RESEARCH PAPER

Method Development and Validation of Stability Indicating UPLC Method For Estetrol and Drospirenone

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Abstract
A simple, rapid, accurate and stability indicating UPLC method was developed and validated for simultaneous estimation of Estetrol and Drospirenone in pharmaceutical dosage form. Chromatographic separation was achieved on a Phenyl column (100 × 2.1 mm, 1.7 µm) employing an isocratic mobile phase composed of 0.1 M ammonium dihydrogen phosphate buffer (adjusted to pH 3.0) and acetonitrile in a 60:40 (v/v) ratio. The analysis was performed at a flow rate of 0.2 mL/min. Estetrol ,Drospirenone they both were detected at 261 nm with good peak symmetry and resolution, and a total chromatographic runtime of 4.0 min. The developed analytical method was validated in accordance with ICH Q2(R2) guidelines by assessing system suitability, specificity, linearity, precision, accuracy, robustness, (LOD), (LOQ), and stability-indicating capability through forced degradation studies .It results showes the both Estetrol and Drospirenone exhibited optimal linear ranges of 14.20–213 □□/□□along with 3–45 µg/mL, respectively, together with correlation coefficient readings of 0.9997 and 0.9994, correspondingly. In the %RSD values obtained in the precision studies were below 2% suggesting good reproducibility. Accuracy studies showed good recoveries of 98.3-100.6% for Estetrol and 98.2-101.6% for Drospirenone. The developed analytical method demonstrated high sensitivity, with LOD and LOQ values of 0.426 µg/mL and 1.42 µg/mL for Estetrol, and 0.09 µg/mL and 0.30 µg/mL for Drospirenone, respectively. Robustness studies showed that the method performance was not significantly affected by deliberate small changes in the chromatographic conditions. Forced degradation studies conducted under acidic, alkaline, oxidative, thermal, and photolytic stress conditions demonstrated the stability-indicating nature of the developed method by achieving effective separation of the degradation products from the analyte peaks. The suggested UPLC method is quick, sensitive, reliable can used for same QC analysis and consistency study related to combined Estetrol and Drospirenone pharmaceutical formulations.
keywords: Estetrol, Drospirenone, UPLC, Stability-indicating technique, Method generation, Method validation, Forced degradation, ICH Q2(R1), Pharmaceutical analysis.
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Introduction

The pharmaceutical industry needs fast, accurate and reliable analytical methods to providing quality, safety, efficacy of drug products during their entire life cycle. Stability-indicating analytical methods are important to identify or quantify active pharmaceutical ingredients (APIs) they presence in degradation products, impurities, excipients [1-3]. These techniques are crucial for formulation development, regulatory compliance, quality control, and stability studies[4-6].

UPLC is the advanced generation in analytical techniques which offers better resolution, sensitivity, analysis time, and solvent use than conventional HPLC. UPLC is an effective separation method that offers better separation and reproducibility of compounds, which is preferable for simultaneous estimation of more than one drug component present in the pharmaceutical

formulations. Estetrol and Drospirenone is a common combination in oral contraceptive pills[2]. Estetrol is a naturally occurring estrogen with selective estrogen receptor activity and desirable pharmacological properties, and Drospirenone is a synthetic progestin that has anti-mineralocorticoid and anti-androgenic effects[1,4,7,8]. The combination is effective for contraception and better tolerated, and is becoming more common in contemporary hormonal therapy. The accurate quantification of these drugs is crucial to preserve product quality and assure patient safety.

Several chromatographic methods for estimating Estetrol and Drospirenone have already been reported, but a simple, rapid, robust and stability indicate UPLC technique that beachieved separate Estetrol and Drospirenone from their degradation products still needs to be developed[9,10]. The present study was undertaken to develop and validate a stability-indicating UPLC method for the simultaneous quantification of Estetrol and Drospirenone in pharmaceutical dosage forms [6,11–13]. The proposed analytical method was validated in accordance with ICH Q2(R2) guidelines by assessing critical validation parameters, including specificity, linearity, precision, accuracy, robustness, limit of detection (LOD), limit of quantification (LOQ), and forced degradation studies. The validation results demonstrated that the method is reliable, stability indicating, and appropriate for routine quality control analysis of pharmaceutical formulations. [12].

