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# Method Development and Analytical Validation of Levonorgestrel Drug By RP-HPLC Method- A Research article

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**ABSTRACT:-** A Simple, reverse Phase High performance liquid chromatographic(RP-HPLC) method has been developed & validated for the Determination of Levonorgestrel in tablet. Levonorgestrel is a second generation synthetic progestogen used as an active ingredient in some hormonal contraceptives, including combined oral contraceptive pills, progestogen only pills, emergency contraceptive pills. The Compounds were separated on an ODS analytical column with a mixtures of water and acetonitrile solution in the ratio(40:60 v/v) as a mobile phase at a flow rate of 1.2 ml/min. Levonorgestrel exhibiting absorption at 243nm and obeyed beers law in the concentration range 3µg/ml.The method was validated for specificity ,accuracy. precision, linearity and robustness.The developed and validated method was successfully used for quantitative analysis Levonorgestrel tablet.total chromatographic analysis time per sample was approximately 10 min with levonorgestrel Eluting with retention time of 3.621,3.281 and 3.617 min respectively. Validation studies reveled the method is specific, rapid, reliable and reproducible calibration plots were linear over the concentration ranges 1.5 to 4.5 µg/ml for Levonorgestrel. The sample solution was stable up to 22 hours. The high recevory and low relative standard deviation confirm the sutaility of the method the determination of Levonorgestrel tablet.The assay results were found to be in good agreement with label claim. The proposed method was simple sensitive, precise, and useful for routine quality control.

**Keywords:** Reverse phase, Levonorgestrel, Validation, Precision, Progestrogen, specificity

**INTRODUCTION:** The ability to provide timely, accurate, and reliable data is central to the discovery, development, and manufacture of pharmaceuticals. Analytical data are used to screen potential drug candidates, aid in the development of drug synthesis, support formulation studies, monitor the stability of bulk pharmaceuticals and formulated products, and test final products for release. HPLC originally referred to the fact that high pressure was needed to generate the flow required for liquid chromatography in packed columns. In the beginning, instrument components only had the capability of generating pressures of 500psi (35 bar). This was called High Pressure Liquid Chromatography (HPLC). HPLC has been rapidly developed with the introduction of new pumping methods, more reliable columns and a variety of detectors. Most of the drugs in multicomponent dosage forms can be analyzed by HPLC methods because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures.

Reversed phase mode is the most popular mode for analytical and preparative separation of compounds of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is nonpolar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent.

**Drug Profile :** Levonorgestrel (Levo) (-)13-ethyl-17-ethinyl-17- hydroxyl-1,2,6,7,8,9,10,11,12,13,14,15,16(– 17-tetradecahydrocyclopenta [a] phenanthren-3-one.

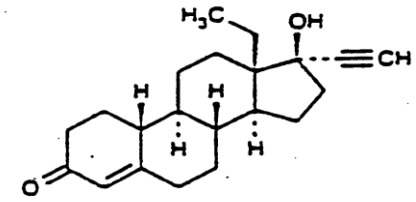


**Fig.1 Chemical Structure of Levonorgestrel**

**Levonorgestrel** (or ***l*-norgestrel** or **d-norgestrel**) is a second generation synthetic progestogen used as an active ingredient in some hormonal contraceptives, including combined oral contraceptive pills, progestogen only pills, emergency contraceptive pills, intrauterine system, contraceptive implants hormone replacement therapy.

In Recent years pharmaceutical Preparations Containing Levonorgestrel available Commercially in india as well as U.S.A. Mainly HPLC analytical Method for estimation of Levonorgestrel in Pharmaceutical Preparation.because HPLC methods have been widely used for routine Quality Control assessment of drug,because of their sensitivity,repeatability and Specificity.

**Objectives of study**

Method Development and Analytical Validation for the estimation of Levonorgestrel in its dosage form**.** Development of HPLC method for the estimation of Levonorgestrel.To develop stability indicating assay method for estimation of Levonorgestrel forced degradation method. Validation of developed analytical method as per ICH guidelines.

