DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF ETHINYL ESTRADIOL AND GESTODENE IN BULK AND PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

Objective: The principal objective of this study is to develop and validate a simple, new, fast, selective, precise, and economic stability-indicating the RP-HPLC method for the simultaneous estimation of Ethinyl estradiol and Gestodene in a bulk and pharmaceutical dosage form.

Methods: The present method was developed and validated on a Waters HPLC system using Phenomenex Gemini C18(250 mm × 4.6 mm i.d., 5 µm particle size) column and mobile phase composition of phosphate buffer: Acetonitrile (75:25 v/v) and the pH was adjusted to 3.6 using dilute orthophosphoric acid. The system was regulated at 1.0 ml/min flow rate at 237 nm UV detection.

Results: The two drugs Ethinyl Estradiol and Gestodene, were eluted at 1.788 min and 3.475 min retention time, respectively. The analytical parameters such as accuracy, precision, linearity, LOD, LOQ, ruggedness, and robustness were used for validating the developed method according to International Conference on Harmonisation [ICH] guidelines. Linearity was exhibited over the concentration range of 10-50µg/ml and 25-125µg/ml for Ethinyl Estradiol and Gestodene, respectively. The method revealed the Limit of Detection and Quantitation values for Ethinyl Estradiol and Gestodene were 1.399µg/ml, 3.909µg/ml and 4.24µg/ml, 11.85µg/ml, respectively. The stress testing was carried out to give rise to degradation products by exposing the drugs to acid, alkali, thermal, oxidative, photolytic, and hydrolytic degradation. The obtained data showed that the content of Active pharmaceutical ingredients and the degradation products were successfully separated without any interference, which confirmed the stability-indicating nature of the developed method.

Conclusion: The new, simple, rapid, selective, precise, and economic stability-indicating RP-HPLC method has been successfully developed and validated. It can be satisfactorily applied for the periodic laboratory quantitative estimation of Ethinyl Estradiol and Gestodene in formulations and active pharmaceutical ingredients.

Keywords: RP-HPLC, Method development, Method validation, Forced degradation studies

INTRODUCTION

Ethinyl Estradiol is chemically (8R,9S, 13S, 14S, 17β)-17-ethynyl-13-methyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a] phenanthrene-3,17-diol [fig. 1], an estrogen drug is used widely in birth control pills in combination with progestins. It is used to treat menopausal symptoms, gynecological disorders, and certain hormone-sensitive cancers [1]. Ethinyl estradiol binds to the estrogen receptor complex, enters the nucleus, and activates the DNA transcription process. It prevents ovulation by decreasing luteinizing hormone, which in turn decreases endometrial vascularization and decreases gonadotrophic hormone. In epididymal tissue, it lowers testosterone levels and prevents prostatic cancer by inhibiting the 5-alpha reductase enzyme. Along with cancer treatment, it is also used for osteoporosis [2].

Fig. 1: Chemical structure of Ethinyl estradiol

Gestodene is chemically (8R,9S,10R,13S,14S,17β)-13-ethyl-17-ethynyl-17-hydroxy-1,2,6,7,8,9,10,11,12,14-decachrycloeptant[a]phenanthrene-3-one [fig. 2], a progestogen hormonal contraceptive used in birth control pills and also used in menopausal hormone therapy [3]. It inhibits growth hormone release from the pituitary gland and suppresses ovulation. It also disrupts fertilization and inhibits implantation [4].

Femovan tablet has 0.03 mg of Ethinyl estradiol and 0.075 mg of Gestodene as active pharmaceutical ingredients. This combination drug is used for contraception and in the treatment of irregular periods. This drug helps to prevent the release and fertilization of the egg. It is also used to treat ovarian cancer.

