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Method Development and Validation of Stability Indicating HPLC Method for Quality Evaluation of Related Substances/Degradation in Levonorgestrel and Ethinyl Estradiol Tablets

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Rajasekhara Reddy*[1], Satyavir[2], S.B.Puranik[3]

[1]Research scholar OPJS University, Churu, Rajasthan, India

[2]Research Guide OPJS University, Churu, Rajasthan, India

[3]Drishti Institute of Distance Learning, Bangalore

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ABSTRACT

To develop the stability indication method for the simultaneous estimation of related substances of Levonorgestrel and Ethinyl estradiol by HPLC. The related substances method is not available in any literature for the combination of Levonorgestrel and Ethinyl Estradiol Tablets. It is very helpful to the estimation and control of impurities in the finished product to avoid the side effects of degradants and impurities. Pharmaceutical analysis plays a very prominent role in quality assurance as well as quality control of bulk drugs and pharmaceutical formulations. The rapid increase in pharmaceutical industries and the production of the drug in various parts of the world has brought a rise in demand for new analytical techniques in the pharmaceutical industries. As a consequence, analytical method development has become the basic activity of analysis. A recent development in analytical methods has resulted from the advancement of analytical instruments. The improvement of the analytical method development and analytical instruments have reduced the time of analysis, increased precision and accuracy, and reduced costs of analysis. As a consequence, most pharmaceutical organizations are investing a huge amount of money for the establishment of advanced analytical laboratories. Analytical techniques are developed and validated for active pharmaceutical ingredients (API), excipients, drug products, degradation products, and related substances, residual solvents, etc. As a result, it has become an integral part of the requirements of the regulatory organization. Analytical method development finally results in official test methods. These methods are used in quality control laboratories to ensure the identity, purity, safety, efficacy, and performance of drug products. Regulatory authorities are placing greater emphasis on analytical methods in manufacturing. Drug approval by regulatory authorities requires the applicant to prove control of the entire process of drug development by using validated analytical methods.

INTRODUCTION

Importance of drug analysis:

'Health is wealth'. It is a vital fact that a healthy body is the desire of every human being. Good health is the first condition to enjoy life and all other things which mankind is having. Nowadays peoples are more concentrating on health. Even governmental bodies of different countries and the World health organization (WHO) is also focusing on the health of the human being. Health care is prevention, treatment, and management of illness and preservation of mental and physical wellbeing. Health care embraces all the goods and services designed to promote health including preventive, curative, and palliative in interventions. The Health care industry is considered an industry or profession which includes people's exercise of skill or judgment or providing of a service related to the prevention or improvement of the health of the individuals or the treatment or care of individuals who are injured, sick, disabled or infirm. The delivery of modern health care depends on an Interdisciplinary Team. The medical model of health focuses on the eradication of illness through diagnosis and effective treatment. A traditional view is that improvement in health results from advancements in medical science. Advancements in medical science bring varieties of medicines. Medicines are a key part of the health care system. The numerous medicines are introducing into the world-market and also, that is increasing every year. These medicines are being either new entities or partial structural modification of the existing one. So, to evaluate the quality and efficacy of these medicines is also an important factor. Right from the beginning of the discovery of any medicine quality and efficacy of the same are checked by quantification means. Quality and efficacy are checked by either observing effect of the drug on various animal models or analytical means. The option of animal models is not practically suitable for every batch of medicine as it requires a long time, high cost, and more man-power. The latter option of an analytical way is more suitable, highly precise, safe, and selective. The analytical way deals with quality standards that are assigned for products to have desirable efficacy of the medicines. A sample representing any batch is analysed for these standards and it is assumed that drug/medicine which is having such standards are having the desired effect on use. Quality control is a concept, which strives to produce a perfect product by a series of measures designed to prevent and eliminate errors at a different stage of production. The decision to release or reject a product is based on one or more types of control action. Analytical methods

validation is an important regulatory requirement in pharmaceutical analysis. High-Performance Liquid Chromatography (HPLC) is commonly used as an analytical technique in developing and validating assay methods for drug products and drug substances (1). Method validation provides documented evidence and a high degree of assurance that an analytical method employed for a specific test, is suitable for its intended use. Over recent years, regulatory authorities have become increasingly aware of the necessity of ensuring that the data submitted to them in applications for marketing approvals have been generated using the validated analytical methodology. The International Conference on Harmonization (ICH) has introduced guidelines for analytical methods validation (2,3). Both United States Food and Drug Administration (USFDA), as well as United States Pharmacopoeia (USP), refer to ICH guidelines (4-7).

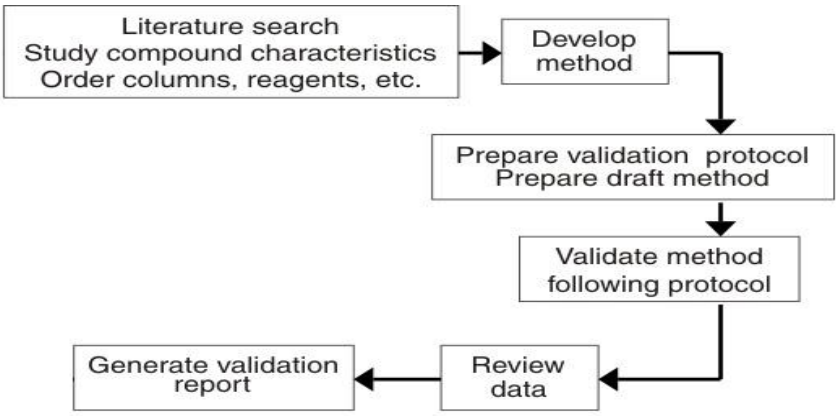
Regulatory authorities are placing greater emphasis on analytical methods in manufacturing.

Drug approval by regulatory authorities requires the applicant to prove control of the entire.

Process of drug development by using validated analytical methods. The philosophy of method development is based on several considerations. There exists today a good practical understanding of chromatographic separation and how it varies with the sample and with experimental conditions. Any systematic approach to HPLC method development should be based on this knowledge of the chromatographic process. In most cases, the desired separation can be achieved easily with only a few experiments. In other cases, considerable experimentation may be needed. A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Ideally, every experiment will contribute to the result so that there are no wasted runs. Usually, this requires that the results of each chromatographic run be assessed before proceeding with the next experiment. Sometimes the chemical structures of the sample components are known, other times this is not the case. The method-development scheme described in this book will usually work in either situation. Finally, method development should be as simple as possible, yet it should allow the use of sophisticated tools such as computer modeling if these are available.

The method development life cycle involves few steps as shown in the following flow chart.

