https://africanjournalofbiomedicalresearch.com/index.php/AJBR
Afr. J. Biomed. Res. Vol. 27(4s) (December 2024); 7994 - 8004
Research Article

Analytical Method Development and Validation of Ethinyl Estradiol and Drospirenone by Using RP-HPLC in Bulk and Pharmaceutical Dosage Form

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Abstract
This approach involves employing Reverse Phase-HPLC (High Performance Liquid Chromatography) to facilitate method development and validation, focusing on the stability and combined tablet formation of Estetrol & Drospirenone. The analysis was conducted using an Agilent Eclipse XDB column (250x4.6 mm, 5 µ) with an Acetonitrile:HSA (70:30) solution, completing the run within 6.0 minutes. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined to be 10mg/L, with a recovery rate ranging from 98% to 102%, indicating satisfactory recovery levels. Validation results confirmed adequacy, with acceptable outcomes for bulk and information analysis. The Relative Standard Deviation (RSD) values below 2.0% demonstrate the accuracy and precision of this approach. Furthermore, a retention formation assay revealed 100.24% presence of the formation. This validated method meets the criteria for global regulatory filing, ensuring specificity, precision, linearity, and accuracy. Linearity analysis conducted at stages ranging from 10% to 150% yielded a regression coefficient of 0.999.

Keywords: Ethinyl Estradiol, Drospirenone, HSA, Acetonitrile, RP-HPLC

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Received: 18/11/2024 *Acceptance: 10/12/2024*

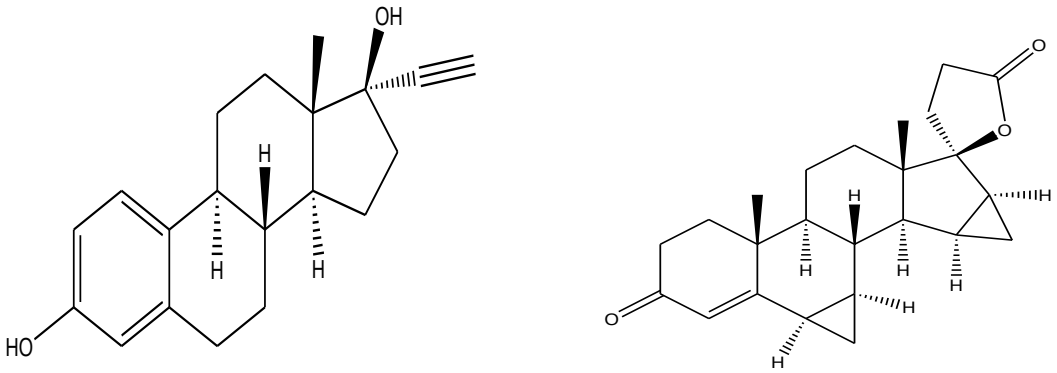
DOI: https://doi.org/10.53555/AJBR.v27i4S.5120

Introduction

Dropirenone is a progestin and antiandrogen medication which is used in birth control pills to prevent pregnancy and in menopausal hormone therapy, among others uses [1]. It is available both alone under the brand name slynd and in combination with an estrogen under the brand name yasmin among the others [2]. The medication is an analog of the drug spiraonolactone [3]. Drospirenone is taken by mouth [4]. Common side effect include acne, headache, breast tenderness, weight increase, and menstrual changes [5]. Drospirenone was patented in 1976 and introduced for medical use in 2000 [6]. It is available widely through the world [7]. The medication is sometimes referred to as a fourth –generation progestin [8]. It is available as a generic medication [9]. Drospirenone is said to more closely resemble bio idental progesterone than other progrestins [10].

Ethinylestradiol (EE) is a widely utilized estrogen medication, frequently combined with progestin for

contraception [11]. Initially employed for various purposes such as managing menopausal symptoms and certain hormone-related cancers, it is commonly administered orally but also available as a patch or vaginal ring [12]. Being a synthetic derivative of natural estrogen, estradiol, EE has distinct characteristics [13]. Developed in the 1930s and introduced for medical use in 1943, it became a staple in birth control pills during the 1960s [14, 15]. Acting either as an estrogen agonist or antagonist, EE interacts with estrogen's biological targets, contributing to its widespread use [16, 17]. Despite its effectiveness, rare but severe side effects like blood clots, liver damage, and uterine cancer have been reported [18]. Presently, EE is a primary component of combined birth control pills, solidifying its status as one of the most commonly employed estrogens [19]. Common side effects include breast tenderness, headaches, fluid retention, and nausea [20].



(a) **(b)**

Chemical structures of (a) Ethinylestradiol (b) Drospirenone

ATERIALS AND METHOD:

CHEMICALS: All experiments were carried out using Acetonitrile of high-performance liquid chromatography (HPLC) grade and internally produced HPLC-Grade Water (Milli Q). Analytical reagent grade ortho phosphoric acid and hexane sulfonic acid provided by Rankem were among the chemicals utilized.

INSTRUMENTION:

The research utilized the Waters e 2695 [Alliance] High-Performance Liquid Chromatography system, controlled by Empower software version 2.0. Eu-tech and Borosil equipment were utilized for experimental procedures. An Ultrasonicator (UCA 701) manufactured by Unichrome was employed in this study. The preparation of the mobile phase, sample, and standard aliquots was carried out using an analytical balance (Sartorius, CP225D).

METHOD OPTIMIZATION:

To optimize the chromatographic conditions, different combinations of Acetonitrile and 0.1% formic acid, as well as Acetonitrile and HAS in the mobile phase, were tested. The composition of the mobile phase was adjusted in each trial to enhance resolution and achieve satisfactory retention time. Ultimately, Acetonitrile and HAS with isocratic elution were selected due to their significant response in dynamic pharmacological components. Throughout the method refinement process, various stationary phases such as X-bridge phenyl and Agilent eclipse XDB columns were evaluated. By these trials the final shape of the peak was much accurate with column of RP-250x4.6 mm, 5 µ by a PDA detector.

VALIDATION PROCEDURE:

The analytical parameters, including system suitability, precision, specificity, accuracy, linearity, robustness, limits of detection (LOD) and quantification (LOQ), forced degradation, and stability, were validated in accordance with the guidelines outlined in ICHQ2(R1).

