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VALIDATED GRADIENT STABILITY INDICATING HPLC METHOD FOR THE DETERMINATION OF RELATED SUBSTANCES OF LEVONORGESTREL IN BULK DRUG AND FORMULATION PRODUCTS

Rohit Dhull*[1] and Satyavir Singh[1]

[1*]Department of Chemistry, OPJS University, Churu, Rajasthan, India.

ABSTRACT

A HPLC method was developed for determination of Related Substances in Levonorgestrel drug substances using Kromasil C18 (250mmx4.6mm, 5µm) column and a mobile phase containing water in gradient combination with acetonitrile (ACN) with flow rate of 1.0mL/min and the chromatogram was monitored using UV Detector at 240nm wavelength. Resolution between Levonorgestrel and its impurities were found more than 1.5 and the stability of test solution and Standard solutions were found upto 24 hours. The LOD and LOQ for Levonorgestrel were observed 0.013% and 0.039%. The analytical method validation of proposed method was performed for various parameters like System suitability, Specificity (interference from Blank and Impurities), Force degradation, LOD and LOQ, Accuracy (Recovery), Linearity, System Precision, Method Precision and Intermediate Precision (Ruggedness) along with Solution stability. The range of method was defined from the data of Precision, Linearity and Accuracy. The Range of method has LOQ to 150% of specification limit. All results for all the validated parameters were found within the acceptance criteria. HPLC method was specific, accurate, precise and suitable for the analysis of Levonorgestrel drug substance and drug products.

KEYWORDS

Levonorgestrel, Kromasil, CAN, Methanol, Stability-indicating, Related substances, ICH guidelines, HPLC and Method Validation.

Author for Correspondence:

Rohit Dhull,
Department of Chemistry,
OPJS University, Churu, Rajasthan, India.

Email: dhull.rohit123@gmail.com

INTRODUCTON

Oral contraceptives are being formulated using an estrogen in a small amount and a synthetic progestin in 5-30 times than the estrogen. Levonorgestrel (or l-norgestrel or d-norgestrel is 13β-ethyl-17β-hydroxy-18, 19-dinor-17α- pregn-4-en-20-yn-3-one (Figure No.1) is a second-generation synthetic progestogen used as an active ingredient in some hormonal contraceptives, including combined oral

contraceptive pills including progestogen only pills and emergency contraceptive pills. Levonorgestrel is also used in preparation/manufacturing of intrauterine system, contraceptive implants and hormone replacement therapy.

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. HPLC analytical methods have been widely used for analysis in quality assessment of active substance (API's) and their related impurities, because of their sensitivity, repeatability and Specificity. So, method for estimation of Levonorgestrel is developed and validated using HPLC instrument.

Various Regulatory requirements from across the world are now being explicitly defined for identification, qualification and control of impurities in drug substances and drug products.

Regulatory guidelines for validation of analytical methods are particularly defined through the International Council for Harmonization of Technical Requirements of Pharmaceuticals for Human Use (ICH). As per ICH recommendation that all impurities present in drug substances and drug products at or above 0.1% level, should be identified through appropriate analytical techniques and methods[1-3]. Numerous analytical methods for the determination of Levonorgestrel in bulk drug as well as in formulations have been reported in literature viz. MS/MS[4], LCMS[5,6], capillary liquid chromatography and capillary electrochromatography[7], UV/fluorescence detection[8], HPTLC[9] and bivariate calibration method and derivative spectrophotometry[10]. Many other stability indicating HPLC[11-13] methods for determination of Levonorgestrel and its related substances (RS) in bulk drug and finished tablets are reported.

METHOD DEVELOPMENT

Instrument, Chemicals and Reagents

Instrumentation

Water alliance 2695 HPLC system with UV and PDA (Photo diode array detector) detector and Kromasil C18 (250mmx4.6mm, 5µm) with a mobile phase containing water in gradient combination with acetonitrile (ACN) at a flow rate of 1.0mL/min. The data were evaluated by Empower2 Software. All the weighing in the experiments was done with Mattler toledo electronic balance capable of measuring with an accuracy of 0.01mg.

Glassware

All the glassware volumetric used in the study was A-grade.