From the literature survey, it was revealed that several methods like RP-HPLC [5, 6], UPLC/MS-MS [7], and stability-indicating RP-HPLC [8] methods are described for the quantitative determination of Ethinyl estradiol and Gestodene. Ethinyl estradiol and...
Materials and methods

Chemicals and reagents

The standard drugs of Ethinyl estradiol and Gestodene were collected as gift samples from Sura labs, Hyderabad. The commercial tablet dosage form FEMOVAN containing 0.03 mg of Ethinyl estradiol and 0.075 mg of Gestodene, marketed by Bayer Zydius Pharma Ltd., was procured from a local pharmacy. Acetonitrile, orthophosphoric acid used for the preparation of the mobile phase (HPLC grade), Methanol (HPLC grade), water (HPLC grade), and Pharma Ltd., was procured from a local pharmacy. Acetonitrile was purchased from Merck India. The standard drugs of Ethinyl estradiol and Gestodene were dissolved in 10 ml of mobile phase, sonicated, and filtered. Further prepared 20µg/ml and 50µg/ml concentration standard solutions were injected five times into the HPLC system, recorded the chromatograms, measured the peak areas, and calculated the % RSD for all five replicate injections on the same day under unchanged operating conditions over a short period and calculated the % RSD.

Instrument used

The present method was quantitatively estimated on a Waters Alliance 2695 separation module HPLC system, and data processing was done using Empower 2 software. The eluates were monitored at 237 nm by 996 Photo-diode array detectors. Sonication’s dissolution and degassing of the solvents and the mobile phase were achieved on Labman digital ultra sonicator. The pH of the solution was adjusted by using a Lab India pH meter.

Methods

Chromatographic conditions

The simultaneous estimation was achieved on Phenomenex Gemini C18(250 mm × 4.6 mm i. d, 5 µm particle size) column with mobile phase composition of phosphate buffer and acetonitrile (75:25 v/v, pH 3.6) adjusted to a flow rate of 1.0 ml/min for a total 8 min run time. The eluates were monitored at 237 nm by a Photo-diode array detector, and the two drugs Ethinyl Estradiol and Gestodene were eluted at 1.786 min and 3.475 min retention time, respectively.

Preparation of stock and working stock solutions

An accurately weighed 10 mg of Ethinyl Estradiol and Gestodene standard drugs were dissolved in 10 ml of mobile phase, sonicated, and filtered. Further prepared 20µg/ml and 50µg/ml concentration working stock solutions of Ethinyl Estradiol and Gestodene respectively, mixed thoroughly, sonicated, and filtered through 0.45µm membrane filter. Introduced the samples into the HPLC system, recorded the chromatograms, and noted the best-optimized conditions to proceed for validation as per ICH guidelines.

Preparation of sample solution

Femovan tablets (containing 0.03 mg Ethinyl estradiol and 0.075 mg Gestodene) were taken and crushed in a mortar using a pestle. An equivalent amount of 10 mg of tablet powder was weighed and dissolved in the diluent, diluted to volume, mixed thoroughly, sonicated, and filtered. Injected the sample in triplicates and calculated the % assay.

Method validation

As per ICH guidelines [10, 11], the parameters checked for method validation are Accuracy, precision, linearity, LOD, LOQ, specificity, and robustness [12-14].

System suitability

For evaluating the system suitability, the mixed working standard solution of Ethinyl Estradiol and Gestodene was injected five times into the HPLC system, recorded the chromatograms, measured the areas, and calculated the % RSD for all five injections in HPLC.

Linearity

The Linearity of the method was determined by plotting the standard curve in the concentration range of 10-50µg/ml and 25-125µg/ml for Ethinyl Estradiol and Gestodene, respectively. The peak areas were noted by injecting each level into the chromatographic system. Plotted a calibration curve of analyte response versus concentration, and by linear regression analysis, the correlation coefficient was calculated.

Accuracy

For evaluating the method’s accuracy, added a pre-analyzed sample solution of 20µg/ml of Ethinyl estradiol and 50µg/ml of Gestodene to a known amount of standard solution of Ethinyl Estradiol (10, 20, and 30µg/ml) and Gestodene (25, 50 and 75µg/ml) respectively. All the solutions were prepared and injected in triplicates. Recorded the chromatograms and measured the peak responses. Calculated the Amount found and Amount added for Ethinyl Estradiol and Gestodene and calculated the individual recovery and mean recovery values.

Precision

The developed method’s precision was evaluated by Intra-assay precision and Intermediate Precision.

Intra-day precision

The repeatability of the method was determined by introducing the standard solution containing 20µg/ml of Ethinyl Estradiol and 50µg/ml of Gestodene for five replicate injections, noted the areas on the same day under unchanged operating conditions over a short period and calculated the % RSD.