Mobile phase preparation:

PREPARATION OF STANDARD SOLUTION:

Precisely weigh and transfer 71mg of Estetrol and 15mg of Drospirenone working standards into a clean, dry 100 ml vacuum flask. Add the appropriate diluent and sonicate until complete dissolution before adjusting the volume to the mark using the same solvent to create the stock solution.Then, pipette an additional 5 mL of the aforementioned stock solutions into a 50 mL vacuum flask and dilute with diluent to achieve the desired concentration levels of 71ppm for Estetrol and 15ppm for Drospirenone.

PREPARATION OF SAMPLE SOLUTION:

After precise weighing, transfer 815 mg of the substance into a clean, dry 100mL vacuum flask. Add the necessary diluent and sonicate the mixture for 30 minutes to ensure complete dissolution. Subsequently, centrifuge the solution for 30 minutes to further dissolve any remaining particles and adjust the volume to the mark using the same solvent. The resulting fluid is then filtered through a 0.45 micron Injection filter to prepare the stock solution.

Next, transfer 5mL of the aforementioned stock solution into a 50 mL vacuum flask and dilute it with diluent up to the mark to achieve the desired

concentrations of 71 ppm for Estetrol and 15 ppm for Drospirenone.

RESULTS AND DISCUSSIONS:

The primary analytical challenge in developing a new method was isolating the active pharmaceutical ingredients. To ensure optimal performance the chromatographic conditions were meticulously fine-tuned.

System suitability: When the standard solution injection system is suited and the USP tailing is reported, the plate count values are shown in the table below and the standard chromatogram was displayed.According to ICH criteria, all system suitability metrics were within acceptable ranges.

Table-1: **System suitability parameters for Estetrol & Drospirenone**

S.no	Parameter	Estetrol	Drospirenone
1	Retention time	3.491	4.085
2	Plate count	5357	6887
3	Tailing factor	1.07	1.14
4	Resolution	----	3.05
5	%RSD	0.35	0.17

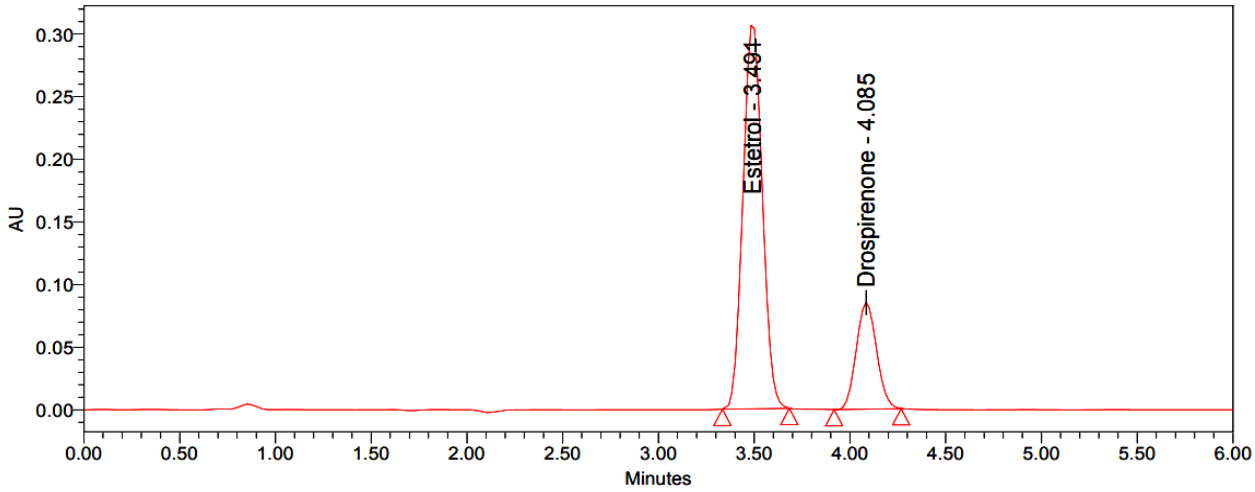


Fig. 1. Chromotogram of standard

SPECIFICITY:

Specificity refers to an analytical method's capacity to quantify a single analyte with no influence from other unknown or blank samples. Three chromatograms were recorded for this purpose: one blank, one standard, and one actual sample. It is clear from the blank chromatogram that the drug reaction was selective, since there is no response at the drug retention times.

