Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 9[9] August 2020 : 71-83

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.876
Universal Impact Factor 0.9804
NAAS Rating 4.95

ORIGINAL ARTICLE **OPEN ACCESS**

Efficient, Cost Effective Analytical Method Validation for Simultaneous Estimation of Related Substances in Ethinyl Estradiol & Drospirenone By HPLC in Combined Dosage Form

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ABSTRACT

A simple, accurate, rapid, precise and gradient High performance liquid chromatographic (HPLC) in-house method was developed and validated for the determination of related substance of Ethinyl estradiol and drospirenone in tablet formulation. The method employs Waters HPLC system on Oyster BDS premium (150mmX4.6mm, 3µm) or equivalent and gradient method with change in flow Rate with respect to time& injection volume 10µL, kept column temperature 30ºC.The detection was carried out at 245 nm for drospirenone &210 nm for Ethinyl estradiol. Retention Time of Ethinyl estradiol and drospirenone were found to be 39 minutes & 46 minutes. This developed method was successfully utilized for the quantitative estimation of Ethinyl estradiol and drospirenone in pharmaceutical dosage forms. Correlation coefficient of ethinylestradiol impurities were found within acceptance criteria (NLT 0.99). Accuracy study were performed for all known impurities spike into sample solution and result found within predefined acceptance criteria. This method was validated for accuracy, precision, linearity and specificity as per ICH guidelines.

Keywords: *Ethinyl Estradiol, Drospirenone, Analytical, method validation, Simultaneous estimation.*

Received 13.04.2020 Revised 18.05.2020 Accepted 11.07.2020

INTRODUCTION

Ethinyl estradiol is also known as ethynyl estradiol (EE) which is a derivative of 17β – estradiol. It is the first orally active semi synthetic steroidal estrogen that is used for the management of menopausal symptoms and female hypogonadism. Ethinyl estradiol is an orally bioactive estrogen used in almost all modern formulations of combined oral contraceptive pills. Chemically it is 19-Nor-17α-pregna-1, 3, 5(10)-trien-20-yne-3, 17-diol [1-2]. Drospirenone is an analogue of the antimineralocorticoid spironolactone that is synthesized from androstenone. Unlike other progestogens, drospirenone, an analogue of spironolactone, has biochemical and pharmacologic profiles similar to endogenous progesterone, especially regarding antimineralocorticoid and antiandrogenic activities. As a combination, oral contraceptive, drospirenone with Ethinyl estradiol, is effective and has positive effects on weight and lipid levels[3-4].So far to our present knowledge, HPLC methods were available in the literature for analyzing Ethinyl estradiol and drospirenone with other drug combinations in pharmaceutical dosage forms[5]. It felt necessary to develop a simple, precise and rapid spectrophotometric method for the quantitative determination of Ethinyl estradiol and drospirenone in combined dosage form. These are hormonal preparation used for reversible suppression of fertility. Because of our alarming population trends, antifertility drugs are the need of the day. In developing countries, particularly, the mortality rate declined and birth rate has increased due to urbanization. In the earlier part of 20th century, methods of contraception used (condoms, diaphragms, spermicidal creams, foam tablets etc.) were intimately related to sexual intercourse, therefore, despised by most couples. These also have higher failure rate. Rock and Pincus announced the successful use of an oral progestin for contraception in 1955. Hormonal contraception refers to birth control methods that act on the endocrine system. Almost all methods are composed of steroid hormones. This type of birth control contains either progestin, estrogen or both. The original hormonal method the combined oral contraceptive pill was first marketed as a contraceptive in 1960[6-7].The validated method is stability-indicating, simple, robust, precise and accurate for the

determination of Drosperinone & ethinylestradiol and degradation impurities in tablet dosage form. The method is capable of separating the peaks due to the degradation products from the main peak. The method was validated as per ICH requirements and thus is useful for routine analysis in quality control laboratories [8-10].

MATERIAL AND METHODS

Apparatus and instruments used in experiment are listed in table 1.

