ANALYTICAL METHOD DEVELOPMENT AND VALIDATION STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ESTETROL AND DROSPIRENONE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Background: Estetrol and Drospirenone are key components in novel oral contraceptive formulations. A reliable and efficient analytical method is essential for their simultaneous estimation in tablet dosage forms to ensure quality control and regulatory compliance. High-performance liquid chromatography (HPLC) offers precision and accuracy in pharmaceutical analysis.

Aim: To develop and validate a precise, accurate, and cost-effective reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of Estetrol and Drospirenone in tablet dosage forms, following ICH guidelines.

Methodology: Chromatographic separation was achieved using a Waters Kromasil C18 column (5 µm, 4.6 x 150 mm) with a mobile phase of formic acid buffer and acetonitrile (70:30). The flow rate was maintained at 1.0 mL/min, and detection was performed at 265 nm. Retention times for Estetrol and Drospirenone were 2.228 min and 2.859 min, respectively. The method was validated for precision, accuracy, linearity, sensitivity, robustness, and specificity.

Conclusion: The developed RP-HPLC method exhibited high precision (%RSD <1.0), accuracy (>99%), and excellent linearity (r^2 = 0.999). Sensitivity was confirmed with low LOD and LOQ values. Stress testing demonstrated robustness and specificity. This validated method is suitable for routine quality control in pharmaceutical industries.

Keywords: Estetrol, Drospirenone, RP-HPLC, Validation, Quality Control.

INTRODUCTION

Estetrol, a synthetic estrogen with the molecular formula $C_{18}H_{24}O_4$ and an approximate molecular weight of 304.38 g/mol, is known for its high selectivity and favorable pharmacokinetic profile. [1,2] Its IUPAC name, estra-1,3,5(10)-triene-3,15α,16α,17β-tetrol, highlights its structural relationship with endogenous estrogens.[3,4] Estetrol primarily functions as a selective estrogen receptor modulator (SERM), making it a key component in hormone therapy and contraceptive formulations. [5,6]

In contrast, Drospirenone, with the molecular formula $C_{24}H_{30}O_3$ and a molecular weight of approximately 366.49 g/mol, is a synthetic progestin derived from spironolactone.[7] Its IUPAC name, 6β,7β,15β,16β-dimethylene-3-oxo-17α-pregn-4-ene-21,17-carbolactone, reflects its anti-mineralocorticoid and anti-androgenic properties.[8,9] Drospirenone is widely used in combination oral contraceptives and hormone replacement therapy due to its ability to counteract estrogen-induced fluid retention and its beneficial cardiovascular effects.[10,11]

The combined use of Estetrol and Drospirenone in pharmaceutical formulations offers a novel approach to contraception and hormone therapy, balancing efficacy with reduced side effects. [12,13] These compounds' complementary pharmacological actions make them an effective alternative to traditional hormonal therapies. [14,15] However, ensuring their stability, efficacy, and quality in pharmaceutical dosage forms necessitates a robust analytical methodology. [16,17]

High-Performance Liquid Chromatography (HPLC), particularly Reverse-Phase HPLC (RP-HPLC), is a widely accepted technique for the simultaneous estimation of Estetrol and Drospirenone in pharmaceutical preparations.[18] This study focuses on the development, validation, and stability-indicating analysis of an RP-HPLC method tailored for these compounds.[19,20] The method adheres to international validation guidelines and evaluates critical parameters such as accuracy, precision, linearity, specificity, robustness, limits of detection (LOD) and quantification (LOQ), system suitability, stability, and forced degradation.[21-23]

Despite advancements in analytical techniques, validated methods for the simultaneous quantification of Estetrol and Drospirenone in bulk and pharmaceutical formulations remain limited. [24,25] This study aims to bridge this gap by establishing a validated RP-HPLC method suitable for routine quality control, stability testing, and regulatory compliance, contributing to the advancement of pharmaceutical quality assurance.[26-28]