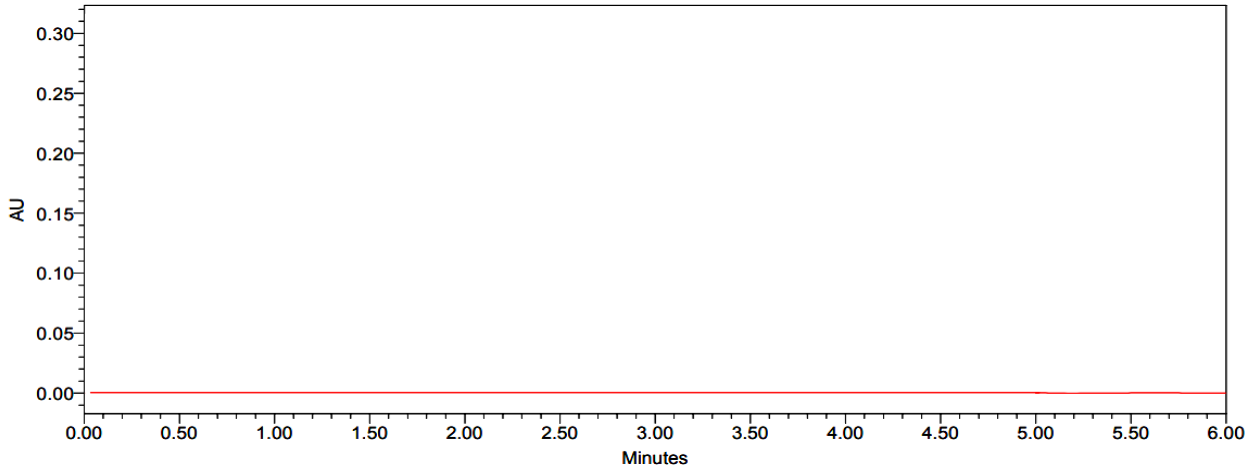


Fig. 2. Chromatogram of blank

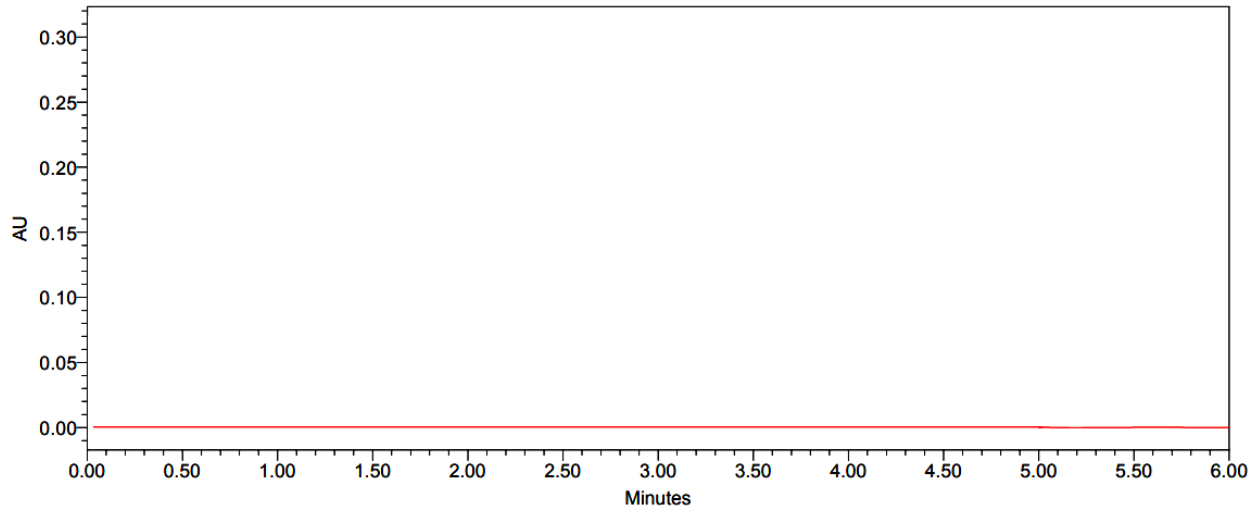


Fig. 3. Chromatogram of placebo

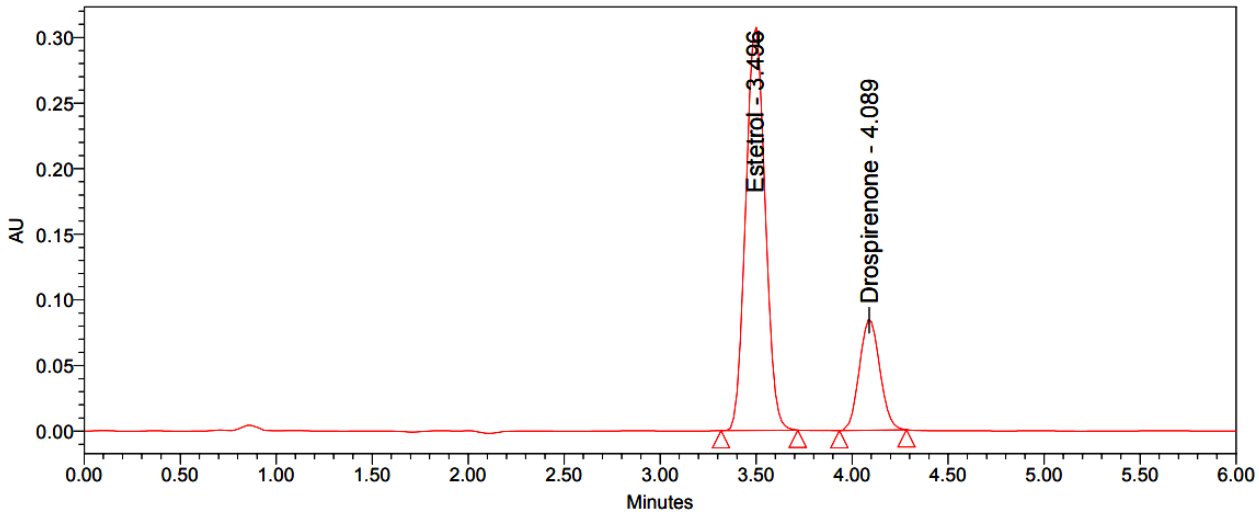


Fig. 4. Optimized chromatogram

Discussion: Estetrol and Drospirenone had retention duration's of 3.496 and 4.089 minutes, respectively. Utilizing this method, we were unable to detect any interference peaks in the blank and placebo throughout the retention periods of these drugs. As a result, this procedure was stated to be particular.

Linearity: The area of linearity peak versus various concentrations has been examined for the drugs as 25, 50, 75,100,125,150% dilutions respectively. Linearity has been performed in the range of 17.75-88.75 ug/ml of Estetrol and 3.75-18.75 ug/ml of Drospirenone. The correlation coefficient was above 0.999 for all.

Table-2: Linearity Results

S.NO	Estetrol		Drospirenone	
	Concentration (µg/ml)	**Peak Response**	**Concentration (µg/ml)**	**Peak Response**
1	17.75	750186	3.75	155337
2	35.50	1420364	7.50	305104
3	53.25	2135729	11.25	468437
4	71.00	2801451	15.00	615224
5	88.75	3632132	18.75	769632
6	106.50	4302856	22.50	912318
Regression equation	y = 40349.19x +365.39		y =40720.61x + 2757.71	
Slope	40349.19		40720.61	
Intercept	365.39		2757.71	
R^2	0.999		0.999	