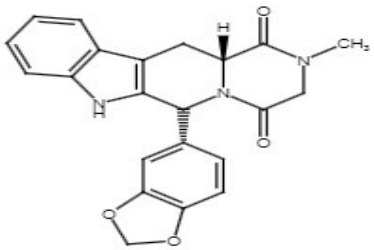
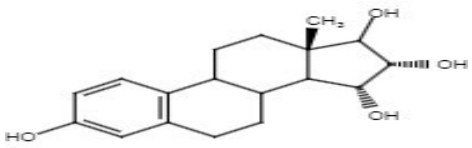


Figure.1:Structure of Drospirenone



Figures. 2: Structure of Estetrol

Materials and Methods:

Instrumentation:

The chromatographic analysis was carried out with UPLC system, which has a photodiode array (PDA) detector and is controlled by the Empower 2 software that acquires and integrates peaks. The separation was performed on a Phenyl column (100 × 2.1 mm, 1.7 µm Particle diameter).Gassing of the MP was performed by an ultrasonic bath sonicator, the Spectra Lab UCB-30. Weights were taken with an electronic analytical balance (Sartorius) and all the volumetric measurements were made in class A borosilicate glassware [14,15].

Materials:

Drospirenone and Estetrol standards were kindly provided by Hetero Drugs, Hyderabad, India [16]. All HPLC grade acetonitrile and Milli-Q water were employed in the study [17]. Ammonium dihydrogen phosphate (analytical reagent grade) and orthophosphoric acid were used for the preparation and pH adjustment of the buffer solution.

Chromatographic Conditions:

Chromatographic resolution was performed by Phenyl column (100 × 2.1 mm, 1.7 µm) used for MP of 0.1 M ammonium dihydrogen phosphate buffer (pH 3.0) and acetonitrile (60:40, v/v). The mobile phase was prepared and subjected to filtration and sonication prior to use. A photodiode array (PDA) detector was used for analyte detection, and the acquired chromatographic data were processed using Empower 2 software.

Preparation of Buffer:

1.15gm of Ammonium dihydrogen phosphate was dissolved in 1 liter HPLC grade water to make 0.1 M ammonium dihydrogen phosphate buffer. Orthophosphoric acid was used to adjust the pH to 3.0. The final solution was filtered using a 0.45 µm membrane filter before being utilized for further analysis.

Preparation of M.P:

The mobile phase consisted of a blend of 0.1 M ammonium dihydrogen phosphate buffer adjusted to pH 3.0 and acetonitrile in a ratio of 60:40 (v/v). Prior to chromatographic analysis, the prepared mobile phase was passed through a 0.45-µm membrane filter to remove particulate matter and subsequently degassed by sonication to ensure optimal chromatographic performance.

Making Standard Solutions:

Drospirenone Standard Stock Solution:

Approximately **30 mg of Drospirenone reference standard** was accurately weighed and transferred into a **100 mL volumetric flask**. An appropriate volume of the mobile phase was added to dissolve the drug, and the solution was sonicated for **15 minutes** to ensure complete dissolution. The final volume was then made up to the calibration mark with the mobile phase to obtain the standard stock solution.

Estetrol Standard Stock Solution:

Accurately weighed quantity of the Estetrol reference standard (approximately 142 mg) was transferred into a 100 mL volumetric flask. The drug was dissolved by sonication with the mobile phase for 15 minutes, after which the solution was

diluted to the mark with the same mobile phase to obtain the standard stock solution.

