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RP-HPLC method development and validation for the simultaneous estimation of segesterone and ethinyl estradiol in bulk and its pharmaceutical dosage form

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Abstract
A simple, accurate, precise and robust RP-HPLC method was developed for the simultaneous estimation of the Segesterone and Ethinyl Estradiol in bulk and its pharmaceutical dosage form. Chromatographic separation was achieved on Phenomenex C_{18} Column (150 x 4.6 mm, 5µ). The optimized mobile phase consist of water and acetonitrile in the ratio 45:55%v/v was pumped through column at a flow rate of 0.7 ml/min. Temperature was maintained at 30 °C. Optimized wavelength selected was 260 nm. Retention time of Segesterone and Ethinyl Estradiol were found to be 2.110 min and 2.654min. %RSD of the Segesterone and Ethinyl Estradiol were and found to be 0.9 and 0.7 respectively. %Recovery was obtained as 99.91% and 98.61% for Segesterone and Ethinyl Estradiol respectively. LOD, LOQ values obtained from regression equations of Segesterone and Ethinyl Estradiol were 0.11, 0.32and 0.05, 0.15 respectively. Regression equation of Ethinyl Estradiol is y = 21000x + 637.6 and y = 25485x + 24862 of Segesterone. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

Keywords: Ethinyl estradiol, segesterone, RP-HPLC, mobile phase, retention time

1. Introduction
Segesterone acetate (SA), also known as Nestorone, is a novel orally inactive but extremely efficient 19-nor-progesterone when administered through implantable, vaginal and transdermal structures. It binds selectively to the progesterone receptor (PR), a transcription factor belonging to the nuclear receptor superfamily, where it acts as an agonist and transactivator. When used in combination with segesterone acetate, ethinyl estradiol potentiates the antigonadotropic of the progestin and prevents irregular shedding of the endometrium 6. Segesterone acetate lacks androgenic activity, and displayed binding affinity to androgen receptors that was 500- to 600-fold less than that of testosterone.

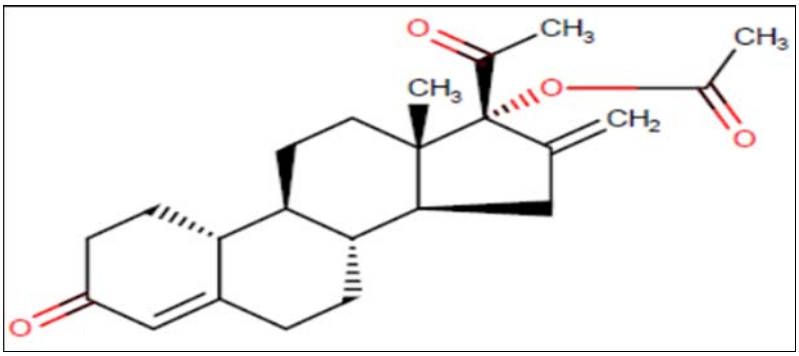

Fig 1: Structure of Segesterone acetate

Ethinyl estradiol has high estrogenic potency when administered orally and is often used as the estrogenic component in oral contraceptives. Estrogens diffuse into their target cells and interact with a protein receptor. Target cells include the female reproductive tract, the mammary gland, the hypothalamus, and the pituitary. Estrogens increase the hepatic synthesis of sex hormone binding globulin (SHBG), thyroid-binding globulin (TBG), and other serum proteins and suppress follicle-stimulating hormone (FSH) from the anterior pituitary.

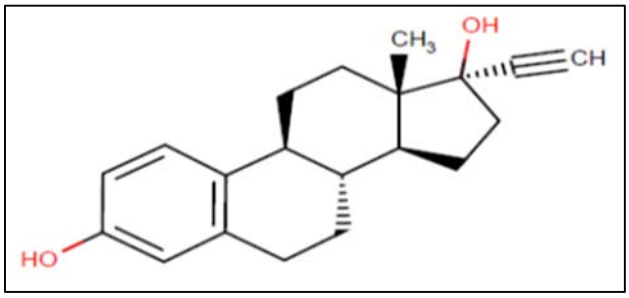

Fig 2: Structure of Ethinyl Estradiol

Literature survey reveals that few analytical methods have been reported for the ethinyl estradiol with different drugs [1-11]. But there is no RP-HPLC method reported for the ethinyl estradiol in combination with segesterone. The present research involves development of a simple, precise, accurate and robust RP-HPLC method with easily available mobile for simultaneous estimation of segesterone and ethinyl estradiol in bulk and pharmaceutical dosage form.