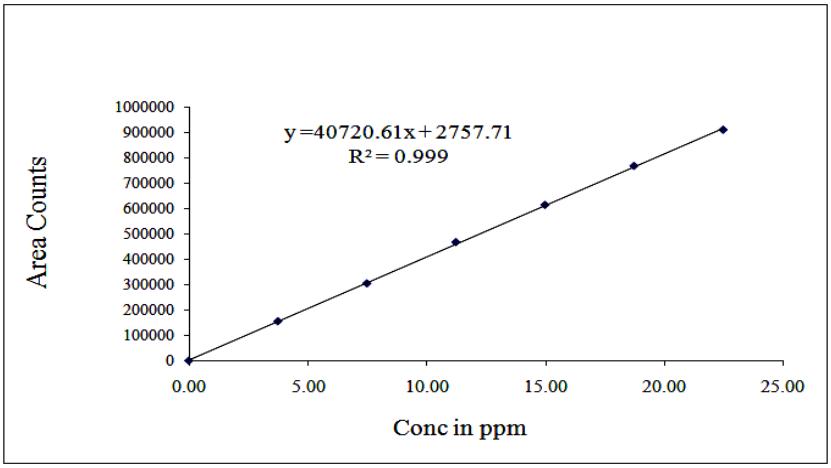


Fig. 5. Calibration curve for Drospirenone at 258 nm

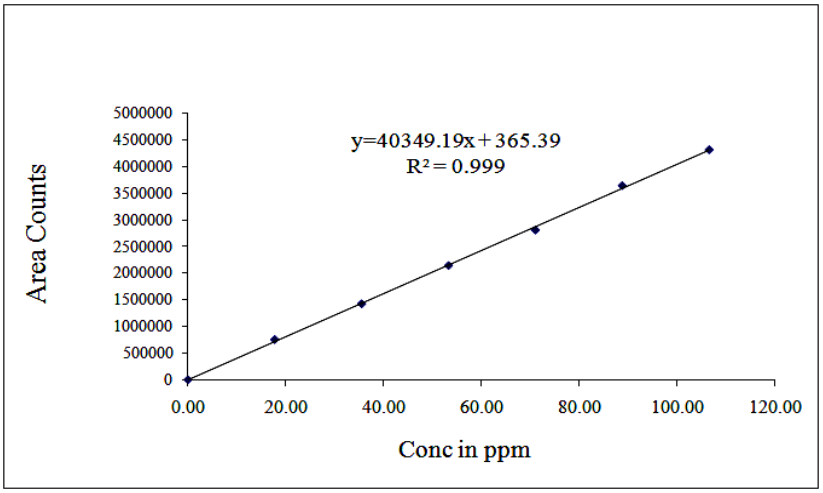


Fig. 6. Calibration curve for Estetrol at 258 nm

Accuracy: Three different concentration levels of 50,100 and 150 percent at a specific limit were used in the process to check the accuracy of this particular method. The method developed was found to be more accurate and reliable.

Table-3: Accuracy outcomes of Estetrol by RP-HPLC method

%Concentration(at specification Level)	Response	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1413221	35.50	35.6	100.3	100.1
100%	2808103	71.00	70.74	99.6	
150%	4245234	106.5	106.94	100.4	

Table-4 :Accuracy outcomes for Drospirenone by RP-HPLC method

Concentration (at specification Level)	Response	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	303463	7.50	7.44	99.2	99.7
100%	611384	15.00	14.99	99.9	
150%	918564	22.50	22.51	100.0	

Discussion: The conventional addition procedure was used to create three levels of Accuracy samples. For each level of accuracy and mean percent, three injections were administered. Estetrol and Drospirenone recovered at rates of 100.1 and 99.7 percent, respectively.