Preparation of Diluent

Methanol (diluents) was injected as blank solution.

Preparation of Standard Solution

Weighed accurately about 100mg of Levonorgestrel standard into 100mL clean and dry volumetric flask, dissolve and make up the volume with diluent. (Stock-I).

Pipette out 1.0mL of Stock-I into 100mL volumetric flask and make volume up to the mark with diluent (Stock-II).

Pipette out 5.0mL of Stock-II into 50mL volumetric flask and make volume up to the mark with diluent. (Approx.concentration 1.0ppm).

Preparation of test solution

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

VALIDATIONOF HPLC METHODS

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 chromatogram is shown in Figure No.1, 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.

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

Specificity and force degradation

The HPLC chromatograms recorded separately for Blank and Levonorgestrel are displayed in Figure No.2, Figure No.3 and Figure No.4 respectively. The tailing factor for Levonorgestrel peak is 0.8. 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 behavior is shown, the results were tabulated in Table No.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 (Figure No.5).

Degradation in basic conditions

Levonorgestrel was found to be degraded to 10% under basic conditions, when treated with 2N 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 Figure No.6.

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 Figure No.7.

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 Figure No.8 and Figure No.9. This suggests that the drug was stable under photolytic conditions exposed for the period of study.

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 Figure No.10.

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 Figure No.11.

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.

Linearity

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 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.

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 No.2 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.

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 No.2. From the results, it can be concluded that the proposed method can quantify small quantity of impurities in Levonorgestrel samples.

Precision and repeatability

Six solutions containing Levonorgestrel (1000µg/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. The results obtained for repeatability studies and for intermediate precision are presented in Table No.3. 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.

Accuracy

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

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. Results are tabulated in Table No.4 and Table No.5.

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 to 24hours when stored at Refrigerator temperature (2-8°C) and room temperature.

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 (±0.02mL/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. Results are summarized in Table No.6 and Table No.7.

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. Tailing factor for Levonorgestrel always ranged from 1 to 1.5, Theoretical plates were ranging from 9000-15000 and the %RSD observed for six replicate injections of Standard solution was observed in the range 0.2-1.6. 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.

RESULTS AND DISCUSSION

In this study, chromatographic conditions such as the wavelength, mobile phase, column, and column

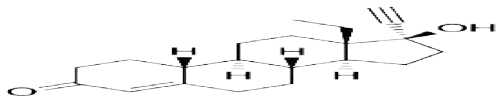
temperature and flow rate were optimized in order to limit run time while obtaining the best possible peak symmetry and resolution. Levonorgestrel was scanned by a UV detector with variable wavelength to determine its most significant UV wavelength for quantitative purposes. The results showed the best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

Too much solvent and matrix interferences were found when the wavelength was less than 220nm. In order to minimize the interferences, a wavelength of 240nm was selected for the detection of Levonorgestrel (Figure No.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 2.0mL/min) were investigated. The best resolution from all the peaks present and degradants products for Levonorgestrel were obtained when flow rate was 1.0mL/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.

Chemical structure of n-nitrosodimethylamine and metformin hydrochloride

Levonorgestrel ***Chemical formula:*** $C_{21}H_{28}O_2$ ***Molecular weight:*** 312.453	

The following reagents and chemicals were used during the evaluation studies

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

Chromatographic Conditions for LC

S.No	Parameters	Description
1	Mobile Phase A	Milli-Q-water
2	Mobile Phase B	Acetonitrile
3	Flow rate	1.0ml/min
4	Column Temp.	25°C
5	Sample temperature	25°C
6	Injection volume	20µl
7	Run time	55 minutes

Gradient Program for LC

S.No	Time (Minutes)	Mobile phase A (% v/v)	Mobile phase B (% v/v)
1	0.00	60	40
2	8.0	55	45
3	35.0	55	45
4	45	60	40
5	55	60	40

Table No.1: Specificity part-B Stress studies representing degradation in various parameters