2. Materials and methods

2.1 Materials

Working standards of segesterone and ethinyl estradiol were obtained from Rankem Labs Pvt ltd, Mumbai. HPLC grade methanol, acetonitrile and water were purchased from E. Merck, Mumbai and AR grade potassium hydrogen phosphate and orthophosphoric acid were purchased from Sd fine Chemicals Pvt Ltd, Mumbai.

2.2 Instruments used

Waters HPLC 2695 System equipped with quaternary pumps, Photo Diode Array detector and Auto sampler integrated with Empower 2 Software. UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2 mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of N Ethinyl Estradiol and Segesterone solutions. pH meter and electronic balance were also used in this study.

2.3 Chromatographic conditions

The Phenomnex C_{18} column ODS (150 x 4.6mm, 5µm) equilibrated with mobile phase acetonitrile and water in the ratio of 55:45 (v/v) was used and the flow rate was maintained at 0.7 mL/min. Chromatographic system was operated at 30^0 c. Detection wavelength was monitored at 293 nm, and the injection volume was 10 µL and run time was kept 5 min.

2.4 Preparation of Standard stock solutions

Accurately weighed 4.35mg of Ethinyl Estradiol, 25.75mg of Segesterone were transferred to individual 25 ml volumetric flasks separately. 3/4 th of diluents was added to both of these flasks and sonicated for 10 minutes. Flasks were made up with diluents and labeled as Standard stock solution 1and 2. (174µg/ml of Ethinyl Estradiol and 1030µg/ml of Segesterone). 100% working standard solution was prepared to the stock solution from 1 ml was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (17.4µg/ml Ethinyl Estradiol of and 103µg/ml of Segesterone).

2.5 Preparation of Sample stock solutions

Equivalent to 17.4mg Ethinyl Estradiol and 103mg of Segesterone was transferred into a 100 ml volumetric flask, 20ml of diluents was added and sonicated for 25min, further the volume was made up with diluent and filtered by HPLC filters (174µg/ml of Ethinyl Estradiol and 1030µg/ml of Segesterone). 100% working sample solution was prepared to the stock solution from 1 ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (17.4µg/ml of Ethinyl Estradiol and 103µg/ml of Segesterone).

2.6 Method validation

The developed analytical method was further subjected to validation in accordance to the ICH guidelines. The evaluated parameters like system suitability, specificity, linearity, precision, accuracy, limit of detection, limit of quantification and robustness [12, 13].

2.6.1 System suitability

The system suitability parameters were assessed by preparing standard solutions of Ethinyl Estradiol (17.4ppm) and Segesterone (103ppm) and the solutions were injected into six replicates and measured parameters like peak tailing, resolution and USP plate count were determined. The % RSD for the area of six standard injections results should not be more than 2%.

2.6.2 Specificity

Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

2.6.3 Linearity

Linearity is ability to obtain test results are directly proportional to the concentration. From the standard stock solution appropriate aliquots of Ethinyl Estradiol and Segesterone were taken in different volumetric flasks and make up with mobile phase up to the mark to obtain final concentrations are 4.35, 8.7, 18 13.05, 17.4, 21.75, 26.1 µg/ml for Ethinyl Estradiol and 25.75, 51.5, 77.25, 103,128.75, 154.5 µg/ml for Segesterone respectively. The solutions are injected into a 10 µl fixed loop system and then recorded the chromatograms. The calibration curve was plotted by concentration Vs Peak area.

2.6.4 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the homogenous sample under the prescribed conditions. The intraday precision was evaluated by analyzing the calibration curves of six replicates of different concentrations of Ethinyl Estradiol and Segesterone within the same day. The inter-day precision was determined by analyzing of six replicates of different concentrations on three different days. The total precision of the method was expressed as the relative standard deviation (%RSD).

2.6.5 Accuracy

Recovery assessment was obtained by using standard addition technique which was by adding known quantities of pure standards at three different levels in 50%, 100% and 150% to the pre analysed sample formulation. From the amount of drug found, amount of drug recovered and percentage recovery were calculated which sense to conformation that the proposed method was accurate.

