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RESEARCH ARTICLE

Development and Validation of a New Robust RP-HPLC Method for the Simultaneous Quantitation of Levonorgestrel and Ethinylestradiol in Bulk and Pharmaceutical Dosage Form

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ABSTRACT

The present study was mainly focused on developing and validating a new isocratic, simple, rapid, precise, accurate, and stable reverse phase high-performance liquid chromatography method for a combination of levonorgestrel and ethinylestradiol. The separation was achieved on a C_{18} µ-bondopak column (250 mm*4.6 mm) using a mobile phase consisting of buffer, water, and acetonitrile in the ratio of 40:20:40 (buffer-0.1% v/v triethylamine pH-3.0 followed by 0.9 mL/min flow rate. The wavelength detection was at 260 nm. The chromatographic retentions were stable at 3.604 min for levonorgestrel and 5.142 min for ethinylestradiol. Linearity concentrations were established in the range of 20–100 µg/mL for levonorgestrel and 10–50 µg/mL for ethinylestradiol and the correlation coefficient for the above drugs was 0.999. The relative standard deviation of inter-and intra-day precision was <2%. The proposed method provides a useful tool for the quantification of levonorgestrel and ethinylestradiol for their assay. This method is simple, accurate, and reproducible and can be successfully employed for routine quality control analysis of drugs in pure as well as in pharmaceutical dosage form. The main advantage of the developed method was its high specificity for the estimation of levonorgestrel and ethinylestradiol.

Keywords: Accuracy, Ethinylestradiol, Levonorgestrel, Validation

INTRODUCTION

Analytical chemistry plays an important role for the development of drugs in pure and also in marketed formulations[1] and also ensures the amount of particular drugs can be easily determined.[2] Levonorgestrel is chemically known as (8R,9S,10R,13S,14S,17R)-13-ethyl-17-ethynyl-17-hydroxy-1,2,6,7,8,9,10,11,12,14,15,16-dodecahydrocyclopenta[a]phenanthren-3-one with chemical formula $C_{21}H_{28}O_2$ and molecular mass of 312.47 g/mol is a hormonal medication and is used in a number of birth control methods.[3] The chemical structure of levonorgestrel is shown in Figure 1. It mainly works by preventing ovulation or fertilization from occurring.[3] In an intrauterine device such as mirena among others, it is effective for the long-term prevention of pregnancy. Various intrauterine levonorgestrel devices are available worldwide, including four FDA-approved intrauterine systems: mirena (52 mg); liletta (52 mg); kyleena (19.5 mg); skyla (13.5 mg); and other levosert (52 mg) and jydess (13.5 mg).[4]

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Ethinylestradiolischemically(8R,9S,13S,14S,17R)-17-ethynyl-13-methyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-3,17-diol with a chemical formula $C_{20}H_{24}O_2$. Veeran *et al*.[5] have mentioned its molecular mass was found to be 296.403 g/mol. AL-Japairai *et al*.[6] mentioned that it is one of the synthetic estrogens that decreases luteinizing hormone to decrease endometrial vascularization and Bao *et al*.[7] have identified that some of the drugs activity mainly depends on design parameters and also on *in vitro* release from intrauterine systems and also some of the drugs such as ethinylestradiol acts by decreasing gonadotrophic hormone to prevent ovulation. The chemical structure of ethinylestradiol is shown in Figure 2.

The present study mainly focuses on developing and validating a reproducible, less cost, simple, and rapid liquid chromatography method for determining levonorgestrel and ethinylestradiol in marketed formulations. Instead of routine analysis, the use of a rapid and uncomplicated method is a matter of highly importance. The present strategy mainly focuses on developing a novel method having a shorter run time and also symmetrical peaks for both of the drugs. The liquid chromatography method was designed and subsequently validated to assess the performance characteristics.[8-13]

