International Journal of Advanced Science and Research
ISSN: 2455-4227, Impact Factor: RJIF 5.12
www.allsciencejournal.com
Volume 2; Issue 2; March 2017; Page No. 31-40

Validated gradient stability indicating HPLC method for the determination of related substances of Levonorgestrel in bulk drug and formulation products

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Abstract
A stability-indicating HPLC method has been developed and validated for the determination of Related Substances by HPLC method for Levonorgestrel was performed to demonstrate that the method is suitable for its intended purpose i.e. to determine the Related Substances in Levonorgestrel drug substances in-house. Simple HPLC chromatographic separation was achieved on a Waters Kromasil C_{18} (250mmx4.6mm, 5µm) with mobile phase containing water in gradient combination with acetonitrile (ACN) at a flow rate of 1.0 mL/min and the eluent was monitored at 240 nm. In the developed method, the resolution of Levonorgestrel from any of impurities was found to be greater than 1.5. The test solution and Standard solutions were found to be stable in the diluent for 24 h. The developed method resolved the drug from its impurities.
Regression analyses indicate correlation coefficient value greater than 0.999 for Levonorgestrel. The LOD & LOQ for Levonorgestrel were observed 0.013% and 0.039%. The high recoveries and low standard deviations confirm the suitability of the method.The method has shown good, consistent recoveries for Levonorgestrel and also for its impurities w.r.t. variation in column temperature and flow. The method was found to be accurate, precise, linear, specific, sensitive, rugged, robust, and stability-indicating.

Keywords: Levonorgestrel; Kromasil; ACN; Methanol``; Stability-indicating; Related substances; ICH guidelines; HPLC

1. Introduction
Oral contraceptives are pharmaceutical formulations containing an estrogen in a small amount and a synthetic progestin in 5-30 times bigger amount. Levonorgestrel (or *l*-norgestrel or d-norgestrel is 13β-ethyl-17β-hydroxy-18, 19-dinor-17α- pregn-4-en-20-yn-3-one (fig.1) 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.

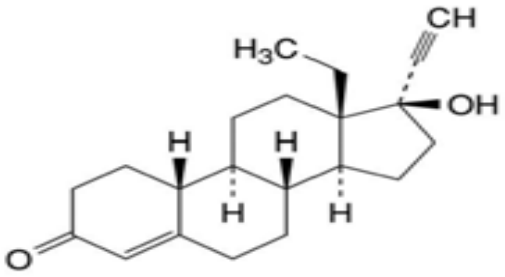

Fig 1: Structure of Levonorgestrel

In Recent years pharmaceutical Preparations Containing Levonorgestrel are available commercially in India as well as other countries. The modern low-dose oral contraceptives require a sensitive, accurate and rapid methods of quantitative determination which is unaffected by the small amount of the estrogen and the large excess of progestogen. Mainly HPLC analytical Method for estimation of Levonorgestrel in Pharmaceutical Preparation are required because HPLC methods have been widely used for routine Quality Control assessment of drug substances and related impurities, because of their sensitivity, repeatability and Specificity.
Regulatory requirements for the identification, qualification, and control of impurities in drug substances and their formulated products are now being explicitly defined, particularly through the International Conference on Harmonization (ICH). It is also recommended by ICH that all routine impurities at or above 0.1% level, should be identified through appropriate analytical methods [7-9]. Numerous analytical methods for the determination of Levonorgestrel in bulk drug as well as in formulations have been reported in literature viz. spectrophotometry [10] and [11], gas chromatography [12], HPTLC [13], HPLC [14-18]. Many other HPLC methods for determination of Levonorgestrel in presence of its related substances (RS) in bulk drug and finished tablets is reported [26] without any comment on the stability indicating potential of the method.
From preceding details of relevant literature it was apparent that a validated method is required to be developed which would be capable for simultaneous determination of Levonorgestrel in the presence of its degrdation impurities, and also serve as stability-indicating. Thus, the aim of current study was to develop and validate an LC method for the determination of Levonorgestrel along with degradation products, in a drug substances and drug product form, in accordance with the ICH guidance document [27].

2. Experimental
2.1. Reagents and chemicals
Qualified standards and samples of Levonorgestrel were

obtained from Surya Pharmaceutical pvt.ltd. Analytical/HPLC grade chemicals and solvents used were obtained from different manufacturers as mentioned in Table:

Table 1

S. No.	Chemicals	Specifications	Manufactures
1.	Acetonitrile	HPLC – Grade	HPLC grade Rankem ltd
2.	Methanol	HPLC – Grade	HPLC grade Rankem ltd
3.	Milli Q Water	Double Distilled	Milli-Q RO system
4.	Hydrochloric acid	AR – Grade	Merck
5.	Sodium hydroxide	AR- Grade	Rankem
6.	Hydrogen Peroxide	AR- Grade	Rankem
7.	Levonorgestrel	Active Pharmaceutical Ingredient	Surya Pharmaceutical India

2.2. Chromatography apparatus and conditions

The chromatograph consisted of Waters alliance 2695 HPLC system with UV and PDA (Photo diode array detector). The data were evaluated by Empower2 Software.

Levonorgestrel was freely soluble in selected analytical solvents like methanol (MeOH). The chromatographic conditions were optimized by different means (using different columns, different buffers and different organic phases). Early chromatographic work was performed with different Inertsil ODS C_{18} and Gemini C_{18} columns as stationary phase and various combinations of buffered (pH 2–7) phases, water & organic phases (Methanol, ACN) mixtures. The flow rate of mobile phase was varied within 0.50–2.0 mL/min. Various isocratic and gradient modes were tried. Wavelength for monitoring the eluent was selected by scanning standard solution of drug within 200–400 nm using PDA detector.

