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Validated RP- HPLC Method for Simultaneous Estimation of Levonorgestrel and Ethinylestradiol in Combined Dosage Form

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Abstract

A simple, accurate, precise, specific, sensitive, reproducible and Reliable RP- HPLC Method was developed for quantitative estimation of Levonorgestrel and Ethinylestradiol in Pharmaceutical Dosage Form. The developed RP- HPLC method with the mobile phase ACN: Water (80: 20) and Qualisilgold-C18 (250X4.6mm, 5µm particle size) as stationary phase with a flow rate of 1.0 mL/minute by using wavelength 225nm in PDA detector. The proposed method was found to be linear in the concentration range of 20.0 to 125.0 µg/mL for Levonorgestrel and 4.0 to 25.0 µg/mL for Ethinylestradiol respectively, and the correlation coefficient was found to be 0.9991 and 0.9982 for Levonorgestrel and Ethinylestradiol respectively. Precision studies showed that the % RSD was within the range of acceptable limits (< 2), and the % Recovery was found to be in the range of 99.82±0.2% for Levonorgestrel and 98.76±0.13% for Ethinylestradiol. The proposed method has been validated as per ICH guidelines.

Keywords: RP- HPLC, Levonorgestrel, Ethinylestradiol potassium, PDA, ICH, Method validation

Introduction

Levonorgestrel (Levo) is 13β-ethyl-17β-hydroxy-18, 19-dinor-17α- pregn-4-en-20-yn-3-one (fig.1) and Ethinylestradiol (EE) is 19-nor-17α-pregna-1,3,5(10)-trien-20yne-3,17β-diol (fig. 2) are oral contraceptive pills available in combined monophasic or multiphasic forms.[1-4] This combination is official in Indian Pharmacopoeia (IP), describes chromatographic method for its estimation. Various methods like Spectrophotometric, HPLC and HPTLC methods for simultaneous estimation of EE and Levo with other drug are reported in literature. The combined dosage forms of Levo and EE available in market for the treatment of prevention of pregnancy. The literature survey reveals that no RP- HPLC method for the simultaneous estimation of Levo and EE in tablet dosage forms. The present manuscript describes a new, simple, linear, precise, accurate, economical and reproducible RP – HPLC method for the simultaneous estimation of Levo and EE in tablet dosage form. [5-10]

Materials and Methods

Levonorgestrel and Ethinylestradiol were obtained as a gift sample from the Aurobindo Pharma Ltd, Hyderabad. Agilent LC-1200 (gradient) chromatograph with PDA detector was used with Ezchrome elite software.

Acetonitrile used to be of HPLC grade, obtained from Merck Chemicals, Mumbai. A commercial tablet formulation (CHOICE®, PHAARMASIA LTD) each containing 0.150mg of Levonorgestrel and 0.03mg of Ethinylestradiol were procured from local pharmacy.

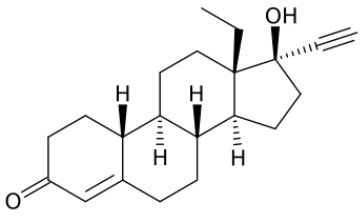

Figure 1: Structure of Levonorgestrel

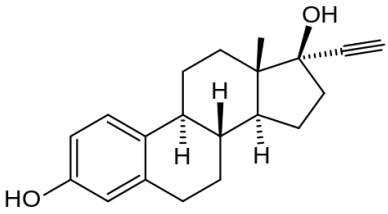

Figure 2: Structure of Ethinylestradiol

Selection of wavelength

UV spectra of Levonorgestrel and Ethinylestradiol potassium were shown that λmax was found at 240 nm and 280 nm respectively. Isobestic points were observed at 225 nm and 264 nm. At 225nm Levonorgestrel and Ethinylestradiol shows maximum absorption, so that wavelength is selected for determinations.