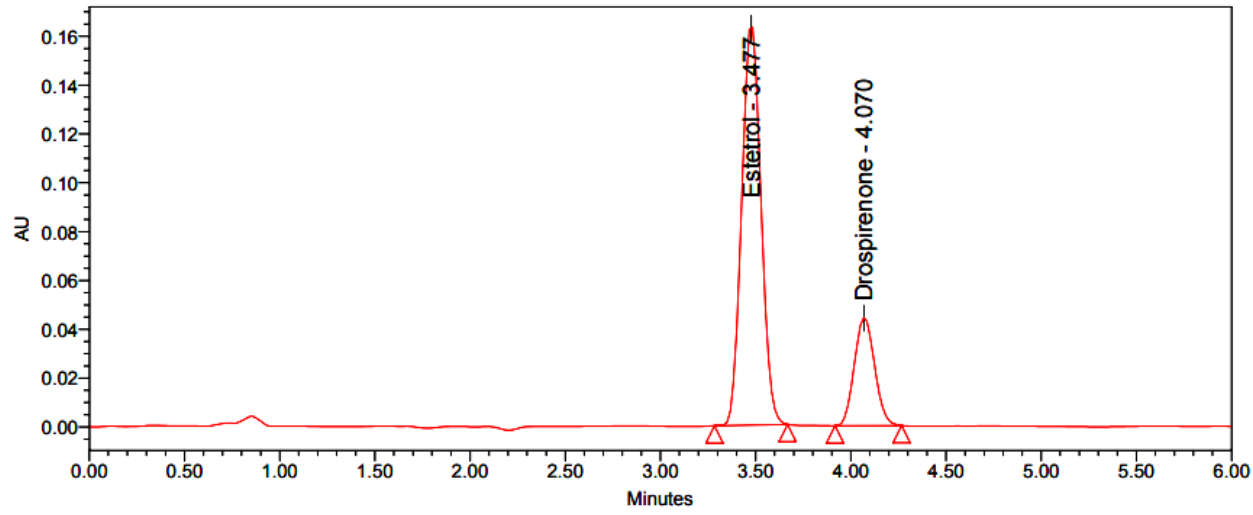


Fig. 7. Accuracy 50% Chromatogram

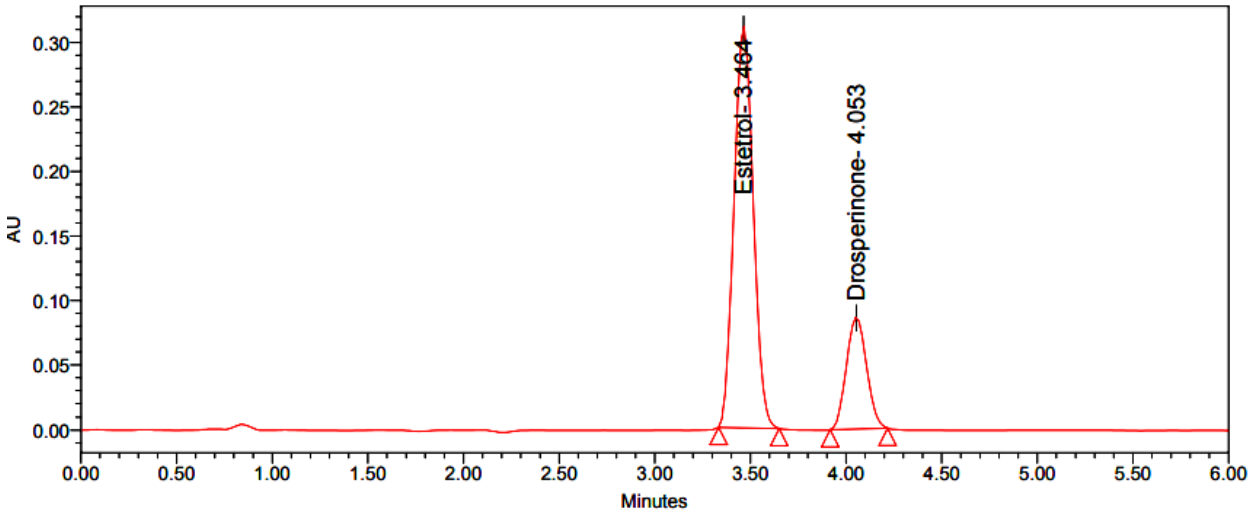


Fig. 8. Accuracy 100% Chromatogram

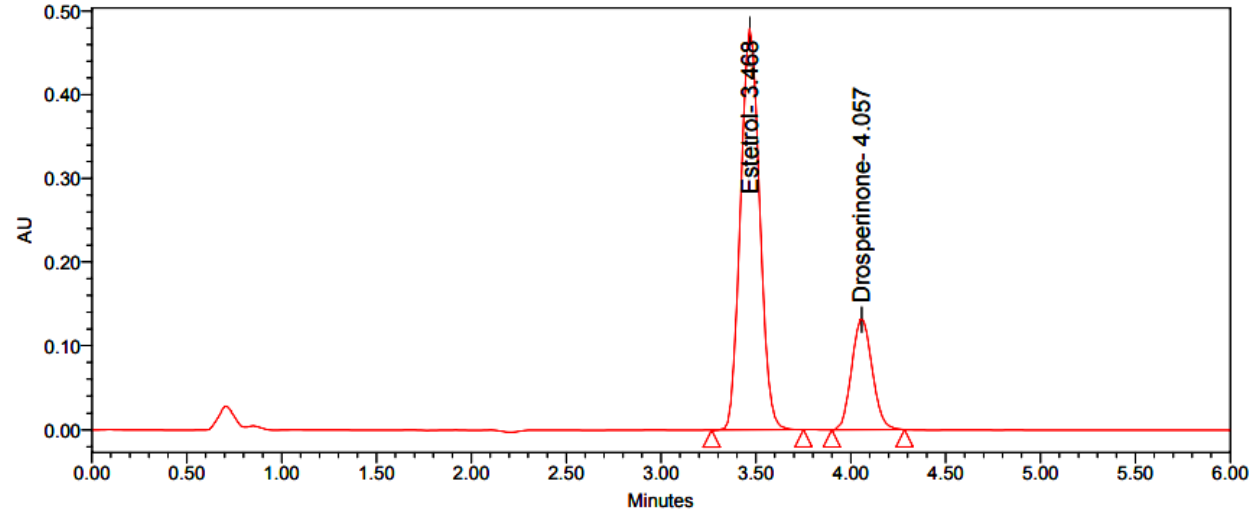


Fig. 9. Accuracy 150% Chromatogram

Precision: In this method prepare six various sample concentration in concentration of Estetrol (70 ug/ml) and Drospirenone (15 ug/ml) injected in UPLC system. The detected % of test results is in the range of 98% to 102%. Peak areas are calculated and used to calculate mean, SD, and %RSD values.

Table-5 System precision table of Estetrol & Drospirenone

S. No	Concentration Estetrol (µg/ml)	Estetrol Response	Concentration of Drospirenone (µg/ml)	Drospirenone Response
1.	71	2807621	15	612354
2.	71	2812861	15	612874
3.	71	2825349	15	611034
4.	71	2809143	15	610341
5.	71	2824786	15	612218
6.	71	2831302	15	613021
Mean	2818510		611974	
S.D	9878.54		1064	
%RSD	0.35		0.17	

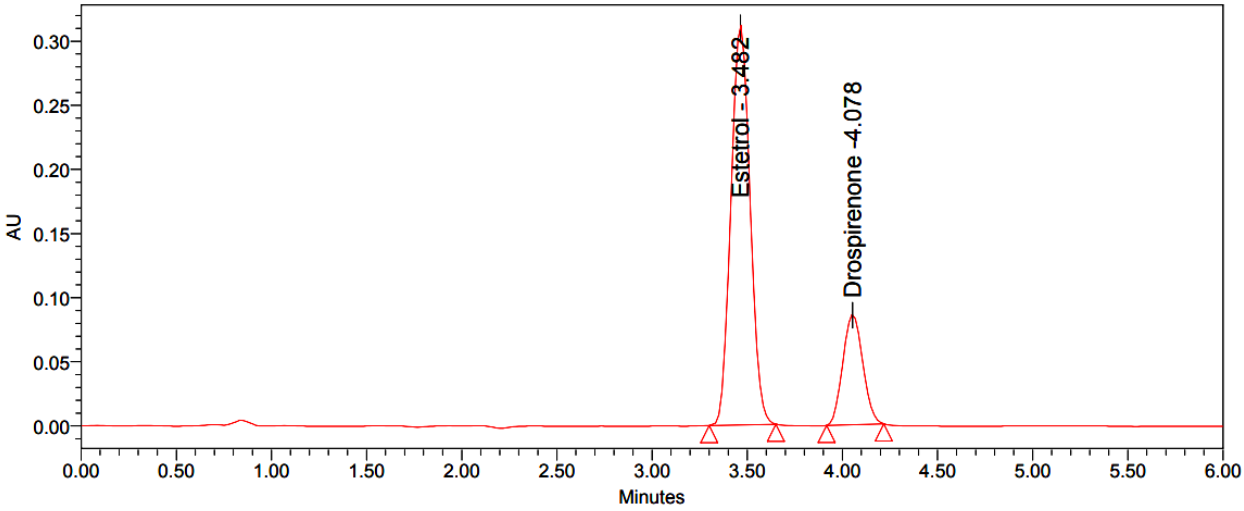


Fig. 10. Chromatogram of method precision

Intermediate precision: Separate instruments were employed on different days and in different locations to conduct independent analyses on six replicates of sample solution. Mean and RSD values were computed from the peak regions, with the findings detailed in the table below. Estetrol (71 ug/ml) and Drospirenone (15 ug/ml) underwent analysis on two distinct days with six separate samples. Consequently it was observed that the current methodology produces highly precise results with RSD results below 2 percent and assay values close to 100 percent.