**Reagents and Materials used**

| S.No. | Chemicals | Specifications | Manufactures |
|---|---|---|---|
| 1. | Acetonitrile | AR – Grade | HPLC grade spectrochem ltd |
| 2. | Methanol | AR – Grade | HPLC grade spectrochem ltd |
| 3. | Milli Q Water | Double Distilled | Milli-QRO system |
| 4. | Hydrochloric acid | AR – Grade | Merck, India |
| 5. | Sodium hydroxide | AR- Grade | Merck, India |
| 6. | Hydrogen Peroxide | AR- Grade | Merck, India |
| 7. | Levonorgestrel | Active Pharmaceutical Ingredient | Bayer schering Pharma,USA |

**Chromatographic condition:-**

Analysis was performed with a HPLC of Waters Equipped with a photodiode array detector and injector walve with 20 µl sample loop.levonorgestrel was seprated on a column on Zorex ODS column with a dimension of C18 (150×4.6mm), 5µm,under reverse phase partition chromatography conditions.the moboile phase was a mixture of water ACN solution in the ratio of (40:60).The

flow rate was 1.2 ml/ min.and the analytes and the internal standard were monitored at 242nm.the system was used in an air condition HPLC laboratory.(20±2ºc).before analysis the mobile was Dgass and filtered through a 0.2 µm filter. sample solution were also filtered through a 0.2 µm filter.The system was eqvilibrated before each injection.

**5.3.1 Mobile phase preparation:**

60 parts of Acetonitrile (HPLC grade) and 40 part of water (HPLC grade) was mixed well and sonicate for 15min to remove the gases impurity. Then filter the mobile by using filtration assembly containing 0.45µ pore filter paper to remove small particals.

**5.3.2 Preparation of Diluents:**

Prepared a mixture of Acetonitrile : water in the ratio of 60:40 which was used as diluents for dilution of standard stock solution.

**5.3.3 Stock solution of Levonorgestrel (150 µg/mL):**

An accurately weighed quantity of Levonorgestrel working/reference standard about 30 mg was transferred into 200 mL volumetric flask and dissolve in diluents and diluted up to the mark with diluent to give a stock solution.150µg/mL

**5.3.4 Working standard solution of Levonorgestrel:**

Accurately pipette out 4 ml of Levonorgestrel stock solution into 200ml volumetric flask and diluted up to mark with diluent. Shake well give a solution having strength 3.0 µg/ml. (Levonorgestrel)

**5.3.5 Sample preparation (100 µg/mL):**

Weighed and transferred 10 intact tablets into a 100.00 ml volumetric flask, add about 70 ml of diluent and sonicate for 30 minutes with intermittent vigorous shaking to disperse tablets completely. Allow it to come to room temperature, dilute up to the mark with diluents and mix. filter the portion of this solution through 0.45µm nylon filter or 0.45µm PVDF membrane filter and use the filtrate, discard first 5ml of solution (concentration of levonorgestrel is 3.0 µg/ml)

**Result and discussion**

**Method Development and optimization:**

Column chemistry ,solvent selectivity ,solvent strength detection of wavelength and flow rate were varied to determine the chromatographic condition giving the best separation the mobile conditions were optimized so the peak from the solvent ,Excipients after each change of mobile phase.reequilibrate by the passage of mobile phase.

To investigate appropriate wavelength for the determination of levonorgestrel solutions of this compound in the mobile phase were scanned by U.V spectrophotometry.in the range of 200-400nm.it was observed that there was no interference from mobile phase or Base line disturbance at 243nm.so it was conclude that 243.nm is the most an appropriate wavelength for the analysis of Levonorgestrel.