S.No	Solid State Stress condition	%Total Impurities	% Degradation	Purity Angle	Purity Threshold	Peak Purity results
1	Control Sample	0.05	--	0.596	1.982	Pass
2	Thermal Degradation (105°C) for 24Hours	0.05	0.00	0.650	1.802	Pass
3	UV Degradation 200Wh/m^2	0.05	0.00	0.709	1.912	Pass
4	Visible Degradation 1.2millon Lux hours	0.05	0.00	0.632	1.703	Pass
5	Humidity Degradation (90% RH at 25°C168Hours)	0.05	0.00	0.637	1.606	Pass
Liquid State Stress condition						
6	Control Sample	0.05	--	0.689	1.784	Pass
7	Stressed with 2N HCl 24hrs at 60° C	7.32	7.27	0.024	0.433	Pass
8	Stressed with 2N NaOH 24hrs at 60° C	9.86	9.81	0.096	0.358	Pass
9	Stressed with 30% H2O2 24hrs at 60°C	3.02	2.97	0.202	0.857	Pass

Table No.2: LOD and LOQ results for Levonorgestrel

S.No	Compound	LOD			LOQ		
		Concentration (mg/ml)	Concentration W.R.T. Sample	s/n	Concentration (mg/ml)	Concentration W.R.T. Sample	s/n
1	Levonorgestrel	0.14	0.015	3.78	0.44	0.044	12.62

Table No.3: Intra-day and intermediate precision of Levonorgestrel (% RSD of *n*=6 injections of test concentration)

S.No	Sample Preparations	Method Precision		Intermediate Precision	
		Single Maximum impurity (%w/w)	Total impurities (%w/w)	Single Maximum impurity (%w/w)	Total impurities (%w/w)
1	Preparation-1	0.05	0.07	0.05	0.07
2	Preparation-2	0.05	0.07	0.05	0.07
3	Preparation-3	0.05	0.07	0.05	0.07
4	Preparation-4	0.05	0.07	0.05	0.07
5	Preparation-5	0.05	0.07	0.05	0.07
6	Preparation-6	0.05	0.07	0.05	0.07

7	Average	0.05	0.07	0.05	0.07
8	% RSD	**0.00**	**0.00**	**0.00**	**0.00**
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**
9	Preparation-1	0.05	0.05	0.07	0.07
10	Preparation-2	0.05	0.05	0.07	0.07
11	Preparation-3	0.05	0.05	0.07	0.07
12	Preparation-4	0.05	0.05	0.07	0.07
13	Preparation-5	0.05	0.05	0.07	0.07
14	Preparation-6	0.05	0.05	0.07	0.07
15	Average	0.05	0.05	0.07	0.07
16	% RSD	0.00	0.00	0.00	0.00
17	Overall %RSD	0.00		0.00	

Table No.4: Results of single maximum impurity in sample solution

S.No	Time (hrs)	Single Max. Impurity			
		At Refrigerated Conditions (2-8°C)		Room temperature	
		Injection Area	Response Ratio	Injection Area	Response Ratio
1	Initial	460172	-	460172	-
2	12 hours	471164	102.4	470856	102.3
3	24 hours	469752	102.0	471462	102.5

Table No.5: Results for total impurities in sample solution

S.No	Time (hrs)	Single Max. Impurity			
		At Refrigerated Conditions (2-8°C)		Room temperature	
		Injection Area	Response Ratio	Injection Area	Response Ratio
1	Initial	701822	-	701822	-
2	12 hours	702984	100.2	696789	99.3
3	24 hours	699896	99.7	699426	99.7

Table No.6: Results for single highest impurity

S.No	Conditions	% of Single Highest impurity	
		% of Single Highest impurity	% Difference W.R.T original condition
1	Original (Method Precision -Sample preparation-1)	0.05	---
2	Flow rate variation –Low Flow (0.8mL/min)	0.05	0.00
3	Flow rate variation-High Flow (1.2mL/min)	0.05	0.00
4	Column oven Temperature Variation 20°C	0.05	0.00
5	Column oven Temperature Variation 30°C	0.05	0.00

Table No.7: Results for sum of impurities

S.No	Conditions	% Sum of All impurities	
		% Sum of All impurities	% Difference W.R.T original condition
1	Original (Method Precision -Sample preparation-1)	0.07	---
2	Flow rate variation –Low Flow (0.8mL/min)	0.07	0.00
3	Flow rate variation-High Flow (1.2mL/min)	0.07	0.00
4	Column oven Temperature Variation 20°C	0.07	0.00
5	Column oven Temperature Variation 30°C	0.07	0.00