2.6.6 LOD & LOQ
LOD is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. LOQ is the lowest concentration of analyte that can be determined with acceptable precision and accuracy. These two parameters were calculated using the formula LOD = 3.3*S D/S and LOQ = 10*SD/S, where SD = standard deviation of response (peak area) and S = slope of the calibration curve.

2.6.7 Robustness
Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines. Robustness conditions like Flow minus (0.6ml/min), Flow plus (0.8ml/min), mobile phase minus, mobile phase plus, temperature minus (25 °C) and temperature plus (35 °C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

3. Results and Discussion
3.1 System suitability
All the system suitability parameters were within the range and satisfactory as per ICH guidelines. According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits. The results were shown in table 1 and fig 3

Table 1: System suitability parameters for Ethinyl Estradiol and Segesterone

S. No	Segesterone			Ethinyl Estradiol			
INJ	RT(min)	USP Plate Count	Tailing	RT(min)	USP Plate Count	Tailing	Resolution
1	2.109	3511	1.27	2.639	5620	1.22	3.6
2	2.111	3619	1.23	2.640	5856	1.21	3.7
3	2.114	3515	1.23	2.642	5860	1.21	3.6
4	2.115	3427	1.23	2.645	5624	1.18	3.6
5	2.119	3354	1.27	2.654	5556	1.21	3.7
6	2.122	3527	1.28	2.654	4916	1.24	3.5

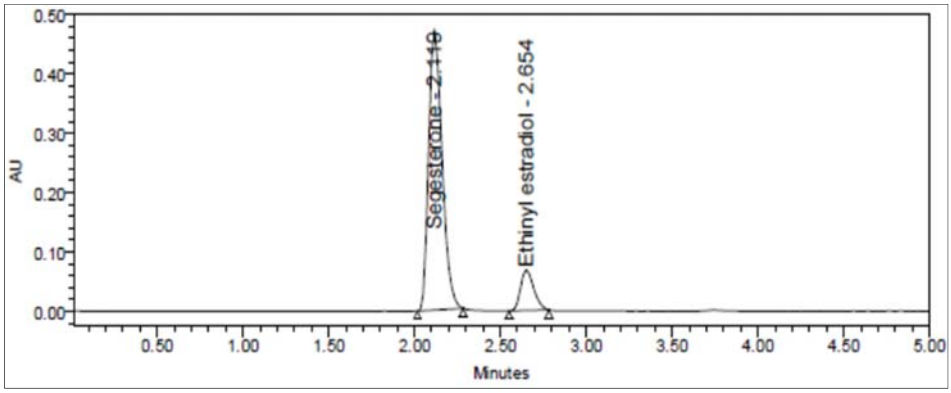

Fig 3: System suitability Chromatogram

3.2 Specificity
Retention times of Segesterone and Ethinyl Estradiol were 2.112 min and 2.647 min, respectively. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific. The specificity data was reported in fig 4.

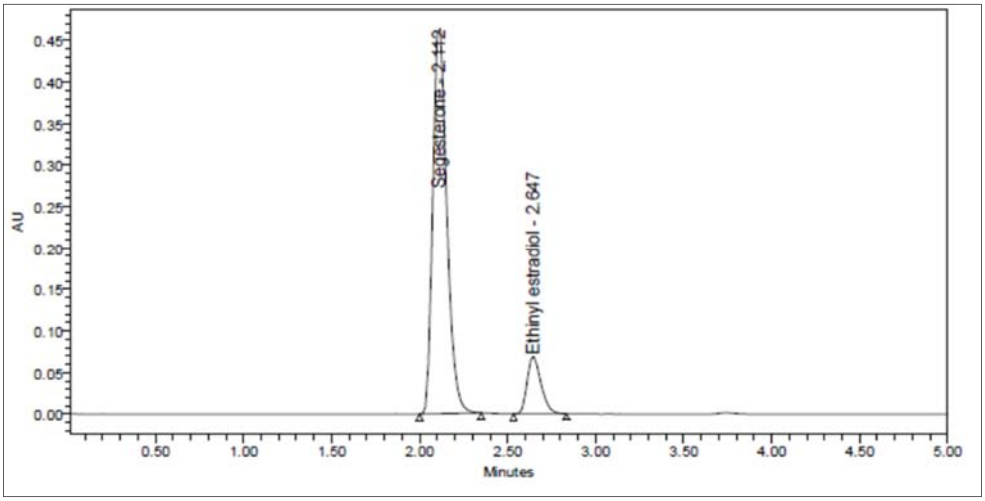

Fig 4: Specificity chromatogram

3.3 Linearity

Six linear concentrations of Ethinyl Estradiol (4.35-26.1µg/ml) and Segesterone (25.75-154.5µg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Ethinyl Estradiol was y = 21000x + 637.6 and of Segesterone was y = 25485x + 24862. Correlation coefficient obtained was 0.999 for the two drugs. The results were explained in table 3 and fig 5 & 6.