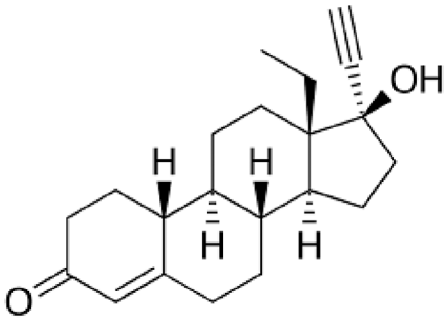

Figure 1: Structure of Levonorgestrel

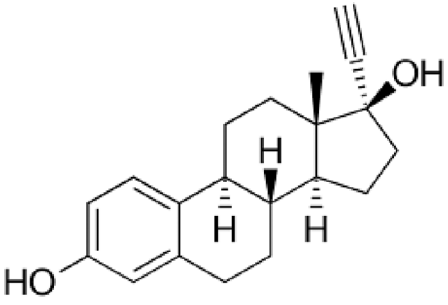

Figure 2: Structure of ethinylestradiol

EXPERIMENTAL

Chemicals and materials

Levonorgestrel and ethinylestradiol reference standards were obtained as gift samples from Spectrum Laboratories, Hyderabad, Telangana, and the marketed formulation Duoluton L produced by Zydus Pharmaceuticals Pvt. Ltd. was obtained from the local market. Acetonitrile and phosphate buffer were purchased from Merck Ltd. (Mumbai, India). Milli-Q water was used throughout the study.

Wavelength detection

Stock solutions of levonorgestrel and ethinylestradiol of 1 mg/mL were prepared in the mobile phase and subsequent dilutions were done to get the final concentrations of 10 µg/mL.[14] Both the solutions were scanned in the range of 200–400 nm by UV spectrophotometer and the spectra were recorded at 260 nm and overlay UV spectrum of levonorgestrel and ethinylestradiol is shown in Figure 3.

Preparation of buffer solution (10 mM Potassium dihydrogen phosphate)

Weigh accurately 1.3609 g of potassium dihydrogen phosphate and transfer it into a 1000 mL volumetric

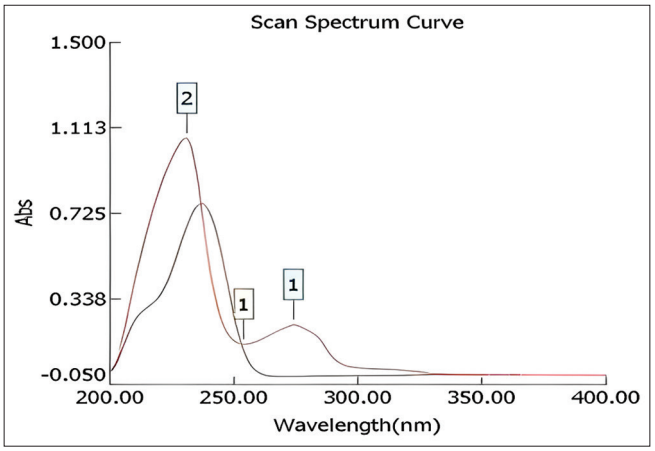

Figure 3: Overlay Spectrum of Levonorgestrel and Ethinylestradiol at 260nm

flask by dissolving in HPLC grade water and the final volume was adjusted up to the mark with the same water up to 1000 mL.

Preparation of mobile phase

Mixture of buffer solution, water, and acetonitrile in the ratio of 40:20:40 (400 mL of phosphate buffer, 200 mL of water, 400 mL of acetonitrile) was transferred into 1000 mL volumetric flask and kept under sonication for 10 min, degassed and filtered through 0.45 um membrane filter.

Diluent preparation

The mobile phase is used as diluent.

Preparation of standard solution of levonorgestrel

A 10mg of levonorgestrel working standard was accurately weighed and transferred into a 10 mL clean dry volumetric flask and add about 7 mL of diluents is added and sonicated to dissolve completely and volume was made up to the mark with the same solvent. Further, 0.3 mL of the above stock solution was pipetted into a 10 mL volumetric flask and diluted up to the mark with diluent

Preparation of standard solution of Ethinylestradiol

A 10 mg of ethinylestradiol working standard was accurately weighed and transferred into a 10 mL clean dry volumetric flask and add about 7 mL of diluents is added and sonicated to dissolve completely and volume was made up to the mark with the same solvent. Further, 0.3 mL of the above stock solution was pipetted into a volumetric flask and diluted up to the mark with diluent.

Preparation of sample solution

Weigh accurately 10 tablets and crushed in a mortar and pestle and weight equivalent to 10 mg of levonorgestrel and ethinylestradiol samples into a 10 mL clean dry volumetric flask and add about 7 mL of diluent and sonicate the above solution to dissolve completely the above samples and made up to the mark with the same solvent. From this pipette, out 0.3 mL of the above stock solution and transfer into 10 mL volumetric flask and fill the diluent up to the mark.