(i) Detection of wavelength

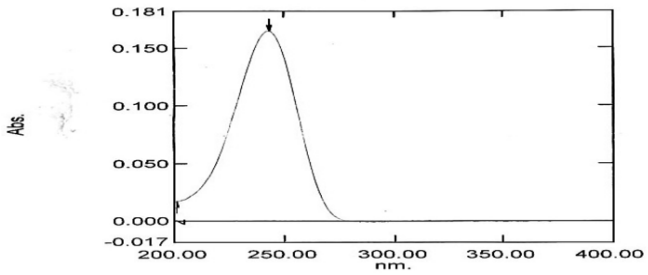


**Fig.A: UV Spectra of levonorgestrel**

**Table 1**

| Sr.no | Wavelength | Absorbance | Maxima |
|---|---|---|---|
| 1. | 243 | 0.164 | 243.4 |

**6.1.3 Determination of solubility of Levonorgestrel**

It was observed that Levonorgestrelwas soluble in water, and freely soluble in methanol, Acetonitrile, 0.1 N HCL,

0.1 N NaOH .Levonorgestrelshowed highest stability below pH7.0. So Diluents chosen was Water:ACN (60:40), which showed good solubility and stability also.

**6.1.4 Selection of Column Temperature**

An inclusion of column temperature (30°C) has minimized day-to-day variation of retention time due to fluctuations in the ambient temperature; along with this peak sharpening and shortening of run time were observed.

**Trials for Development:-**

**Trials taken for Method Optimization:**

**Trial 1:** In first trial we have selected water :Methanol in the ratio of (50:50) and column used was Phenomenex LUNA (250×4.6), 5µ,injection volume was 10 µl,flow was 0.8 ml/min. it was observed that due to higher length of the column run time increases .Run time increases more than 10 min.

**Trial 2 :** In second trial we have selected water:acetonitrile in the ratio of (80:20) and column used was Kromasil C8 (150×4.6), 5µ,injection volume was 25 µl,flow was 1.0 ml/min. it was observed that peak Shape was good but retention time is still very long.

**Trial 3 :** In third trial we have selected water:acetonitrile in the ratio of (40:60) and column used was Zorbax C18 (150×4.6), 5µ,injection volume was 50 µl,flow was 1.2 ml/min. it was observed that peak Shape was also good short retention time,Stable baseline.

So,**Trial 3** was taken into consideration and further Analytical Method Development For The Estimation of Levonorgestrel in dosage form By HPLC was demonstrated

**METHOD VALIDATION :-**

**Specificity:**

Prepare the blank, Placebo Solution, Standard Solution at Specification Level and inject as per methodology. There should not be any interference from blank and placebo peaks with main peak.

**Accuracy:**

Prepare sample solution to achieve the concentration in the range of 50%,100% and 150% with respect to specification limit and inject as per methodology Accuracy for individual and mean at each level should be between 98.0% to 102.0% with RSD not more than 2.0%.

**Method Precision:**

Prepare the Blank (Diluent), Diluted Standard solution at Specification level and inject as per methodology .Inject six injections of Diluted Standard solution. The RSD of Assay of five sample preparations should not be more than 2.0%.

**Linearity:**

Inject standard solution of levonorgestrel in the range of 50% to 150% of concentration with respect to specification level. Process data and plot the graph of area vs Concentration.Calculate Correlation Coefficient and % Y-intercept for levonorgestrel.

**Robustness:**

Prepare diluted Standard solution at the specification level of levonorgestrel tablets and analyse under the following deliberate variation in Chromatographic conditions.

a) Change in Flow Rate (± 0.3 ml/min)
   (i.e 0.9 ml and 1.5 ml)
b) Change in Column Temp (± 3ºC)
   (i.e 33ºC and 27ºC)

**Solution Stability:**

Prepare the Diluent, Diluted Standard Solution, and freshly diluted standard at specification level as per methodology. Inject the solutions initially and at different time interval upto 22 hrs by storing the solution at room temperature .Calculate the difference between initial area of diluted standard solution and area of diluted standard solution at each time interval with respect to initial area.