Intermediate precision

The intermediate precision of the method was evaluated by injecting the standard solution containing 20 µg/ml and 50 µg/ml of Ethinyl Estradiol and Gestodene, respectively, for five times. Measured the areas and calculated the %RSD for the five replicate injections on different days under unchanged operating conditions.

Robustness

The robustness of the developed method was evaluated by deliberate variations in flow rate and mobile phase organic composition.

Effect of the slight change in flow rate

Examined the solution at 0.9 ml/min and 1.1 ml/min in rather than 1.0 ml/min, under unchanged operating conditions, 20 µl of the mixed standard solution was injected, recorded the chromatograms, and compared with an optimized chromatogram.

Effect of the slight change of percent organic mobile phase

The sample was analyzed by varying the percentage of organic mobile phase composition in the ratio of 70:30, 80:20 instead of 75:25, under identical conditions. Injected into the HPLC, recorded the chromatograms and compared with an optimized chromatogram.

Detection limit

The Ethinyl estradiol and Gestodene LOD values were quantitated by using the formula- 

\[ \text{LOD} = \frac{3.3 \times \sigma}{S} \]

Where, 

\( \sigma \) = Standard deviation of the intercept 
\( S \) = Slope of the calibration curve

Limit of quantitation

The quantitation limit of an analyte in the samples was quantified by using the formula-

\[ \text{LOQ} = 10 \times \sigma/S \]

Where, 

\( \sigma \) = Standard deviation of the response 
\( S \) = Slope of the calibration curve

Forced degradation studies

According to the ICH guidelines, stress testing was carried out upon exposure to extreme stress conditions of acid, base, peroxide,
thermal, UV, and hydrolytic degradation. Later studied the main peak of the drug for peak purity by calculating the percentage of degraded amount and percentage of the active amount.

Acid degradation
Added 1 ml of 2N HCl to 1 ml of Ethinyl estradiol and Gestodene stock solutions and refluxed at 60 °C for 30 min. Later 2N NaOH was added to neutralize the solutions and diluted to volume to obtain 20μg/ml and 50μg/ml solutions, respectively. Cooled the solutions to room temperature and filtered. Injected the sample into the HPLC system and recorded the chromatograms.

Alkaline degradation
Added 1 ml of 2N sodium hydroxide to 1 ml of Ethinyl estradiol and Gestodene stock solutions and kept for 30 min at 60 °C. Later diluted to volume to obtain 20μg/ml and 50μg/ml solution, respectively. Cooled and then filtered through a 0.45μm membrane filter. Injected into the HPLC system and recorded the chromatograms.

Oxidation degradation
Added 1 ml of 20% Hydrogen peroxide to 1 ml of Ethinylestradiol and Gestodene stock solutions and refluxed at 60 °C for 30 min. Later 1 ml of 2N HCl was added for neutralization and diluted to volume to obtain 20μg/ml and 50μg/ml solutions Ethinyl estradiol and Gestodene, respectively. Cooled and filtered using 0.45μm membrane filter. Injected into the HPLC system and recorded the chromatograms.

Photodegradation
For this study, the stock solutions were exposed to UV light for 1d or 200Watt-hm-2 in a photostability chamber and later diluted to volume to obtain 20μg/ml and 50μg/ml solutions Ethinyl estradiol and Gestodene, respectively, cooled and filtered using a 0.45μm membrane filter. Injected the solutions into the HPLC system and recorded the chromatograms.

Water degradation studies
Added 1 ml of distilled water to 1 ml of stock solution of Ethinyl estradiol and Gestodene and kept aside at 60 °C for 30 min. Later, diluted to volume to obtain 20μg/ml and 50μg/ml solutions of Ethinyl estradiol and Gestodene, respectively. Filtered the solutions and injected the mixed standard into the HPLC system, and recorded the chromatograms.