Table-6 Intermediate Precision (Day variation) for Estetrol and Drospirenone by RP-HPLC method

S. No.	Estetrol Response		Drospirenone Response	
	Day-1	Day-2	Day-1	Day-2
1	2811234	2847261	610430	615861
2	2801328	2855047	615320	613217
3	2841384	2826598	613461	614659
4	2823784	2831429	611312	612988
5	2833128	2843221	612761	615124
6	2813354	2838944	617124	618512
Average	2820702	2840417	613401	615060
Standard Deviation	14903.94	10417.85	2495.887	2020.526
%RSD	0.53	0.37	0.41	0.33

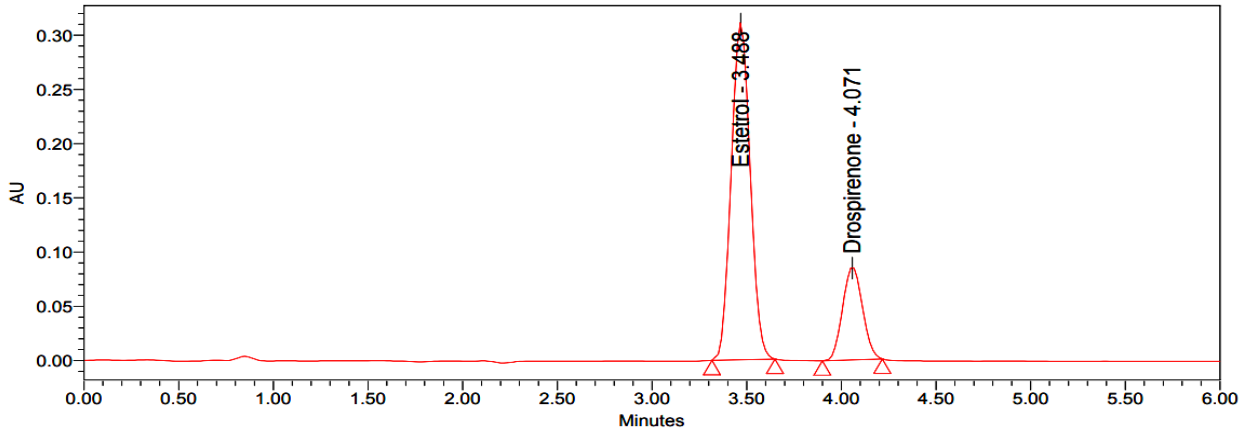


Fig. 11. Inter day precision chromatogram

LOD AND LOQ:

The ICH criteria were used to compute the limit of detection (LOD) and limit of quantification (LOQ) of the medication.