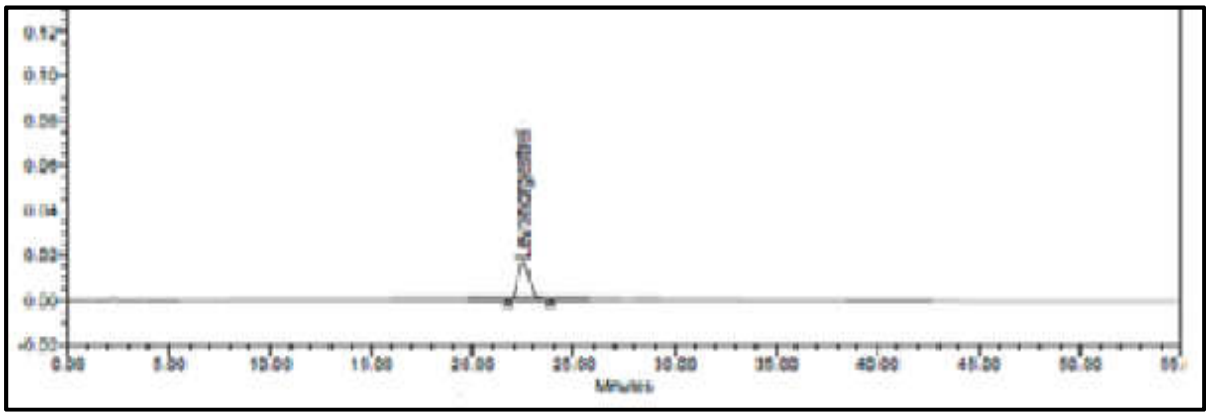


Figure No.1: Chromatogram for Standard (1ppm)