**Table 2: Chromatographic data of Trial 1**

| Sr.no | Peak name | RT | Area | USP Plate count | USP tailing | % Area |
|---|---|---|---|---|---|---|
| 1 | Levonorgestrel | 11.460 | 478886 | 4502 | 1.09 | 100.00 |

**Trial 2:**

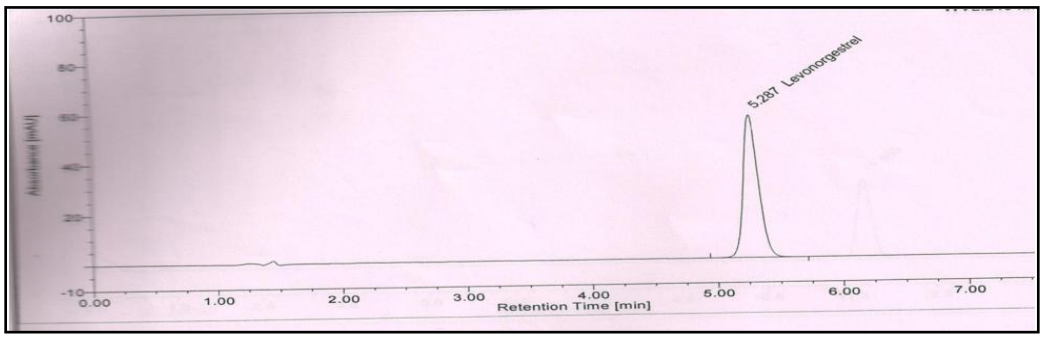


**Fig C: Chromatogram of Trial 2 (Absorbance vs Retention time)**

**Table 3: Chromatographic data of Trial 2**

| Sr.no | Peak name | RT | Area | USP Plate count | USP tailing | % Area |
|---|---|---|---|---|---|---|
| 1 | Levonorgestrel | 5.287 | 479435 | 4678 | 1.04 | 100.00 |

**Trial 3**

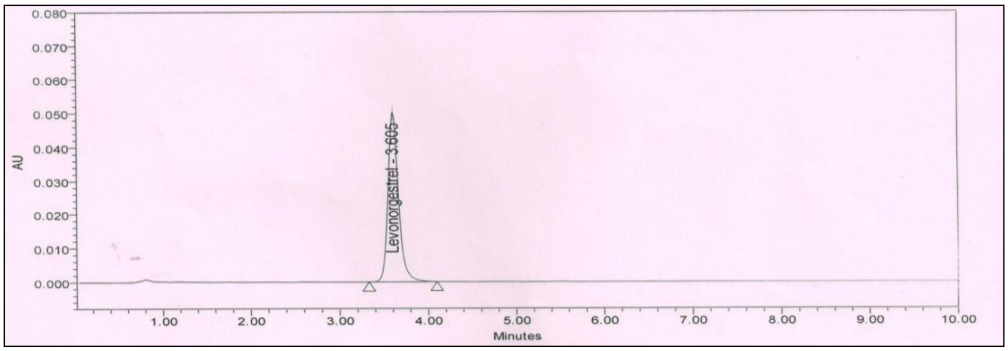


**Fig D: Chromatogram of Trial (Absorbance vs Retention time)**

**Table 4 : Chromatographic data of Trial 4**

| Sr.no | Peak name | RT | Area | USP Plate count | USP tailing | % Area |
|---|---|---|---|---|---|---|
| 1 | Levonorgestrel | 3.605 | 412738 | 4820 | 1.19 | 100.00 |

So,**Trial 3** was taken into consideration and further Analytical Method Development For The Estimation of Levonorgestrel in dosage form By HPLC was demonstrated.