All noted measurements were performed with an injection volume of20 µL and UV detection at 240 nm of samples dissolved in a diluent; Mobile phase-A [Water, filtered through 0.22µm filter; Mobile phase-B [ACN].The optimized gradient programme is Time(min)/%Mobile Phase-B (0 min/40% ,8min/45% ,35min/45% ,45min/40% ,and 55min/40%).

2.3. Preparation of solutions

2.3.1. Preparation of Blank solution

Methanol (diluents) was injected as blank solution.

2.3.2. Preparation of laboratory mixture solutions

Appropriate amounts of active pharmaceutical ingredient Levonorgestrel (1000 µg/mL as stock solution) and (1 µg/mL) were individually prepared by dissolving their appropriate amounts in the diluent.

2.3.3. Preparation of sample solution

An amount of 50mg of active pharmaceutical ingredient (Levonorgestrel) was transferred to a 50mL volumetric flask. Diluent (40 mL) was added to it and sonicated for 5 min with intermittent shaking and diluted to volume with the diluent.

2.4. System suitability

System suitability parameters were evaluated for the verification of the analytical system is working properly and suitable to get reliable results. Parameters such as peak tailing factor, Theoritical plates and % RSD of area obtained from standard solutions of Levonorgestrel were evaluated.

2.6. Analytical method validation

2.6.1. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc [12]. Specificity can be assessed in two parts.

In one part of specificity, there should be no- interference of blank peak with Levonorgestrel peak.

Purity angle should be less than Purity Threshold in standard solution and sample solution. Levonorgestrel peak should not have any Flag in purity results. In other part of specificity drug was exposed to different stress conditions[13] like hydrolysis conditions in all the pH ranges (Acid ,Alkaline and Neutral water) and oxidative conditions at a concentration of 1 mg/mL. The drug samples were also subjected to thermal and photolytic degradation. The stressed samples were analysed on HPLC with Photo diode array detector and calculations were done using standard solution. The Peak purity evaluation and mass balance studies were done for each type of stress study.

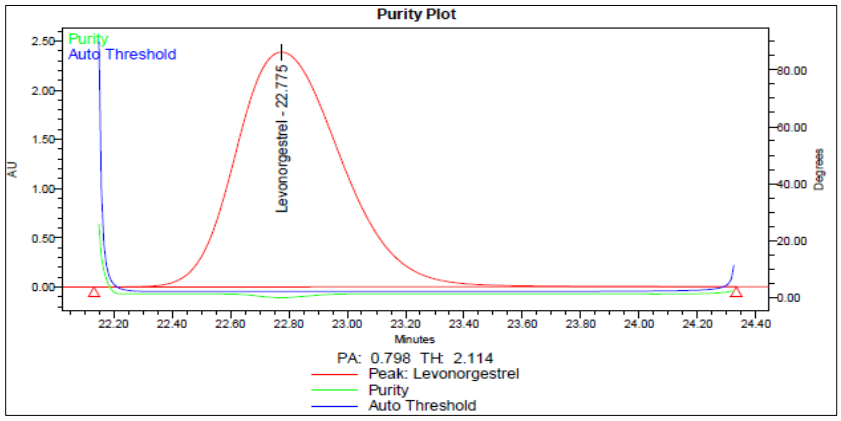

Fig 2

2.6.2. Linearity and Range

Linearity of the method was performed according to ICH Quality guidelines. Suitable solutions with appropriate known concentrations were prepared from stock solution of Levonorgestrel. Stock solution was diluted with the diluent to get solutions containing target concentrations. Linearity of Levonorgestrel was determined over a range of obtained limit of quantification (shown in Table.1) to 150% of specification limit (range was inclusive of concentrations at LOQ, 50, 80, 90,100, 110, 120, and 150%).Calibration curve was obtained by plotting the peak areas of Levonorgestrel versus its corresponding concentration. A graph of peak area vs. concentration (µg/mL) was plotted. Values of the coefficient of correlation, regression and slope of the calibration curve were calculated.

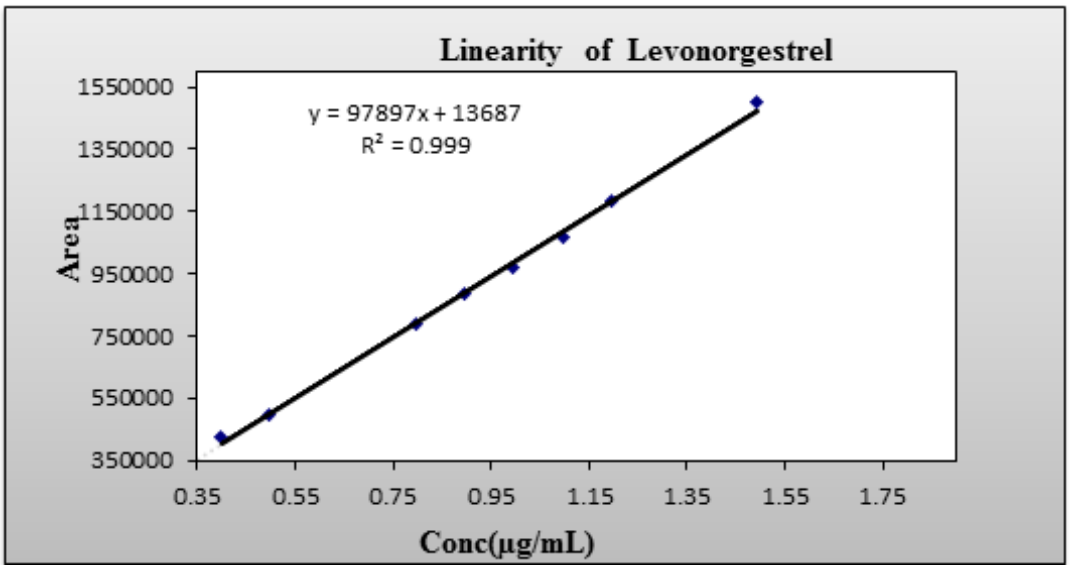

Fig 3

Table 2: Linearity parameters of the calibration curves for Levonorgestrel.