Method development life cycle

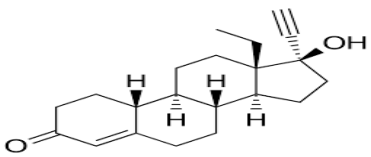
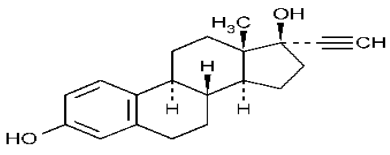


Trails for Related substances in Levonorgestrel and Ethinyl Estradiol Tablets:

General Information (Drug Product)

Name of Drug Product	Levonorgestrel and Ethinylestradiol Tablets
Description of Drug Product	Circular, white to off white, uncoated, biconvex tablets, plain on both sides.
Pharmacopeial Status of DP	An official in United States Pharmacopoeia (USP)
Method Reference	In-House

General Information of (Drug Substance)

Name of Drug Substance	Levonorgestrel	Ethinylestradiol
Description of Drug Substance	White or almost white, crystalline powder	White or slightly yellowish-white, crystalline powder
Chemical Name	13-ethyl-17-hydroxy-l 8,19- dinor-17a-pregn-4-en-20-yn-3-one	19-Nor-17a-pregna-1,3,5(10)- trien -20-yne-3,17-diol
Structure		
Molecular Formula	$C_{21}H_2S0_2$	$C_{20}H_{24}0_2$
Molecular Weight	312.5	296.40
CAS Nos.	797-63-7	57-63-6

The solubility of Drug substances:

Solubility is the concentration of a solute when the solvent has dissolved all the solute than it can at a given temperature. A useful definition of solubility is the concentration of solute in a saturated solution at equilibrium. Saturated solution concentration is the solubility of the solute at that temperature. Solubility is a physical property, and values for the solubility of pure substances are found in the literature. Some substances can dissolve in a higher concentration than could be attained at equilibrium, have increased solubility, and form supersaturated solutions.

Solubility Test for Levonorgestrel:

In water, it was observed: 2.055mg/ml at Room temperature The solubility of Levonorgestrel is approximately 0.2 mg/ml in ethanol, and approximately 5 mg/ml in DMSO and DMF. Levonorgestrel is sparingly soluble in aqueous buffers.

Solubility Test for Ethinyl Estradiol:

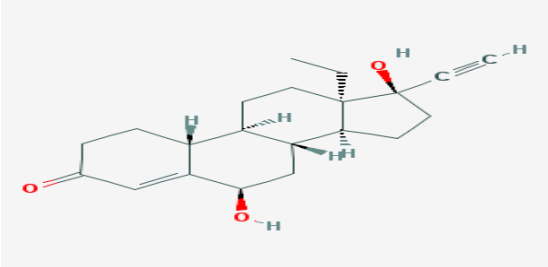
Solubility: 1 part in 6 of ethanol, 1 in 4 of ether, 1 in 5 of acetone, 1 in 4 of dioxane, and 1 in 20 of chloroform. Soluble in vegetable oils, and solutions of fixed alkali hydroxides.

In water, 11.3 mg/L at 27 deg C. No related substances method is available for determination/quantification of impurities present in LG and EE tablets which is specific and stability-indicating nature.

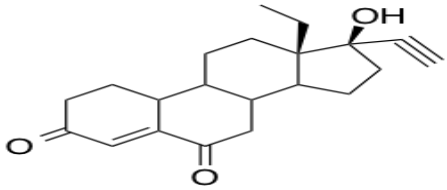
For low potent drugs, quantification of impurities at a precise level at low test concentration is quite difficult. To get a satisfactory area counts it is mandated to prepare the test solution at high concentration, which in turn requires more number of tablets. When the sample is injected at a high test concentration level, more amount of placebo will also go into the column and after a repeated number of injections, there would be a probable chance of column bleeding or choking due to placebo. Hence it is a difficult task to the chromatographer to develop a method on low dose Finished products for related substances method.

Levonorgestrel Impurities:

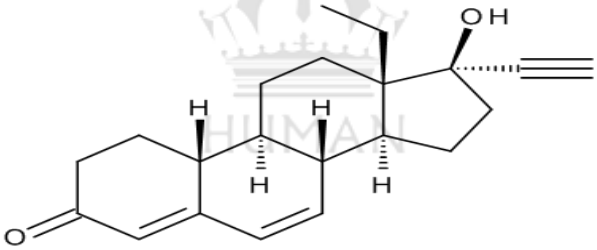
A) 6-Beta-Hydroxy Levenorgestrel (LG-I-1)



B) 6-Keto Levenorgestrel (LG-I-2)

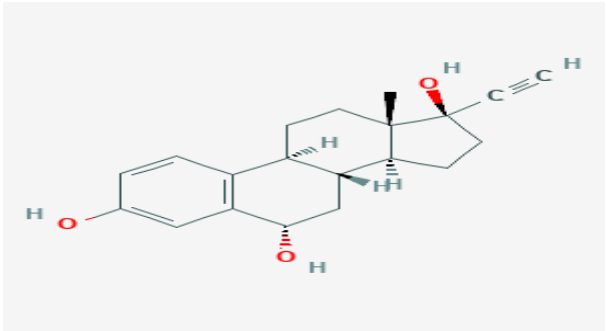


C) Delta-6-Levonorgestrel

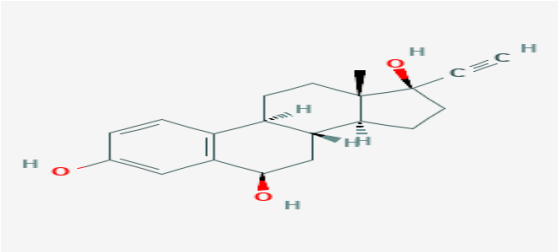


Ethinyl Estradiol Impurities:

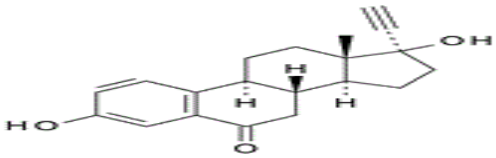
A) 6-alpha-hydroxy Ethinyl Estradiol:



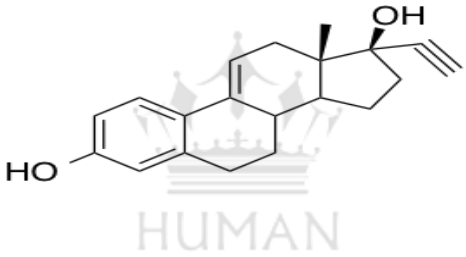
B) 6-beta-hydroxy Ethinyl Estradiol



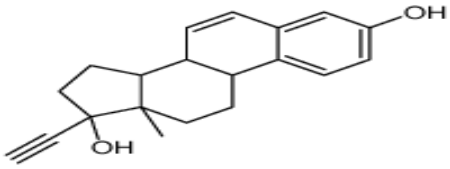
C) 6 keto- Ethinyl Estradiol



D) Delta-9,11- Ethinyl Estradiol



E) Delta-6-Ethinyl Estradiol



Initiation of Method Development for Related substances:

Method development work was initiated to separate LG and EE impurities to meet the stability-indicating requirements by using the HPLC method. LG drug is found to be nonpolar than EE. A literature survey and some monograph references on this product indicated that these compounds did not require any buffer for mobile phase preparation.

Considering this fact, trials were directly taken using water and acetonitrile as Mobile phase A and B.