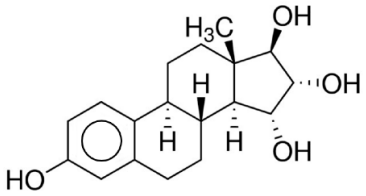

Figure 1. Chemical structure of Estetrol

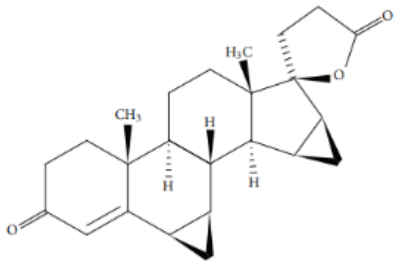

Figure 2. Chemical structure of Drospirenone

MATERIALS AND METHODS

Materials and Reagents

Estetrol and Drospirenone were used as active pharmaceutical ingredients (APIs). The combination formulation of Estetrol and Drospirenone (Nextstellis) was analyzed along with analytical-grade distilled water, acetonitrile, phosphate buffer, methanol, potassium dihydrogen orthophosphate buffer, and ortho-phosphoric acid. All chemicals and reagents were of analytical grade and procured from Rankem Chemicals.

Instrumentation

A UV-VIS spectrophotometer (PG Instruments T60), equipped with 2 mm and 10 mm bandwidth options and matched quartz cells, was utilized for absorbance measurements, operating through UV Win 6 Software. HPLC analyses were conducted using a WATERS HPLC 2695 SYSTEM, featuring quaternary pumps, a Photodiode Array detector, and an autosampler, all controlled by Empower 2 Software. Additional instruments included a Denver Electronic Balance, a BVK Enterprises pH Meter, and an Ultrasonicator from BVK Enterprises, ensuring precise analytical performance.

Methodology

Diluent Selection and Buffer Preparation:

The diluent selected based on drug solubility was a mixture of Water and Buffer in a ratio of 80:20. The 0.01N $(NH_4)_3PO_4$ buffer was prepared by dissolving 1.32g of Dibasic ammonium phosphate in 1000ml of Milli-Q water, degassing, and adjusting the pH to 6.8 using diluted OPA.

Stock Solutions Preparation:

- **Standard Stock Solutions:** 14.2mg of Estetrol and 3mg of Drospirenone were dissolved in a 25ml volumetric flask with 3/4 diluent and sonicated. The volume was then made up with diluent (568µg/ml Estetrol and 120µg/ml Drospirenone).
- **Standard Working Solutions:** 1ml from each stock solution was pipetted into a 10ml

volumetric flask and diluted (56.8µg/ml Estetrol and 12µg/ml Drospirenone).

- **Sample Stock Solutions:** 10 tablets were weighed, and the tablet equivalent was dissolved in 50ml of diluent. After sonication, the solution was filtered (284µg/ml Estetrol and 60µg/ml Drospirenone).
- **Sample Working Solutions:** 2ml of filtered stock solution was diluted to 10ml with diluent (56.8µg/ml Estetrol and 12µg/ml Drospirenone).

Validation:

- **System Suitability:** Six injections of the standard solutions were made, and parameters like peak tailing, resolution, and USP plate count were evaluated. The % RSD for area was < 2%.
- **Specificity:** The method was free from interference, with no peaks at the retention times of Estetrol and Drospirenone.
- **Precision:** Six injections of sample solutions were performed. % RSD for the area was < 2%.
- **Linearity:** Standard solutions were prepared at varying concentrations (25%, 50%, 75%, 100%, 125%, 150%), and the calibration curve was linear for both drugs.
- **Accuracy:** Spiked solutions (50%, 100%, 150%) were prepared. % Recovery was within 98-102%.
- **Robustness:** Method variations such as flow rate, mobile phase ratio, and temperature showed no significant impact on results.
- **LOD/LOQ:** Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined based on the signal-to-noise ratio.

Degradation Studies:

- **Oxidation:** Hydrogen peroxide (20%) was added to the stock solutions, kept for 30 minutes at 60°C, and analyzed.
- **Acid Degradation:** 2N Hydrochloric acid was added to the stock solutions, refluxed for 30 minutes at 60°C, and analyzed.
- **Alkali Degradation:** 2N Sodium hydroxide was added to the stock solutions, refluxed for 30 minutes at 60°C, and analyzed.
- **Dry Heat:** The drug solution was placed at 105°C for 1 hour, then analyzed.
- **Photo Stability:** The sample was exposed to UV light for 1 day, and chromatograms were recorded.
- **Neutral Degradation:** The sample was refluxed in water at 60°C for 1 hour, followed by analysis.