Compound	Linearity range (µg/mL)	*R*	Slope	Intercept
Levonorgestrel	0.40-1.50	0.9991	978972.96	13686.98

2.6.3. Precision

Six solutions containing Levonorgestrel (1000 µg/mL) were prepared. Chromatography was performed by HPLC and value of % RSD was calculated for Percentage of highest impurity and total impurities by considering peak area for Levonorgestrel standard and each impurity. In a similar way intermediate precision of the method was also evaluated on different day by another analyst in the same laboratory.

2.6.4. Limit of detection (LOD) and limit of quantification (LOQ)

Limit of Detection (LOD) and Limit of Quantitation (LOQ) level for Levonorgestrel were determined based on Signal to Noise ratio. The Limit of detection (LOD) and Limit of Quantitation (LOQ) Level of Levonorgestrel were derived based on the concentration of LOD and LOQ solutions. The LOD and LOQ were estimated by signal-to-noise ratio of 3:1 and 10:1, respectively, injecting a series of diluted solutions with known concentrations.

2.6.5. Accuracy

Accuracy studies were done for triplicate at concentration levels of LOQ, 100 and 150% of Levonorgestrel (1000 µg/mL) to evaluate the recovery and accuracy of the proposed method.

2.6.6. Stability of analytical solutions

The Sample solution was prepared as directed in the methodology and was stored at refrigerator temperature (2-8°C) and room temperature. The stored solutions were injected at initial, 12 hrs and 24 hrs. The difference in percentage of impurities obtained with initial results was calculated.

2.6.7. Robustness

The method was subjected with little variations by changing the mobile phase flow rate (±0.02 mL/min), and varying the temperature from normal (20°C-30°C). Chromatograms of Levonorgestrel and Standard Solution solutions were evaluated by applying system suitability parameters with the robustness changes made.

3. Results and discussion

3.1. Development of the stability-indicating HPLC method

In this study, chromatographic conditions such as the wavelength, mobile phase, column, and column temperature and flow rate were optimized in order to limit run time while obtaining the best possible peak symmetry and resolution. Levonorgestrel was scanned by a UV detector with variable wavelength to determine its most significant UV wavelength for quantitative purposes. The results showed the best absorption was 240 nm for Levonorgestrel.

Too much solvent and matrix interferences were found when the wavelength was less than 220 nm. In order to minimize the interferences, a wavelength of 240 nm was selected for the detection of Levonorgestrel (Fig. 2).The peak shapes of Levonorgestrel were not sharp and symmetrical when a mobile phase consisted of MeOH–water, and in combination with Gemini C18, Inertsil C18 and Kromasil C18 columns. Peak shapes were significantly improved with mobile phase of ACN–water. Still peak shapes with Gemini C18 and Inertsil C18 column were not significantly good. However, the peak shapes with Kromasil C18 columns were looking good but needed to be optimized by using applying different mobile phase ratios for ACN-Water. Good peak shape and reasonable retention time with measurable resolution were obtained by applying gradient program. Different mobile phase flow rates (0.5, 1.0 and 2.0 mL/min) were investigated. The best resolution from all the peaks present and degradants products for Levonorgestrel were obtained when flow rate was 1.0 mL/min.

Based on the development study a simple mobile phase filtered and degassed water is selected as mobile phase-A and Acetonitrile as counterpart viz. mobile phase-B. The chromatographic elution is performed in Kromasil C_{18} columns. The separations were achieved by modifying different gradients programs. The finalized method has a gradient composition of Time(min)/%Mobile Phase-B (0 min/40% ,8min/45% ,35min/45% ,45min/40% ,and 55min/40%) with Kromasil C_{18} (250mmx4.6mm, 5µm) column, 25°C as column oven temperature, injection volume 20µL,column temperature 25°C and eluent is monitored at 240nm.

3.2. System suitability

System suitability is the primary requirement of the any methodology, to ensure that the working conditions are fit for its intended use. The chromatography was performed with Kromasil C18 (250mm length, 4.6mm I.D and 5.0µm particle size) with gradient mentioned in chromatography conditions. A representative is shown in Fig.2, which shows tailing factor for Levonorgestrel is less than 2.0, Theoretical Plates are more than 5000. The response ratio obtained for two 0.1% Levonorgestrel standard injections is between 0.95 to 1.05.

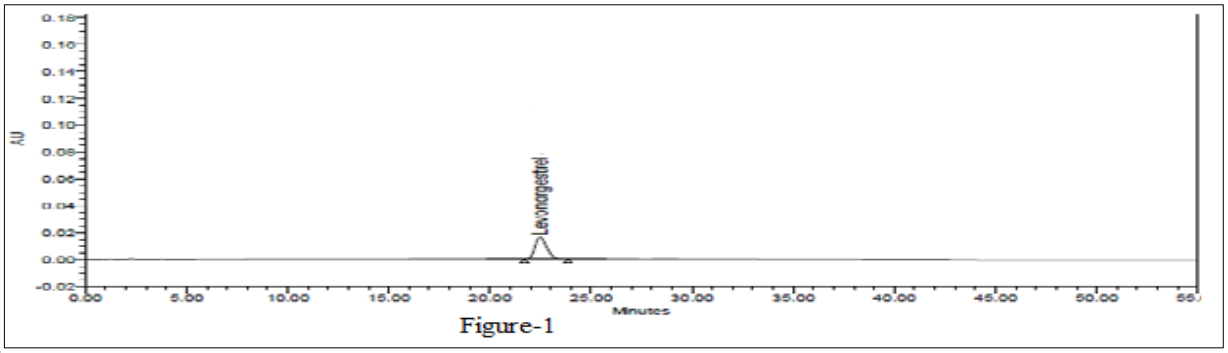

Fig 4: Tailing factor, a parameter that ICH guidelines consider as a factor to be controlled, was within the established limits.