Table: 1 Apparatus / Instruments used

S.No.	Instruments	Make	Software	Detector/ Model No.	Separation Module
1	HPLC	Waters Alliance	Empower 2	UV/2996	2695
2	HPLC	Waters Alliance	Empower 2	PDA/2998	2695
3	Weight balance	Mettler Toledo	NA	ML204	NA
4	Sonicator	Lab India	NA	NA	NA

Working standards: Pharmaceutical grade Ethinyl estradiol and drospirenone working standards, sample & impurities were kindly supplied as a gift sample by Alpa Laboratories Private Limited, Sector 3, Pithampur, Madhya Pradesh, India.

Methodology: Reagents and Solvents: Water (HPLC grade, Milli Q or equivalent), Acetonitrile (HPLC grade, JT Baker or equivalent) Methanol (HPLC grade, JT Baker or equivalent).

Diluent preparation: Prepare a degassed and filtered mixture of water and Acetonitrile (30: 70, v/v).

Mobile phase A: water

Mobile phase B: Acetonitrile: Methanol (60:40 v/v)

Chromatographic conditions:

Column	:	Oyster BDS premium (150mmX4.6mm, 3µm) or equivalent
Column Temperature	:	30°C
Wavelength	:	210 nm and 245 nm
Injection Volume	:	10µL
Retention time of		
a) Ethinyl Estradiol		About 39 minutes
b) Drospirenone	:	About 46 minutes
	:	

Gradient programme:

Time (min.)	Flow rate (mL/min.)	Mobile Phase A (%)	Mobile Phase B (%)
0.0	1.10	72	28
45	1.10	54	46
55	1.10	45	55
65	1.50	05	95
80	1.50	05	95
85	1.10	72	28
95	1.10	72	28

Preparation of Estrone stock Solution: Weigh accurately about 3 mg of Estrone impurity standard and transfer it into a 100-mL volumetric flask. Add about 70 mL of Acetonitrile and sonicate to dissolve. Allow it to come to room temperature, make up to the mark with Acetonitrile and mix. Label this solution as Solution A. (Concentration: 30 µg per mL).

Preparation of Ethinylestradiol Stock Solution: Weigh accurately about 30 mg of Ethinyl Estradiol working standard and transfer it into 50 mL volumetric flask. Add about 30 mL of diluent and sonicate to dissolve. Allow it to come to room temperature, make up to the mark with diluent and mix. Label this solution as Solution B. (Concentration: 600 µg per mL) Further dilute 2.0 mL of the above Solution B to 20 mL with diluent and mix. Label this solution as Solution C. (Concentration: 60 µg per mL).

Preparation of Drospirenone Standard Stock Solution: Weigh accurately about 50 mg of Drospirenone working standard and transfer it into 25 mL volumetric flask. Add about 15 mL of diluent and sonicate to dissolve, allow it to come to room temperature, make up to the mark with diluent and mix. Label this solution as Solution D. (Concentration: 2000 µg per mL).

Preparation of Diluted Standard Solution: Pipette out 1.0 mL of Solution C and 3.0 mL of Solution D into a 100mL volumetric flask, dilute up to the mark with diluent and mix. (Concentration of Drospirenone is 60 µg per mL and Ethinyl Estradiol is 0.6 µg per mL)

Preparation of System suitability Solution: Weigh accurately about 60 mg of Drospirenone working standard and transfer it into a 10mL volumetric flask. Add about 5 mL of diluent and sonicate to dissolve. Allow it to come to room temperature. Add 0.2 mL of solution A, 1.0 mL of solution B and make up to the mark with diluent and mix. (Concentration of Drospirenone is 6000 µg per mL, Ethinyl Estradiol is 60 µg per mL and Estrone is 0.6 µg per mL).

Preparation of sensitivity solution: Dilute 2.0 mL of the above diluted standard solution to 20 mL with diluent and mix. (Concentration of Drospirenone is 6 µg per mL and Ethinyl Estradiol is 0.06 µg per mL).

