

METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF SEGESTERONE & ETINYL ESTRADIOL BY RP-HPLC METHOD**Butt Khadheeja*, Dr. Devanaboyina Narendra and Gadi Vijaya Lakshmi**

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ABSTRACT

A simple, Accurate, precise method was developed for the simultaneous estimation of the Segesterone and Ethinyl Estradiol in syrup dosage form. Chromatogram was run through AgilentC18 150 x 4.6 mm, 5 μ . Mobile phase containing Buffer 0.01N KH₂PO₄ (5ph): Acetonitrile taken in the ratio 65:35 was pumped through column at a flow rate of 1.0ml/min. Buffer used in this method was 0.01N KH₂PO₄. Temperature was maintained at 30°C. Optimized wavelength selected was 260nm. A simple, Accurate, precise method was developed for the simultaneous estimation of the Segesterone and Ethinyl Estradiol in syrup dosage form. Retention time of Segesterone and Ethinyl Estradiol were found to be 2.302 min and 3.324min. %RSD of the Segesterone and Ethinyl Estradiol were found to be 0.6 and 1.0 respectively. %Recovery was obtained as 100.33% and 99.45% for Segesterone and Ethinyl Estradiol respectively. LOD, LOQ values obtained from regression equations of Segesterone and Ethinyl Estradiol were 0.91, 2.75 and 0.04, 0.11 respectively. Regression equation of Ethinyl Estradiol is $y = 14306x + 2432.1$. And $y = 7317.8x + 2228.5$ of Segesterone. Retention times were decreased and 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.**INTRODUCTION**

Chemically Segesterone acetate (SGS) selectively binds to the progesterone receptor (PR), a transcription factor belonging to the nuclear receptor superfamily, where it acts as an agonist and transactivator 5. According to the findings from docking experiments, it adopts the same docking position within the PR ligand-binding domain (LBD) as progesterone but due to additional stabilizing contacts between 17 α -acetoxy and 16-methylene groups and PR LBD, segesterone acetate display higher potency than progesterone 5. As with other progestins, segesterone acetate prevents ovulation by blocking the midcycle surge in luteinizing hormone (LH) secretion, thereby inhibiting the development of ovarian follicles 6. 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 4. It does not display binding affinity toward estrogen receptors 4. When the relative binding affinities of segesterone acetate to human steroid receptors were investigated in vitro, it was demonstrated that segesterone acetate binds to the glucocorticoid receptor 3. However, segesterone acetate did not exert any

glucocorticoid activity in the in vivo assays showing no increase in liver glycogen and tyrosine transaminase TAT 3. Structure of the SGS was shown in figure 1 (A).^[1]

Chemically Ethinyl estradiol (EED) is a 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. This cascade is initiated by initially binding to the estrogen receptors. The combination of an estrogen with a progestin suppresses the hypothalamic-pituitary system, decreasing the secretion of gonadotropin-releasing hormone (GnRH). Structure of the EED was shown in figure 1 (B).^[2]

Literature survey reveals there are several methods to estimated these drugs in single or in combination of two or three drugs.^[5-9] But there is only very few HPLC methods are available for simultaneous estimation of SGS and EED, so the scope of developing and validating an analytical method is to ensure a suitable method for a

particular analyte to be more specific, accurate and precise. The main objective for that is to improve the

conditions and parameters, which should be followed in the development and validation processes.

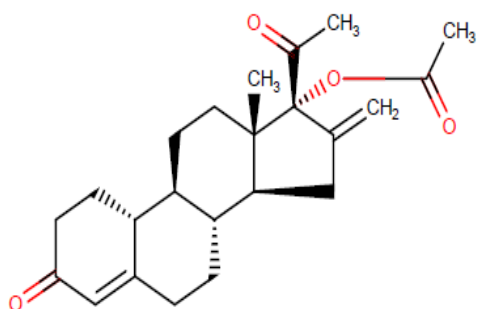
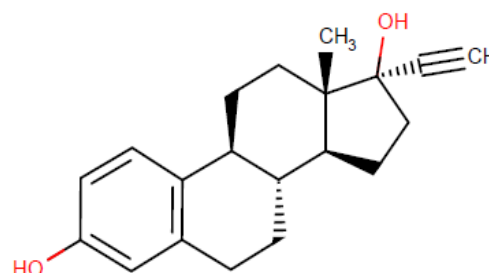


Figure 1: Structure of (A) Segesterone.



(B) Ethinyl Estradiol.

MATERIALS AND METHODS

• **Reagents and Chemicals:** Ethinyl Estradiol and Segesterone pure drugs (API), Combination Ethinyl Estradiol and Segesterone (Annovera), Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem.