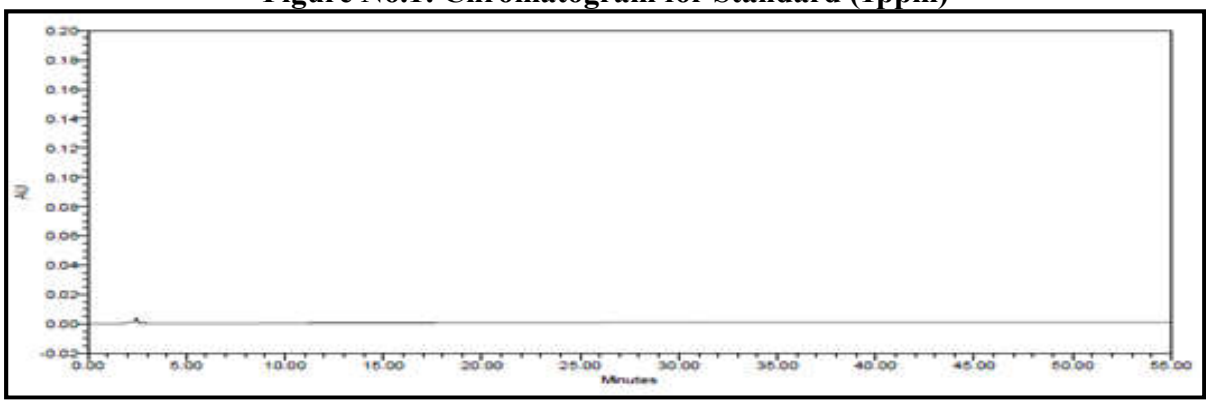


Figure No.2: Chromatogram for Blank

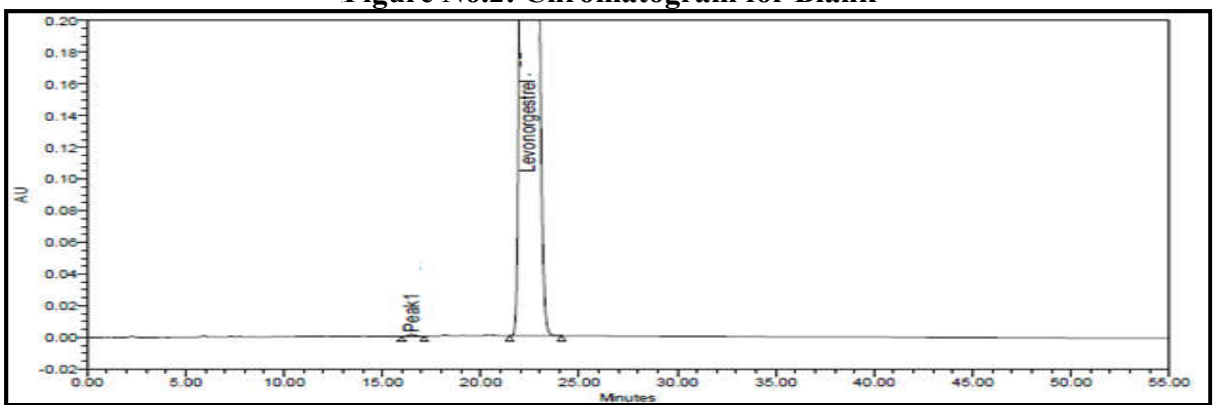


Figure No.3: Chromatogram and Purity plot of Sample Solution

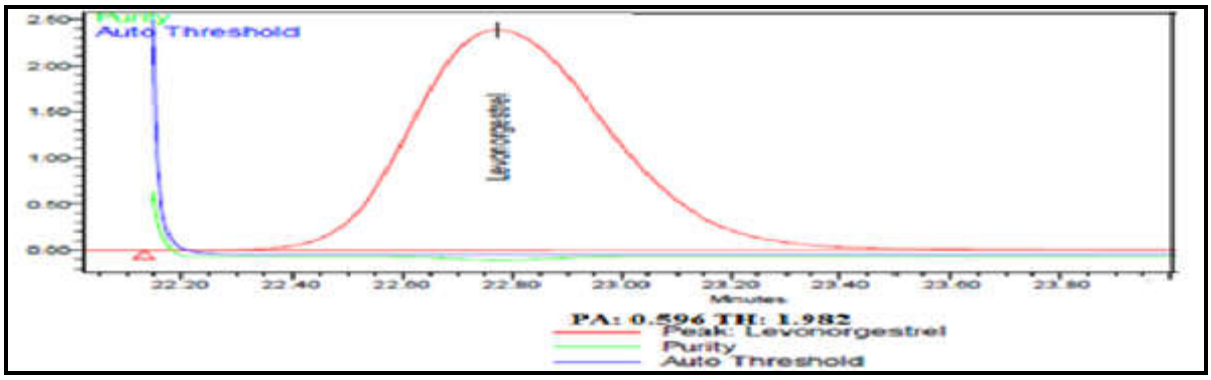


Figure No.4: Purity plot for levonorgestrel

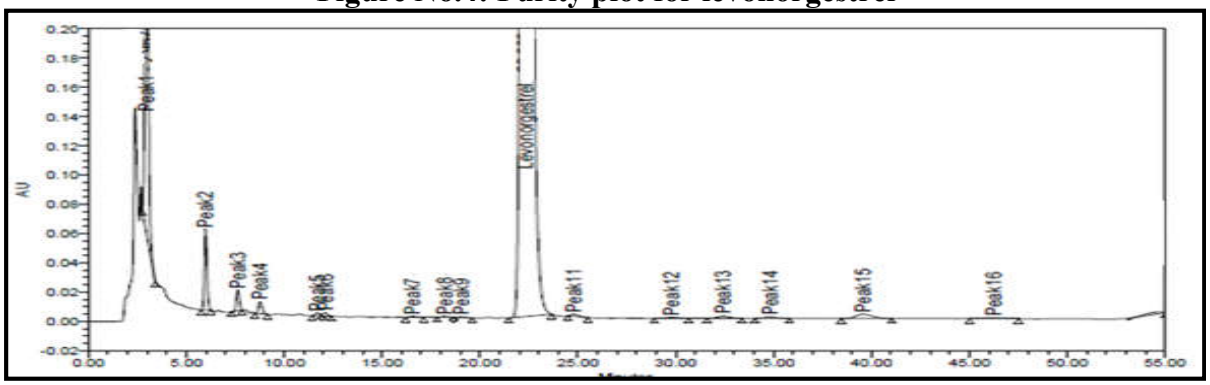


Figure No.5: Chromatograms of acid stressed samples treated with 2N HCl at 60°C for 24 hours