Selection of Mobile phase

Trial 1 Mobile phase: 60 ACN: 40 water, Detection wavelength: 225 nm, Drawback: Peak broadening.

Trial 2 Mobile phase: 70 ACN: 30 water, Detection wavelength: 225 nm, Drawback: Two peaks show good separation and peak shape also good. In order to decrease analysis time, further I tried with increasing organic strength.

Trial 3 Mobile phase: 80 ACN: 20 water Detection wavelength: 225 nm, both peaks eluted with adequate separation and meet all system suitability parameters, so trail 3 is selected for determinations. The chromatogram was shown in figure. 3

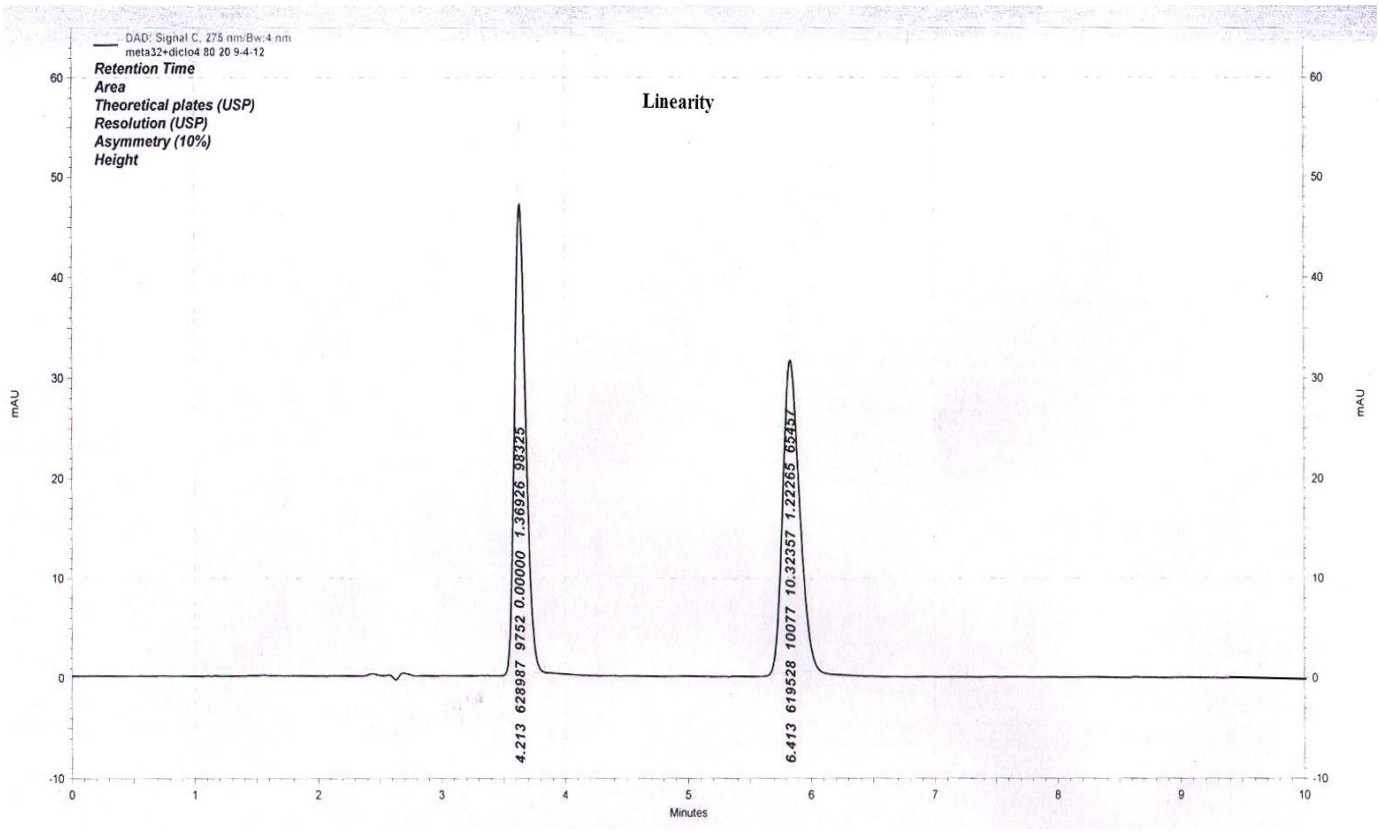

Figure 3: Optimized chromatogram for Levonorgestrel and Ethinylestradiol

Preparation of standard drug solutions

A stock solution of Levonorgestrel and Ethinylestradiol were prepared by dissolving 10 mg of each in separate 10 ml volumetric flasks with small quantities of ACN. The mixture was sonicated for 15 min and then makes up the volume with ACN. From the stock solution 100 µg/ml of Levonorgestrel and Ethinylestradiol were prepared by pipette out 1ml of the stock solution into a 10 ml flask and make up to volume with mobile phase (ACN 80: water 20).

Chromatographic conditions

The mobile phase consisting of ACN and water in the ratio of 80: 20 was filtered before use through a 0.45µm membrane filter and degassed in an ultrasonicator for 10 min. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1.0 ml/min and the injection volume was 20 µL. The column temperature was maintained at 25^0C. The eluents were monitored at 275 nm.

Calibration of standards

Two separate calibration plots were constructed for each component. Concentration range of 20-125 µg/ml for Levonorgestrel and 4-25 µg/ml for Ethinylestradiol were prepared for each component by pippeting different volumes of each stock solution and made up to the mark with mobile phase (ACN 80: water 20).

Method validation