RESULTS AND DISCUSSION

Method development
For developing the present method, various columns like Symmetry and Zodiac columns were tried. But finally, Phenomenex Gemini C18(25×0.46 cm, 5 μm) was confirmed to be optimal since all the parameters are within the acceptance criteria like resolution, peak symmetry, and theoretical plates. Various mobile phases tried were water: methanol, Water: Acetonitrile, Phosphate buffer: Methanol, Phosphate buffer: Acetonitrile by varying proportions and at last, the Phosphate buffer: Acetonitrile (75:25 v/v) and the pH was adjusted to 3.6 using dilute orthophosphoric acid by maintaining the system at 1.0 ml/min flow rate at 237 nm UV detection was finalized as optimal. The optimized chromatogram of Ethinyl estradiol and Gestodene was displayed in fig. 3 and resulted in table 1.

In comparison to the previously reported methods [5, 8], the retention times of Ethinyl estradiol and Gestodene were observed to be more and required more analysis time for quantification. But the present established method requires lesser analysis time and consumes lesser solvents showing retention times of Ethinyl estradiol and Gestodene at 1.768 min and 3.475 min, which is more advantageous in pharmaceutical industries. This revealed that the developed method could be suitably applied for routine laboratory analysis.

Method validation
System suitability
All the efficiency parameters like theoretical plates were observed to be more than 7000 for Ethinyl estradiol and Gestodene drugs. The peak tailing was not more than 2.0. The %RSD for the five replicate injections was not more than 2.0 and ensured that the entire testing system and chemicals used could generate an accurate and precise result by showing all the efficiency parameters within the specified limits. Reported the results in Tables 2 and 3.

Linearity
The proposed method was confirmed to be linear in the concentration range of 10-50μg/ml and 25-125μg/ml for Ethinyl Estradiol and Gestodene, respectively, showing a correlation coefficient of 0.999, which was analyzed by linear regression analysis. The results proved that the analyte response is proportional to the analyte concentration in the selected concentration range. The calibration graphs of Ethinyl Estradiol and Gestodene are depicted in fig. 4 and fig. 5 and data in Tables 4 and 5, respectively.

Accuracy
The mean recovery values obtained at 50%, 100%, and 150% levels are 99.84% and 99.92% for Ethinyl estradiol and Gestodene, respectively, which are within the acceptance criteria. Thus, it confirmed the method’s accuracy and reported the results in Tables 6 and 7.

Precision
The obtained %Relative standard deviation of intra-day precision and intermediate precision of Ethinyl estradiol and Gestodene was found to be not more than 2.0, which is within the acceptance criteria, indicating that the developed method is precise. Reported the measured results of intra-day precision in Tables 8, 9 and intermediate precision in Tables 10, 11, 12, and 13.

Robustness
The results revealed that the method is robust upon slight changes in flow rate conditions and even by the ±5% organic phase for the %Assay of Ethinyl estradiol and Gestodene in Femovan tablets is 11.848µg/ml for Gestodene, respectively. Thus, the present method successfully applied for the assay of pharmaceutical dosage forms, and reported the results in Tables 16, 17, 18, and 19.

Degradation studies
Upon degradation study, observed that Ethinyl estradiol and Gestodene had undergone degradation under all stress conditions. The calculated % degraded amount was within the acceptance criteria. It was observed that the drug was more susceptible to photolysis showing the highest degradation. The successful separation of the obtained degradation products from the active pharmaceutical ingredients without any interference confirmed the specificity. Thus, it proved the stable nature of the developed
method. Presented the acidic, basic, oxidative, thermal, photolytic, and hydrolytic degradation chromatograms in fig. 6, 7, 8, 9, 10, and 11, respectively. Reported the calculated results for Ethinyl estradiol and Gestodene in tables 20 and 21, respectively.

All the method validation parameters are within the acceptance criteria and assured sufficient precision and accuracy. A good linear relationship was observed in the concentration range of 10-50µg/ml and 25-125µg/ml for Ethinyl estradiol and Gestodene, respectively. The detection limit for Ethinyl estradiol was found to be 1.399µg/ml indicating the high sensitivity of the developed method compared to the reported method [6]. The recovery of the analyte was also found to be more than the reported methods [5, 8], indicating a high degree of accuracy. The non-interference of the degraded products with the active pharmaceutical ingredients revealed the stability-indicating nature of the developed method.