**6 System suitability:**

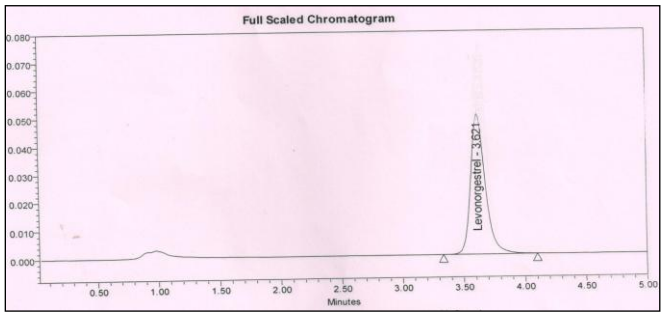


**Figure E : Chromatogram of Standard for System Suitability(Absorbance vs time)**

**Table 5: Chromatographic data of Standard for system suitability**

| Sr.no | Peak name | RT | Area | USP Plate count | USP tailing | % Area |
|---|---|---|---|---|---|---|
| 1 | Levonorgestrel | 3.621 | 409425 | 4843 | 1.19 | 100.00 |

## Result

Results system suitability were recorded in Table No.6.15.The %RSD of Area, Theoretical Plates, Asymmetry was calculated.

1.Specificity :

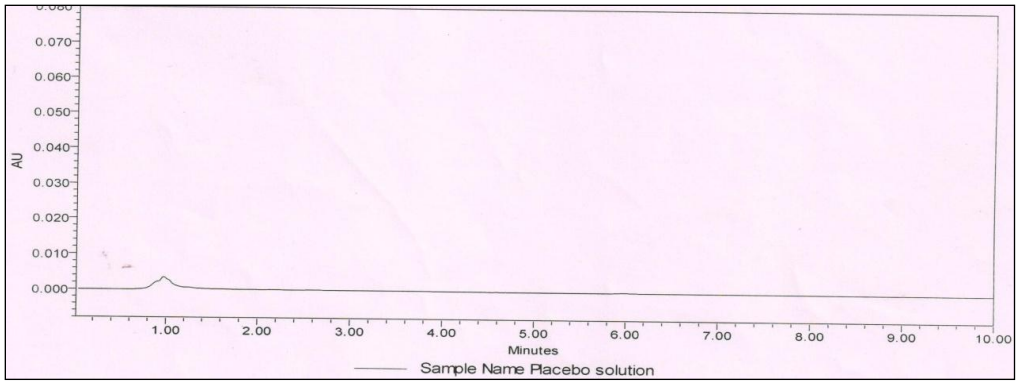


**Fig F: Chromatogram of placebo Solution (Absorbance vs Retention time)**

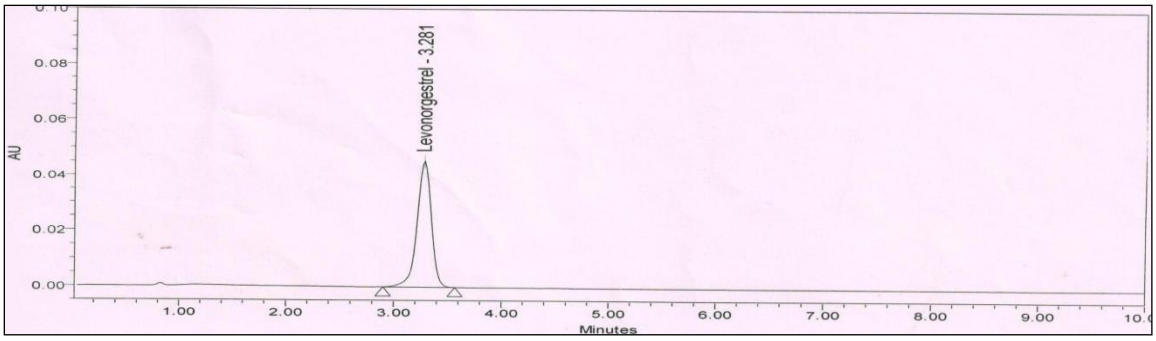


**Fig G: Chromatogram of Standard Solution (Absorbance vs Retention time)**

**Table 6: Chromatographic data of Standard Solution**

| Sr.no | Peak name | RT | Area | USP Plate count | USP tailing | % Area |
|---|---|---|---|---|---|---|
| **1** | **Levonorgestrel** | **3.281** | **416333** | **3162** | **0.92** | **100.00** |