Instrumentation: HPLC (waters 2695) system with Empower-2 software and 2996 module photo diode array detector equipped with a quaternary solvent delivery pump, automatic sampler unit, Agilent C18 (4.6 x 150mm, 5 μ m). As part of experimentation, additional equipment such as sonicator (ultrasonic cleaner power sonic 420), pH meter, vacuum oven (wadegati), water bath and other glassware were used for the present investigation.

Chromatographic conditions: The Agilent C18 (4.6 x 150mm, 5 μ m) column was used for analytical separation. Potassium dihydrogen ortho phosphate and one drop of triethyl amine in every 100ml of Acetonitrile and 0.01N NH₂ PO₄ was taken in the ratio of (35:45% v/v) mobile phase for the investigation with a flow rate of a 0.8ml/min. The temperature was maintained at 30°C. The injection volume was 10 μ l and the UV detection was achieved at 260nm.

Preparation of potassium dihydrogen ortho phosphate buffer (pH:3.0): Accurately weighed 1.36gm of Potassium dihydrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then PH adjusted to 3.45 with dil. Orthophosphoric acid solution.

Preparation of mobile phase

Buffer: Water - in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water

Preparation of mixture Standard stock solution: Accurately weighed 8.7mg of Ethinyl Estradiol, 51.5mg of Segesterone and transferred to individual 50ml 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 1 and 2. (174 μ g/ml of Ethinyl Estradiol and 1030 μ g/ml of Segesterone).

Preparation of Sample (Tablet) stock solutions: : Equivalent to 17.4mg Ethinyl Estradiol and 103mg of Segesterone was transferred into a 100ml volumetric flask, 20ml of diluents was added and sonicated for 25min, further the volume was made up with diluent and filtered by HPLC filters (174 μ g/ml of Ethinyl Estradiol and 1030 μ g/ml of Segesterone).

Optimized chromatographic conditions

Column Used : Agilent C18 (4.6 x 150mm, 5 μ m)

Mobile phase : Acetonitrile : 0.01N NH₂ PO₄ (35:65 v/v)

Flow rate : 0.8ml/min

Wavelength : 260.0 nm

Temperature : 30°C

Injection Volume : 10.0 μ l

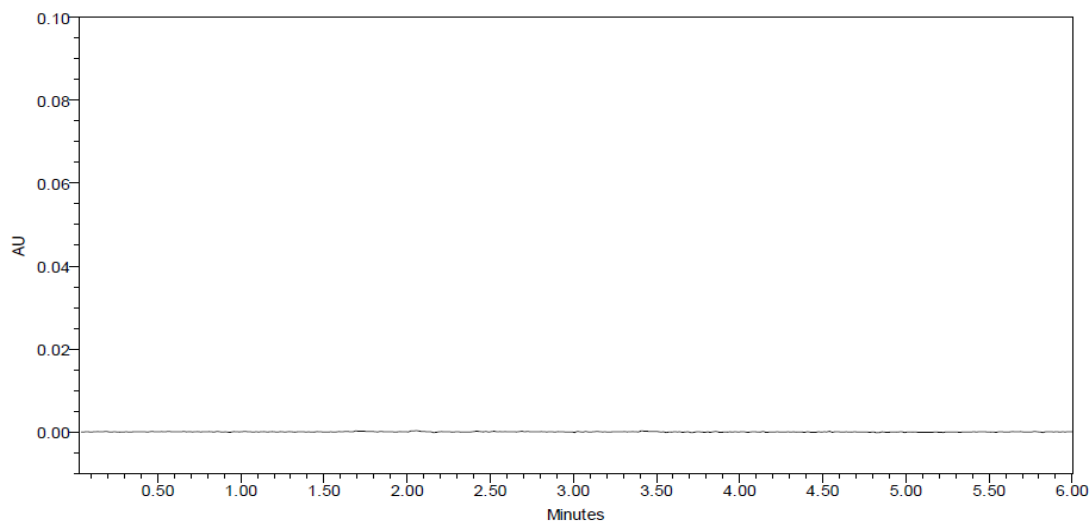


Figure 2: Blank chromatogram.