![Fig. 3: Optimized chromatogram of ethinyl estradiol and gestodene](image)

**Table 1: Optimized chromatogram result**

| S. No. | Drug            | Retention time (min) | Area of the peak | Height of the Peak | Resolution | Peak asymmetry | No. of theoretical plates |
|--------|-----------------|----------------------|------------------|-------------------|------------|----------------|--------------------------|
| 1      | Ethinylestradiol| 1.788                | 558647           | 7658              | 1.26       | 7854           |                         |
| 2      | Gestodene       | 3.475                | 7986585          | 48546             | 7.12       | 1.35           | 8865                     |

**Table 2: System suitability data of Ethinyl estradiol**

| Drug         | Retention time (min) | Analyte response | Theoretical plates(N) | Peak asymmetry |
|--------------|----------------------|------------------|-----------------------|----------------|
| Ethinyl estradiol |                     |                  |                       |                |
| 1            | 1.792                | 556985           | 7896                  | 1.28           |
| 2            | 1.793                | 557849           | 7896                  | 1.28           |
| 3            | 1.794                | 559865           | 7824                  | 1.29           |
| 4            | 1.791                | 558498           | 7869                  | 1.27           |
| 5            | 1.791                | 558498           | 7869                  | 1.27           |

*Mean (n=5), ±SD (n=5), % RSD (n=5)

**Table 3: System suitability data of gestodene**

| Drug | Retention time (min) | Area of the peak | Theoretical plates(N) | Peak asymmetry | Resolution |
|------|----------------------|------------------|-----------------------|----------------|------------|
| Gestodene |                     |                  |                       |                |            |
| 1    | 3.438                | 798695           | 8856                  | 1.34           | 7.16       |
| 2    | 3.446                | 798698           | 8896                  | 1.39           | 7.15       |
| 3    | 3.444                | 798695           | 8896                  | 1.39           | 7.15       |
| 4    | 3.440                | 7984874          | 8874                  | 1.34           | 7.16       |
| 5    | 3.442                | 798698           | 8859                  | 1.38           | 7.15       |

*Mean (n=5), ±SD (n=5), % RSD (n=5)

**Table 4: Linearity data of ethinylestradiol**

| Concentration µg/ml | Average peak Area |
|---------------------|-------------------|
| 10                  | 253898            |
| 20                  | 501647            |
| 30                  | 751256            |
| 40                  | 985789            |
| 50                  | 1235898           |
Table 5: Linearity data of gestodene

| Concentration µg/ml | Average peak area |
|---------------------|-------------------|
| 25                  | 3252897           |
| 50                  | 6316585           |
| 75                  | 9438787           |
| 100                 | 12387436          |
| 125                 | 15365874          |

Table 6: Ethinyl estradiol accuracy data

| Drug            | Spiking level | Peak area | *Average area (n=3) | Amount added (µg/ml) | Amount obtained (µg/ml) | *Percentage recovery | Average recovery (n=9) |
|-----------------|---------------|-----------|---------------------|----------------------|-------------------------|----------------------|------------------------|
| Ethinyl estradiol | 50%           | 253848    | 253526              | 10                   | 9.89                    | 98.9%                | 99.84%                 |
|                  | 100%          | 252856    | 251858.67           | 20                   | 20.04                   | 100.2%               |                        |
|                  | 150%          | 501563    | 501689              | 30                   | 30.13                   | 100.43%              |                        |

*Mean of three determinations
Table 7: Gestodene accuracy data

| Drug    | Spiking level | Peak area (μg/ml) | *Average area (n=3) | Amount added (μg/ml) | Amount found (μg/ml) | *Percentage recovery (n=3) | Average recovery |
|---------|---------------|-------------------|---------------------|---------------------|---------------------|---------------------------|-----------------|
| Gestodene | 50%           | 33145.35          | 33151.76.67         | 25                  | 25.183              | 100.7%                    | 99.92%          |
|         | 100%          | 62874.97          | 62845.38.67         | 50                  | 49.68               | 99.37%                    |                 |
|         | 150%          | 93287.48          | 93232.85            | 75                  | 74.76               | 99.68%                    |                 |