Preparation of Placebo Solution: Weigh accurately about placebo blend equivalent to 20 tablets or 20 placebo tablets, transfer into a 20mL volumetric flask, add accurately 10.0 mL of diluent with the help of pipette, stopper well and sonicate for 15 minutes at room temperature with intermittent shakings. Allow it to come to room temperature and centrifuge the solution for 3 minutes at about 3000 rpm. Filter the supernatant solution through 0.45µm nylon membrane filter, discard first 2 mL of the filtrate and inject.

Preparation of Sample Solution: Weigh accurately about 20 tablets, transfer into a 20mL volumetric flask, add accurately 10.0 mL of diluent with the help of pipette, stopper well and sonicate for 15 minutes at room temperature with intermittent shakings. Allow it to come to room temperature and centrifuge the solution for 3 minutes at about 3000 rpm. Filter the supernatant solution through 0.45µm nylon membrane filter, discard first 2 mL of the filtrate and inject.

Note: 1**)** Do not make up the Sample solutions to the volume.

2) Sonicator temperature should not exceed 25°C

Procedure: Inject the specified volume of Diluent, Sensitivity solution, System suitability solution, Diluted standard solution and Sample solution into the chromatograph as mentioned in the Injection sequence table and record the chromatograms. Disregard peaks due to diluent and placebo solution**.** Disregard the impurity peaks below LOQ level for both Drospirenone and Ethinyl Estradiol. Calculate all known impurities of Ethinyl Estradiol against Ethinyl Estradiol diluted standard area at 210 nm. Calculate all unknown impurities due to Drospirenone at 245 nm and Ethinyl Estradiol at 210 nm against corresponding diluted standards areas at corresponding wave lengths. Calculate any other impurity at 245 nm against Drospirenone diluted standard area at 245 nm and calculate any other impurity at 210 nm against Ethinyl Estradiol diluted standard area at 210 nm. Any other impurity showing response at both wavelength 210 nm and 245 nm will be calculated at wavelength where it shows maximum area response against Drospirenone diluted standard area at respective wavelength.

RRT, RF and LOQ values for known impurities of Ethinyl Estradiol:

Name of the Impurity	RRT w.r.t Ethinyl Estradiol	RF
6-alpha Hydroxy Ethinyl Estradiol	about 0.26	0.94
6-beta Hydroxy Ethinyl Estradiol	about 0.35	1.35
6-keto-Ethinyl Estradiol	about 0.50	1.09
Δ9,11-Ethinyl Estradiol	about 0.92	0.88
Δ6-Ethinyl Estradiol	about 0.94	0.84
Ethinyl Estradiol	about 1.00	1.00
Estrone#	about 0.96	1.01
17β Ethinyl Estradiol#	about 1.15	1.02
Drospirenone	NA	NA

These are process impurities and not part of finished product specification. But these impurities to be considered for calculation of total impurities.

Evaluation of System suitability:

Sensitivity solution: The area ratio of sensitivity solution to diluted standard solution should be 0.08 to 0.12 for Drospirenone at 245 nm and 210 nm and for Ethinyl Estradiol at 210 nm.

System suitability solution: Resolution between Estrone peak and Ethinyl Estradiol should not be less than 2.0 and tailing factor for Drospirenone at 245 nm and 210 nm should not be more than 3.0 and for Ethinyl Estradiol at 210 nm should not be more than 2.0.

Diluted standard solution: The relative standard deviation of six replicate injections for Drospirenone at 245 nm and 210 nm and for Ethinyl Estradiol at 210 nm should not be more than 5.0%.

RESULTS AND DISCUSSION

Specificity: Blank (diluent), sensitivity solution, system suitability solution, placebo solution, diluted standard solution, all known impurity solutions individually, sample solution and sample solution spiked with all known impurities at specification level were prepared and injected into the HPLC equipped with a photodiode array detector and analyzed as per above mentioned methodology. The retention time of the peak of Drospirenone and Ethinyl Estradiol in sample solution was obtained at 43.86 min. and 36.86 min. respectively. The retention time of the peak of Drospirenone and Ethinyl Estradiol in diluted standard solution was obtained at 44.51 min. and 36.83 min. respectively. The retention time of all the known impurities spiked in sample solution were found to be comparable with those injected individually. Results are tabulated in Table 2.