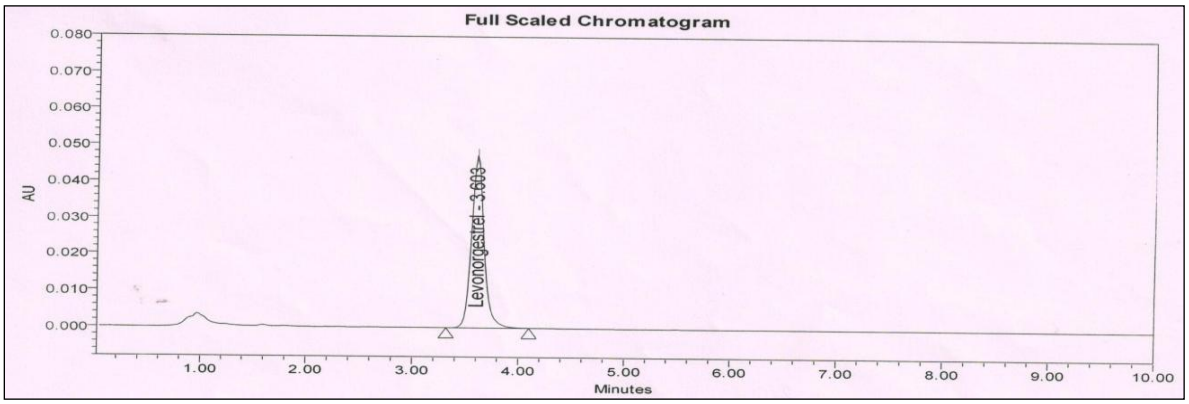


**Fig H .Chromatogram of Sample Solution (Absorbance vs time)**

**Table 7 : Chromatographic data of Sample Solution**.

| Sr.no | Peak name | RT | Area | USP Plate count | USP tailing | % Area |
|---|---|---|---|---|---|---|
| 1 | Levonorgestrel | 3.603 | 388150 | 4764 | 1.19 | 100.00 |

**Conclusion:** There was no interference from blank and placebo. The peak purity index was 1.so the method passes the specificity.

2.Accuracy:

a) 50% Accuracy Level

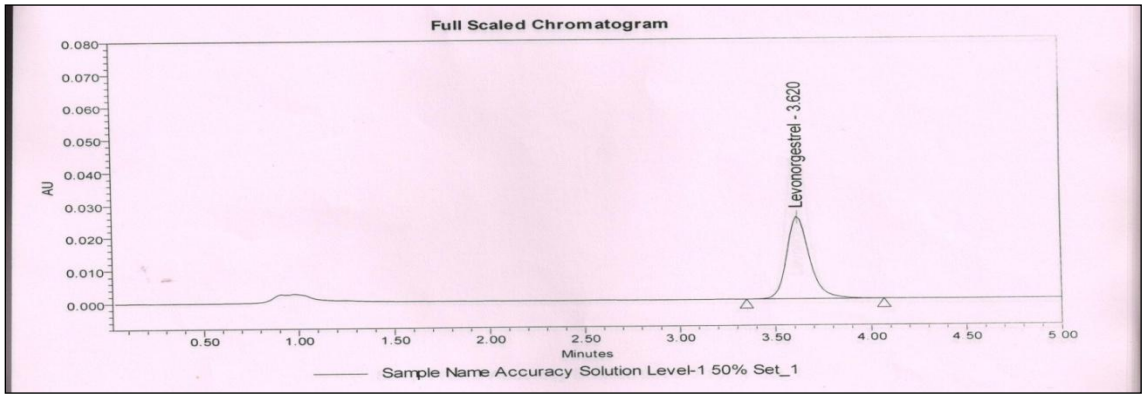


**Fig I :Chromatogram of levonorgestrel at 50% Level (Absorbance vs time)**

**Table 8 : Chromatographic data of Levonorgestrel at 50% Level**

| Sr.no | Peak name | RT | Area | USP Plate count | USP tailing | % Area |
|---|---|---|---|---|---|---|
| 1 | Levonorgestrel | 3.620 | 205683 | 4830 | 1.19 | 100.00 |