3.3. Specificity

The HPLC chromatograms recorded separately for Blank and Levonorgestrel are displayed in Fig.3 and Fig.4 respectively. The tailing factor for Posaconazole peak is 1.0. Peak purity graph shows that there is no interference in Levonorgestrel peak with respect to components present in sample matrix. Thus the HPLC method presented in this study is specific for Levonorgestrel. To have stability indicating nature of the method, forced degradation studies of Levonorgestrel evaluated and the following degradation behaviour is shown, the results were tabulated in Table-2

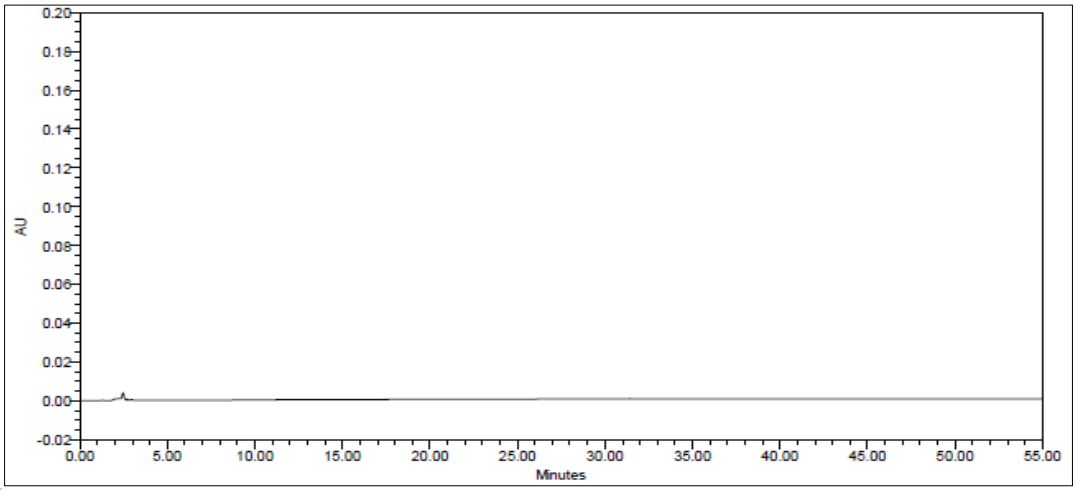

Fig 5

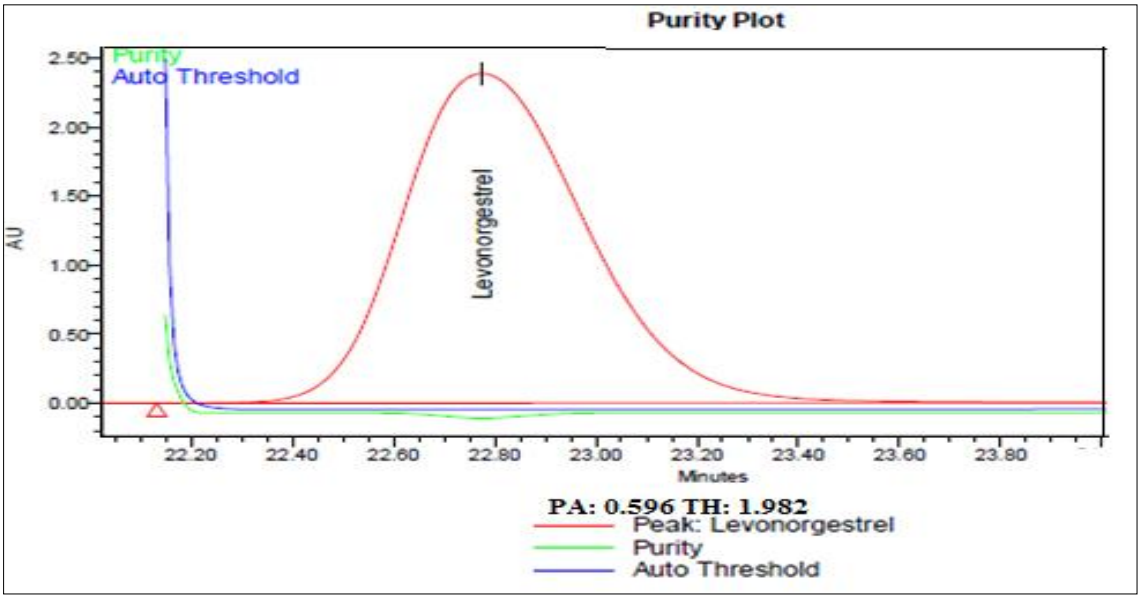

Fig 6: Specificity part-B (stress) studies representing degradation in various parameters.

Solid State Stress condition	%Total Impurities	% Degradation	Purity Angle	Purity Threshold	Peak Purity results
Control Sample	0.05	--	0.596	1.982	Pass
Thermal Degradation (105°C) for 24Hours	0.05	0.00	0.650	1.802	Pass
UV Degradation 200Wh/m^2	0.05	0.00	0.709	1.912	Pass
Visible Degradation 1.2millon Lux hours	0.05	0.00	0.632	1.703	Pass
Humidity Degradation (90% RH at 25°C168Hours)	0.05	0.00	0.637	1.606	Pass
Liquid State Stress condition					
Control Sample	0.05	--	0.689	1.784	Pass
Stressed with 2N HCl 24hrs at 60° C	7.32	7.27	0.024	0.433	Pass
Stressed with 2N NaOH 24hrs at 60° C	9.86	9.81	0.096	0.358	Pass
Stressed with 30% H2O2 24hrs at 60°C	3.02	2.97	0.202	0.857	Pass

3.4.1. Degradation in acidic conditions

Levonorgestrel was observed to be degraded to about 7% in acidic conditions, when treated with 2N HCl for 24hours at 60 ° C. The chromatogram obtained on analyzing the stability sample displayed more than ten peaks for degradation products; degradation product as shown in (Fig. 7B).