Combined Standard Working Solution:

A mixed standard stock solution was prepared by accurately weighing 30 mg of Drospirenone and 142 mg of Estetrol into a 100 mL volumetric flask. The drugs were dissolved in the mobile phase with the aid of sonication for 15 minutes, after which the volume was made up to the mark using the same mobile phase. Subsequently, 5 mL of the stock solution was transferred to a 50 mL volumetric flask and diluted to volume with the mobile phase to obtain a working standard solution containing 14.2 µg/mL of Estetrol and 3.0 µg/mL of Drospirenone.

Validation Studies:

The developed UPLC method for the simultaneous determination of Drospirenone and Estetrol was validated in accordance with the International Council for Harmonisation (ICH) Q2(R2) guidelines. Validation of the method included the evaluation of system suitability, specificity, linearity, precision, accuracy, robustness, LOD, LOQ, forced degradation studies, and assay of the commercial tablet formulation.

System Suitability:

Before sample analysis the performance of chromatographic system is verified by assessing system performance evaluation. Optimized chromatographic conditions were used and six replicate injections of a working standard solution containing Estetrol(14.2µg/mL) and Drospirenone(3.0 µg/mL) were made. The chromatograms were assessed in terms of retention time, theoretical plates, tailing factor, resolution and reproducibility of the area of peaks. The following criteria were used to decide if the chromatograms passed or failed: 3000 or more theoretical plates, a tailing factor of ≤ 2.0 was obtained, %RSD of peak area less than or equal to 2.0%, and a resolution greater than 1.5.

Selectivity:

Specificity of the developed method was evaluated by analyzing blank, placebo, standard, and sample solutions. The same diluent and excipients were used to prepare blank and placebo solutions which were analyzed using the same chromatographic conditions without the active pharmaceutical ingredients. Interference was monitored on the chromatograms at the retention times of Drospirenone and Estetrol. It was found that the developed method was specific due to the absence of interfering peaks.

Linearity:

The method was linearized by testing a set of mixed standard solutions obtained by suitable dilution of the standard sampleinMP. Drospirenone was tested at concentrations between 3 and 45 µg/mL and Estetrol was tested at a concentration range between 14.2 and 213 µg/mL. All concentrations were injected three times and calibration curves were drawn with analyte concentration versus peak area. The calibration curve was evaluated by linear regression analysis, from which the slope, intercept, regression equation, and coefficient of determination (R^2) were derived.

Repeatability :

To evaluate system precision, six replicate injections of the mixed standard solution were analyzed using the UPLC system. The relative standard deviation (%RSD) of the peak responses both the batch of the pharmaceutical formulation and when analyzed under the same chromatographic conditions. Assay values were calculated and %RSD of the analytes was calculated.

Precision is the technique was evaluated by preparing six independent samples solutions from the same batch of the pharmaceutical formulation and then analyzing these under the same chromatographic conditions. The assay values were calculated, and the %RSD was determined to check the repeatability of the assay.

Accuracy:

The accuracy is the method used to check the standard addition method. Pre-analyzed sample solutions were spiked with known amounts of Drospirenone and Estetrol reference standards at 50%, 100% and 150% of the target assay concentration. The three concentrations were replicated and subjected to the proposed chromatographic method. To examine the accuracy of the method, the percentage recovery, mean recovery and %RSD was calculated.

Robustness:

The robustness of the developed method was assessed by introducing deliberate variations to the chromatographic conditions. The flow rate was altered by ±0.1 mL/min from the optimized value, while the proportion of the organic component in the mobile phase was varied by ±5% to evaluate the method's reliability under small but intentional changes in analytical conditions. Peak area, retention time and %RSD were calculated for sample solutions under each modified condition and then evaluated. The method was judged to be robust if there were no significant changes to the assay results or instrument stability parameters.

LOD and LOQ:

The sensitivity of the method was determined by estimating the LOD and LOQ. These values were calculated from the calibration curve using the equations:

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

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

In this equation, σ corresponds to the standard deviation of the response, while S represents the slope of the calibration curve. Thecalculated values were experimentally verified by injecting dilute

standard solutions and evaluating the signal-to-noise ratio.