**Table 9 : Accuracy of Assay at 50 % level**

| Sample No. | Amount spiked (mg) | Amount recovered (mg) | %Recovery |
|---|---|---|---|
| 1 | 1.30 | 1.32 | 101.6 |
| 2 | 1.30 | 1.32 | 101.6 |

b) 150% Accuracy level:

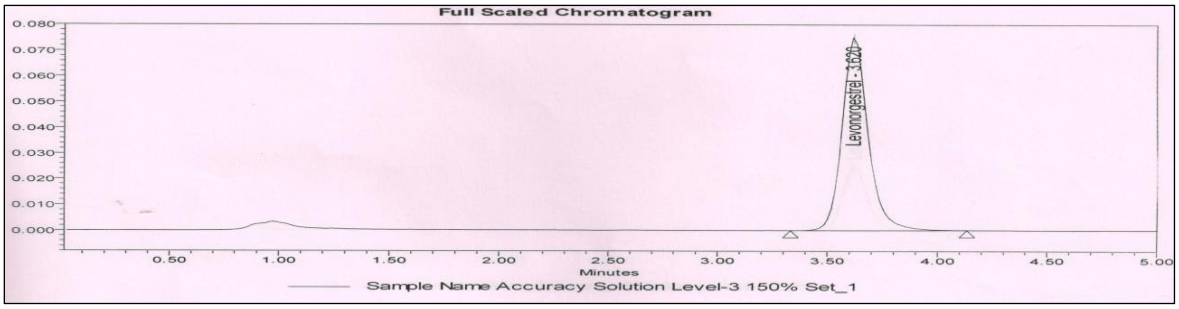


**Fig.J : Chromatogram of levonorgestrel at 150% Level (Absorbance vs time)**

**Table 10 : Chromatographic data of Levonorgestrel at 150% Level**

| Sr.no | Peak name | RT | Area | USP Plate count | USP tailing | % Area |
|---|---|---|---|---|---|---|
| 1 | Levonorgestrel | 3.620 | 614772 | 4833 | 1.19 | 100.00 |

**Table 11 : Accuracy of Assay at 150 % level**

| Sample No. | Amount spiked (mg) | Amount recovered (mg) | %Recovery |
|---|---|---|---|
| 1 | 3.80 | 3.83 | 100.8 |
| 2 | 3.80 | 3.82 | 101.1 |

**Discussion:**

The % Accuracy is within limit (98.0 – 102.0 %) with %RSD less than 2%.So the method is accurate.

**3.Method Precision:**

**Acceptance Criteria:**

The RSD of Assay of five sample preparations should not be more than 2.0%.

**Table 12 : Acceptance Criteria**

| Sr. No. | Standard reading | Test reading | Assay (mg/ tab) |
|---|---|---|---|
| 1 | 412738 | 388150 | 99.7 |
| 2 | 412736 | 387947 | 100.3 |
| 3 | 412735 | 388713 | 100.9 |
| 4 | 412732 | 388915 | 99.8 |
| 5 | 412804 | 388155 | 100.6 |
| 6 | 412988 | 388706 | 99.8 |

**Discussion:**

The RSD of Assay of six sample preparations was found to be 2.0%.So the method was passes the precision test.

**4. Linearity and range:**

**Acceptance criteria:**

The correlation coefficient value should not be less than 0.995 over the working range.