*Mean of three determinations

Table 8: Repeatability data of Ethinyl estradiol

| Drug    | Injection | Retention time (min) | Peak area (μg/ml) | No. of theoretical plates | Peak asymmetry |
|---------|-----------|----------------------|-------------------|---------------------------|----------------|
| Ethinyl | 1         | 1.789                | 558748            | 7896                      | 1.26           |
| estradiol | 2        | 1.780                | 558698            | 7845                      | 1.28           |
|         | 3         | 1.790                | 558475            | 7892                      | 1.29           |
|         | 4         | 1.791                | 558698            | 7849                      | 1.27           |
|         | 5         | 1.792                | 558265            | 7829                      | 1.28           |

*Mean (n=5) ±SD (n=5) = 203.8816

%RSD (n=5) = 0.0365

*Mean of five determinations, SD: Standard Deviation, %RSD: Relative Standard Deviation

Table 9: Repeatability data of gestodene

| Drug    | Injection | Retention time (min) | Peak area (μg/ml) | No. of theoretical plates | Peak asymmetry |
|---------|-----------|----------------------|-------------------|---------------------------|----------------|
| Gestodene | 1         | 3.408                | 7986985           | 8856                      | 1.36           |
|         | 2         | 3.414                | 7985487           | 8849                      | 1.37           |
|         | 3         | 3.419                | 7985468           | 8874                      | 1.39           |
|         | 4         | 3.428                | 7968547           | 8957                      | 1.38           |
|         | 5         | 3.435                | 7982564           | 8965                      | 1.37           |

*Mean (n=5) ±SD (n=5) = 203.8816

%RSD (n=5) = 0.0365

*Mean of five determinations, SD: Standard Deviation, %RSD: Relative Standard Deviation

Table 10: Day 1 Intermediate precision data of ethinylestradiol

| Drug    | Injection | Retention time (min) | Peak area (μg/ml) | No. of theoretical plates | Peak asymmetry |
|---------|-----------|----------------------|-------------------|---------------------------|----------------|
| Ethinyl | 1         | 1.792                | 558965            | 7899                      | 1.29           |
| estradiol | 2        | 1.789                | 558476            | 7895                      | 1.28           |
|         | 3         | 1.787                | 558947            | 7829                      | 1.27           |

*Mean (n=3) ±SD (n=3) = 277.2742

%RSD (n=3) = 0.04962

*Mean of three determinations, SD: Standard Deviation, %RSD: Relative Standard Deviation

Table 11: Day 1 Intermediate precision data of gestodene

| Drug    | Injection | Retention time (min) | Peak area (μg/ml) | No. of theoretical plates | Peak asymmetry |
|---------|-----------|----------------------|-------------------|---------------------------|----------------|
| Gestodene | 1         | 3.435                | 7986986           | 8849                      | 1.38           |
|         | 2         | 3.477                | 7985985           | 8879                      | 1.37           |
|         | 3         | 3.482                | 7989654           | 8896                      | 1.39           |

*Mean (n=3) ±SD (n=3) = 50712.01

%RSD (n=3) = 0.637309

*Mean of three determinations, SD: Standard Deviation, %RSD: Relative Standard Deviation

Table 12: Day 2 Intermediate precision data of ethinyl estradiol

| Drug    | Injection | Retention time (min) | Peak area (μg/ml) | No. of theoretical plates | Peak asymmetry |
|---------|-----------|----------------------|-------------------|---------------------------|----------------|
| Ethinyl | 1         | 1.793                | 568965            | 7989                      | 1.28           |
| estradiol | 2        | 1.789                | 569854            | 7986                      | 1.29           |
|         | 3         | 1.790                | 569878            | 7994                      | 1.28           |

*Mean (n=3) ±SD (n=3) = 520.331

%RSD (n=3) = 0.091356

*Mean of three determinations, SD: Standard Deviation, %RSD: Relative Standard Deviation
Table 13: Day 2 Intermediate precision data of gestodene

| Drug          | Injection | Retention time (min) | Peak area  | No. of theoretical plates | Peak asymmetry |
|---------------|-----------|----------------------|------------|---------------------------|----------------|
| Gestodene     | 1         | 3.478                | 8045652    | 8987                      | 1.38           |
|               | 2         | 3.473                | 8065879    | 8959                      | 1.39           |
|               | 3         | 3.474                | 8075847    | 8937                      | 1.37           |
| *Mean (n=3)   |           | 3.476                | 8062459    | 15385.22                  | 0.190825       |
| ±SD (n=3)     |           |                      |            |                           |                |
| %RSD          |           |                      |            |                           |                |