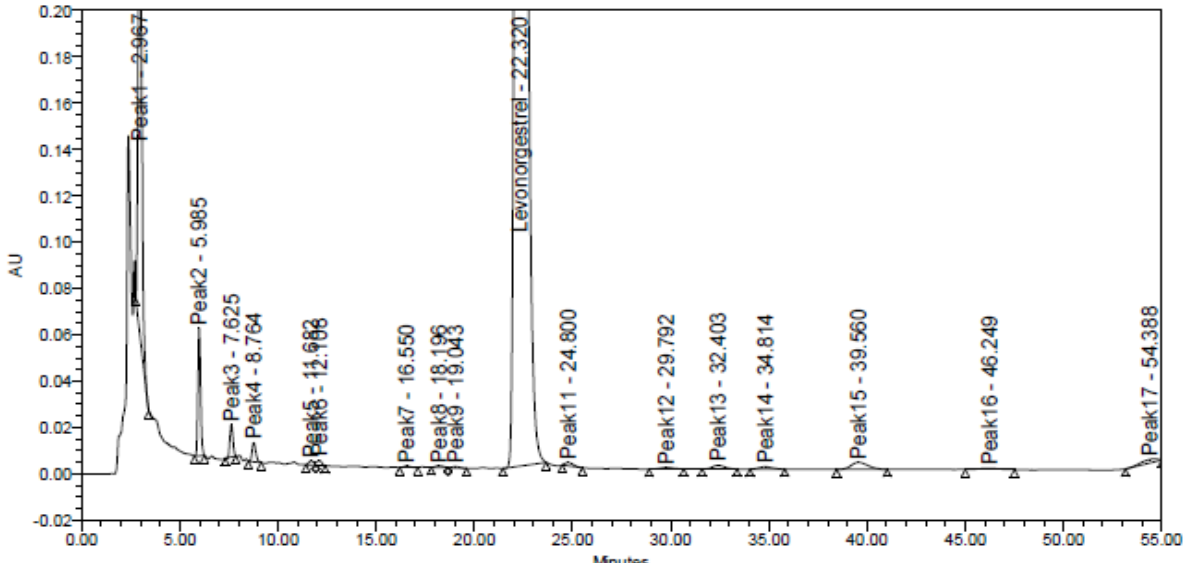

Fig 7: Chromatograms of acid stressed samples treated with 2N HCl at 60 ° C for 24Hours.

3.4.2. Degradation in basic conditions

Levonorgestrel was found to be degraded to 10% under basic conditions, when treated with @N NaOH for 24hours at 60 ° C. The chromatogram obtained on analyzing the stability sample displayed more than five peaks for degradation products; degradation product as shown in Fig. 8B.

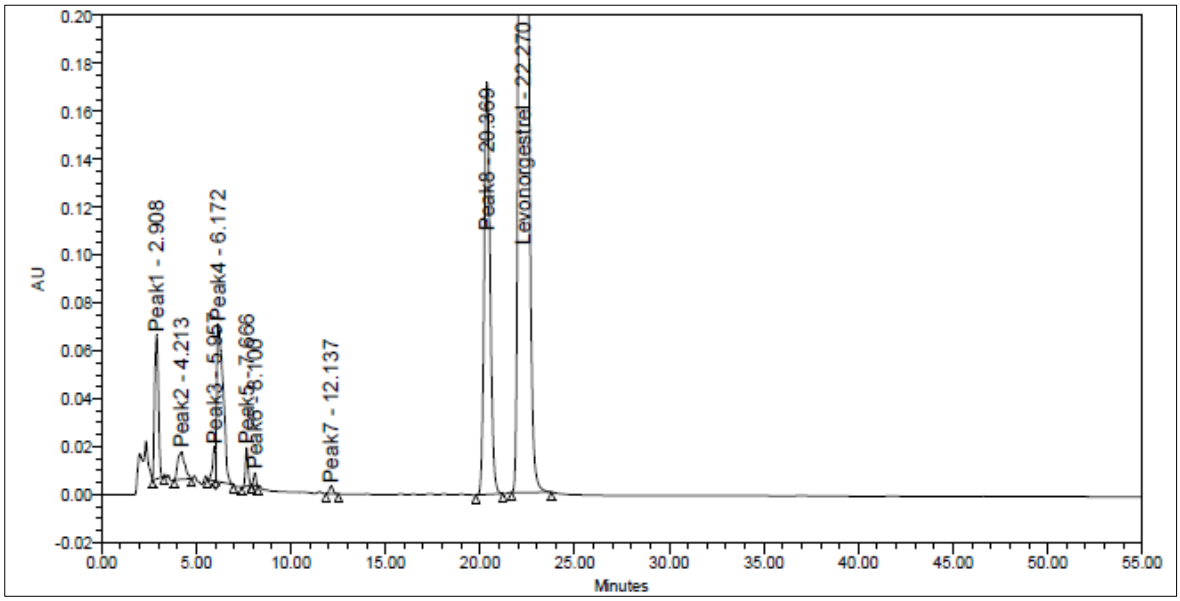

Fig 8: Chromatograms of alkali stressed samples treated with 2n NaOH at 60 ° C for 24hours

3.4.3. Degradation under oxidative conditions

Levonorgestrel was observed to be degraded to about 3% Oxidative conditions, when treated with 30%H2O2 for 24hours at 60 ° C. The chromatogram obtained on analyzing the stability sample displayed more than five small peaks for degradation products; degradation product as shown in (Fig. 9B).