Table 3: Linearity table for Ethinyl Estradiol and Segesterone.

Ethinyl Estradiol		Segesterone	
Conc (µg/mL)	**Peak area**	**Conc (µg/mL)**	**Peak area**
0	0	0	0
4.35	90736	25.75	708904
8.7	183986	51.5	1342290
13.05	277182	77.25	1956884
17.4	368201	103	2674816
21.75	453039	128.75	3356416
26.1	549652	154.5	3915688

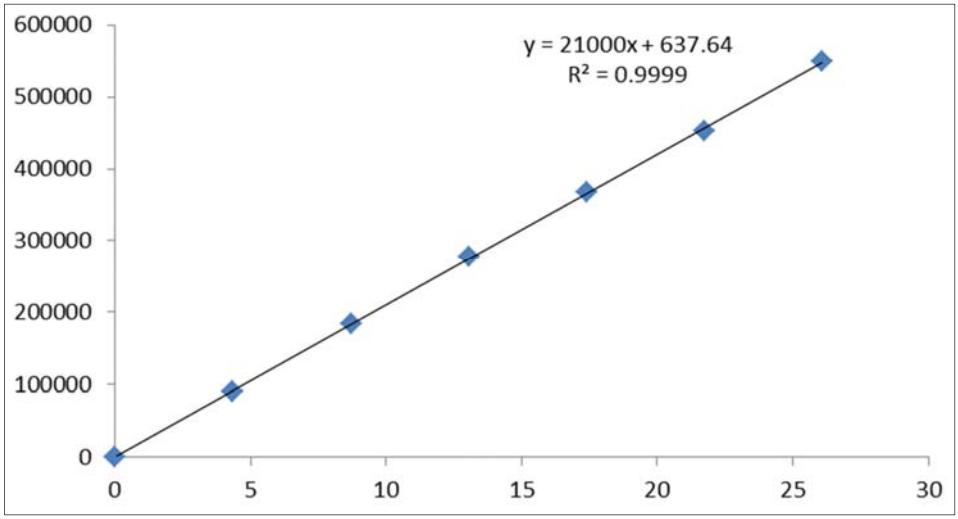

Fig 5: Calibration curve of Ethinyl Estradiol

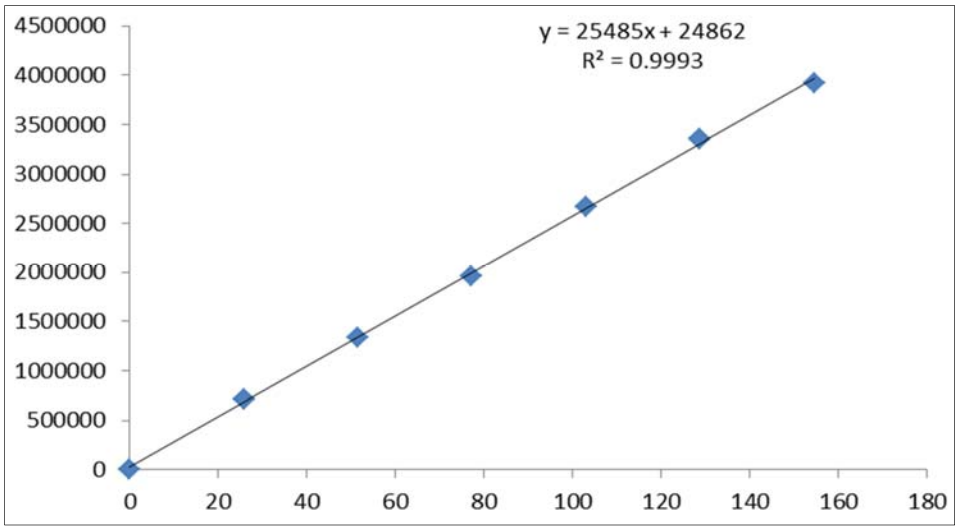

Fig 6: Calibration curve of Segesterone

3.4 Precision

Multiple sampling from a sample stock solution six replicates of same concentrations were prepared and individually injected into the chromatographic system. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.7% and 0.9% for intraday precision and 0.6% and 0.7% for inter day precision respectively for Ethinyl Estradiol and Segesterone. As the limit of Precision was less than "2" the system precision was passed in this method. The results were shown in table 4.