The developed method was validated as per International Conference on Harmonization (ICH) guidelines. The validation parameters are,

Specificity

For determining specificity of the method, a tablet dosage form was analyzed. The chromatograms were examined to determine if compounds of interest co-elute with each other or with any additional excipients peaks. Injections of the marketed product revealed the absence of interferences with the elution of the drug. These results demonstrate that there was no interference from other materials in the tablet formulation therefore, confirm the specificity of the method.

Linearity

The linearity of calibration curves in pure solution was checked over the ranges of 20-125 µg/ml for Levonorgestrel and 4-25 µg/ml for Ethinylestradiol. the calibration curves were linear in the studied range and equations of the regression analysis were obtained for Levonorgestrel and Ethinylestradiol and the linearity plots were shown in figure no. 5& 6. The mean± Standard Deviation (SD), slope, intercept, and correlation coefficient of standard curves (N=3) were calculated and the results were shown in table no. 1 and 2.

Table 1: Linearity data for Ethinylestradiol

S. No	Concentration of EE (µg/ml)	Peak area (mean ± SD)	%RSD
1	4	215796 ± 841	0.3898
2	6	310910 ± 716	0.2300
3	10	554431± 2776	0.5000
4	15	794457 ± 3043	0.3831
5	20	1044095 ± 6255	0.6255
6	25	1361975 ± 6924	0.5084
Regression equation		Y = 53774x – 3376	
Intercept		53774	
Slope		3376	
Correlation coefficient		0.9982	

Table 2: Linearity data for Levonorgestrel

S. No	Concentration of Levo (µg/ml)	Peak area (mean ± SD)	%RSD
1	20	1622620 ± 2821.25	0.1730
2	30	2377375 ± 10949	0. 4605
3	50	3918875 ± 14665	0.3740
4	75	5977368 ± 21575	0.3626
5	100	7988290 ± 4874	0.0610
6	125	10286013 ± 9533	0.092
Regression equation		Y = 82055.41x- 108604	
Intercept		82055.41	
Slope		108604	
Correlation coefficient		0.9991	