Chromatographic Conditions:

Column	5µm, C18, 250 x 4.6mm
Column Temperature	25ºC
Sample Temperature	15ºC
Flow rate	1.0 mL/minute
Wavelength	PDA (200nm to 400nm)
Injection Volume	50µ1
Run time	125 minutes

Preparation of Diluent:

Prepare a mixture of filtered Water and Acetonitrile (30: 70, v/v).

Preparation of Mobile Phase:

Preparation of Mobile phase A: Water

Preparation of Mobile phase B: Acetonitrile and Methanol(90:10 v/v)

Gradient program:

Time (min.)	**Mobile Phase A (%) (Water)**	**Mobile Phase B (%) (Methanol: Acetonitrile, 90 : 10 v/v)**
0.0	91.0	11.0
2.0	91.0	11.0
5.0	61.0	39.0
8.0	55.0	45.0
20.0	52.0	48.0
42.0	48.0	52.0
70.0	42.0	58.0
80.0	35.0	65.0
85.0	30.0	70.0
92.0	20.0	80.0
112.0	15.0	85.0
113.0	91.0	11.0
125.0	91.0	11.0

RESULTS AND DISCUSSION

All the impurities are injected individually including both the Standards All are separated with each other.

Selection of Wavelength for Drug product:

Ethinyl estradiol impurities spectrums observed and noticed all impurities absorption maximum at 215nm and Baseline noise slightly more when compared with 210nm, no significant change in the absorption of all Ethinyl estradiol impurities between both wavelengths. Ethinyl estradiol impurities spectrums observed and noticed all impurities absorption maximum at 240nm and Baseline noise slightly more when compared with 254nm, no significant change in the absorption of all Levonorgestrel impurities between both wavelengths. Hence 210nm and 254nm selected for the estimation of Ethinyl Estradiol impurities and Levonorgestrel impurities respectively.

Finalization of Sample Concentration:

Based on the ICH Reporting threshold limit for impurities, Levonorgestrel impurities are injected 0.05% level and Ethinyl estradiol injected 0.1% of the below respective concentrations. Levonorgestrel is 150ppm and Ethinyl Estradiol is 30ppm All the impurities are visible and can be quantifiable. Hence below sample preparation was finalized.

Test Solution: Preparation of Sample solution:

(For 0.15 mg/0.03 mg) Weigh and transfer 10 intact tablets into 10.00 mL volumetric flask, add about 5 ml of diluent and sonicate for about 30 minutes with intermittent shaking to disperse tablets completely. Allow it to come to room temperature, dilute up to the mark with diluent and mix. Centrifuge the sample at around 3500 rpm for about 10 minutes. Filter the supernatant solution through a 0.45 µm Nylon filter, discarding the first 2 ml of the filtrate and inject.

(Concentration of Levonorgestrel is 150.0 µg/ mL and Ethinyl Estradiol 30.0 µg/ mL)

(For 0.15 mg/0.02 mg):

Weigh and transfer 15 intact tablets into 10.00 mL volumetric flask, add about 5 ml of diluent

and sonicate for about 30 minutes with intermittent shaking to disperse tablets completely.

Allow it to come to room temperature, dilute up to the mark with diluent and mix. Centrifuge the sample at around 3500 rpm for about 10 minutes. Filter the supernatant solution through 0.45 µm Nylon filter, discarding the first 2 ml of the filtrate and inject.

LOQ Values are finalized based on the daily dose of drug substances and the ICH Reporting threshold limit.

LOQ is 0.05% for Levonorgestrel and 0.1% Ethinyl Estradiol impurities and should prove precision and Accuracy for all impurities at the specified concentrations in the Method Validation.

Based on the above considerations below test method was finalized.

Sr. No.	**Name of the Impurity of Levonorgestrel**	**RRTw.r.t Levonorgestrel**	**Limit (%w/w)**
1	6-Beta-hydroxy Levonorgestrel(LG-I-1)	About 0.34	NMT 1.0
2	6-keto-Levonorgestrel(LG-I-2)	About 0.50	
3	66-Levonorgestrel(LG-I-3)	About 0.92	
Sr. No.	Name of the Impurity of Ethinyl Estradiol	RRTw.r.t Ethinyl Estradiol	NMT 1.0
1	6-alpha-hydroxy Ethinyl Estradiol(EE-I-1)	About 0.30	
2	6-beta-hydroxy Ethinyl Estradiol(EE-I-2)	About 0.42	
3	6 keto- Ethinyl Estradiol(EE-I-3)	About 0.52	
4	Delta-9,11- Ethinyl Estradiol(EE-I-4)	About 0.90	
5	Delta-6- Ethinyl Estradiol(EE-I-5)	About 0.94	
6	Maximum Unknown Impurity	---	

Methodology for Related Substances of Levonorgestrel and Ethinyl Estradiol

Specifications:

Reagents and Solvents:

Water (HPLC grade, Milli Q or equivalent)

Acetonitrile (HPLC grade, Make: J.T. Baker or equivalent)

Methanol (HPLC grade, Make: J.T. Baker or equivalent)

Points of emphasis:

Samples should be centrifuged before filtration to avoid clogging of the membrane filter.

Sample preparation flasks should be properly stoppered with lids to avoid solvent loss.

Wash the column first with Water: Acetonitrile: Methanol (80: 10: 10, v/v) for 60 minutes and followed by Water: Acetonitrile: Methanol (20: 40: 40, v/v) for 60 minutes after each analysis and store the column in the final solvent mixture.

Use Water: Acetonitrile (40: 60, v/v) as needle wash.

The method is sensitive to change in wavelength, temperature, and organic composition

Chromatographic Conditions:

Column	5µm, C18, 250 x 4.6 mm
Column Temperature	25 °C
Sample Temperature	15ºC
Flow rate	1.0 mL/minute
Wavelength For Ethinyl Estradiol For Levonorgestrel	 210 nm 254 nm
Injection Volume	50µ1
Retention time of Ethinyl Estradiol Levonorgestrel	 About 60.0 minutes About 76.0 minutes
Run time	125 minutes

Preparation of Diluent:

Prepare a mixture of filtered Water and Acetonitrile (30: 70, v/v).

Preparation of Mobile Phase:

Preparation of Mobile phase A: Water

Preparation of Mobile phase B: Acetonitrile and Methanol (90:10 v/v)

Gradient program:

Time (min.)	Mobile Phase A (%) (Water)	Mobile Phase B (%) (Methanol: Acetonitrile, 90 : 10 v/v)
0.0	91.0	11.0
2.0	91.0	11.0
5.0	61.0	39.0
8.0	55.0	45.0
20.0	52.0	48.0
42.0	48.0	52.0
70.0	42.0	58.0
80.0	35.0	65.0
85.0	30.0	70.0
92.0	20.0	80.0
112.0	15.0	85.0
113.0	91.0	11.0
125.0	91.0	11.0

Preparation of diluted standard solution:

Weigh and transfer accurately about 30.00 mg of Levonorgestrel working standard and 6.00 mg of Ethinyl Estradiol working standard into 200.00 mL volumetric flask. Add about 80 mL of diluent and sonicate to dissolve. Allow it to come to room temperature, dilute up to the mark with diluent and mix. Pipette out 1.00 mL of this solution into 100.00 mL volumetric flask, dilute up to the mark with diluent and mix. (Concentration of Levonorgestrel is 1.5 µg / mL and Ethinyl Estradiol is 0.3 µg/ mL) Preparation of Sensitivity solution: Pipette out 5.00 mL of Diluted standard solution into 100.00 mL volumetric flask, dilute up to the mark with diluent and mix.(Concentration of Levonorgestrel is 0.15 µg/ mL and Ethinyl Estradiol is 0.03 µg/ mL) Preparation of Impurity standard stock solution: Weigh and transfer accurately about 1.5 mg of 17P- Ethinyl Estradiol impurity standard and 1.5 mg of Estrone

impurity standard into 50.00 mL volumetric flask. Add about 40 mL of diluent and sonicate to dissolve. Allow it to come to room temperature, dilute up to the mark with diluent and mix. (Concentration of l 7P-Ethinyl Estradiol is 30.0 µg/ mL and Estrone is 30.0 µg/ mL).