RESULTS:

RP-HPLC Method Development for Estetrol/Drospirenone Analysis

The chromatographic method for the analysis of Estetrol and Drospirenone was developed by

optimizing various parameters such as mobile phase composition, column selection, flow rate, and injection volume. Initial trials (Trial 1 to Trial 4) involved adjustments to the mobile phase ratios and stationary phase.

In Trial 1, using a mobile phase of 0.1% OPA: Methanol (50:50 v/v) with an Agilent C18 column resulted in elution of both Estetrol and Drospirenone. However, the column efficiency was below the acceptance criteria, prompting further investigation. Trial 2 with a mobile phase of Methanol: Ammonium formate (50:50 v/v) showed elution of both peaks, but the plate count was below the required limit (<2000). Trial 3, using a 0.1% Formic acid: Acetonitrile (55:45 v/v) mobile phase and Kromasil C18 column, resulted in the peaks eluting in the void volume, suggesting the need for further refinement. Trial 4 with 0.01N KH2PO4: Acetonitrile (70:30 v/v) as the mobile phase showed both peaks but at higher elution times than expected from the literature.

Finally, the optimized method was developed by adjusting the mobile phase to 0.1% Formic acid: Acetonitrile (70:30 v/v) with a Kromasil C18 column. This configuration provided a flow rate of 1.0 ml/min, a column temperature of 30°C, and a run time of 5.0 minutes. The results showed good resolution, with Estetrol and Drospirenone eluting at 2.228 min and 2.859 min, respectively. The method met the required criteria for resolution, theoretical plate count, and tailing factor, making it suitable for further validation.

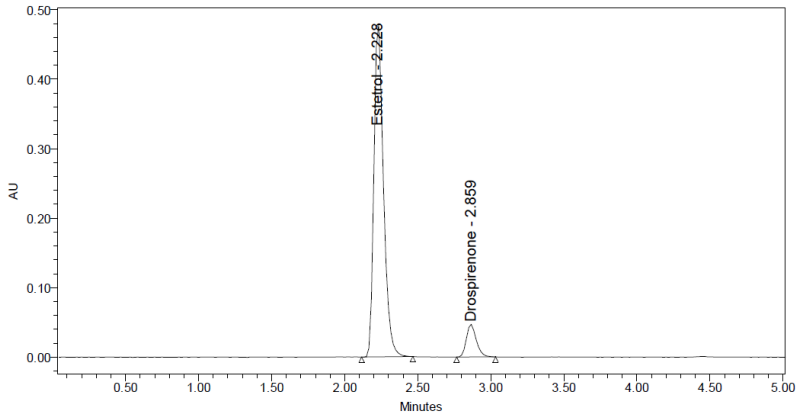

Figure 3. Optimized chromatogram of Estetrol and Drospirenone

RP-HPLC METHOD VALIDATION

System Suitability

The system suitability parameters were evaluated and found to be within the acceptable range as per ICH guidelines. All criteria, including resolution, plate count, and tailing factor, were satisfactory. Therefore, the developed method is considered suitable for accurate analysis.

Table 1. System suitability parameters for Estetrol and Drospirenone

S. no	**Estetrol**			**Drospirenone**			
Inj	RT (min)	USP Plate Count	Tailing	RT (min)	USP Plate Count	Tailing	Resolution
1	2.219	6524	1.24	2.794	7817	1.24	4.6
2	2.219	6652	1.23	2.795	7721	1.24	4.6
3	2.221	6598	1.24	2.800	7824	1.23	4.6
4	2.222	6602	1.24	2.801	7834	1.24	4.5
5	2.223	6588	1.23	2.801	7798	1.23	4.6
6	2.223	6606	1.23	2.809	7835	1.24	4.6

Acceptance Criteria: 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.