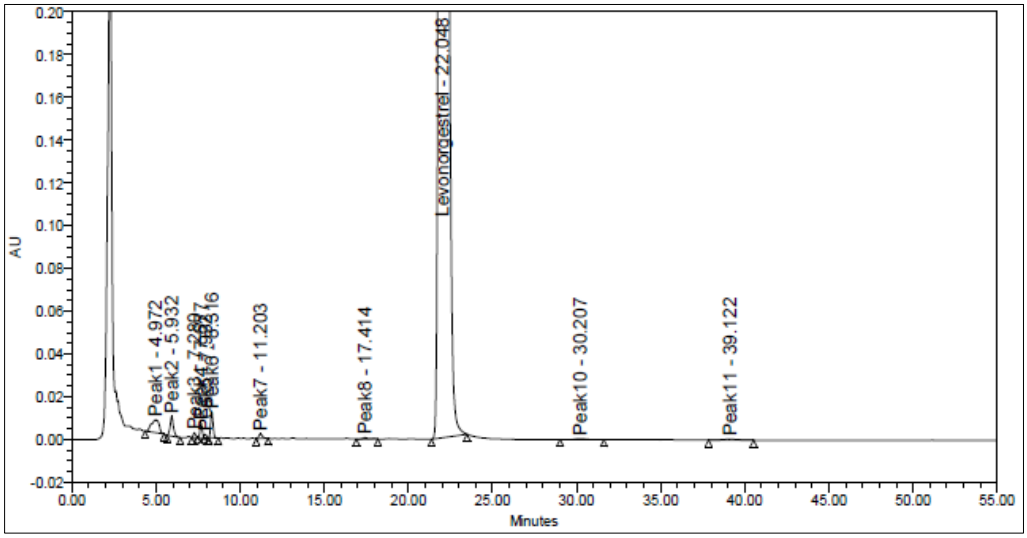

Fig 9: Chromatograms of peroxide stressed samples treated with 30% H_2O_2 refluxed at 60 ° C for 24 hours

3.4.4. Degradation in photolytic conditions

Levonorgestrel was found to be practically stable under the exposed conditions with an overall illumination of 1.2 million lx h with near-UV energy≥200 Wh/m^2; the chromatogram is given in Fig. 10. This suggests that the drug was stable under photolytic conditions exposed for the period of study.
Figure 10.

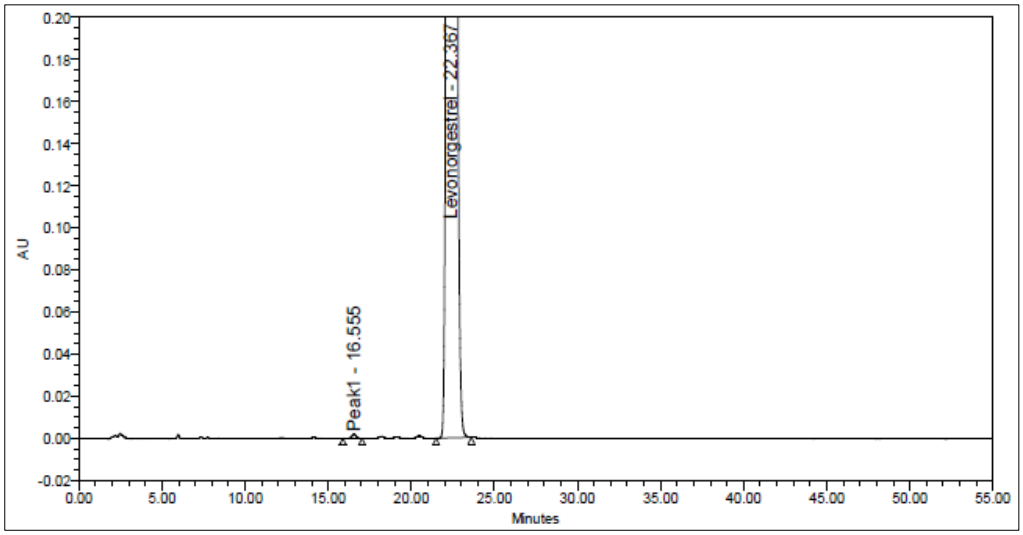

Fig 10

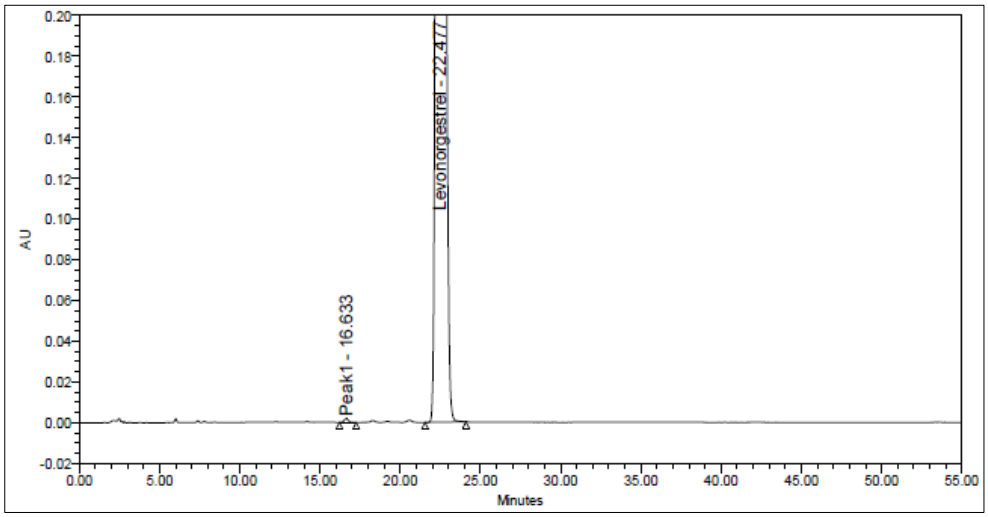

Fig 11: Chromatogram of photo stressed sample.