Chromatographic conditions

The liquid chromatographic analysis was performed using a Shimadzu HPLC system equipped with a PDA Detector. Chromatographic separation of both the drugs was achieved using a C_{18} µ-bondopak column having dimensions of 250*4.6 mm, 5 µm particle size, and in an isocratic mode with mobile containing a mixture of phosphate buffer, water, acetonitrile in the ratio of 40:20:40 (v/v). Elution of drugs was carried out at room temperature with a flow rate of 0.9 mL/min, injection volume of 20 µL, and a total run time of 12 min. Before injecting blank and drug solution, the chromatographic system was equilibrated for 80 min with the degassed mobile phase. The blank solution was filtered through 0.22 µm nylon filter and injected to check the solvent interference.

Procedure

20 µL of the standard, samples are injected into the chromatographic system and the areas for levonorgestrel and ethinylestradiol peaks are measured and the % assay is calculated using the formulae.

RESULTS

Method development

Initially, mobile phase optimization was carried out with shimadzu C_{18} µ-bondopak column using buffer, acetonitrile, and water at different concentrations. During these trials, the peaks are not eluted properly. Finally, the mobile ratio was changed to 40:20:40 v/v at a flow rate of 0.9 mL/min. With this composition of mobile phase, the peaks were eluted satisfactorily. The peaks were well separated by following chromatographic parameters. The

run time of the method was 12 min. The detection wavelength was at 260 nm. The prescribed method was validated as per the ICH guidelines.

System suitability

The retention times of levonorgestrel and ethinylestradiol were found to be 3.6 min and 5.1 min, respectively. No major deviations were observed in the retention times during the analysis. The percentage of related standard deviation (%RSD) of the lowest concentration of individual six peaks was <2%. System suitability parameters are summarized. The chromatogram of the standard solution is shown in Figure 4 and the chromatogram of the sample solution is shown in Figure 5

Linearity

The peaks were eluted at different concentrations of standard solution and the calibration curve was plotted using peak area against concentration. The regression coefficient was found to be 0.999 for the above two drugs. The linear concentrations of levonorgestrel and ethinylestradiol are in the range of 10–50 µg/mL for ethinylestradiol and 20–100 µg/