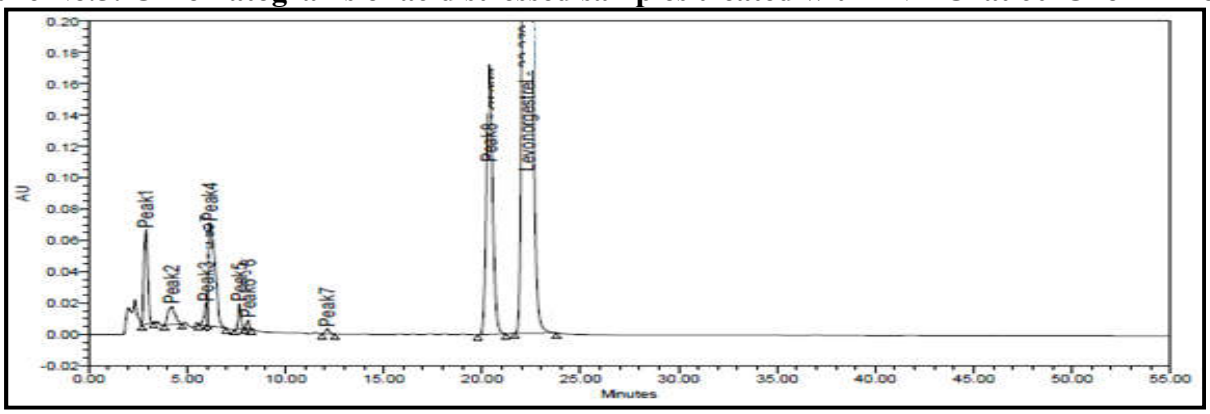


Figure No.6: Chromatograms of alkali stressed samples treated with 2N NaOH at 60°C for 24hours

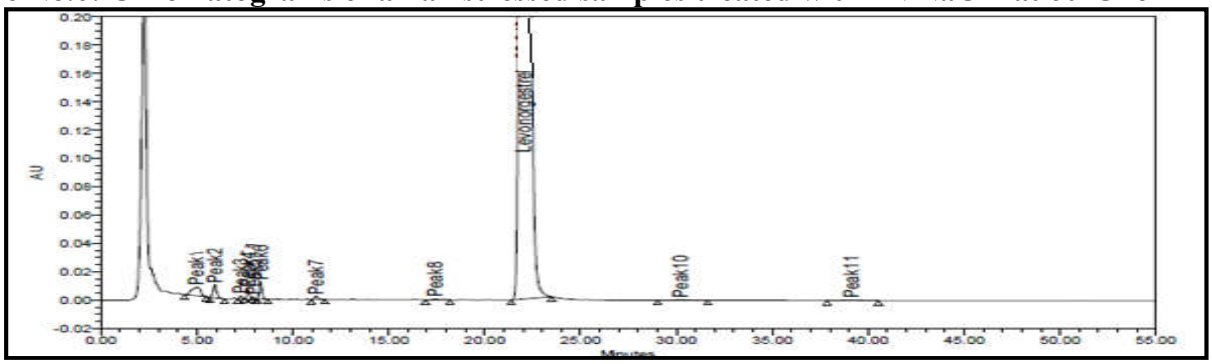


Figure No.7: Chromatograms of peroxide stressed samples treated with 30% H_2O_2 refluxed at 60°C for 24 hours