Specificity

The specificity of the method was confirmed as no interfering peaks were observed in the blank and placebo chromatograms at the retention times of Estetrol (2.228 min) and Drospirenone (2.859 min). Figures 4 and 5 show the absence of any interference, supporting the method's specificity. Thus, the method is deemed specific for Estetrol/Drospirenone analysis.

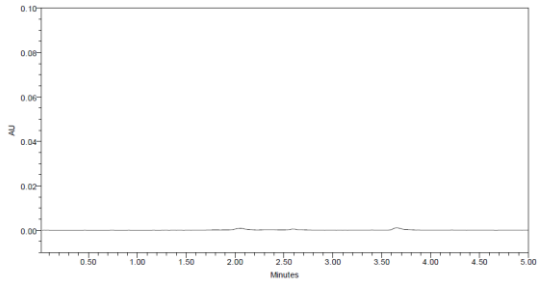

Figure 4. Chromatogram of blank.

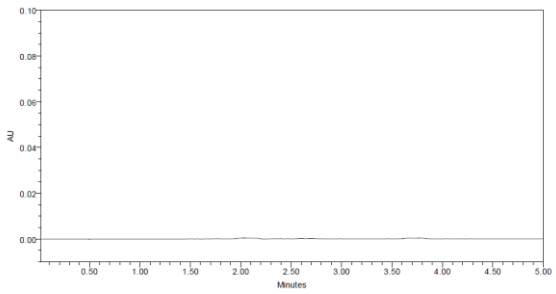

Figure 5. Chromatogram of placebo.

LINEARITY

Six linear concentrations of Drospirenone (3-18µg/ml) and Estetrol (14.2-85.2µg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Drospirenone was y = 69652x + 1303.1 and of Estetrol was y = 62844x + 50224. Correlation coefficient obtained was 0.999 for the two drugs.

Table 2. Linearity table for Estetrol and Drospirenone.

Drospirenone		Estetrol	
Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area
0	0	0	0
3	205654	14.2	917400
6	419696	28.4	1833932
9	626503	42.6	2742056
12	841549	56.8	3644845
15	1063378	71	4548363
18	1239134	85.2	5354900