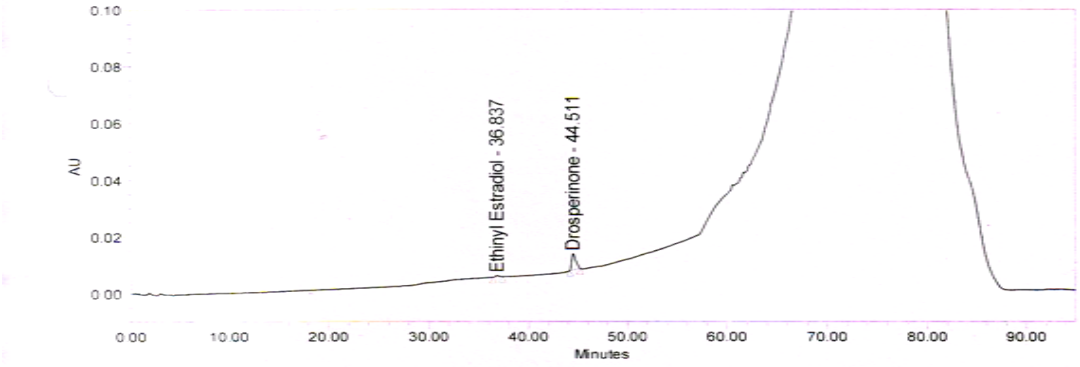
Table 2: Individual Identification

Impurities/Active	Individual Injection	Spiked Sample	
	RT (min)	RT (min)	RRT
Drospirenone and its impurities			
Ethinyl Estradiol and its impurities			
6α Hydroxy Ethinyl Estradiol	9.606	9.637	0.25
6β Hydroxy Ethinyl Estradiol	13.009	12.973	0.35
6 Keto Ethinyl Estradiol	18.379	18.326	0.49
Δ9,11Ethinyl Estradiol	34.029	33.866	0.92
Ethinyl Estradiol	36.878	36.872	1.00
Δ6 Ethinyl Estradiol	34.991	34.952	0.95

Interference Study: Blank (diluent), sensitivity solution, system suitability solution, placebo solution, diluted standard solution, all known impurity solutions individually, sample solution and sample solution spiked with all known impurities at specification level were prepared and injected into the HPLC equipped with a photodiode array detector and analyzed as per methodology. Blank (diluent) and placebo solution I did not show interference at the retention time of Drospirenone, Ethinyl Estradiol and their impurities. Peak purity of Drospirenone, Ethinyl Estradiol and their impurities in spiked sample solution passed. Results are tabulated in table 3 and figure 1 to figure 4.