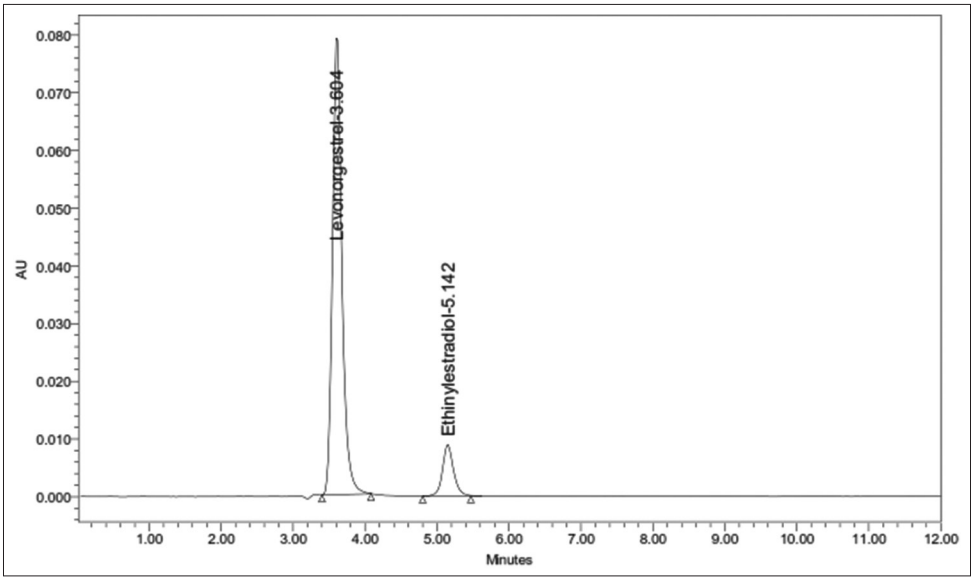

Figure 4: Chromatogram of Standard Solution

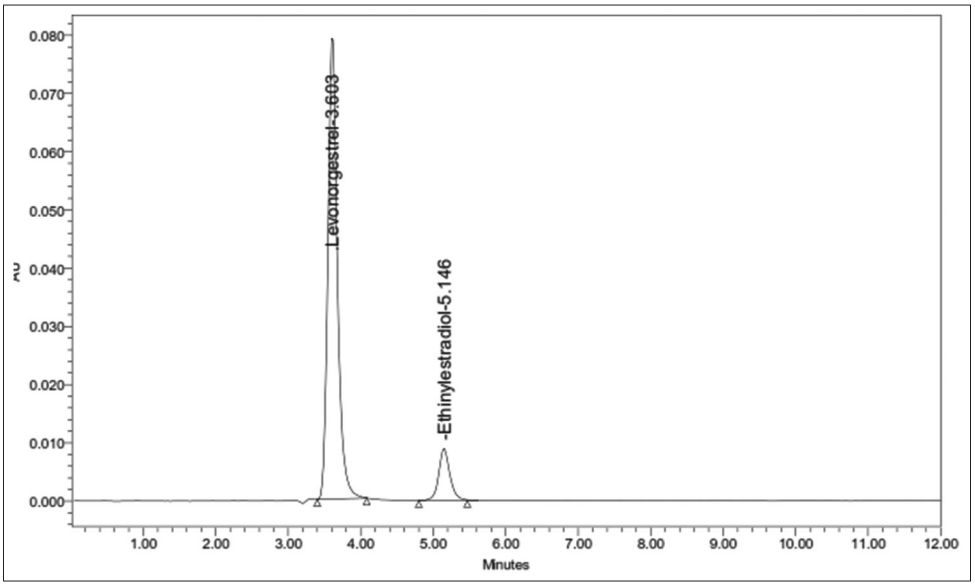

Figure 5: Chromatogram of Sample Solution

mL for levonorgestrel, respectively. The linearity results of levonorgestrel are tabulated in Table 1 and linearity results of ethinylestradiol are tabulated in Table 2. Calibration graphs of levonorgestrel and ethinylestradiol are shown in Figures 6 and 7.

Accuracy

The accuracy of a method mainly depends on the recovery estimation study. Recovery a study was performed by spiking the known amount of standard drug corresponding to 50,100,150% of label claim had been added to the marketed drug sample. Recovery studies were found to be satisfactory and are in the range of 98–102%. The results of accuracy for levonorgestrel are tabulated in Table 3 and for ethinylestradiol accuracy results are tabulated in Table 4. The result has shown that the proposed method was accurate. Hence, the developed method can be adopted in industry and as well as academics for the assay of levonorgestrel and ethinylestradiol.

Precision

Precision studies indicate that the developed method has accepted values of validation for the estimation of the above drugs in combination. The repeatability results of levonorgestrel are tabulated in Table 5 and ethinylestradiol are tabulated in Table 6. The %RSD value was found to be <2. Deviations were not observed in inter- and

Table 1: Linearity values of levonorgestrel

Injection no.	Concentration (µg/mL)	Peak area
1	20	756893
2	40	1583786
3	60	2241779
4	80	2997571
5	100	3744463

Table 2: Linearity values of ethinylestradiol

Injection no.	Concentration (µg/mL)	Peak area
1	10	496895
2	20	993771
3	30	1490657
4	40	2017542
5	50	2484427

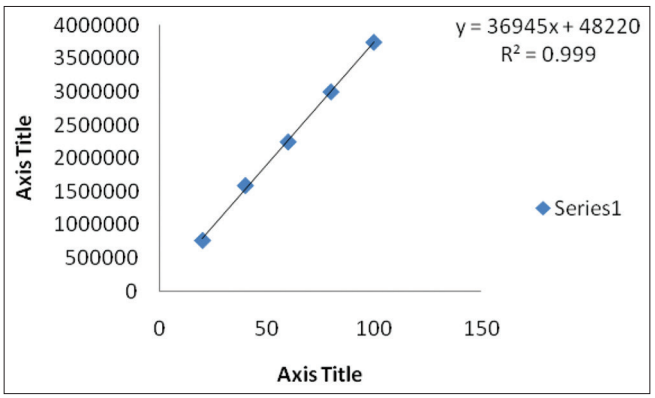

Figure 6: Calibration Graph of Levonorgestrel

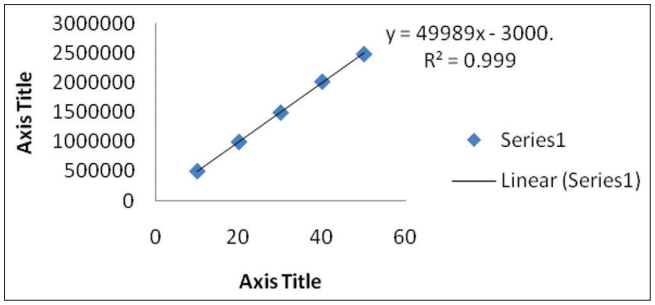

Figure 7: Calibration Graph of Ethinylestradiol

Table 3: Accuracy results of levonorgestrel

% Con	Area	Amount added	Amount found	% Recovery	Mean recovery
50	3513866	5	5.10	101.3	100.5%
100	4735089	10	9.94	99.4	
150	5911797	15	14.8	99.2	

Table 4: Accuracy results of ethinylestradiol

% Con	Area	Amount added	Amount found	% Recovery	Mean recovery
50	2332745	5	5.10	101.8	100.5%
100	3132694	10	9.99	99.9	
150	3918996	15	14.9	99.1	

intra-day analysis which reveals that the proposed method is more precise.