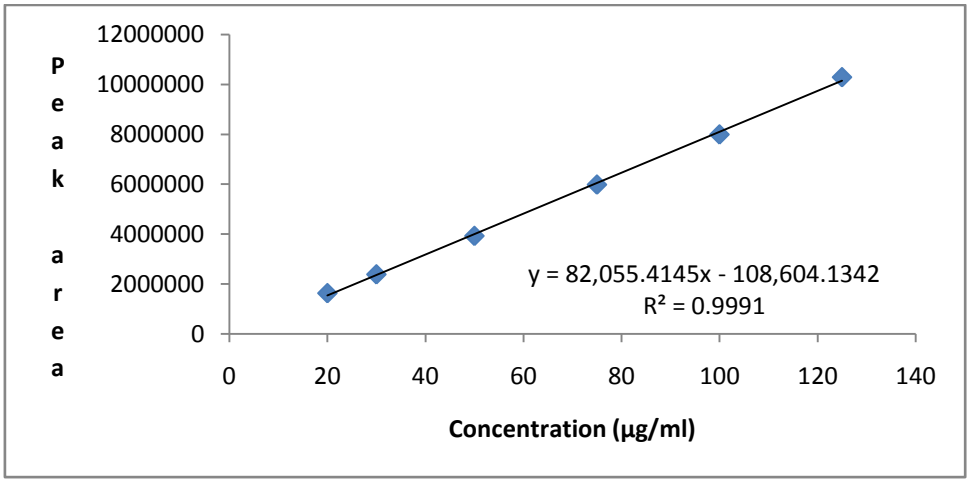

Figure 5: Linearity plot of Levonorgestrel

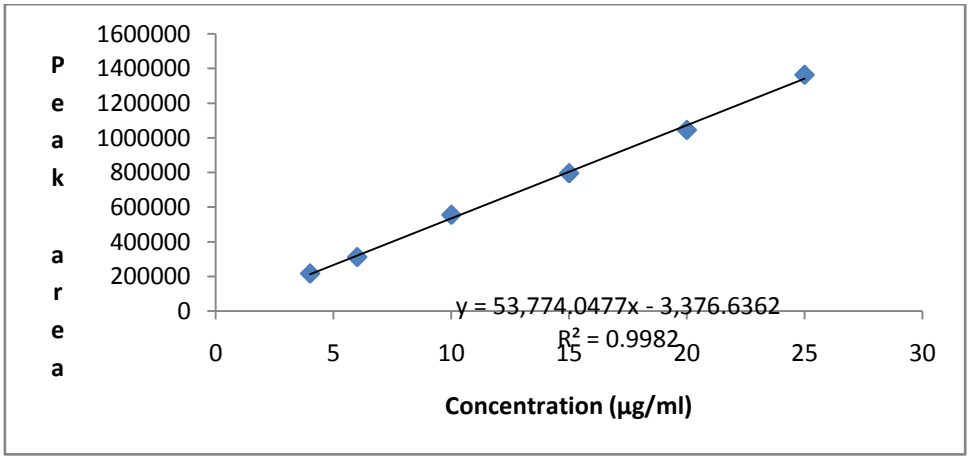

Figure 6: Linearity plot of Ethinylestradiol

Accuracy (Recovery study)

Accuracy of the method was determined by recovery experiments. To the formulation, the reference standards of the drug were added at the level of 80%, 100%, 120%. The recovery studies were carried out 3 times and the % Recovery and % RSD of the recovery of Levonorgestrel and Ethinylestradiol were calculated and the results were shown in table no. 3.