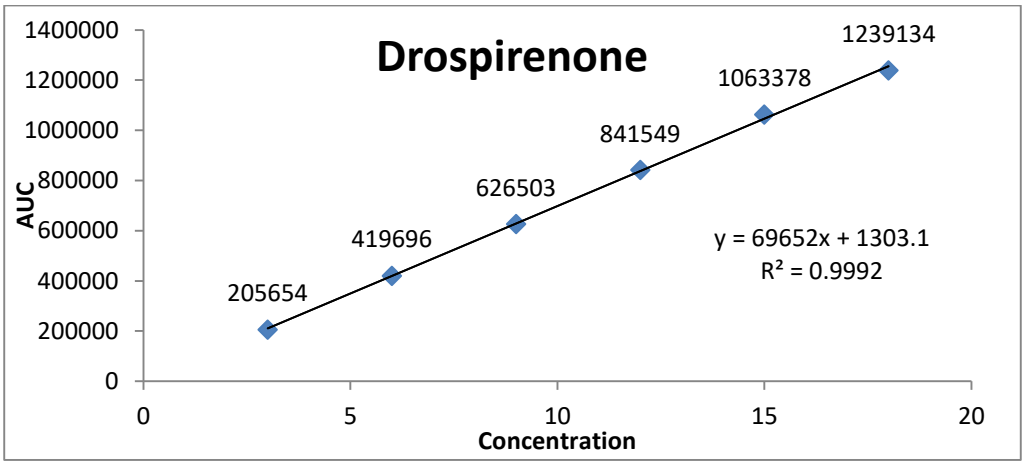

Figure 6. Calibration curve of Drospirenone

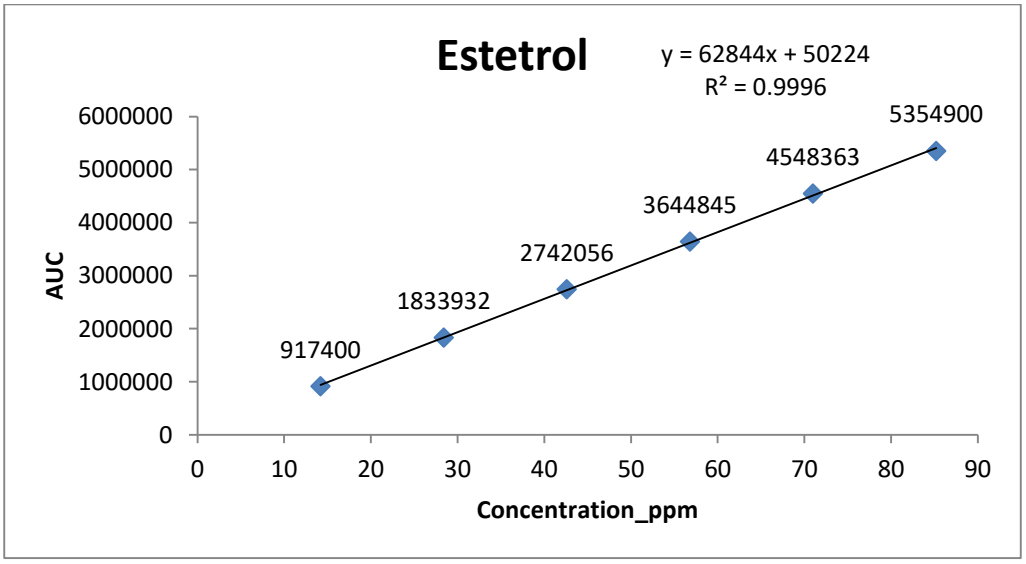

Figure 7. Calibration curve of Estetrol

Acceptance Criteria: The method demonstrated good linearity within the specified range of 3-18 µg/ml for Drospirenone and 7.1-42.6 µg/ml for Estetrol. The regression coefficients (R^2 = 0.999) for both analytes confirm a strong linear relationship between concentration and response, meeting the acceptance criterion of R < 1.

Precision:

System Precision:

From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 0.4%and 0.4% respectively for Estetrol and Drospirenone. As the limit of Precision was less than "2" the system precision was passed in this method.

Table 3. System precision table of Estetrol and Drospirenone

S.no	Area of Drospirenone	Area of Estetrol
1	837701	3666589
2	833639	3640207
3	833039	3629764
4	839145	3655394
5	831810	3624661
6	839914	3644100
Avg	835875	3643453
Stdev	3461.4	15680.2
%RSD	0.4	0.4

Repeatability:

A multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.8% and 0.4% respectively for Estetrol and Drospirenone. As the limit of Precision was less than "2" the system precision was passed in this method.

Table 4. Repeatability table of Estetrol and Drospirenone

S.no	Area of Estetrol	Area of Drospirenone
1	3656745	838185
2	3608492	832659
3	3651631	833947

4	3685171	830311
5	3661446	839488
6	3689760	836141
Avg	3658874	835122
Stdev	29145.9	3466.3
%RSD	0.8	0.4

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2%.

Intermediate precision (Day Precision):

Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given on the next day of the sample preparation and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.6% and 0.2% respectively for Estetrol and Drospirenone. As the limit of Precision was less than "2" the system precision was passed in this method.

Table 5. Intermediate precision table of Estetrol and Drospirenone

S.no	Area of Drospirenone	Area of Estetrol
1	832369	3599163
2	833294	3561297
3	836269	3569412
4	835377	3603335
5	834112	3565558
6	831254	3601258
Avg	833779	3583337
Stdev	1868.9	19835.8
%RSD	0.2	0.6

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2%.

Accuracy:

Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.89% and 99.52% for Estetrol and Drospirenone respectively.