Specificity

The most important aspect of this method was its usage in the formulation analysis. Hence, the marketed formulations were collected and analyzed by employing the above method. The test samples are prepared by weighing a known amount of sample and diluted with the mobile phase. Later the solution was filtered through a membrane filter and the solution was further diluted to get the equivalent concentration like that of the standard solution. The two samples are injected separately and the concentration was calculated as per the standard formula. The results were found to be within the acceptable range.

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

LOD and LOQ were calculated for sensitivity measurement by dividing K* standard deviation of the peak response area with slope, where k =3.3 and 10 for LOD and LOQ. Sensitivity means lowest amount of sample detected with signal response, i.e., 3:1 and 10:1, calculated in terms of %RSD should not be more than 10%. LOD chromatogram for levonorgestrel and ethinylestradiol is shown in Figure 8 and LOQ chromatogram for levonorgestrel and ethinylestradiol is shown in Figure 9.

Table 5: Repeatability results of levonorgestrel

Injection	Area
Injection-1	2235418
Injection-2	2240677
Injection-3	2249491
Injection-4	2245823
Injection-5	2251693
Average	2244605
Standard deviation	6657.7
%RSD	0.33

RSD: Related standard deviation

Table 6: Repeatability results of ethinylestradiol

Injection	Area
Injection-1	1501418
Injection-2	1486941
Injection-3	1490657
Injection-4	1487327
Injection-5	1490385
Average	1491347
Standard deviation	5882.5
%RSD	0.39

RSD: Related standard deviation

Robustness

The robustness of the method was studied by injecting the standard solution with variation in the optimized conditions of the method. It includes change in flow rate and also column temperature. The values indicate there are no significant deviations in results. System suitability results (flow rate change) for levonorgestrel and ethinylestradiol were tabulated below in Tables 7 and 8. System suitability results (change in mobile phase composition) for levonorgestrel and ethinylestradiol are shown in Tables 9 and 10.

Table 7: System suitability results (flow rate change) for levonorgestrel

S. No.	Flow rate (mL/min)	System suitability results	
		USP plate count	USP tailing
1.	0.8	884.4	1.57
2.	1.0	1235.1	1.1
3.	1.2	969.3	1.6

Table 8: System suitability results (flow rate change) for ethinylestradiol

S. No.	Flow rate (mL/min)	System suitability results	
		USP plate count	USP tailing
1.	0.8	1748.5	1.23
2.	1.0	1548.2	1.2
3.	1.2	1948.1	1.2

Table 9: System suitability results (mobile phase) for levonorgestrel

S. No.	Change in mobile phase composition	System suitability results	
		USP plate count	USP tailing
1.	10% less	2257.1	1.51
2.	Actual	2457.2	1.12
3.	10% more	2633.1	1.61