Table 3: Recovery report of Levo and EE

Concentration of standard	Recovery level	Amount of drug added (µg/ml)	Total amount of drug (µg/ml)	Amount found (µg/ml) n=3 (mean±SD)	% recovery	%RSD
Levonorgestrel (50 µg/ml)	80%	40	90	39.96±0.04	99.825±0.20	0.01012
	100%	50	100	50.273±0.155	100.56±0.294	0.3083
	120%	60	110	59.43±0.234	99.05±0.767	0.3846
Ethinylestradiol (10 µg/ml)	80%	8	18	8.1±0.05	101.25±0.625	0.3674
	100%	10	20	10.14±0.07	101.4±0.12	0.6903
	120%	12	22	12.18±0.1123	101.5±0.36	0.9225

Assay of CHOICE® Tablets

Twenty tablets were weighed, finely powdered and an accurately weighed sample of powdered tablets equivalent to 2.5 mg of Levonorgestrel and 0.5 mg of Ethinylestradiol were extracted with 40 ml of ACN. The flask was sonicated for 45 min and volume was made up to the mark with water. The above solution was filtered using whatman filter paper, to obtain 50 µg/ml of Levonorgestrel and 10 µg/ml of Ethinylestradiol. The solution was injected under above chromatographic conditions and peak area was measured. The assay chromatogram was shown in figure. 4. The assay procedure was made triplicate (n=3) and weight of sample taken for assay was calculated. The percentage of drug found in formulation, mean and standard deviation in the formulation was calculated. The results were shown in table no. 4.

Table 4: Assay report of *choice*® (Levonorgestrel and Ethinylestradiol)

Formulation *choice*®	Labeled claim (mg)	Peak area mean±SD (n=3)	Amount found (mg) mean±SD n=3	Assay	%RSD
Levonorgestrel	0.15 (50µg)	3991186±4794	0.1497±0.13	99.82±0.2%	0.092
Ethinylestradiol	0.03 (10µg)	576381±2294	0.02963±0.133	98.76±0.13%	0.155