Forced Degradation Studies:

The stability-indicating performance of the proposed method was confirmed by subjecting the mixed standard solution to a range of stress conditions as recommended by the ICH guidelines.

- Acidic degradation: Samples were treated with 3 N hydrochloric acid and refluxed at 60°C for 1 h, following neutralization with sodium hydroxide.
- Alkaline degradation: Samples were treated with 2 N sodium hydroxide and refluxed at 60°C for 1 h, following neutralization with hydrochloric acid.
- Oxidative degradation: Samples were treated with 30% hydrogen peroxide and temperature upto 60°C for 1 h.
- Thermal degradation: Samples were exposed to 105°C for 72 h.
- Photolytic degradation: Samples were exposed to ultraviolet light (200 Wh/m²) and visible light (1.2millionlux hours) according to ICH photostability guidelines.

Following stress treatment, the sample was diluted and analyzed using the optimized UPLC technique. Peak purity was evaluated using PDA detection to confirm that the analyte peaks were free from co-eluting degradation products.

Assay of Pharmaceutical Formulation:

For assay determination, a quantity of finely powdered tablets equivalent to the labeled amounts of Drospirenone and Estetrol was accurately weighed and transferred into a 100 mL volumetric flask. The analytes were dissolved in the mobile phase with sonication for 15 minutes, after which the solution was filtered through a 0.45 µm membrane filter. The filtrate was then diluted to the desired concentration using the mobile phase before being injected into the UPLC system for analysis. The assay of each drug was calculated using the corresponding calibration equation obtained from the linearitystudy.

RESULTS and DISCUSSION:

Analytical System Suitability:

System suitability was established by performing six replicate injections of the mixed working standard solution prior to sample analysis. Key chromatographic parameters, including retention time, theoretical plate count, tailing factor, resolution, and percentage relative standard deviation (%RSD) of peak areas, were assessed to verify the performance of the chromatographic system. The system was considered suitable when the theoretical plate count exceeded 3000, the tailing factor was ≤2.0, the resolution between peaks was greater than 1.5, and the %RSD of peak areas remained below 2.0%[6,17,18].

Table 1.System suitability parameters

Parameter	Estetrol	Drospirenone
Theoretical plates (n)	3325	7241
Tailing factor (T)	1.14	1.03
%RSD	0.318	0.278
Resolution (R)	N/A	7.53

Specificity:

Specificity was assessed by analyzing blank, placebo, standard and sample preparations under the optimized chromatographic conditions. No interfering peaks were observed at the retention times of Estetrol and Drospirenone, confirming that the formulation excipients did not interfere with the determination of the analytes[17].

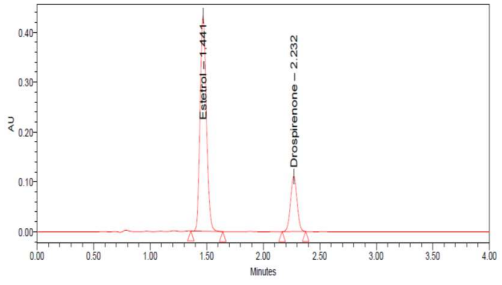


Figure 3. Representative chromatograph of mixed standard solution.

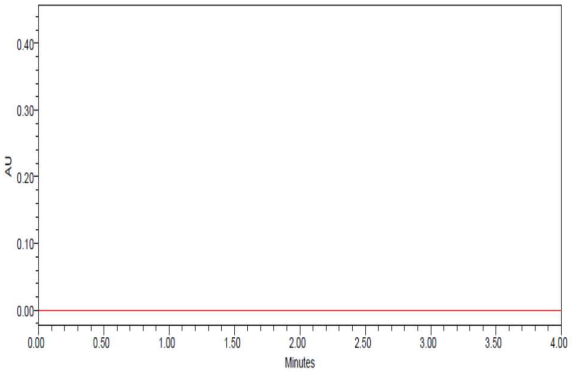


Figure 4. Chromatograph of blank solution.