Preparation of System suitability solution:

Weigh and transfer accurately about 7.5 mg of Levonorgestrel working standard and 1.5 mg of Ethinyl Estradiol working standard into 50.00 mL volumetric flask. Add about 40 mL of diluent and sonicate to dissolve. Allow it to come to room temperature, add quantitatively 0.5 mL of Impurity standard stock solution, dilute up to the mark with diluent and mix.

(Concentration of Levonorgestrel is 150.0 µg/ mL, Ethinyl Estradiol is 30.0 µg/ mL, 17P-Ethinyl Estradiol is 0.3 µg/ mL and Estrone is 0.3 µg/ mL).

Preparation of Placebo Solution-I: (For 0.15 mg/ 0.03 mg) (Placebo without both Levonorgestrel and Ethinyl Estradiol) Weigh and transfer accurately about placebo blend equivalent to 10 tablets or 10 placebo tablets into10.00 mL volumetric flask, add about 5 mL of diluent and sonicate for about 30 minutes with intermittent shaking to disperse tablets completely. Allow it to come to room temperature, dilute up to the mark with diluent and mix. Centrifuge this solution at about 3500 rpm for 10 minutes. Filter the supernatant solution through a 0.45 µm Nylon filter, discarding the first 2 ml of the filtrate and inject.

Preparation of Placebo Solution-II: (For 0.15 mg/ 0.03 mg) (Placebo without Levonorgestrel and with Ethinyl Estradiol) Weigh and transfer accurately about placebo blend equivalent to 10 tablets or 10 placebo tablets into 10.00 mL volumetric flask, add about 5 mL of diluent and sonicate for about 30 minutes with intermittent shaking to disperse tablets completely. Allow it to come to room temperature, dilute up to the mark with diluent and mix. Centrifuge this solution at about 3500 rpm for 10 minutes. Filter the supernatant solution through a 0.45 µm Nylon filter, discarding the first 2 ml of the filtrate and inject.

Preparation of Sample solution:

(For 0.15 mg/0.03 mg)

Weigh and transfer 10 intact tablets into 10.00 mL volumetric flask, add about 5 ml of diluent and sonicate for about 30 minutes with intermittent shaking to disperse tablets completely. Allow it to come to room temperature, dilute up to the mark with diluent and mix. Centrifuge

the sample at around 3500 rpm for about 10 minutes. Filter the supernatant solution through a 0.45 µm Nylon filter, discarding the first 2 ml of the filtrate and inject. (Concentration of Levonorgestrel is 150.0 µg/ mL and Ethinyl Estradiol 30.0 µg/ mL).

Note: Control Sample and Placebo preparations (For 0.15mg/0.02mg): 15Tablets transfer into 20ml volumetric flask and follow the same procedure like higher strength.

Procedure:

Inject the specified volume of Diluent, Sensitivity solution, System suitability solution, Diluted standard solution, placebo solutions, and Sample solution into the chromatograph as mentioned in the Injection sequence table and record the chromatograms. Disregard peaks due to diluent and placebo solution-I.

Disregard the impurity peaks below the LOQ level for both Levonorgestrel and Ethinyl Estradiol.

Calculate all known impurities of Levonorgestrel against Levonorgestrel diluted standard area at the respective wavelength.

Calculate all known impurities of Ethinyl Estradiol against the Ethinyl Estradiol diluted standard area at 210 nm.

Calculate all unknown impurities at 254 nm against Levonorgestrel diluted standards areas at corresponding wavelengths with the help of Placebo solutions - II and III.

Calculate all unknown impurities at 210 nm against Ethinyl Estradiol diluted standards areas at corresponding wavelengths with the help of Placebo solutions - II and III.

Any other impurity showing response at both wavelength 210 nm and 254 nm will be calculated at a wavelength where it shows maximum area response against Levonorgestrel diluted standard area at the respective wavelength. Any known or unknown impurity will be calculated only once at the respective wavelength. If the single impurity showing response at both wavelength then it will not be considered again in the calculation at another wavelength.

Any other impurity showing response at 210 nm should be calculated against Ethinyl Estradiol diluted standard area.

Sr. No.	Solutions to be injected	Number of Injections
1.	Diluent	1
2.	Sensitivity solution	1
3.	System suitability solution	1
4.	Diluent	1
5.	Diluted standard solution	6
6.	Diluent	1
7.	Placebo solution-I	1
8.	Placebo solution-II	1
9.	Placebo solution-III	1
10.	Sample solution	1
11.	Diluent	1
12.	Bracketing standard	1

RRT, RF, and Limit of Quantitation (LOQ) level of known impurities of Levonorgestrel:

Sr. No.	Known impurities	RRT (about)	RF	Wavelength	%w/w LOQ HS
1.	Levonorgestrel	1.00	1.0	254nm	0.05
2.	6-Beta-hydroxy Levonorgestrel(LG-I-1)	0.34	1.6	254nm	0.05
3.	6-keto-Levonorgestrel(LG-I-2)	0.48	1.7	254nm	0.05
4.	Delta-6-Levonorgestrel(LG-I-3)	0.94	3.5	254nm	0.05

RRT, RF, and Limit of Quantitation (LOQ) level of known impurities of Ethinyl Estradiol:

Sr. No.	Known impurities	RRT	RF	Wavelength	%w/w LOQ
1.	Ethinyl Estradiol	1.00	1.0	210nm	0.10
2.	6-alpha-hydroxy Ethinyl Estradiol(EE-I-1)	0.32	0.8	210nm	0.10
3.	6-beta-hydroxy Ethinyl Estradiol(EE-I-2)	0.37	1.0	210nm	0.10
4.	6 keto- Ethinyl Estradiol(EE-I-3)	0.50	0.7	210mn	0.10
5.	Delta-9,11- Ethinyl Estradiol(EE-I-4)	0.85	0.9	210 nm	0.10
6.	Delta-6- Ethinyl Estradiol(EE-I-5)	0.93	0.8	210nm	0.10

Evaluation of System suitability:

System suitability solution:

The tailing factor for Ethinyl Estradiol and Levonorgestrel peak should not be more than 2.0 at both wavelengths.