3.4.5. Thermal degradation

Levonorgestrel was found to be practically stable with dry heat as no degradation was observed when exposed to thermal heat at 105 ° C for 24 hours; the chromatogram is given in Fig. 11.

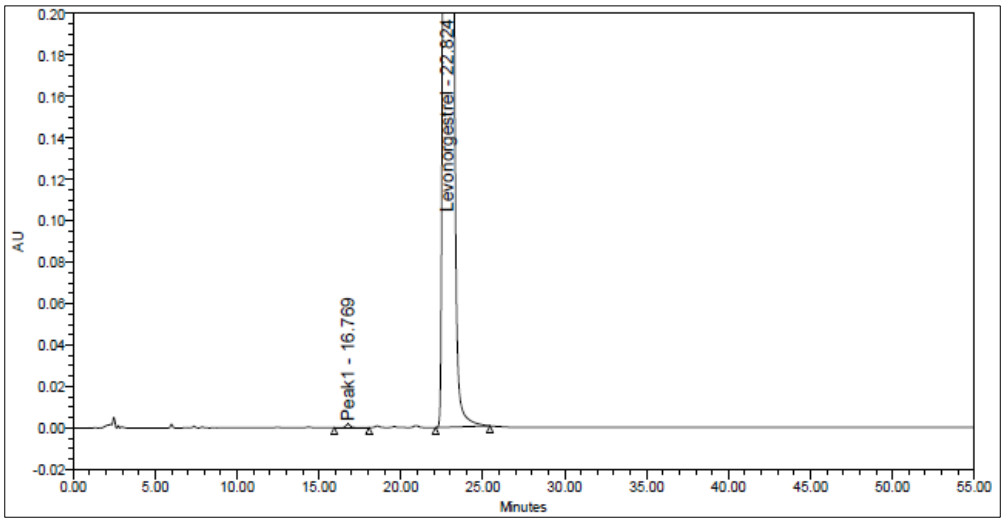

Fig 12: Chromatogram of thermal stressed sample.

3.4.6. Degradation under Humidity conditions

Levonorgestrel was found to have a negligible degradation of about 0.20% when treated under 90% RH at 25°C for 168Hours under neutral conditions; the chromatogram is given in Fig. 12.

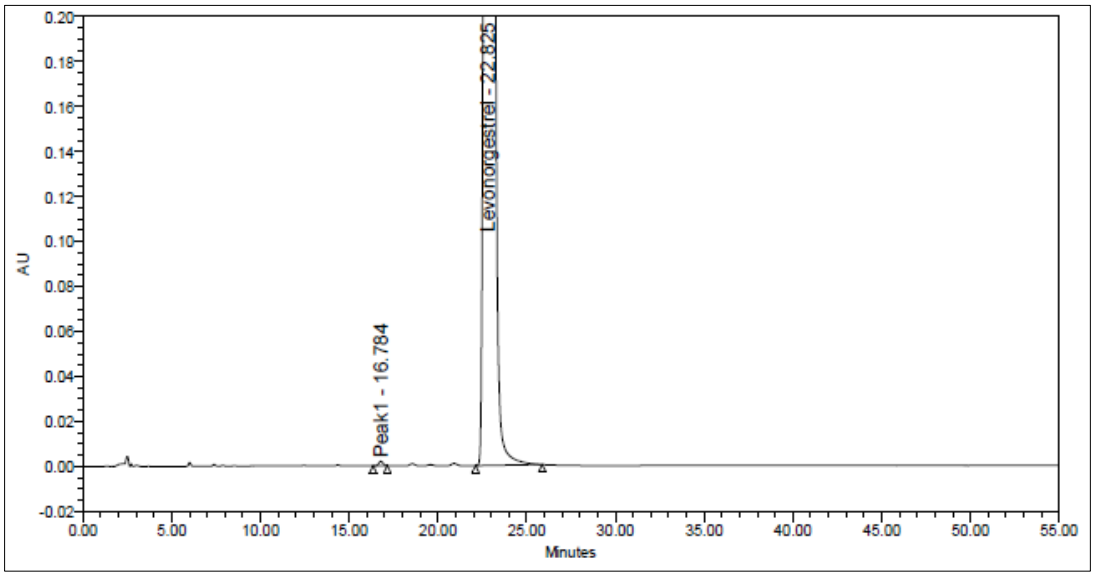

Fig 13: Chromatogram of neutral stressed sample under humidity

3.5. Linearity

Calibration curves for Levonorgestrel, examined in pure solutions as well as in the laboratory mixture solutions, were found to be linear; correlation coefficients≥0.999 in all the cases. Table 1 enlists the linearity parameters of the calibration curves for Levonorgestrel in laboratory mixture. Statistical treatment of the linearity data of Levonorgestrel shows a linear response between lower levels to highest level. In addition, the analysis of residuals shows values randomly scattered around zero, which fits well within the linear model. The origin of linearity curve was within the lower and the upper limit of 95% that gives high degree of confidence to the value obtained for intercept.

3.6. LOD and LOQ

LOD and LOQ, as a measure of method sensitivity, were provided for degradation products and impurity calculated by means of signal-to-noise ratio. The LOD and LOQ for Levonorgestrel are tabulated in Table 4. From the results, it can be concluded that the proposed method can quantify small quantity of impurities in Levonorgestrel samples.

Table 3: LOD and LOQ results for Levonorgestrel.