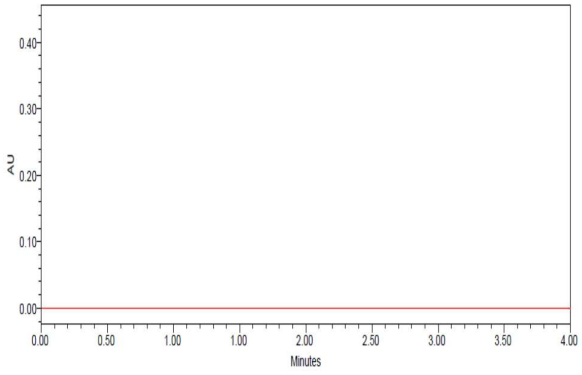


Figure 5. Chromatograph of placebo solution.

Precision:

System precision and method precision were used to determine the precision (The stability of both the analytical system and the developed method was evaluated independently.), where six replicate injections were analysed. The RSD values for both analytes were calculated and the values were within the acceptable range of 2.0%.

Table 2.Precision results

injection	Drospirenone				Estetrol			
	Method Precision		Intermediate precision		Method Precision		Intermediate precision	
	Mean Area counts	% Assay	Mean Area counts	% Assay	Mean Area counts	% Assay	Mean Area counts	% Assay
1	894314	100.2	890420	99.8	3362891	100.4	3322889	99.2
2	892141	99.9	895346	100.3	3313789	99	3353794	100.1
3	885739	99.2	893047	100.0	3335810	99.6	3305815	98.7
4	899352	100.7	899359	100.8	3351608	100.1	3374611	100.8
5	891343	99.8	888940	99.6	3389396	101.2	3393597	101.3
6	893561	100.1	887936	99.5	3375312	100.8	3341324	99.8
	Mean	100	Mean	100	Mean	100.2	Mean	100
	% RSD	0.50	% RSD	0.49	% RSD	0.80	% RSD	0.97

Accuracy:

The accuracy of the developed method was evaluated using the standard addition technique at three concentration levels, namely 50%, 100%, and 150% of the target concentration. Each concentration level was analyzed in triplicate, and the percentage recovery was calculated to assess the accuracy of the method.

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Table 3. Accuracy results

Estetrol					
Concentration of spiked level	**Amount added (mg)**	**Amount found (mg)**	**Percent Recovery**	**Mean (%) Recovery**	**% RSD**
50%	7.1	7.09	99.9	99.5	1.10
	7.1	7.13	100.4		
	7.1	6.98	98.3		
100%	14.2	14.29	100.6	100.1	0.46
	14.2	14.16	99.7		
	14.2	14.21	100.1		
150%	21.3	21.18	99.4	99.0	0.53
	21.3	20.96	98.4		
	7.1	21.1	99.1		

Drospirenone					
Concentration of spiked level	**Amount added (mg)**	**Amount found (mg)**	**Percent Recovery**	**Mean (%) Recovery**	**% RSD**
50%	1.5	1.5	100.0	99.6	0.39
	1.5	1.49	99.3		

	1.5	1.49	99.3		
100%	3	3.02	100.7	100.2	0.51
	3	3.01	100.3		
	3	2.99	99.7		
150%	4.5	4.57	101.6	100.1	1.72
	4.5	4.42	98.2		
	4.5	4.53	100.7		

Linearity:

The linearity of the proposed method was established using mixed standard solutions over concentration ranges of 14.2–213 µg/mL for Estetrol and 3–45 µg/mL forDrospirenone, demonstrating a proportional relationship between analyte concentration and detector response throughout the specified ranges.. Peak area was plotted against concentration to construct calibration curves and regression analysis was done.