Table 6. Accuracy table of Drospirenone

% Level	Amount Spiked(µg/mL)	Amount recovered(µg/mL)	% Recovery	Mean% Recovery
50%	6	5.96	99.29	99.52%
	6	5.92	98.65	
	6	5.95	99.21	
100%	12	11.92	99.29	
	12	11.91	99.27	
	12	11.89	99.07	
150%	18	18.21	101.19	
	18	17.98	99.86	
	18	17.97	99.81	

Table 7. Accuracy table of Estetrol

% Level	AmountSpiked(µg/mL)	Amount recovered(µg/mL)	% Recovery	Mean% Recovery
50%	28.4	28.50	100.35	99.89%
	28.4	28.31	99.68	
	28.4	28.48	100.28	
100%	56.8	56.65	99.74	
	56.8	56.74	99.90	
	56.8	56.82	100.04	
150%	85.2	85.88	100.79	
	85.2	84.47	99.14	
	85.2	84.46	99.13	

Acceptance Criteria: The accuracy of the method was determined through % recovery studies, with results of 99.52% for Drospirenone and 99.89% for Estetrol, meeting the acceptance criteria of 98-102%. This indicates the method is reliable and provides accurate quantification.

Sensitivity:

The sensitivity of the method was evaluated for both Estetrol and Drospirenone, with the LOD and LOQ values as follows: Drospirenone (LOD: 0.03, LOQ: 0.08) and Estetrol (LOD: 0.20, LOQ: 0.61). Chromatograms for both LOD and LOQ standards illustrate the method's capacity to detect and quantify the compounds at low concentrations. These results confirm that the method possesses adequate sensitivity for both drugs.

Table 8. Sensitivity table of Estetrol and Drospirenone

Molecule	LOD	LOQ
Drospirenone	0.03	0.08
Estetrol	0.20	0.61

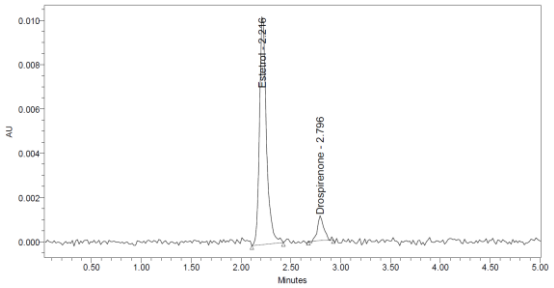

Figure 8. LOD Chromatogram of Standard

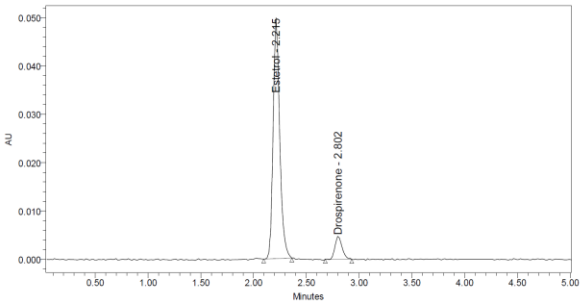

Figure 9. LOQ Chromatogram of Standard

Acceptance Criteria: LOD (Limit of Detection): 0.03 µg/ml for Drospirenone and 0.20 µg/ml for Estetrol, within the acceptable range (NMT 3). **LOQ (Limit of Quantification):** 0.08 µg/ml for Drospirenone and 0.61 µg/ml for Estetrol, also meeting the limit (NMT 10).

Robustness:

Robustness conditions like Flow minus (1.1ml/min), Flow plus (1.3ml/min), mobile phase minus (65B:35A), mobile phase plus (75B:25A), temperature minus (21°C) and temperature plus (31°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.

Table 9. Robustness data for Estetrol and Drospirenone.

S.no	Condition	%RSD of Drospirenone	%RSD of Estetrol
1	Flow rate (-) 1.0ml/min	0.4	0.7
2	Flow rate (+) 1.3ml/min	0.2	0.7
3	Mobile phase (-) 65B:35A	0.3	0.6
4	Mobile phase (+) 75B:25A	0.4	0.3
5	Temperature (-) 21°C	0.3	0.9
6	Temperature (+) 31°C	0.3	0.8

Acceptance Criteria The robustness of the method was evaluated by making small variations in flow rate (FM), column temperature (TP), mobile phase composition (MP), and other parameters. The %RSD values ranged from 0.2% to 0.9%, well within the acceptance limit of NMT 2.0%, indicating that the method is robust and can withstand minor variations without affecting the results significantly.