LOD = 3.3 X σ /S

LOQ = 10 X σ /S

Table-7 Sensitivity parameters (LOD & LOQ) by RP-HPLC

Drug Name	LOD(µg/ml)	LOQ(µg/ml)
Estetrol	0.21	0.71
Drospirenone	0.05	0.15

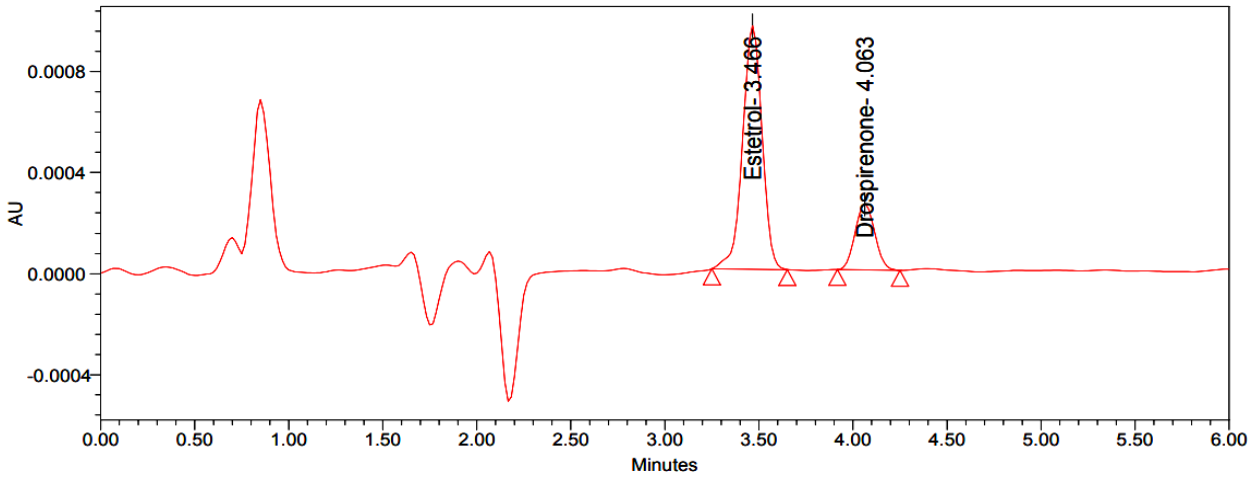


Fig. 12. LOD chromatogram

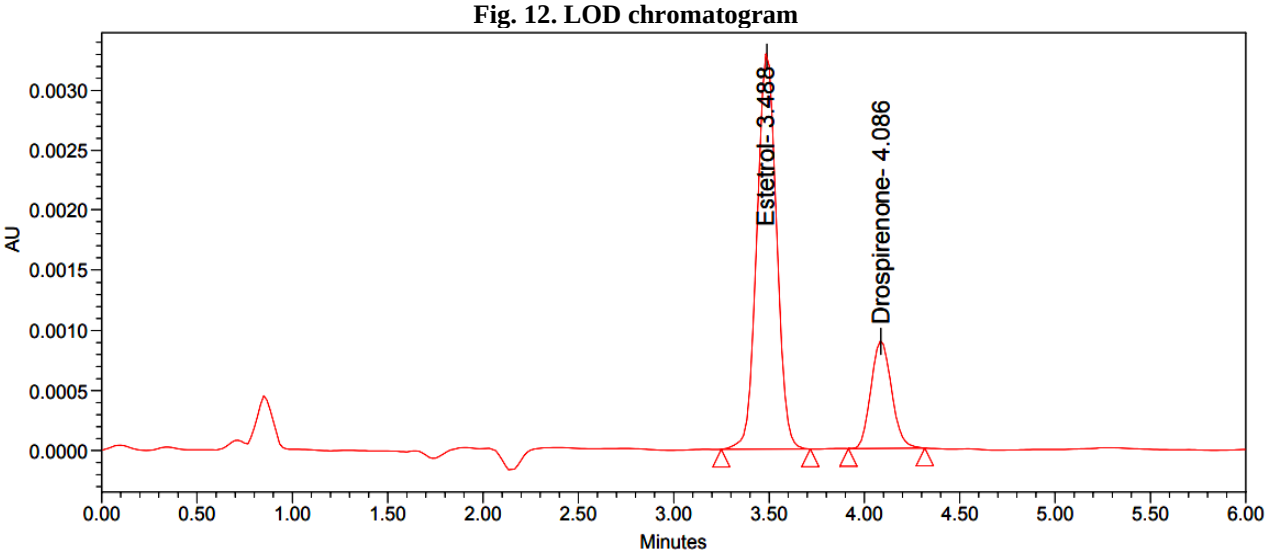


Fig. 13. LOQ Chromatogram

ROBUSTNESS: A purposeful change in the flow rate, the composition of the mobile phase, and the variation in temperature were done in order to assess the method's robustness.**A.** Flowrates ranged from 0.9 ml/min to 1.1 ml/min in this experiment. Solution that has been widely accepted Method flow rate was used to manufacture 71ppm of Estetrol and 15ppm of Drospirenone for analysis. Based on the data shown above, it's clear that the approach was considerably impacted by the flow rate variability. As a result, even a 10% decrease in flow rate doesn't affect the method's robustness.

B. Organic Phase Ratio Variation. Analysis of a standard solution of 71ppm Estetrol and 15ppm Drospirenone was performed utilising the changed mobile phase ratio.

Table-8 Robustness results of Estetrol by RP-HPLC

Parameter	Estetrol					
	Condition	**Retention time(min)**	**Peak Response**	**Resolution**	**Tailing**	**Plate count**
Flow rate Change (mL/min)	Less flow (0.9ml)	5.268	3091586		1.08	5423
	Actual (1ml)	3.496	2807621		1.05	5369
	More flow (1.1ml)	3.121	2786390		1.11	5311
Organic Phase change	Less Org (63:37)	5.242	3109598		1.16	5398
	Actual (70:30)	3.491	2812861		1.07	5374
	More Org (77:23)	2.542	2536745		1.02	5301

Table-9 Robustness results of Drospirenone by RP-HPLC

Parameter	**Drospirenone**					
	Condition	**Retention time(min)**	**Peak Response**	**Resolution**	**Tailing**	**Plate count**
Flow rate Change (mL/min)	Less flow (0.9ml)	5.978	638468	2.96	1.20	6895
	Actual (1ml)	4.089	612354	3.02	1.18	6870
	More flow (1.1ml)	3.657	596116	2.81	1.12	6822
Organic Phase change	Less Org (63:37)	5.986	651565	2.90	1.17	6921
	More Org (77:23)	3.029	583443	2.64	1.10	6814

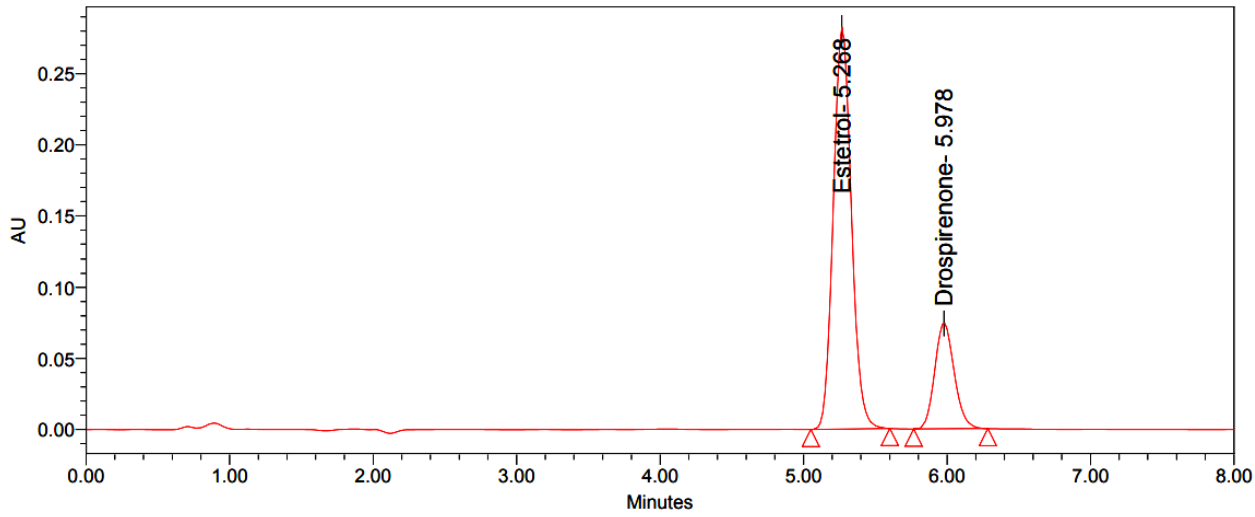


Fig. 14. Less flow rate chromatogram (0.9ml)