Table 4. Linearity data

Estetrol		**Drospirenone**	
Concentrtion of (µg/mL)	**Mean Peak area (n=6)**	**Concentration of (µg/mL)**	**Mean Peak area (n=6)**
14.20	417808	3.00	132581
35.50	844753	7.50	255651
71.00	1691560	15.00	468128
106.50	2582175	22.50	687473
142.00	3366503	30.00	895033
177.50	4087600	37.50	1095570
213.00	5022881	45.00	1306743

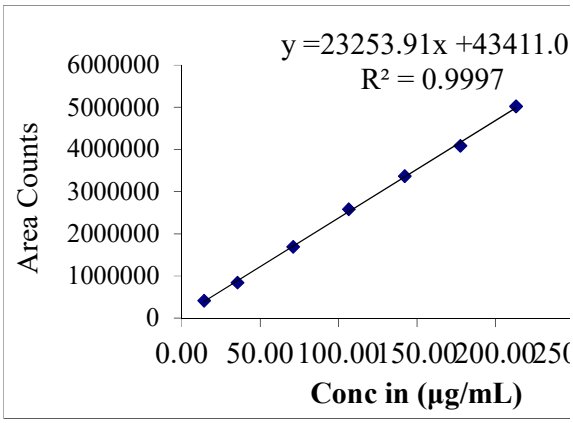

Figure 6. Calibration curve of Estetrol.

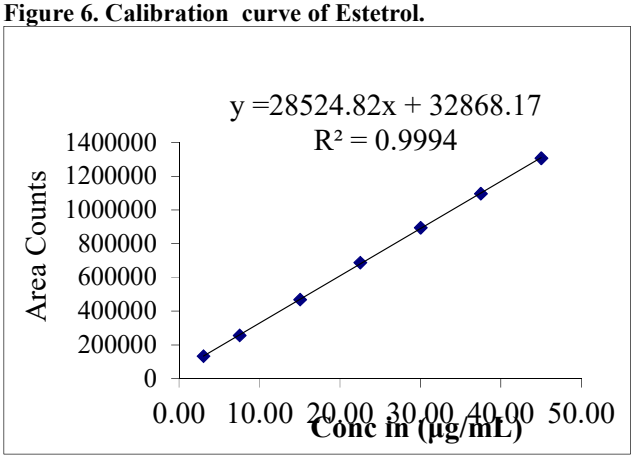

Figure 7. Calibration curve of Drospirenone.

LOD and LOQ:

Limit of Detection and Limit of Quantification were determined from above calibration curve was based upon the standard deviation of the calibration curve's response and the calibration curve's slope.

LOD = 3.3 × (σ/S)

LOQ = 10 × (σ/S)

The limit of detection (LOD) and limit of quantification (LOQ) were determined using the standard deviation of the response (σ) and the slope of the calibration curve (S). The calculated values were further confirmed experimentally by injecting standard solutions at low concentration levels and evaluating the corresponding signal-to-noise (S/N) ratios

Table 5. LOD and LOQ

Parameter	Estetrol	Drospirenone
LOD (µg/mL)	0.426	0.09
LOQ (µg/mL)	1.42	0.3

Robustness:

The robustness of the developed method was evaluated by introducing deliberate, minor variations in the flow rate and mobile phase composition. These intentional modifications did not produce any significant effect on the chromatographic performance, and the %RSD values remained within the acceptable limits, demonstrating the robustness and reliability of the method. [17,18,].**Table 6. Robustness Results**

Degradation Studies:

To establish the stability-indicating capability of the proposed method, forced degradation studies were performed under acidic, alkaline, oxidative, thermal, and photolytic stress conditions. Peak purity analysis was carried out to evaluate the integrity of the analyte peaks, confirming that they remained spectrally pure and were completely resolved from the corresponding degradation products. [20].