ASSAY

The assay data for Drospirenone and Estetrol show the analysis of sample areas with their corresponding standard areas, along with the calculated percentage assay. For Drospirenone, the average assay is 99.71%, with a %RSD of 0.4, indicating good consistency. Estetrol's assay results show an average of 100.22%, with a slightly higher %RSD of 0.8, reflecting more variation in sample areas.

Table 10. Assay Data of Drospirenone

S.no	Standard Area	Sample area	% Assay
1	837701	838185	100.08
2	833639	832659	99.42
3	833039	833947	99.57
4	839145	830311	99.14
5	831810	839488	100.23
6	839914	836141	99.83
Avg	835875	835122	99.71
Stdev	3461.4	3466.3	0.4
%RSD	0.4	0.4	0.4

Table 11. Assay Data of Estetrol

S.no	Standard Area	Sample area	% Assay
1	3666589	3656745	100.16
2	3640207	3608492	98.84
3	3629764	3651631	100.02
4	3655394	3685171	100.94
5	3624661	3661446	100.29
6	3644100	3689760	101.07
Avg	3643453	3658874	100.22
Stdev	15680.2	29145.9	0.80
%RSD	0.4	0.8	0.8

Acceptance Criteria: The accuracy of the method was determined through % recovery studies, with results of 99.52% for Drospirenone and 99.89% for Estetrol, meeting the acceptance criteria of 98-102%. This indicates the method is reliable and provides accurate quantification.

Degradation data

The degradation data for Drospirenone and Estetrol shows minimal degradation across all conditions, with both compounds retaining high recovery percentages. Drospirenone generally exhibits slightly lower degradation than Estetrol, particularly under acid and base

conditions.

Table 12. Degradation Data of Drospirenone and Estetrol

Type of degradation	**Drospirenone**		**Estetrol**	
	%Recovered	% Degraded	%Recovered	% Degraded
Acid	93.78	6.22	94.80	5.20
Base	95.69	4.31	95.52	4.48
Peroxide	95.69	4.31	96.89	3.11
Thermal	98.60	1.40	98.13	1.87
UV	98.41	1.59	98.41	1.59
Water	99.16	0.84	99.25	0.75