Theoretical plate for Ethinyl Estradiol and Levonorgestrel peak should not be less than 20000 at both the wavelengths.

Diluted standard solution: The relative standard deviation of six replicate injections for Levonorgestrel at (254 nm) and Ethinyl Estradiol at 210 nm should not be more than 5.0%.

Formula:

$$\frac{\text{Impurity Area} \times \text{Standard concertation} \times \text{Potency of standard} \times 1 \times \text{Label claim}}{\text{Standard Area} \quad \text{Sample concentration} \quad \text{RRF} \quad \text{Avg.Wt}}$$

Note 1) Levonorgestrel related substances (Known and Unknown) Calculate against Levonorgestrel standard.

2) Ethinyl Estradiol related substances (Known and Unknown) Calculate against Levonorgestrel standard.

Other unknown impurities calculate against Ethinyl Estradiol.

Summarised validation Results for the Finished Product:

1) Specificity: As part of specificity Blank, Placebo, Standard, Un-spiked sample, Spiked sample (spiked with all impurities at specification level) and Individual impurities were injected and the results were found the below:

<table>
<tr><th>Sr. No.</th><th>Validation Parameter</th><th colspan="3">Results</th><th>Acceptance Criteria</th></tr>
<tr><td>1.0</td><td colspan="5">Specificity</td></tr>
<tr><td rowspan="5">1.1</td><td rowspan="3">Identification</td><td colspan="3">Retention time (min)</td><td rowspan="3">The difference in the retention time of the peak of Levonorgestrel and Ethinyl Estradiol obtained in the unspiked sample solution and spiked sample solution should be NMT ± 10 %, when compared with that obtained in diluted standard solution (1st injection from six replicate injections for system suitability)</td></tr>
<tr><td colspan="2">Sample solution</td><td rowspan="2">Diluted standard</td></tr>
<tr><td>Un-spiked</td><td>Spiked</td></tr>
<tr><td>Levonorgestrel (254 nm)</td><td>75.212</td><td>75.016</td><td>74.961</td></tr>
<tr><td>Ethinyl Estradiol (210 nm)</td><td>59.993</td><td>59.993</td><td>59.989</td></tr>
<tr><td></td><td colspan="3">The retention time of all the known impurities spiked in the Sample solution was found comparable with those injected individually.</td><td>For impurities eluting before 10 min.: The difference in the retention times of all known impurities of Levonorgestrel and Ethinyl Estradiol in the spiked sample, the solution should be not more than ± 1.0 minute with that obtained in individual impurity standard solutions.

For impurities eluting after 10min.: The difference in the retention times of all known impurities of Levonorgestrel and Ethinyl Estradiol in the spiked sample, the solution should be not more than ± 10.0 % with that obtained 111 individual impurity standard solutions</td></tr>
</table>

Method Precision and Intermediate Precision Results for all Impurities:

Both the analysts performed Method Precision and Intermediate precision in different days, on different instruments by using different columns. Six spiked samples prepared at 100% specification level and injected. Results find the below:

Sample	6-beta-hydroxy Levonorgestrel(LG-I-1)		6-keto-Levonorgestrel(LG-I-2)		Delta-6-Levonorgestrel(LG-I-3)	
	M.P.	I.P.	M.P.	I.P.	M.P.	I.P.
	(%w/w)	(%w/w)	(%w/w)	(%w/w)	(%w/w)	(%w/w)
1	0.95	0.94	I.I1	I.I1	1.31	1.32
2	0.97	0.93	1.11	1.11	1.31	1.32
3	0.97	0.94	1.11	1.21	1.31	1.32
4	0.95	0.93	1.11	1.11	1.31	1.32
5	0.96	0.94	1.11	1.11	1.31	1.32
6	0.96	0.94	1.11	1.11	1.31	1.32
Overall Mean	0.95		1.12		1.32	
SD (n=6+6)	0.014		0.028		0.005	
%RSD (n=6+6)	1.5		2.6		0.4	

S.no	6-alpha-hydroxy Ethinyl Estradiol(EE-I-1)		6-beta-hydroxy Ethinyl Estradiol(EE-I-2)		6 keto- Ethinyl Estradiol(EE-I-3)	
	M.P.	I.P.	M.P.	I.P.	M.P.	I.P.
	(%w/w)	(%w/w)	(%w/w)	(%w/w)	(%w/w)	(%w/w)
1	0.90	0.93	1.02	1.11	0.88	0.89
2	0.91	0.90	1.02	1.11	0.88	0.88
3	0.91	0.91	1.02	1.11	0.89	0.87
4	0.92	0.88	1.02	1.11	0.87	0.87
5	0.92	0.91	1.02	1.11	0.87	0.87
6	0.92	0.90	1.02	1.11	0.88	0.87
Overall Mean	0.91		1.1		0.88	
SD (n=6+6)	0.013		0.047		0.008	
¾RSD (n=6+6)	1.4		4.4		0.9	

Sr. No.	Delta-9,11- Ethinyl Estradiol(EE-I-4)		Delta-6- Ethinyl Estradiol(EE-I-5)	
	M.P.	I.P.	M.P.	I.P.
	(%w/w)	(%w/w)	(%w/w)	(%w/w)
1	1.29	1.23	0.99	1.11
2	1.29	1.23	0.99	1.11
3	1.29	1.23	0.99	1.11
4	1.29	1.23	0.99	1.01
5	1.29	1.23	0.99	1.01
6	1.29	1.23	0.99	1.11
Overall Mean (n=6+6)	1.26		1.03	
SD (n=6+6)	0.031		0.057	
%RSD (n=6+6)	2.5		5.5	

Precision at LOQ:

Precision at LOQ was performed for all impurities and the results are found below:

Precision for Limit of Quantification of Ethinyl Estradiol impurities:

Sr. No.	Peak Area Counts					
	6-alpha-hydroxy Ethinyl Estradiol(EE-I-1)	6-beta-hydroxy Ethinyl Estradiol(EE-I-2)	6 keto- Ethinyl Estradiol(EE-I-3)	Delta-9,11-Ethinyl Estradiol(EE-I-4)	Delta-6- Ethinyl Estradiol(EE-I-5)	Ethinyl Estradiol
	0.10%w/w	0.10%w/w	0.10% w/w	0.10%w/w	0.10% w/w	0.10% w/w
1	5133	5111	5123	4955	5111	6665
2	5050	5023	5088	5234	5109	6897
3	5233	5071	5123	5098	5089	6766
4	5333	5145	5003	5345	5189	6567
5	4998	5122	5082	5102	5113	6633
6	5111	5099	5144	5005	5220	6367
Mean	5143	5095	5093.8	5123.2	5138.5	6649.2
SD	122.47	43.03	50.28	144.78	52.76	179.97
%RSD	2.4	0.8	1.0	2.8	1.0	2.7

Precision for Limit of Quantitation of Levenorgestral impurities:

Sr. No.	Peak Area Counts			
	6B-hydroxy Levonorgestrel(LG-I-1)	6-keto-Levonorgestrel(LG-I-2)	Delta-6-Levonorgestrel(LG-I-3)	Levonorgestrcl
	0.05 %w/w	0.05 %w/w	0.05 % w/w	0.05%w/w
1	6021	5800	2945	12089
2	6546	5871	3021	12221
3	5678	5634	3115	12377
4	5789	5545	3045	12365
5	6234	5805	2956	12209
6	6118	5923	3567	12190
Mean	6064	5763	3108	12242
SD	313.24	144.61	233.21	110.45
%RSD	5.2	2.5	7.5	0.9

System Precision

Six replicate injections of the diluted standard solution were injected into the chromatograph.