*Mean of three determinations, SD: Standard Deviation, %RSD: Relative Standard Deviation

Table 14: Robustness data of ethinyl estradiol

| Slightly changed parameters | Peak area  | Retention time (min) | No. of theoretical plates | Peak asymmetry |
|-----------------------------|------------|----------------------|---------------------------|----------------|
| Flow rate 1.0 (ml/min)      |            |                      |                          |                |
|                             | 558647     | 1.788                | 7854                      | 1.26           |
| 0.9                         | 636589     | 1.867                | 7978                      | 1.27           |
| 0.9 More flow rate of 0.9 ml/min | 535685 | 1.744                | 7576                      | 1.39           |
| % of Acetonitrile 20        | 540576     | 1.821                | 7367                      | 1.37           |
| 30                          | 525874     | 1.874                | 7296                      | 1.28           |

Table 15: Robustness study data of gestodene

| Slightly changed parameters | Peak area  | Retention time (min) | No. of theoretical plates | Peak asymmetry |
|-----------------------------|------------|----------------------|---------------------------|----------------|
| Flow rate 1.0 (ml/min)      |            |                      |                          |                |
|                             | 7986585    | 3.475                | 8865                      | 1.35           |
| 0.9                         | 8265847    | 3.724                | 9152                      | 1.49           |
| 1.1                         | 7658745    | 3.097                | 8685                      | 1.38           |
| % of Acetonitrile 20        | 7758498    | 6.242                | 8475                      | 1.37           |
| 30                          | 7659854    | 2.402                | 8369                      | 1.36           |

Table 16: Assay data of Ethinyl estradiol standard

| Drug          | Injection | Retention time (min) | Peak area  | No. of theoretical plates | Peak asymmetry |
|---------------|-----------|----------------------|------------|---------------------------|----------------|
| Standard      | 1         | 1.791                | 558698     | 7854                      | 1.26           |
| Ethinyl       | 2         | 1.794                | 558674     | 7822                      | 1.28           |
| estradiol     | 3         | 1.793                | 558694     | 7895                      | 1.29           |
| 4              | 1.792     | 558748               | 7826       | 1.27                      |                |
| 5              | 1.788     | 558992               | 7849       | 1.26                      |                |
| *Mean (n=5)   |           | 558755.2             |            |                           |                |
| ±SD (n=5)     |           |                      |            |                           |                |
| %RSD (n=5)    |           | 0.021257             |            |                           |                |

*Mean of five determinations, SD: Standard Deviation, %RSD: Relative Standard Deviation

Table 17: Assay data of gestodene standard

| Drug          | Injection | Retention time (min) | Peak area  | No. of theoretical plates | Peak asymmetry | Resolution |
|---------------|-----------|----------------------|------------|---------------------------|----------------|------------|
| Gestodene     | 1         | 3.442                | 7986852    | 8857                      | 1.36           | 7.15       |
| standard      | 2         | 3.440                | 7985685    | 8874                      | 1.34           | 7.14       |
|               | 3         | 3.444                | 7984573    | 8892                      | 1.35           | 7.16       |
|               | 4         | 3.446                | 7986365    | 8849                      | 1.39           | 7.15       |
|               | 5         | 3.438                | 7989856    | 8825                      | 1.35           | 7.18       |
| *Mean (n=5)   |           | 7986666              |            |                           | 7.15           |            |
| ±SD (n=5)     |           | 1977.644             |            |                           | 7.15           |            |
| %RSD (n=5)    |           | 0.024762             |            |                           | 7.15           |            |

*Mean of five determinations, SD: Standard Deviation, %RSD: Relative Standard Deviation