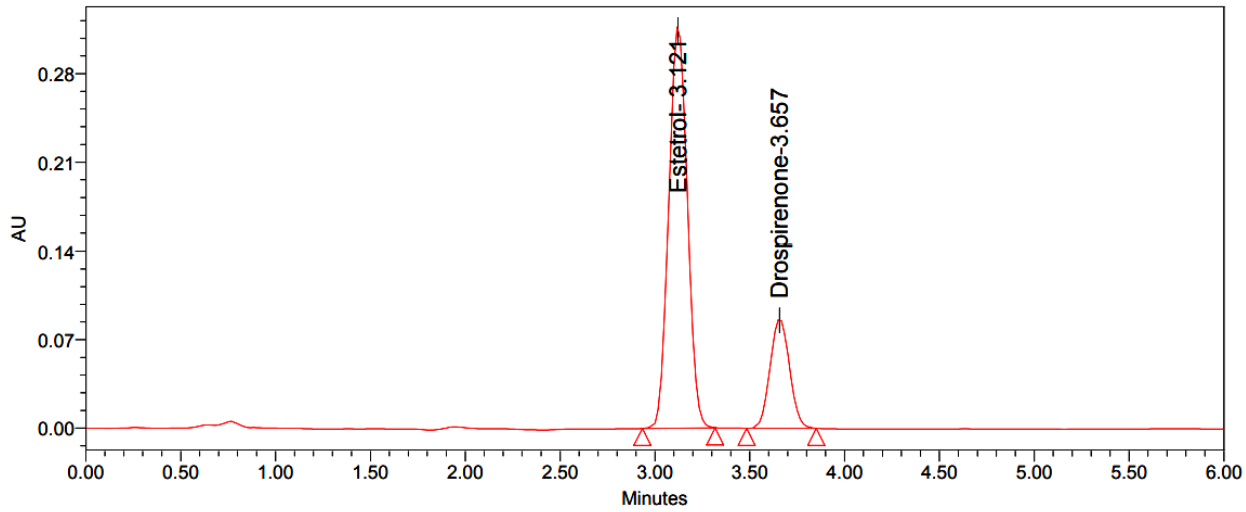


Fig. 15. More flow rate chromatogram (1.1ml)

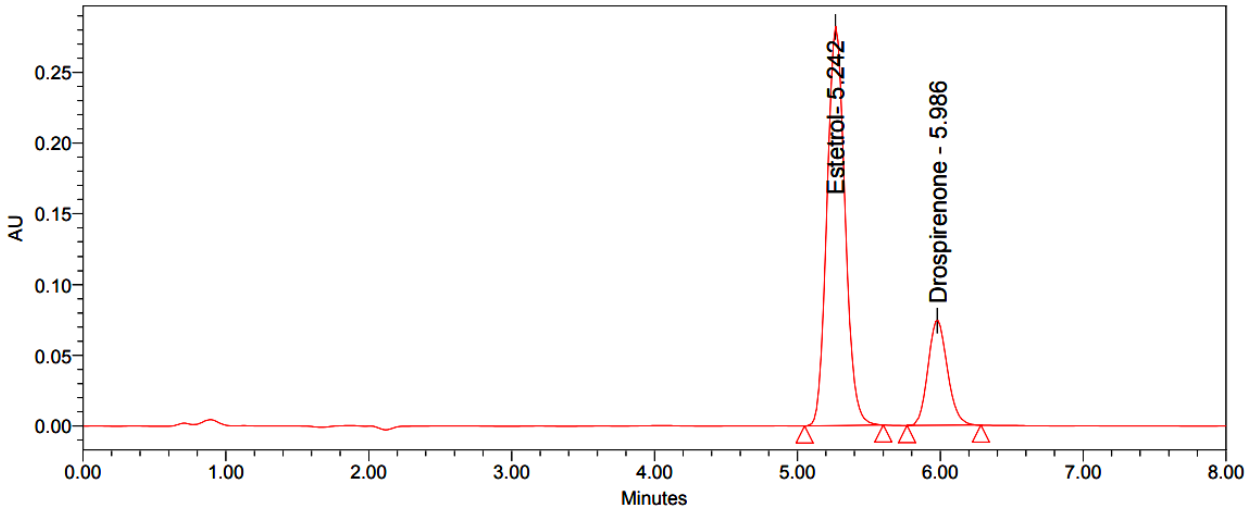


Fig. 16. Less organic phase chromatogram (63:37)

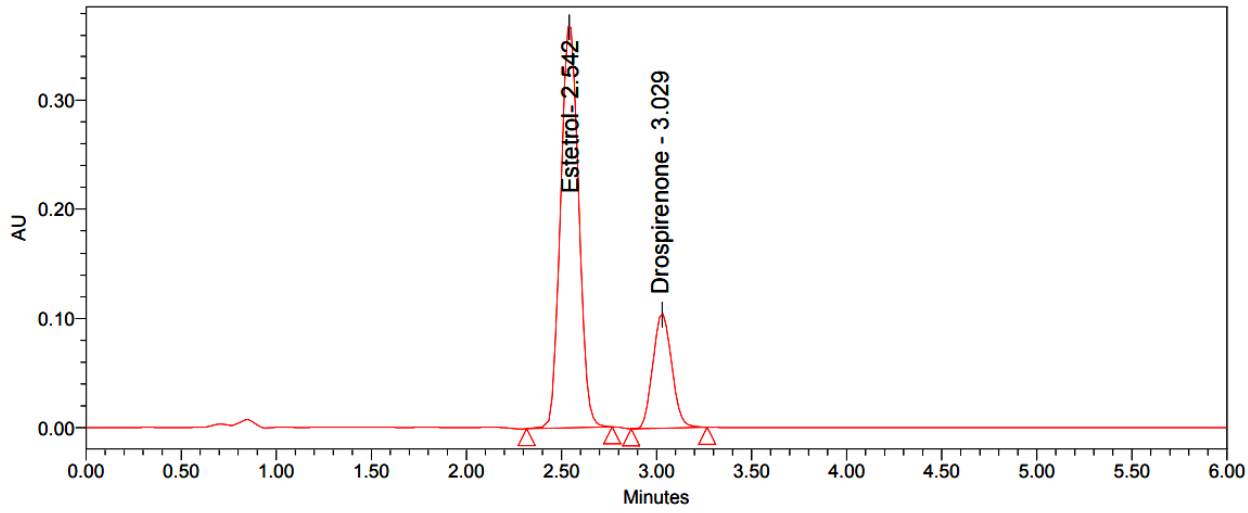


Fig. 17. More organic phase chromatogram (77:23)

CONCLUSION:

The developed method was accurate, precise and reliable for the concurrent analysis of Estetrol and Drospirenone in pharmaceutical methods. This method was validated for linearity, accuracy, precision, robustness, forced degradation of Estetrol and Drospirenone. The RSD values for all parameters were found to be less 2, which indicates the validity of method and results obtained by this method are in fair agreement. Finally this method can be used for better analysis of Estetrol and Drospirenone.

ACKNOWLEDGMENT: The authors are thankful to Dr. M DAVID RAJU for his
encouragement and support to complete this research work.

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