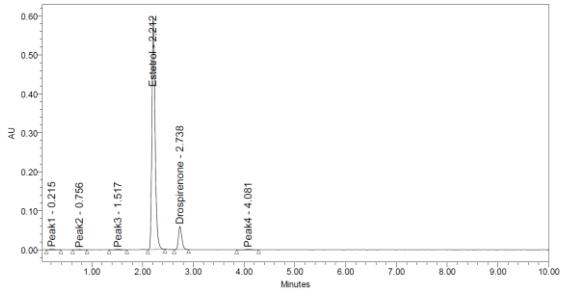

Figure 10. Chromatogram of Acid degradation

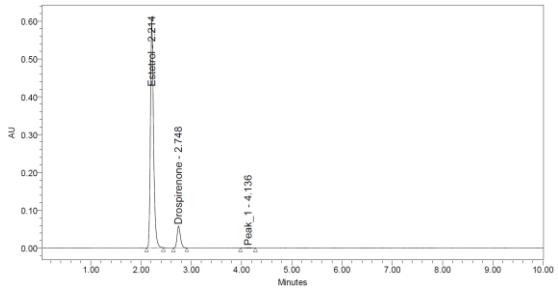

Figure 11. Chromatogram of Base degradation

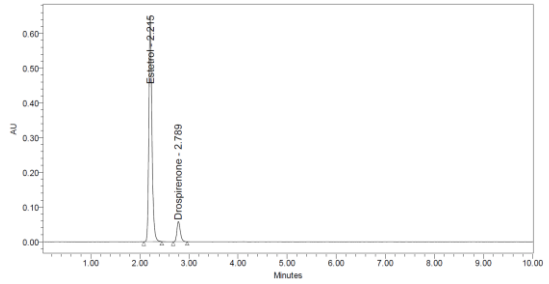

Figure 12. Chromatogram of Peroxide degradation

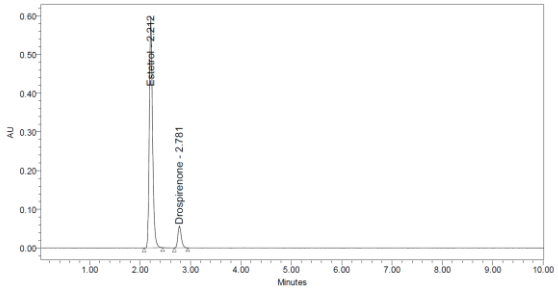

Figure 13. Chromatogram of Thermal degradation

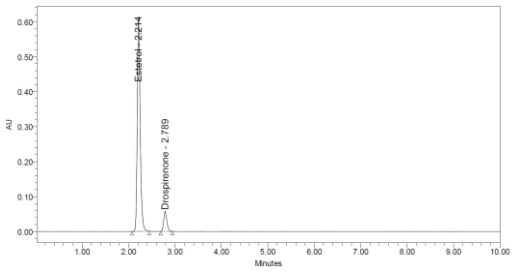

Figure 14. Chromatogram of UV degradation

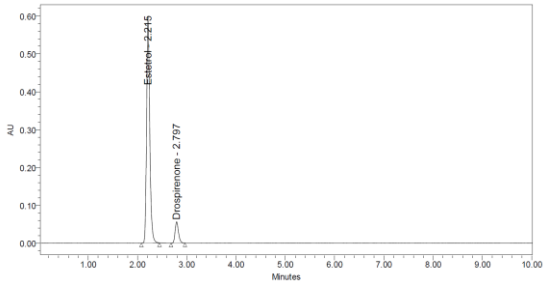

Figure 15. Chromatogram of Water degradation

Acceptance Criteria: The forced degradation study confirmed that Drospirenone and Estetrol remained stable under various stress conditions, with minimal degradation observed. The

highest degradation was seen under acidic conditions (6.22% for Drospirenone, 5.20% for Estetrol), while the least occurred in water (0.84% and 0.75%, respectively). These results indicate the method's stability-indicating capability, ensuring reliable quantification even in the presence of degraded products.

SUMMARY

Table 13. Parameters Data of Drospirenone and Estetrol

Parameters		**Drospirenone**	**Estetrol**	**LIMIT**
Linearity Range(µg/ml)		3-18 µg/ml	7.1-42.6 µg/ml	R< 1
Regression coefficient		0.999	0.999	
Slope(m)		68985	68038	
Intercept(c)		1303.1	50224	
Regression equation (Y=mx+c)		y = 69652x + 1303.1	y = 62844x + 50224	
Assay (% mean assay)		99.71%	100.22%	90-110%
Specificity		Specific	Specific	No interference of any peak
System precision %RSD		0.4	0.4	NMT 2.0%
Method precision %RSD		0.4	0.8	NMT 2.0%
Accuracy %recovery		99.52%	99.89%	98-102%
LOD		0.03	0.20	NMT 3
LOQ		0.08	0.61	NMT 10
Robustness	**FM**	0.4	0.7	%RSD NMT 2.0
	FP	0.2	0.7	
	MM	0.3	0.6	
	MP	0.4	0.3	
	TM	0.3	0.9	
	TP	0.3	0.8	

CONCLUSION

A simple, Accurate, precise method was developed for the simultaneous estimation of the Estetrol and Drospirenone in Tablet dosage form. Retention time of Estetrol and Drospirenone were found to be 2.228min and 2.859min. %RSD of the Estetrol and Drospirenone were found to be 0.8 and 0.4 respectively. %Recovery was obtained as 99.89% and 99.52% for Estetrol and Drospirenone respectively. LOD, LOQ values obtained from Estetrol and Drospirenone regression equations were 0.20, 0.61 and 0.03, 0.08 respectively. Regression equation of Drospirenone is y = 69652x + 1303.1 and y = 62844x + 50224 of Estetrol. Retention times were decreased, and the run time was decreased, so the method developed was simple and economical which can be adopted in regular Quality control tests in Industries.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest for this work.

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