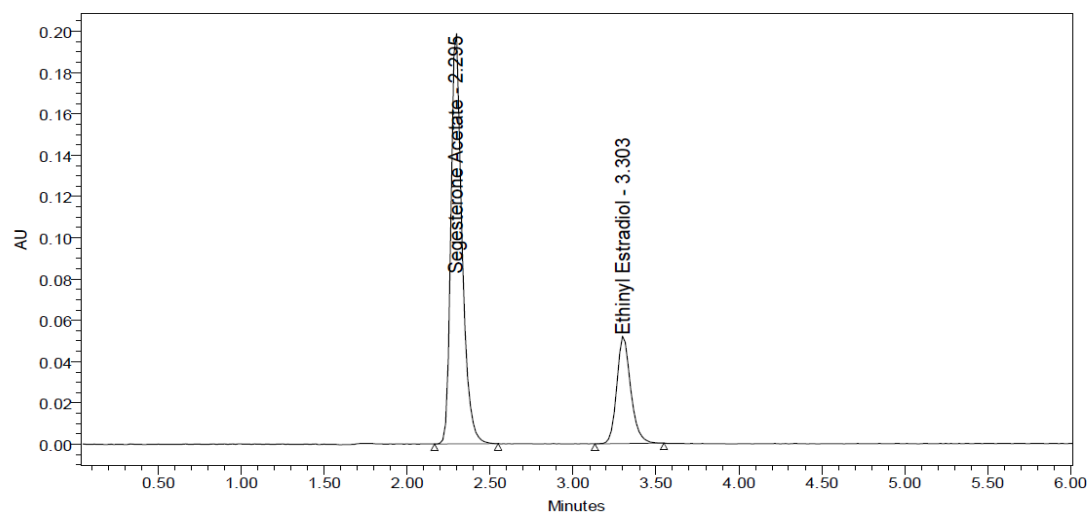


Figure 3: Chromatogram of standard mixture of SGS & EED.

| | Peak Name | RT | Area | USP Tailing | USP Resolution | USP Plate Count |
|---|-------------------|-------|--------|-------------|----------------|-----------------|
| 1 | Segesterone | 2.295 | 243678 | 1.28 | 6 | 6424 |
| 2 | Ethinyl Estradiol | 3.303 | 751512 | 1.16 | 7.6 | 7681 |

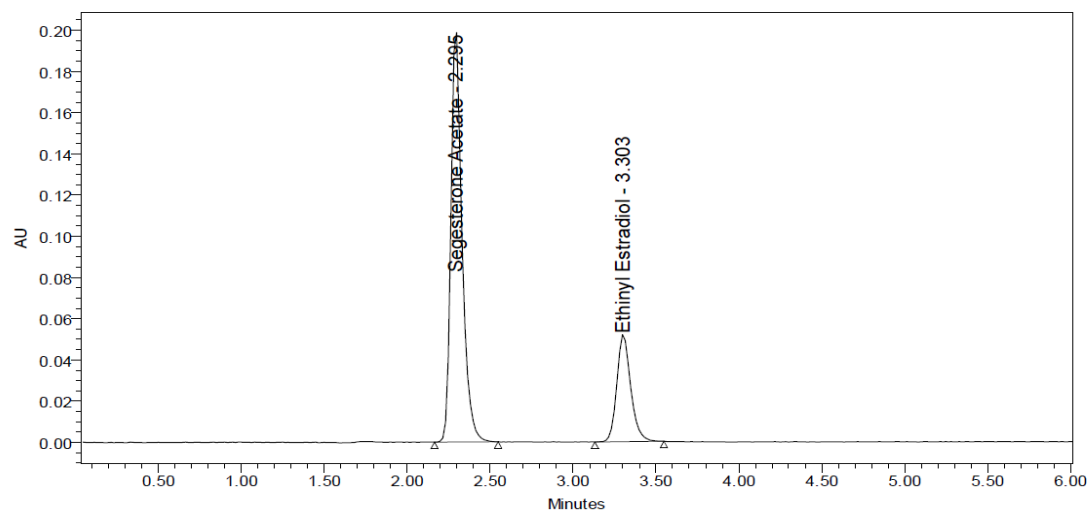


Figure 4: Chromatogram of sample mixture of SGS & EED.

VALIDATION

The above optimized chromatographic method has been validated for the assay of SGS & EED using the following parameters [International Conference on Harmonization (ICH) 1995]. Linearity was studied to find out the relationship of concentration with Peak area. Six different concentrations of Segesterone and Ethinyl Estradiol (SGS & EED) drug mixtures respectively. Each concentration of solution was injected into the HPLC and chromatogram was recorded. The calibration graph was constructed by plotting the peak versus the final concentration of the each drug ($\mu\text{g/ml}$) and the corresponding regression equation derived. Precision was studied to find out variations in the test methods of mixtures of Segesterone (51.5mg)+ Ethinyl Estradiol (8.7mg) respectively. The precision of each method was ascertained separately from the peak area by actual determination of five replicates of a fixed amount of Segesterone (51.5mg)+ Ethinyl Estradiol (8.7mg) respectively. The %RSD (percentage relative standard deviation) was calculated for precision and ruggedness. The accuracy of the method was shown by analyzing the model mixtures containing 80,100 and 120% of Segesterone and Ethinyl Estradiol. After the measurement, the Amount found and individual recoveries were calculated. Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated based on the linearity data using the formulae $\text{LOD} = 3.3 \times \text{standard deviation} / \text{slope}$; $\text{LOQ} = 10 \times \text{standard deviation} / \text{slope}$. Robustness was performed by following the same method with different flow rate.

