass in control sample and spiked sample for Norethindrone Acetate and Ethinyl Estradiol Peaks
1.4	Forced degradation studies	Norethindrone Acetate and Ethinyl Estradiol peaks should be homogeneous and there should be no co-eluting peaks. Peak purity for Norethindrone Acetate and Ethinyl Estradiol Peaks should pass.
2.	Accuracy (Recovery)	Mean recovery should be in the range of 98.0% to 102.0%. The RSD should not be more than 2.0%.
3.	Precision	
3.1	System Precision	RSD should not be more than 2.0%.
3.2	Method Precision	RSD should not be more than 2.0%.
4.	STABILITY OF ANALYTICAL SOLUTION	Standard solution: Assay of old standard against freshly prepared is between 98.0-102.0%. Sample solution: Correlation of old sample solution against initial Assay is between 98.0-102.0%.
5.	SYSTEM SUITABILITY	**Standard solution** The relative standard deviation of five replicate injections should not be more than 2.0% for both Ethinyl Estradiol and Norethindrone Acetate .The theoretical plates should not be less than 2000 for both the peaks.

Fig 1. How was the product designed to have these attributes



CONCLUSION

The test method is validated for Specificity, Linearity and Range, Precision, Accuracy (Recovery), Ruggedness, Stability of Analytical solution, Filter equivalency and Robustness and found to be meeting the predetermined acceptance criteria. The validated method is Specific, Linear, Precise, Accurate, Rugged and Robust for Assay of Norethindrone Acetate and Ethinyl Estradiol in Norethindrone Acetate and Ethinyl Estradiol Tablets USP. Hence this method can be introduced into routine use for the assay of Norethindrone Acetate and Ethinyl Estradiol in Norethindrone Acetate and Ethinyl Estradiol Tablets USP.

ACKNOWLEDGEMENT

I take this opportunity to acknowledge my indebtness to all those who helped and encouraged me to complete this article. It is a moment of happiness for me and my family completion of this article for master degree in Pharmacy.

This work would have not been in its present form without the cooperation and coordination of my academic guide Mr. Rajveer Bhaskar (Assistant Professor, Pharmaceutics) R. C Patel Institude of Pharmaceutical & Research Centre & who always helped me whenever I needed. It is indeed a matter of pride and honor for me to have worked under his active and intellectual supervision. I am thankful for affection and good wishes throughout the research work.

It's my privilege to acknowledge my deep indebtedness to Dr. S.G.Surana, Principal R. C Patel Institude of Pharmaceutical & Research Centre.

H S Mahajan Head of department of pharmaceutics & QA for providing the necessary facilities, requirements and excellence guidance to complete the dissertation work and allowing me to carry out my work in the multidisciplinary setup of this institute.

Personnel who encourage me a lot and who were always besides of me in every condition that are my roommates Chetan, Pravin, Kamlesh, Umesh, Mahesh & Pankaj

My deepest gratitude goes to My Family, My Sisters for their unflagging love and support throughout my life; this dissertation is simply impossible without them. I

am indebted to My Father for his care and love. I cannot ask for more from My Mother as she is simply perfect. I have no suitable word that can fully describe her everlasting love to me.

Thank you God for my life, helping me and making me realize that someone somewhere is watching me groom. You have made my life more beautiful. May your name be exalted, honored, and glorified.

REFERENCES

Analytical Methods: A Statistical perspective on the ICH Q2A and Q2B Guidelines for Validation of Analytical Methods. *BioPharmInternational.* 2006: 1-6.

ICH Guidelines Q2B: Validation of Analytical Procedures Methodology, Center for Drug Evaluation and Research, Rockville. 1997: 1-10.

ICH Harmonized Tripartite Guidelines. Stability Testing of New Drug Substances and Products Q1A (R2) current step 4 version parent guideline. 2003

Kailas Bansal, Pankaj Pant, Rama Therdana Rao P, VenkateswarReddi M, KumudPadhee, AjitSathapathi, Prithipal Singh Kochhar, UV Spectrophotometric Method for the Estimation of Norethindrone inImmediate Release Tablet,Formulation, Research and Development Department, Jagsonpal Pharmaceuticals Limited, ,SIDCUL, Rudrapur, Uttarakhand, India. *International Journal of Pharmaceutical Sciences and Drug Research*. 3(2); 2011: 127-128.

Lloyd RS, Joseph JK, John WD. John wiley and sons, inc, publication, Introduction to modern liquid chromatography. 532-565.

Murali Krishna P, Thirupathi Rao B, Kishore Kumar R and Venkateswarlu P**.** *International Journal of Chem.Tech Research*. 3(1); 2011: 143-148.

Skoog DA, Holler FG, Nieman TA. Principles of Instrumental Analysis, Thomson Brooks/Cole Asia Pvt. Ltd., *Singapore*. 2004: 4-7.

U.S. Food and Drug Administration. Guidance for Industry, Q3B Impurities in New Drug Products. 2006.

United States Pharmacopeia, USP32-NF27, U.S. Pharmacopeial Convention, Rockville,2009: 3105