Sr. No.	Levonorgestrel area (254 nm)	Ethinyl Estradiol area (210 nm)
1	191231	37533
2	190222	36922
3	192881	36701
4	192134	37000
5	192675	37221
6	190009	37421
Mean	191525	37133
SD	1234.068	316.07
%RSD	0.6	0.9

Solution Stability:

Solution stability was performed on Standard up to 120 Hours and Spiked sample up to 96 hours at 15 degrees. Solutions are stable.

Mobile Phase Stability:

During Standard and Sample solution Mobile phase was used up to 5Days at Benchtop, Retention times and Bracketing standard solution results are very consistent. No

Haziness was observed.

Filter validation:

Similarity factor between filtered and unfiltered standard area was observed 1.02 for Ethinyl Estradiol and 1.01 for Levonorgestrel. The spiked sample solution was injected Centrifuged and Nylon Filtered Samples. All the impurities % difference between Centrifuged and Nylon filter samples are below 5.

Based on the results Nylon filter is suitable for Standard and Sample solutions.

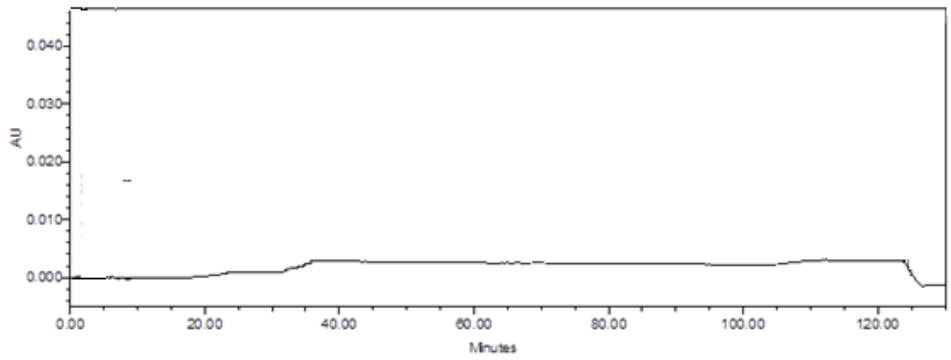
Robustness:

To evaluate robustness, following small but deliberate variations were made in the analytical method parameters and sample (Levonorgestrel and Ethinyl Estradiol Tablets) spiked with known impurities were analyzed.Change in the flow rate of mobile phase by± 10 % of 1.00 mL/min (i.e. 0.90 mL/min and 1.10mL/min). Increase in the temperature of the column oven by+ 3°C of 25°C (28°C). Change in the wavelength by ± 5 nm of 210 nm and 254 nm (i.e. 205 nm, 215 nm, and 249 nm, 259).

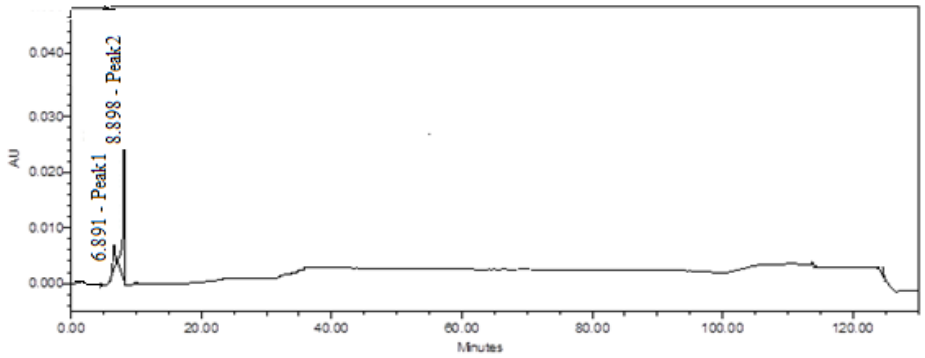
Change in the organic Acetonitrile component of mobile phase B by± 10 % relative of Methanol and Acetonitrile (90: 10, v/v). [i.e. Methanol and Acetonitrile (91: 09, v/v) for the decrease in acetonitrile component and Methanol and Acetonitrile (89: 11, v/v) for an increase in acetonitrile component]

Reference Chromatograms:

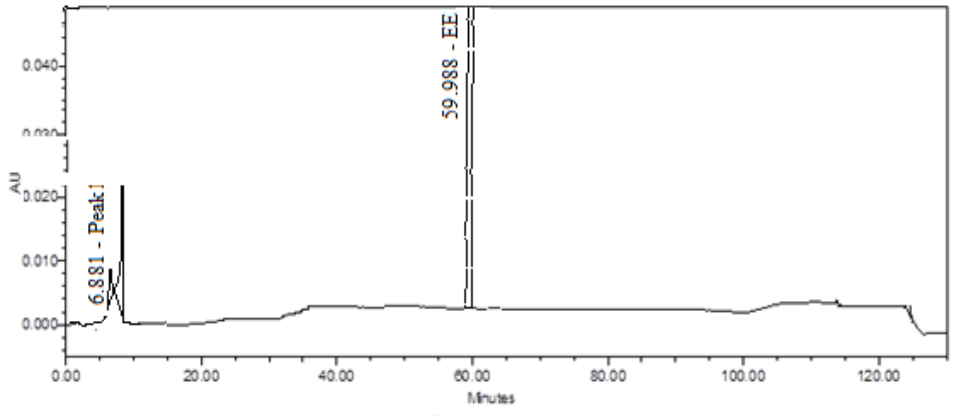
1. Chromatogram of Blank at 210nm



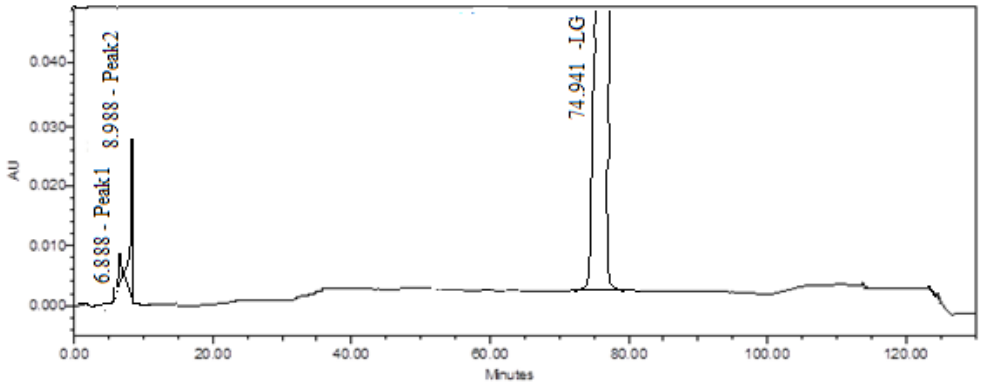
2. Chromatogram of Placebo-I at 210 nm (Placebo without any drug)