RESULTS AND DISCUSSION

The regression equation for SGS was found to be $y = 7317.8x + 2228.5$ (slope, intercept and correlation coefficient were found to be 7317.8, 2228.5 and 0.999 respectively) and linear over beer's range of 25.75-154.5 $\mu\text{g/ml}$. The regression equation for EED was found to be $y = 14306x + 2432.1$ (slope, intercept and correlation coefficient were found to be 14306, 2432.1 and 0.999

respectively) and linear over beer's range of 4.35-26.1 $\mu\text{g/ml}$. Linearity graph of SGS & EED were shown in Figure 5 & 6 respectively. Linearity data was shown in table 1. The precision and ruggedness were determined using the % RSD of the peak area for six replicate preparations of the drug. %RSD of system precision for Segesterone and Ethinyl Estradiol were and found to be 0.9 and 1.2 respectively. %RSD of method precision for Segesterone and Ethinyl Estradiol were and found to be 0.6 and 1.0 respectively. % recovery was obtained as 100.33% and 99.45% for Segesterone and Ethinyl Estradiol respectively. The calculated RSD values were less than 2. Precision and ruggedness data are presented in Table 2. In order to verify the accuracy of the described method, recovery studies were carried out by analyzing model mixtures contained 50%, 100% and 150% of standard solution of drug SGS & EED and along with 5 $\mu\text{g/mL}$ of placebo solution within the linearity ranges. The mean percentage recoveries were found to be 99.49% and 99.55% w/w for 50%, 100% and 150% respectively. The results of accuracy were shown that the developed method have a good percentage recovery at different concentrations of drugs. LOD for SGS & EED was found to be 0.91 $\mu\text{g/ml}$ and 0.04 $\mu\text{g/ml}$ respectively. LOQ for SGS & EED was found to be 2.75 $\mu\text{g/ml}$ and 0.11 $\mu\text{g/ml}$ respectively. Summary of all the validation parameter shown in table 6.

Degradation

Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed the limits of degradation.

CONCLUSION

A simple, accurate, precise method was developed for the simultaneous estimation of the Segesterone and Ethinyl Estradiol in Tablet dosage form was developed and the proposed method as suitable for routine analysis of SGS & EED.

Table 1: Linearity table for SGS & EED.

| Segesterone | | Ethinyl Estradiol | |
|---------------------------|-----------|---------------------------|-----------|
| Conc ($\mu\text{g/mL}$) | Peak area | Conc ($\mu\text{g/mL}$) | Peak area |
| 0 | 0 | 0 | 0 |
| 25.75 | 186429 | 4.35 | 63069 |
| 51.5 | 381094 | 8.7 | 131372 |
| 77.25 | 575943 | 13.05 | 190647 |
| 103 | 759204 | 17.4 | 251593 |
| 128.75 | 939205 | 21.75 | 313281 |
| 154.5 | 1130822 | 26.1 | 373962 |

Table 2: System precision table of SGS & EED.

| S. No | Area of Segesterone | Area of Ethinyl Estradiol |
|-------|---------------------|---------------------------|
| 1. | 754659 | 245364 |
| 2. | 755664 | 239434 |
| 3. | 739597 | 242933 |
| 4. | 747388 | 247677 |
| 5. | 754142 | 244324 |
| 6. | 757619 | 242335 |
| Mean | 751512 | 243678 |
| S.D | 6787.2 | 2815.0 |
| %RSD | 0.9 | 1.2 |

Table 6: summary of validation data of EED & SGS.