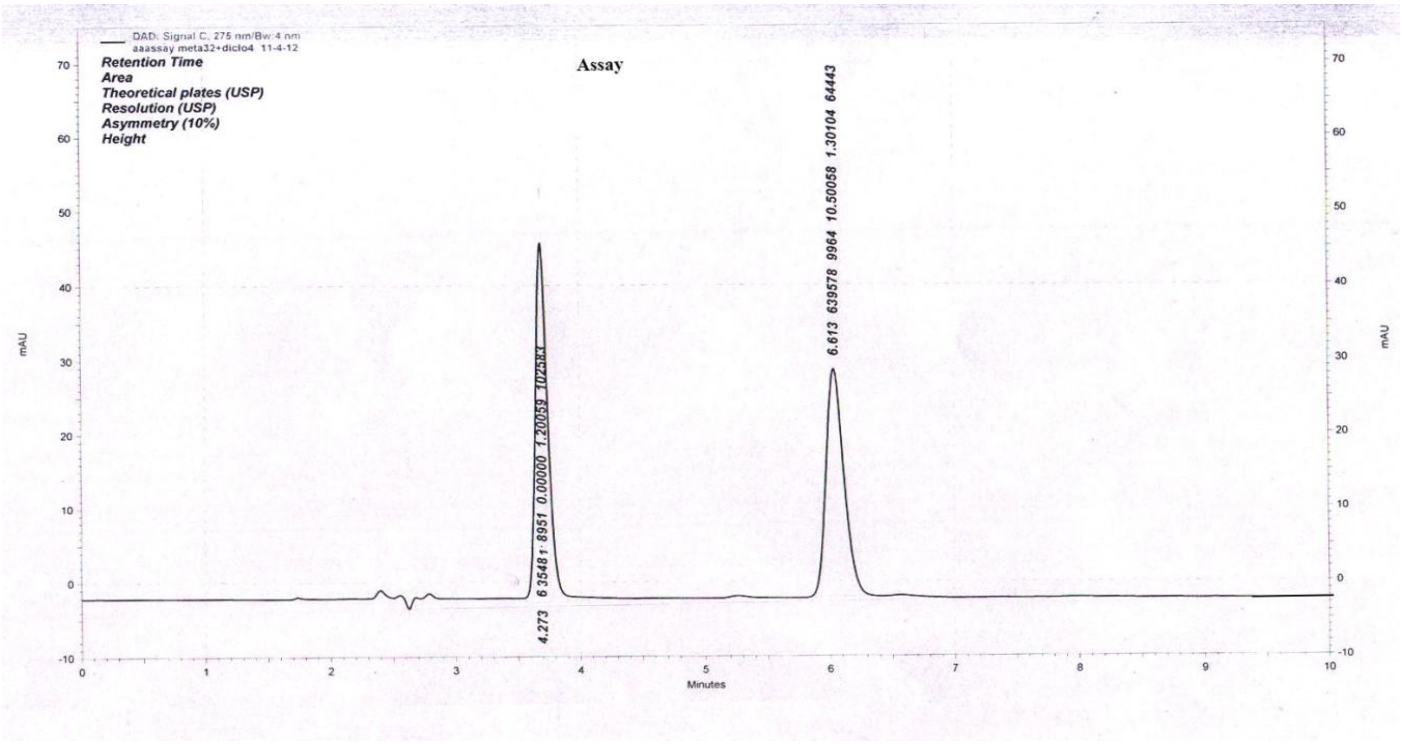

Figure 4: Assay chromatogram of Levonorgestrel and Ethinylestradiol

Precision

The system precision was studied by six replicate measurements of single concentration or three replicate measurements of three different concentrations and the results were shown in table no. 11. To assess the precision of the method, the intraday (3 times) and Interday (3 days) measurements of two drugs were completed with computation of % RSD for replicate samples (n=3) using concentrations of 20, 50 and 125 µg/ml of Levonorgestrel and 4, 10 and 25 µg/ml for Ethinylestradiol. Both intraday and inter day results were calibrated with standard curve concurrently prepared on the day of analysis and were shown in table no. 5.

Table 5: Intraday and Interday precision data of Levo and EE

Drug	Concentration (µg/ml)	Peak (peak area±SD) n=3		%RSD	
		Intraday	Interday	Intraday	Interday
Levonorgestrel	20	1666111±14509	1653218±18234	0.87	1.109
	50	3794684±19471	3746517±68119	0.51	1.818
	125	10286783±20891	10177183±154998	0.20	1.523
Ethinylestradiol	4	215900±1361	214332±2217	0.63	1.034
	10	554329±2989	549491±6841	0.54	1.2451
	25	1352046±10108	1340399±16471	0.75	1.228

Robustness

The Robustness of the method was evaluated by analyzing the system suitability standards and evaluating system suitability parameter data after varying pump flow rate, mobile phase ratio and detection wavelength. All of the alterations caused a significant change in peak area and Retention time. The results were shown in table no. 6.

Table 6: Robustness data of Levo and EE

Parameter	Modification	Retention time (mean±SD) n=3		Asymmetry	
		Levo	EE	Levo	EE
Flow rate (ml/min)	0.9	5.36±0.075	4.29±0.105	1.29	1.27
	1.0*	4.85±0.003	3.972±0.01	1.26	1.18
	1.1	4.25±0.13	3.58±0.04	1.34	1.27
Mobile phase (ACN: water)	78:22	4.525±0.035	3.69±0.04	1.26	1.27
	80:20*	4.85±0.003	3.972±0.01	1.26	1.18
	82:18	4.65±0.02	3.861±0.007	1.29	1.28
Wavelength (nm)	223	4.835±0.012	3.69±0.04	1.29	1.37
	225*	4.85±0.003	3.972±0.01	1.26	1.18
	227	4.835±0.012	3.973±0.002	1.34	1.28

* Optimized method

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD was expressed by establishing the minimum level at which the analyte can be reliably detected. LOQ was considered as the lowest concentration of analyte in standards that can be reproducibly measured with acceptable precision. The LOD and LOQ were calculated by standard deviation of y-intercept and slope of the linearity curves. The results were shown in table no. 8.