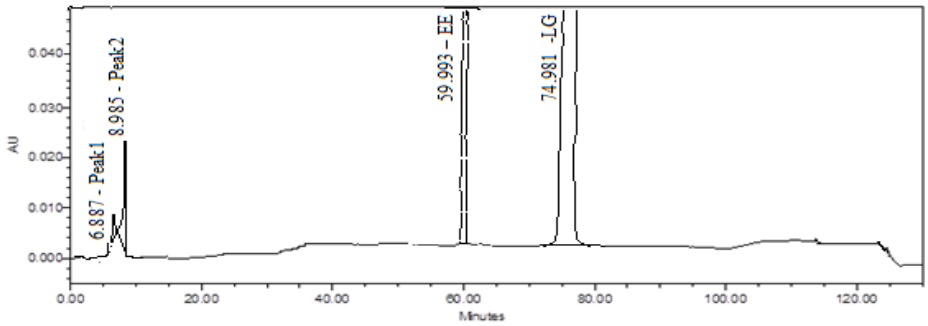
3. Chromatogram of Placebo-II at 210 nm (Placebo with Ethinyl Estradiol)



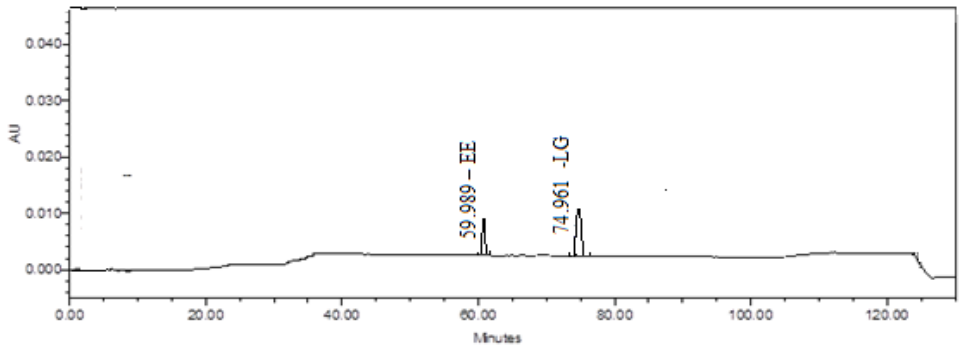
4. Chromatogram of Placebo-II at 210 nm (Placebo with Levonorgestrel)



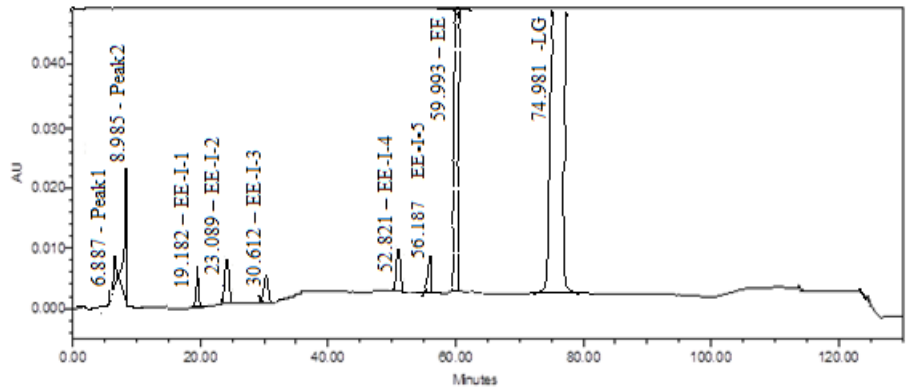
5. Chromatogram of Control Sample at 210nm



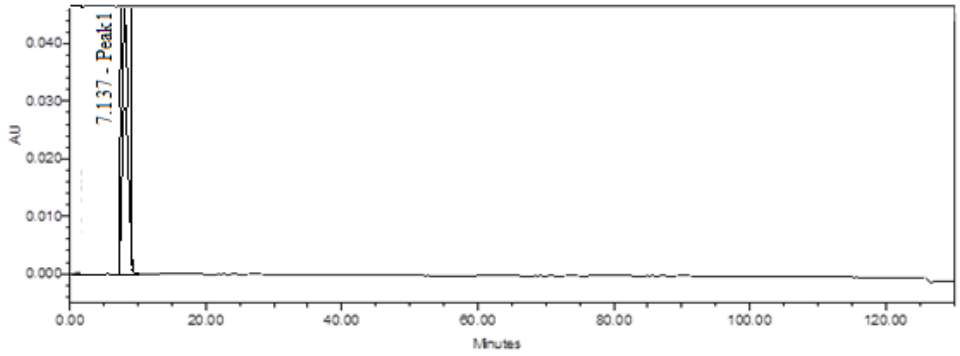
6. Chromatogram of Standard at 210nm



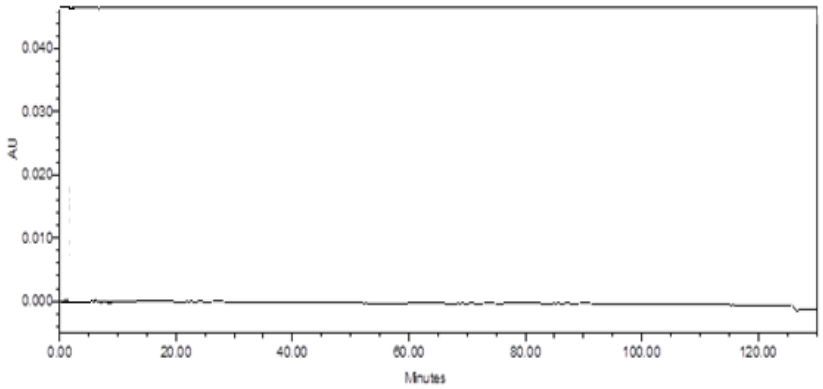
7. Chromatogram of Spiked Sample with Ethinyl estradiol impurities at 210nm



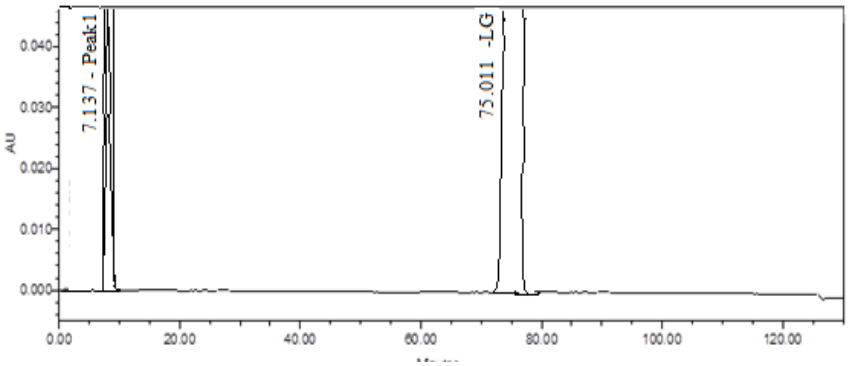
8. Chromatogram of Plain Placebo at 254nm for Levonorgestrel