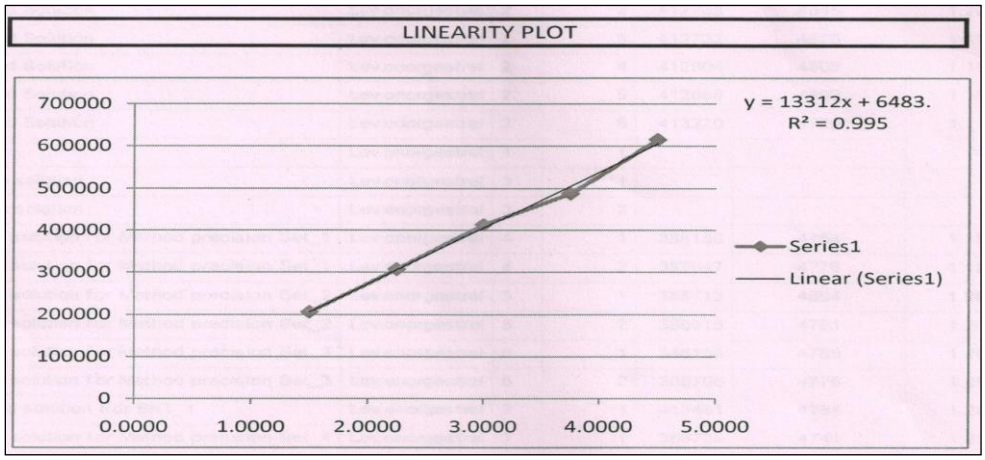


**Figure K: Calibration curve of Levonorgestrel**

**Table 13: Linearity of Levonorgestrel by RP-HPLC Method**

| Linearity level | Levonorgestrel | |
|---|---|---|
| | Conc. ( µg/mL) | Mean area |
| 1 | 1.50 | 206176 |
| 2 | 2.25 | 306917 |
| 3 | 3.00 | 413186 |
| 4 | 3.75 | 488385 |
| 5 | 4.50 | 614671 |
| Correlation coefficient($R^2$) | 0.99781214 | |
| **Slope of regression line** | 133127 | |
| **Y-intercept** | 6483.3 | |

**Conclusion:**

From the calibration curve of Levonorgestrel, the correlation coefficient was found to be 0.999.so the method is linear in the conc. range 1.5-4.5 ug /mL.

**6.4 SUMMARY AND CONCLUSION:**

**A) Summary**

**Table 14 : Summary**

| Sr.No. | Validation Parameter | Acceptance Criteria | Results |
|---|---|---|---|
| 1. | Specificity | 1.There Should not be any interference from blank and Placebo peaks with the main Peak. 2.The Peak Purity index Should be equal to or more than 0.990 | 1.There was no interference from blank and Placebo. 2.The peak purity index was 1.So,passes the Specificity |
| 2. | Recovery(Accuracy) | 1.Accuracy and individual and mean at each level should be between 98.0% to 102% 2.% RSD should not more than 2.0% | 1.The % Accuracy was found to be 101.6%, 100.8%. 2.%RSD was found to be 0.3 %. |
| 3. | Precision | 1.The RSD of Assay of five Sample Preparation Should not be more than 2.0% | 1.The RSD of Assay of five Sample Preparation was found to be 2.0% |
| 4. | Linearity and Range | 1.The Correlation Coefficient value Should not be less than 0.995 over the working range. | 1.The Correlation coefficient was found to be 0.999. |

## B) CONCLUSION:-

The Test method was validated for Specificity, Linearity, Precision, Accuracy (Recovery),Stability of analytical Solution, and Robustness Parameter found to be meeting the predetermined acceptance criteria. The validated method is Specific, Linear ,Precise, Accurate and Robust over Specified Range for determination of Assay of Levonorgestrel in Levonorgestrel Tablets (0.03 mg).

Hence, this method can be used for Q.C Release and Stability analysis for the determination of the assay of Levonorgestrel in Levonorgestrel Tablet. (0.03 mg)

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