Table 7. Forced degradation results

Treatment	%Label claim	%Degradation	Peak purity		Pass/fail
			Purity angle	Purity threshold	
Estetrol					
Control	100	0	0.258	3.145	Pass
Acid	83.7	16.3	0.471	3.703	Pass
Alkali	85.5	14.5	0.154	3.948	Pass
Peroxide	85.1	14.9	0.304	3.096	Pass

Variations	Tadalafil		Drospirenone	
	Mean Area Counts	% Label Claim	Mean Area Counts	% Label Claim
Flow rate 0.4 mL/min	3426947	100.0	909861	100.1
Flow rate 0.4 mL/min	3418429	99.7	905557	99.7
Flow rate 0.4 mL/min	3437178	100.3	904243	99.5
%RSD		**0.3**		0.31
Flow rate 0.0.5 mL/min	3127452	98.7	889123	100.3
Flow rate 0.0.5 mL/min	3174461	100.2	880542	99.3
Flow rate 0.0.5 mL/min	3148241	99.4	886349	99.9
%RSD		**0.76**		0.50
Mobile phase 35:65	3561411	100.3	913373	99.9
Mobile phase 35:65	3533419	99.5	919989	100.6
Mobile phase 35:65	3585420	101.0	915235	100.1
%RSD		**0.75**		0.36
Mobile phase 45:55	3057416	100.4	861178	99.8
Mobile phase 45:55	3020866	99.2	863247	100.0
Mobilephase 45:55	3010921	99.8	869743	100.8
%RSD		**0.84**		0.53

Thermal	89.8	10.2	0.178	3.175	Pass
Photo	99.1	0.9	0.283	3.565	Pass
	Drospirenone				
Control	100	0	1.576	7.754	Pass
Acid	84.1	15.9	1.854	7.636	Pass
Alkali	83	17	1.817	7.551	Pass
Peroxide	83.3	16.7	1.937	7.474	Pass
Thermal	86.8	13.2	1.741	7.355	Pass
Photolysis	98.2	1.8	1.612	7.849	Pass

Conclusion:

A simple, rapid, precise, robust and stability indicating UPLC method was successfully developed and validated for simultaneous quantitation of Estetrol and Drospirenone in pharmaceutical dosage form. The separation was performed on a Phenyl column (100 × 2.1 mm, 1.7 µm) with a mobile phase of 0.1 M ammonium dihydrogen phosphate buffer (pH 3.0) and acetonitrile (40:60, v/v). The analysis was carried out at 260 nm, and at a flow rate of 0.2 mL/min. The optimized chromatographic conditions showed good separation of Estetrol and Drospirenone with retention times of 1.44 min and 2.23 min respectively and total chromatographic run time of 4 min.

The developed analytical method was validated according to the ICH Q2(R1) guidelines and the method was found to be satisfactory for all the validation parameters such as system suitability, specificity, linearity, precision, accuracy, robustness, sensitivity and assay. Excellent linearity was obtained for both the calibration curves of Estetrol (r = 0.9997) and Drospirenone (r = 0.9994). The method was found to be precise and accurate as indicated by %RSD values < 2% and percentage recovery of 98.2% to 101.6%. Moreover, the LOD and LOQ values for both analytes obtained were very low, which suggests that the method is very sensitive and appropriate for routine quality control analysis.

The developed method was tested under forced degradation conditions such as acidic, alkaline, oxidative, thermal and photolytic stress to prove the stability indicating capability of the method. The analytes were completely separated from their degradation products without interference under all stress conditions. The results of specificity and peak purity analyses verified the stability of the analyte peaks and confirmed the high specificity of the method. Additionally, the method was successfully applied to the assay of a marketed pharmaceutical formulation with the results of the assay being 99.8% for Estetrol and 100.3% for Drospirenone.

The developed UPLC method is simple, reliable and cost effective and can be used for the routine QC analysis, assay determination and stability studies of Estetrol and Drospirenone in both bulk drug substance and pharmaceutical dosage forms.

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Conflict of Interest:

The authors have no conflicts of interest to declare for the publication of this manuscript. The study was conducted independently and no financial, commercial or personal relationships were seen to have influenced the study design, data collection, data analysis or preparation of the manuscript.

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