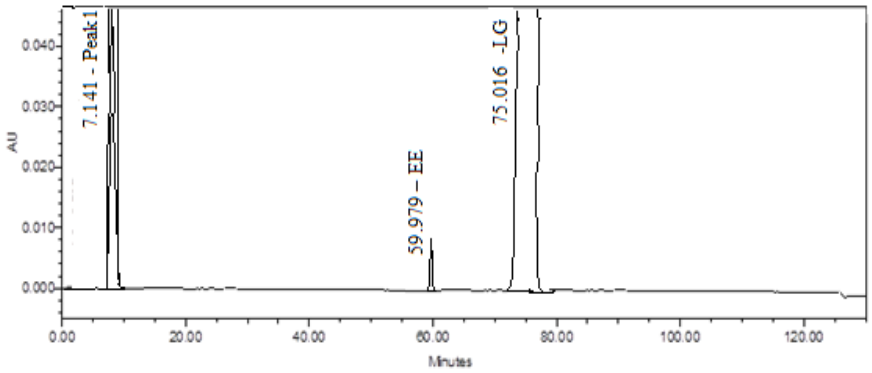
9. Chromatogram of Blank at 254nm for Levonorgestrel



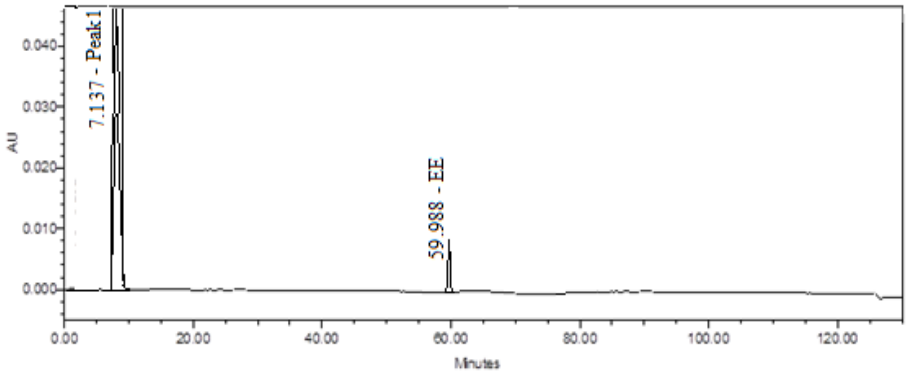
10. Chromatogram of Placebo with Levonorgestrel at 254 nm



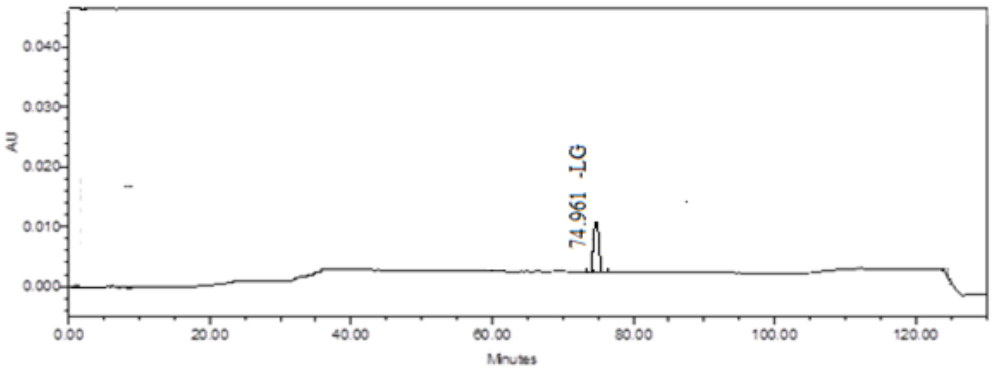
11. Chromatogram of Control Sample at 254 nm



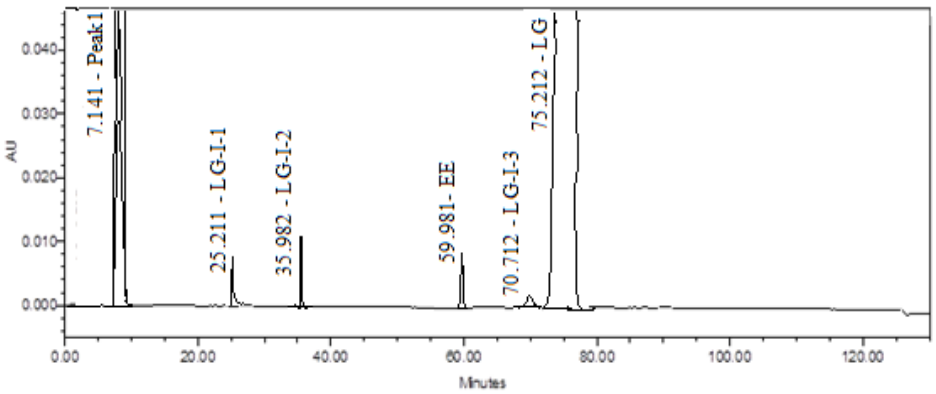
12. Chromatogram of Placebo with Ethinyl estradiol at 254 nm



13. Chromatogram of Standard at 254 nm for Levonorgestrel



14. Chromatogram of Spiked sample (Spiked with Levonorgestrel impurities) at 254nm



CONCLUSION

Disregard the impurity peaks below the LOQ level for both Levonorgestrel and Ethinyl Estradiol. Calculate all known impurities of Levonorgestrel against Levonorgestrel diluted standard area at the respective wavelength. Calculate all known impurities of Ethinyl Estradiol against the Ethinyl Estradiol diluted standard area at 210 nm. Calculate all unknown impurities at 254 nm against Levonorgestrel diluted standards areas at corresponding wavelengths with the help of Placebo solutions - II and III. Calculate all unknown impurities at 210 nm against Ethinyl Estradiol diluted standards areas at corresponding wavelengths with the help of Placebo solutions - II and III. Any other impurity showing response at both wavelength 210 nm and 254 nm will be calculated at a wavelength where it shows maximum area response against Levonorgestrel diluted standard area at the respective wavelength. Any known or unknown impurity will be calculated only once at the respective wavelength. If the single impurity showing response at both wavelength then it will not be considered again in the calculation at another wavelength. Any other impurity showing response at 210 nm should be calculated against Ethinyl Estradiol diluted standard area. The Proposed HPLC method for related substances for Levonorgestrel and Ethinyl Estradiol is developed and validated. The method is found to be specific. The Method is also Stability indicating as evidenced by forced degradation studies. The method is found to be Linear in the specified range. Hence the method is Accurate. System suitability parameters are not affected by changing the robustness conditions and hence the method found to be robust. System suitability is established and recorded. Hence, this method can be used for stability and routine analysis.

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