Table 18: Assay data of ethinyl estradiol sample

| Drug          | Injection | Retention time (min) | Peak area  | No. of theoretical plates | Peak asymmetry |
|---------------|-----------|----------------------|------------|---------------------------|----------------|
| Ethinyl       | 1         | 1.791                | 562453     | 7965                      | 1.28           |
| estradiol     | 2         | 1.791                | 563124     | 7982                      | 1.29           |
| Sample        | 3         | 1.794                | 563256     | 7985                      | 1.29           |
| *Mean (n=3)   |           | 562944.33            |            |                           |                |
| ±SD (n=3)     |           | 430.59               |            |                           |                |
| %RSD (n=3)    |           | 0.076                |            |                           |                |

*Mean of three determinations, SD: Standard Deviation, %RSD: Relative Standard Deviation
Table 19: Assay data of gestodene sample

| Drug          | Injection | Retention time (min) | Peak area | No. of theoretical plates | Peak asymmetry |
|---------------|-----------|----------------------|-----------|---------------------------|----------------|
| Gestodene     | 1         | 3.434                | 8012432   | 7264                      | 1.39           |
| sample        | 2         | 3.442                | 8023654   | 7285                      | 1.38           |
|               | 3         | 3.440                | 8012543   | 7293                      | 1.37           |

*Mean (n=3)  
±SD (n=3)   
%RSD (n=3)  
8016209.67  
6447.22     
0.08

*Mean of three determinations, SD: Standard Deviation, %RSD: Relative Standard Deviation

Fig. 6: Ethinylestradiol and Gestodene acidic degradation chromatogram

Fig. 7: Ethinyl estradiol and gestodene alkaline degradation chromatogram

Fig. 8: Ethinyl estradiol and gestodene oxidative degradation chromatogram
Fig. 9: Ethinylestradiol and gestodene thermal degradation chromatogram

Fig. 10: Ethinyl estradiol and gestodene photolytic degradation chromatogram

Fig. 11: Ethinyl estradiol and gestodene hydrolytic degradation chromatogram

Table 20: Forced degradation studies data for Ethinyl estradiol

| S. No. | Stress condition | Peak area  | % of degraded amount | % of active amount | Total % of amount |
|--------|------------------|------------|----------------------|-------------------|------------------|
| 1      | Standard         | 558647     | 0                    | 100%              | 100%             |
| 2      | Acidic           | 476693.48  | 14.67                | 85.33             | 100%             |
| 3      | Basic            | 515351.85  | 7.75                 | 92.25             | 100%             |
| 4      | Oxidative        | 497307.55  | 10.98                | 89.02             | 100%             |
| 5      | Thermal          | 486413.94  | 12.93                | 87.07             | 100%             |
| 6      | Photolytic       | 395186.88  | 29.74                | 70.74             | 100%             |
| 7      | Water            | 491832.81  | 11.96                | 88.04             | 100%             |

Table 21: Forced degradation studies data for gestodene

| S. No. | Stress condition | Peak area  | % of degraded amount | % of active amount | Total % of amount |
|--------|------------------|------------|----------------------|-------------------|------------------|
| 1      | Standard         | 7986585    | 0                    | 100%              | 100%             |
| 2      | Acidic           | 6733489.81 | 15.69                | 84.31             | 100%             |
| 3      | Basic            | 7271785.64 | 8.95                 | 91.05             | 100%             |
| 4      | Oxidative        | 7032188.09 | 11.05                | 88.05             | 100%             |
| 5      | Thermal          | 6880442.98 | 13.85                | 86.15             | 100%             |
| 6      | Photolytic       | 5154541.96 | 35.46                | 64.54             | 100%             |
| 7      | Hydrolysis       | 7108059.31 | 10.99                | 89.01             | 100%             |
CONCLUSION

The established present method revealed that it is simple, selective, specific, and can generate accurate and precise results. Moreover, the shorter duration of analysis time and lesser mobile phase consumption confirmed that the method is rapid and economical. The successful separation of the forced degradation products from the active pharmaceutical ingredients without any interference confirmed the stability-indicating nature of the developed method. Hereupon, the present method can be satisfactorily applied for the routine laboratory simultaneous estimation of Ethinyl estradiol and Gestodene.

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AUTHORS CONTRIBUTIONS

We declare that the author's present work was done and is an original work. D. Suchitra has carried out the research work. Professor Battu Satyanarayana guided, proofread the manuscript, suggested the necessary corrections, and helped in writing the manuscript.

CONFLICT OF INTERESTS

Declared none

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