Table 4: Precision results for Ethinyl Estradiol and Segesterone

	Intraday		Inter day	
S. No	**Area of Ethinyl Estradiol**	**Area of Segesterone**	**Area of Ethinyl Estradiol**	**Area of Segesterone**
1.	370187	2673796	354621	2396892
2.	365161	2659013	353532	2407029
3.	369768	2699965	356559	2398743
4.	365026	2645774	352308	2371535
5.	365577	2632469	352123	2418266
6.	364742	2651511	357313	2380107
Mean	366744	2660421	354409	2395429
S.D	2522.9	23748.0	2168.4	17175.0
%RSD	0.7	0.9	0.6	0.7

3.5 Accuracy

Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were prepared and injected to the HPLC system then calculate the mean %recovery and it was obtained as 98.61% for Ethinyl Estradiol and 99.91% for Segesterone respectively. The results were given in table 5 & 6.

Table 5: Accuracy table of Ethinyl Estradiol

Spike Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	8.7	8.608	98.95	98.61%
	8.7	8.680	99.77	
	8.7	8.580	98.62	
100%	17.4	17.064	98.07	
	17.4	17.072	98.12	
	17.4	17.064	98.07	
150%	26.1	25.642	98.24	
	26.1	25.778	98.77	
	26.1	25.811	98.89	

Table 6: Accuracy table of Segesterone

Spike Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	51.5	51.81	100.60	99.91%
	51.5	51.35	99.71	
	51.5	50.85	98.73	
100%	103	102.88	99.88	
	103	102.72	99.73	
	103	102.15	99.17	
150%	154.5	155.43	100.60	
	154.5	155.05	100.36	
	154.5	155.09	100.38	

3.6 LOD & LOQ

Signal-to-noise ratios of 3:1 and 10:1 were obtained for the LOD and LOQ respectively. The LOD was found to be 0.05 for ethinyl estradiol and 0.11 for segesterone and LOQ was found to be 0.15 for ethinyl estradiol and 0.32 for segesterone.

3.7 Robustness

Robustness conditions like Flow minus (0.6ml/min), Flow plus (0.8ml/min), mobile phase minus (60B:40A), mobile phase plus (50B:50A), temperature minus (25 °C) and temperature plus (35 °C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit. The results were shown in table 7.

Table 7: Robustness data for Ethinyl Estradiol and Segesterone

S.no	Condition	%RSD of Segesterone	%RSD of Ethinyl Estradiol
1	Flow rate (-) 0.6ml/min	0.5	0.9
2	Flow rate (+) 0.8ml/min	0.3	0.6
3	Mobile phase (-) 60B:40A	0.3	1.1
4	Mobile phase (+) 60B:50A	0.2	1.1
5	Temperature (-) 25 °C	0.3	0.3
6	Temperature (+) 35 °C	0.2	0.4

3.8 Analysis of marketed formulation

Marketed formulation of ethinyl estradiol and segesterone combination was available with brand name of ANNOVERA and bearing the label claim Ethinyl Estradiol 17.4mg, Segesterone 103mg. Assay was performed with the above formulation. Average % Assay for Ethinyl estradiol and Segesterone obtained was100.42% and 99.75% respectively.

3.9 Discussion

Many works reviewed the use of C18 column for separation

and acetonitrile used as a main solvent. The developed method was compared to the past reported works it showing good resolution and shorter run time. Present developed method was newer, simple, precise, accurate, robust and cost effective RP-HPLC method for the simultaneous estimation of ethinyl estradiol and segesterone in pharmaceutical dosage form.

4. Conclusion

The proposed work concluded that simple, specific, precise, accurate and robust RP-HPLC method can be used for simultaneous analysis ethinyl estradiol and segesterone in bulk samples and its dosage form. The result of the study follows the protocol of ICH guidelines and it can be successfully applied for the simultaneous estimation of the marketed products of ethinyl estradiol and segesterone in bulk samples and its combined dosage form.

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