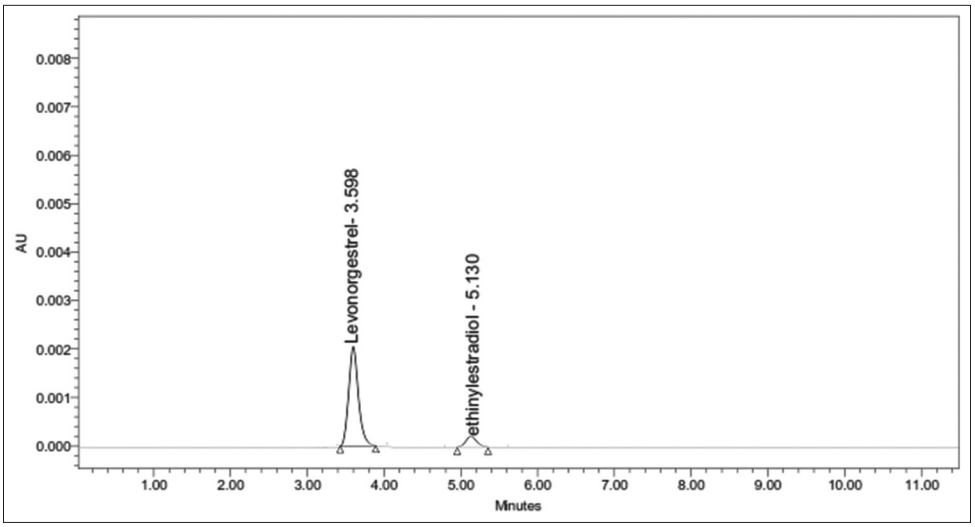

Figure 8: LOD Chromatogram of Levonorgestrel and Ethinylestradiol

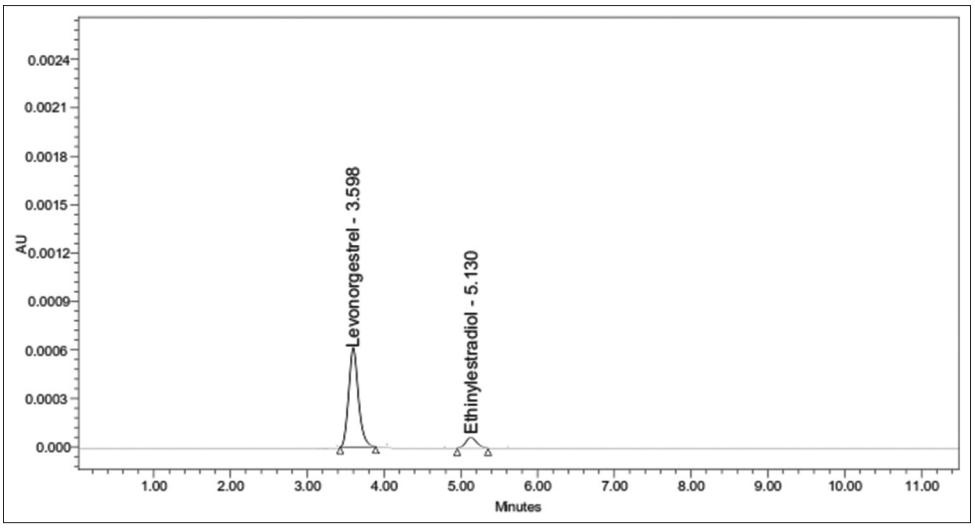

Figure 9: LOQ Chromatogram of Levonorgestrel and Ethinylestradiol

Table 10: System suitability results (mobile phase) for ethinylestradiol

S. No.	Change in mobile phase composition	System suitability results	
		USP plate count	USP tailing
1.	10% less	2357.1	1.22
2.	Actual	2501.3	1.2
3.	10% More	2799.2	1.2

Ruggedness

Ruggedness was deliberate by changing the instruments, columns, solvents, and their proportions. No major changes are observed during the analysis and hence the above proposed method is a rugged method. The ruggedness results for levonorgestrel are shown in Table 11 and for ethinylestradiol are tabulated in Table 12.

Table 11: Ruggedness results of levonorgestrel

Injection No.	Area
1	2194759
2	2195701
3	2196192
4	2195327
5	2200952
Average	2196586
Standard deviation	2496.1
%RSD	0.11

RSD: Related standard deviation

DISCUSSION

The marketed pharmaceutical dosage forms contain the above-mentioned drugs and hence it is most important to have a mono method for the

Table 12: Ruggedness results of ethinylestradiol

Injection no.	Area
1	1456297
2	1457423
3	1456514
4	1454578
5	1451484
Average	1455258
Standard deviation	2347.5
%RSD	0.16

RSD: Related standard deviation

determination of the above-mentioned ingredients. By keeping in mind, the above criteria a simple, rapid method has been developed for the estimation of the above drugs. The results obtained clearly reflects that the method is rugged and sensitive. The method developed has cleared all the validation parameters indicating that the method can be employed for routine quality control analysis.

CONCLUSION

Few liquid chromatography methods were available in the literature for the determination of the levonorgestrel and ethinylestradiol. However, majority of them are reported with single or with other combinations. The proposed method was a simple, sensitive, accurate, and rapid method for the assay of these drugs. The method developed shows lesser run time and an isocratic method has been developed for the separation of drugs. The proposed liquid chromatography method simultaneously determines the amount of these drugs in pure and marketed formulations. The method is simple, accurate, precise, and cost-effective. This method can be employed for routine quality control analysis of the above-mentioned drugs.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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