Compound	LOD			LOQ		
	Concentration (mg/ml)	Concentration w.r.t. Sample	s/n	Concentration (mg/ml)	Concentration w.r.t. Sample	s/n
Levonorgestrel	0.14	0.015	3.78	0.44	0.044	12.62

3.7. Precision and repeatability

The results obtained for repeatability studies and for intermediate precision are presented in Table 5. Values of % RSD for system precision of Levonorgestrel were 0.0. Method precision has a % RSD 0.0 for repeatability and 0.0 for intermediate precision, which comply with the acceptance criteria.

Intra-day and intermediate precision of Levonorgestrel (% RSD of *n*=6 injections of test concentration).

Table 4

Sample Preparations	Method Precision		Intermediate Precision	
	Single Maximum impurity(%w/w)	Total impurities (%w/w)	Single Maximum impurity(%w/w)	Total impurities (%w/w)
Preparation-1	0.05	0.07	0.05	0.07
Preparation-2	0.05	0.07	0.05	0.07
Preparation-3	0.05	0.07	0.05	0.07
Preparation-4	0.05	0.07	0.05	0.07
Preparation-5	0.05	0.07	0.05	0.07
Preparation-6	0.05	0.07	0.05	0.07
Average	0.05	0.07	0.05	0.07
% RSD	0.00	0.00	0.00	0.00

Table 5

Sample Preparations	Comparison Table (Method VS Intermediate Precision)			
	Single Maximum impurity(%w/w)		Total impurities(%w/w)	
	Method Precision	Intermediate Precision	Method Precision	Intermediate Precision
Preparation-1	0.05	0.05	0.07	0.07
Preparation-2	0.05	0.05	0.07	0.07
Preparation-3	0.05	0.05	0.07	0.07
Preparation-4	0.05	0.05	0.07	0.07
Preparation-5	0.05	0.05	0.07	0.07
Preparation-6	0.05	0.05	0.07	0.07
Average	0.05	0.05	0.07	0.07
% RSD	0.00	0.00	0.00	0.00
Overall %RSD	0.00		0.00	

3.8. Accuracy

Accuracy of method can be inferred with the help of Specificity, Method Precision and Linearity.

3.9. Stability in analytical solution

Sample solution was prepared as directed in the methodology and was stored at refrigerator temperature (2-8°C) and room temperature. The stored solutions were injected at initial, 12 hrs and 24 hrs.

Table 6: Results of Single Maximum Impurity in sample solution

Time (hrs)	Single Max. Impurity			
	At Refrigerated Conditions (2-8°C)		Room temperature	
	Injection Area	Response Ratio	Injection Area	Response Ratio
Initial	460172	-	460172	-
12 hours	471164	102.4	470856	102.3
24 hours	469752	102.0	471462	102.5

Table 7: Results for Total Impurities in sample solution

Time (hrs)	Single Max. Impurity			
	At Refrigerated Conditions (2-8°C)		Room temperature	
	Injection Area	Response Ratio	Injection Area	Response Ratio
Initial	701822	-	701822	-
12 hours	702984	100.2	696789	99.3
24 hours	699896	99.7	699426	99.7

The response ratio's w.r.t. initial for Single Max. And Total Impurities in sample solution at different time intervals are in the range of 95% to 105% when stored at Refrigerated conditions (2-8°C) and room temperature for 24hours, which are well within the acceptance criteria of not more than ±5.0 % variation. Based on the obtained data it is concluded that sample solution can be stored and used up to24hours when stored at Refrigerator temperature (2-8°C) and room temperature.

3.10. Robustness

The robustness of the method is verified for the method by changing small variations of chromatographic conditions by changing the mobile phase flow rate (±0.02 mL/min), and changing the temperature from normal (±5˚C). Chromatograms of Levonorgestrel standards and sample solutions were evaluated by applying system Suitability parameters with the robustness changes made. There are no variations observed in system suitability Criteria and results obtained for as such sample.

Table 8: Results for Single Highest impurity

Conditions	% of Single Highest impurity	
	% of Single Highest impurity	% Difference w.r.t original condition.
Original (Method Precision -Sample preparation-1)	0.05	---
Flow rate variation –Low Flow (0.8 mL/min)	0.05	0.00
Flow rate variation-High Flow (1.2mL/min)	0.05	0.00
Column oven Temperature Variation 20°C	0.05	0.00
Column oven Temperature Variation 30°C	0.05	0.00

Table 9: Results for Sum of Impurities

Conditions	*% Sum of All impurities*	
	% Sum of All impurities	*% Difference w.r.t original condition.*
Original (Method Precision -Sample preparation-1)	0.07	---
Flow rate variation –Low Flow (0.8 mL/min)	0.07	0.00
Flow rate variation-High Flow (1.2mL/min)	0.07	0.00

Column oven Temperature Variation 20°C	0.07	0.00
Column oven Temperature Variation 30°C	0.07	0.00

Method robustness checked after deliberate alterations of flow and temperature shows that the changes of the operational parameters do not lead to essential changes of the performance of the chromatographic system; results are displayed in Table 8. Tailing factor for Levonorgestrel always ranged from 1 to 1.5 and the components were well separated. The percent recoveries of Levonorgestrel were good and did not show a significant change when the critical parameters were modified. Considering the results of modifications in the system suitability parameters and the specificity of the method, it would be concluded that the method conditions are robust.

4. Conclusion

The proposed HPLC method for estimation of related substances for Levonorgestrel is analyzed in drug substance as per ICH guidelines. The method is found to be specific for the estimation of known, unknown impurities and degradation products. The method is also stability indicating as evident from results obtained when method applied to stability samples. The assay utilized a previously unreported set of conditions, including a gradient ramp and simple mobile phases, to effect the separation without using an ion-pair reagent. LOD and LOQ, established by this method, are lesser than earlier reported methods. The method is found to be linear in the specified range, precise and robust. Accuracy of the method is also established for the formulation. Hence, the proposed method stands validated and may be used for routine and stability sample analysis.

5. References

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