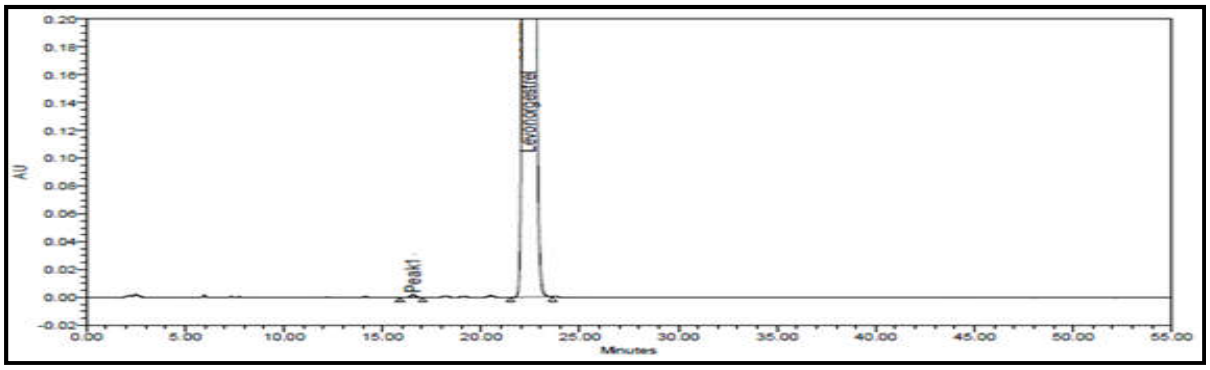


Figure No.8: Chromatogram for UV light stressed Sample

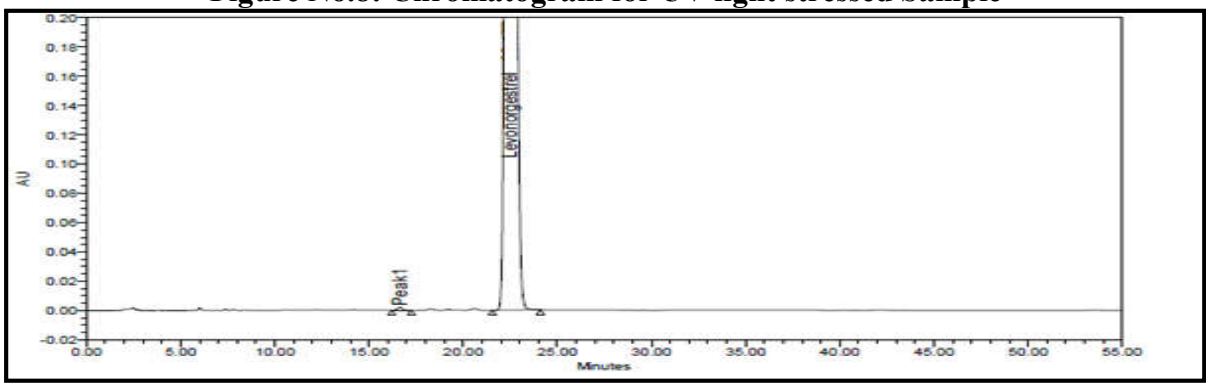


Figure No.9: Chromatogram for white light stressed Sample

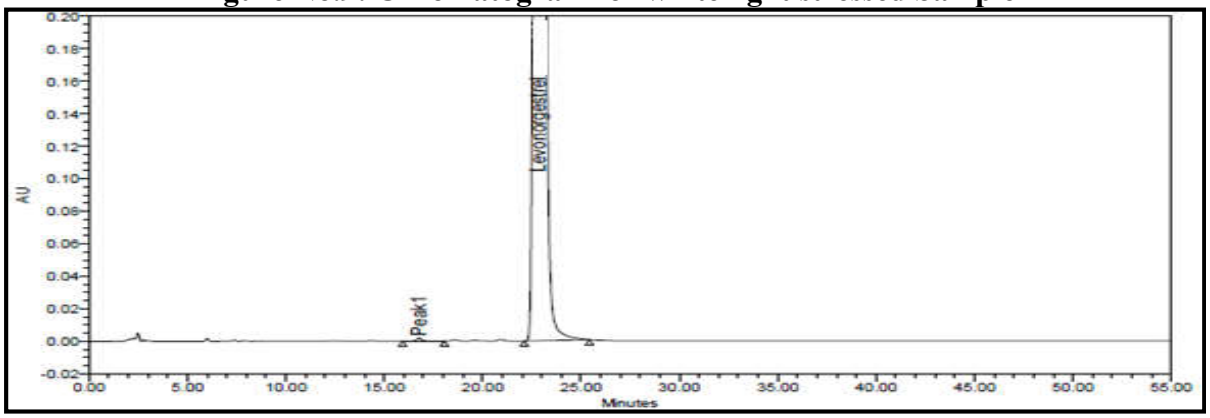


Figure No.10: Chromatogram for thermal degradation

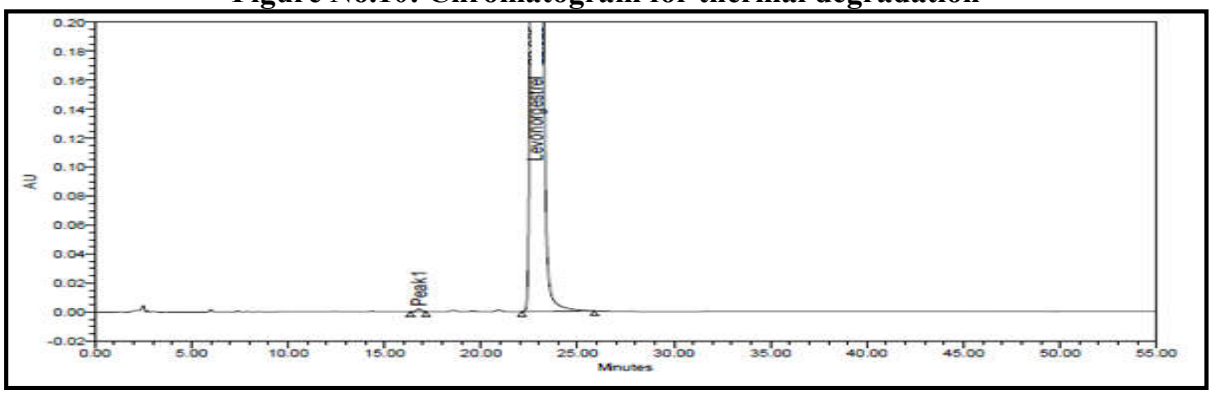


Figure No.11: Chromatogram for humidity degradation

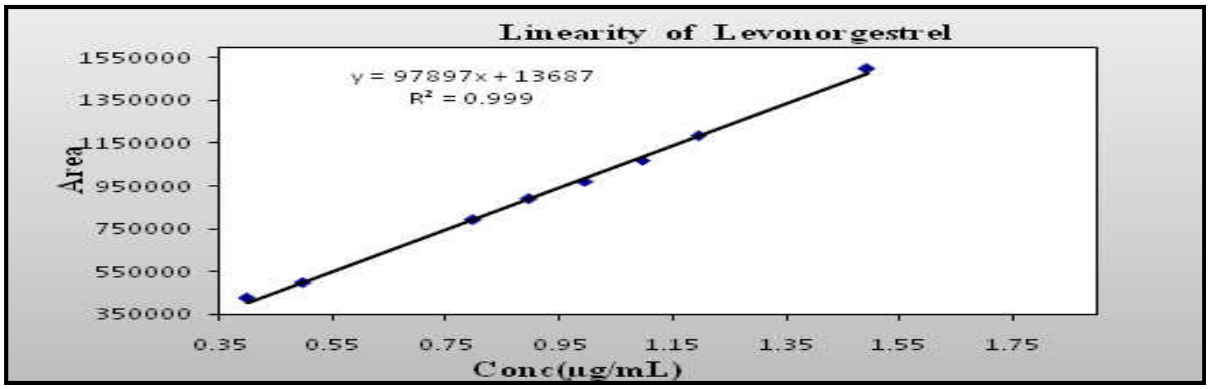


Figure No.12: Linearity curve

CONCLUSION

The proposed HPLC method for estimation of related substances for Levonorgestrel is validated in drug substance as per ICH guidelines. The method is found to be specific and suitable for the analysis. This method can produce Specific, Precise and Accurate results during regular analysis of product. Method is specific to determine the related substances as no interference is observed from Blank and unknown compounds at the retention time of Levonorgestrel and known impurities. The range of analytical method is suitable to determine the Organic impurities in the range of LOQ to 150% of specification level. Hence, the proposed method is validated and can produce Specific, Precise and Accurate during analysis.

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CONFLICT OF INTEREST

We declare that we have no conflict of Interest.

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