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

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

Where, σ = the standard deviation of the response and

S = slope of the calibration curve

System suitability

It is defined as tests to measure the method that can generate result of acceptable accuracy and precision. The system suitability was carried out after the method development and validation have been completed. For this, parameters like Plate number (N), Resolution (R), tailing factor, Capacity factor, HETP, Peak symmetry of samples were measured. The results were shown in table no. 7.

Table 7: System suitability data of Levo and EE

Parameter	Levo	EE	Acceptance criteria
Retention time (min)	4.85	3.973	6-8
Peak area (mean±SD) n=3	3991186±4794	576381±2294	-
Plate count	11643	11031	>2000
Tailing factor	1.01	1.04	≤2.0
Asymmetry (10%)	1.26	1.40	0.9-1.2
Resolution	--	5.29	>1.5
Capacity factor	0.92	0.56	1-20

Results and Discussion

The present work was aimed at developing new validated RP-HPLC Method for Levonorgestrel and Ethinylestradiol. In the present work the RP-HPLC method development was done for Levonorgestrel and Ethinylestradiol by using ACN: Water (80: 20) as mobile phase and detection was performed at 225 nm. The system suitability parameters like Plate number (N), Resolution (R), tailing factor, Capacity factor, HETP, Peak symmetry of samples were within the acceptance limits. Linearity curves were obtained between peak area and concentrations of Levo and EE in the concentration ranges of 20-125 µg/ml and 4-25 µg/ml, with R2 value 0.9991and 0.9982 respectively. The linearity of the calibration curve was validated by the high values of correlation coefficient of regression. The RSD values for Intraday and Interday precision of Levo and EE were found to be less than 2%. LOD and LOQ values for Levo were found to be 66.19 ng/ml and 200.6 ng/ml respectively. LOD and LOQ values for EE were found to be 32.45 ng/ml and 98.36 ng/ml respectively. These data show that method is sensitive for the determination of Levo and EE. The recovery experiment was performed by the standard addition method. The recoveries of Meta and Diclo were found to be in the range of 99.82±0.2% and 98.76±0.13% respectively. The results of recovery studies indicate that the proposed method is highly accurate.

The complete summary of the RP- HPLC method was represented in Table. 8. The proposed validated RP-HPLC method was successfully applied to combined dosage form (tablet). Hence the proposed method can be used as alternative method to already reported methods and provide a wide choice for the routine determination of the above mentioned drugs.

Table 8: Summary of the method

Parameter	Levonorgestrel	Ethinylestradiol
Retention time	4.5 min	3.973 min
Run time	07 min	
Assay	99.82±0.2%	98.76±0.13%
Specificity	Response of analyte with respect to blank is well resolved	
Linearity	20-125 µg/ml	4-25 µg/ml
LOD	66.19 ng/ml	32.45 ng/ml
LOQ	200.6 ng/ml	98.36 ng/ml

Accuracy	99.91±0.91 %	101.37±0.12 %
System precision	% RSD <2	% RSD <2
Precision	% RSD <2	% RSD <2

Conclusion

RP-HPLC Method was developed for the Simultaneous Estimation of Levonorgestrel and Ethinylestradiol. The HPLC used was Agilent 1200 (Gradient) PDA detector with Rheodyne injector of 20µL volume and column Qualisil gold C18 (250X4.6mm, 5µm). The mobile phase comprised of ACN: Water in the ratio of 80:20 v/v and flow rate of 1ml/min with detection at 225nm produced peaks for Levonorgestrel and Ethinylestradiol in the chromatogram with retention times of 4.5 and 3.973 respectively. The HPLC method was validated for various parameters like Linearity, Assay, Accuracy, Precision, Robustness, Specificity, LOD and LOQ as per ICH guidelines. The proposed method was applied for determination of Levonorgestrel and Ethinylestradiol in marketed formulation. The Assay results confirmed to the label claim of the formulation. Hence the proposed method was found to be satisfactory and could be used for the routine analysis of Levonorgestrel and Ethinylestradiol potassium in their marketed tablet dosage formulations.

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