| Parameters | Ethinyl Estradiol | Segesterone | LIMIT |
|---|----------------------------|------------------------------|-----------------------------|
| Linearity Range($\mu\text{g/ml}$) | 4.35-26.1 $\mu\text{g/ml}$ | 25.75-154.5 $\mu\text{g/ml}$ | R < 1 |
| Regression coefficient | 0.999 | 0.999 | |
| Slope(m) | 14306 | 7317.8 | |
| Intercept(c) | 2432.1 | 2228.5 | |
| Regression equation ($Y=mx+c$) | $y = 14306x + 2432.1$ | $y = 7317.8x + 2228.5$ | |
| Assay (% mean assay) | 99.55% | 99.49% | 90-110% |
| Specificity | Specific | Specific | No interference of any peak |
| System precision %RSD | 1.2 | 0.9 | NMT 2.0% |
| Method precision %RSD | 1.0 | 0.6 | NMT 2.0% |
| Accuracy%recovery | 99.45% | 100.33% | 98-102% |
| LOD | 0.04 | 0.91 | NMT 3 |
| LOQ | 0.11 | 2.75 | NMT 10 |
| Robustness | FM | 0.3 | %RSD NMT 2.0 |
| | FP | 0.2 | |
| | MM | 1.1 | |
| | MP | 0.3 | |
| | TM | 1.0 | |
| | TP | 1.0 | |

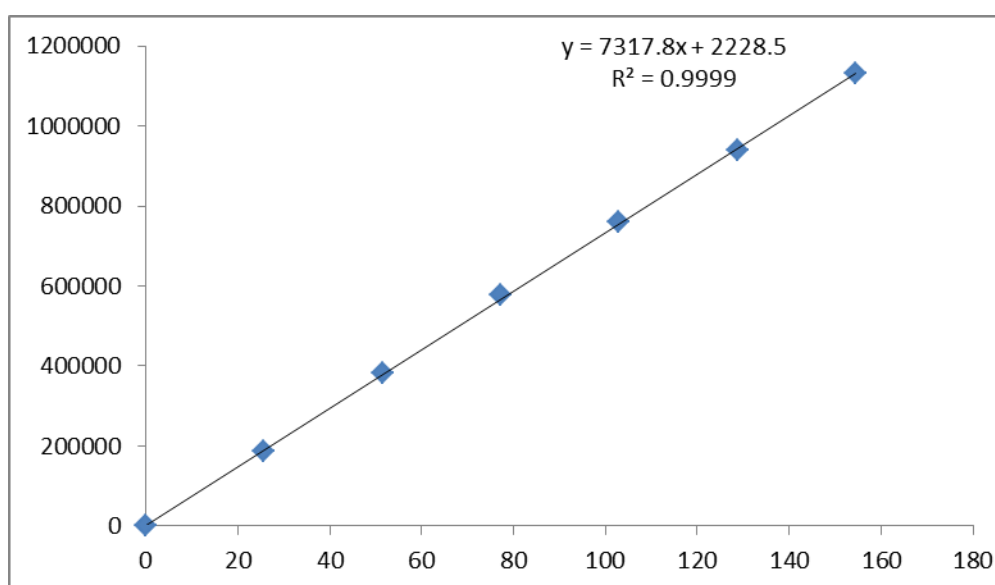


Fig No 7: Linearity curve of Segesterone.

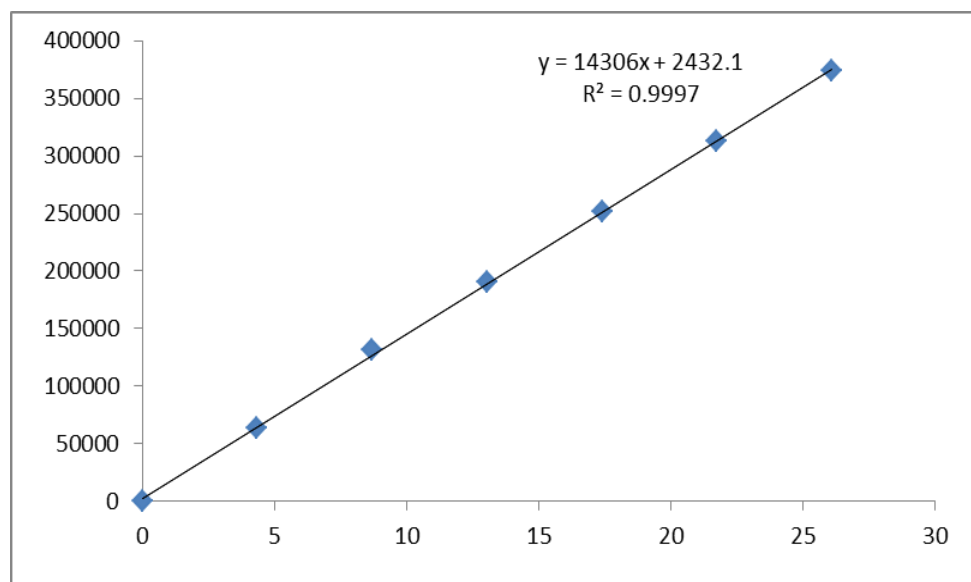


Fig No 8: Linearity curve of Ethinyl Estradiol.

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