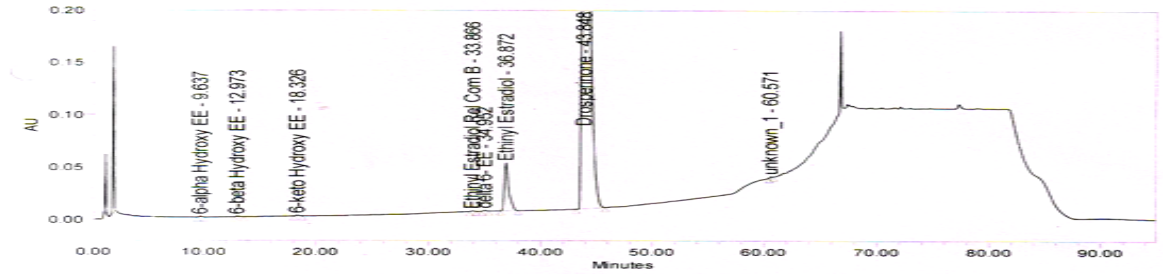
Table 3: Interference Study

Impurities/Active	Peak Purity			
	Individual Injection		Spiked Sample	
	PA	PT	PA	PT
Ethinyl Estradiol and its impurities				
6α Hydroxy Ethinyl Estradiol	21.371	31.540	20.106	28.100
6β Hydroxy Ethinyl Estradiol	24.418	37.252	23.580	33.402
6 Keto Ethinyl Estradiol	14.402	21.467	10.684	13.203
Δ9,11Ethinyl Estradiol	17.747	24.529	10.313	14.211
Δ6 Ethinyl Estradiol	14.689	22.497	11.779	15.717
Ethinyl Estradiol	32.784	53.026	0.437	0.697



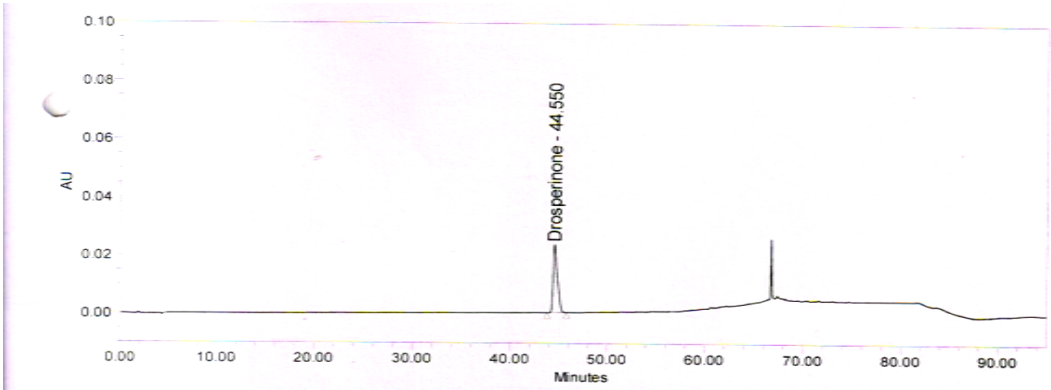
	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	Purity1 Angle	Purity1 Threshold
1	Ethinyl Estradiol	36.837	14505	8.10	62796	1.23	23.867	34.588
2	Drosperinone	44.511	164478	91.90	50633	1.60	0.110	0.308

Figure 1: chromatogram of Standard at 210nm



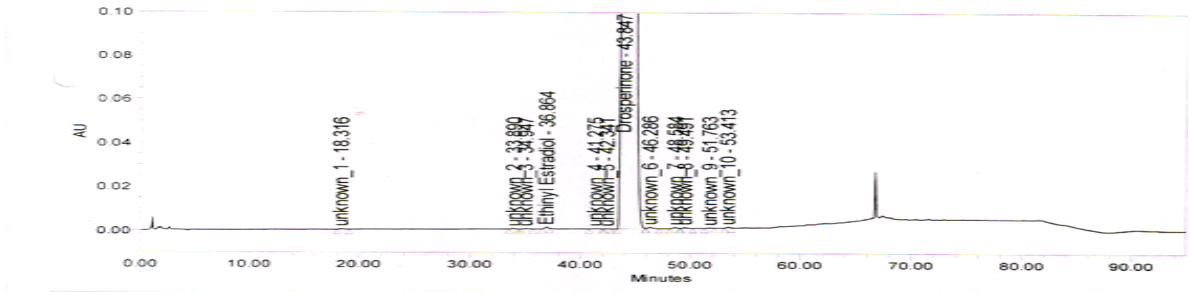
	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	Purity1 Angle	Purity1 Threshold
1	6-alpha Hydroxy EE	9.637	11953	0.07	8923	1.36	20.106	28.100
2	6-beta Hydroxy EE	12.973	11618	0.07	10679	1.85	23.580	33.402
3	6-keto Hydroxy EE	18.326	15505	0.09	22480	1.36	10.684	13.203
4	Ethinyl Estradiol Rel Com B	33.866	8596	0.05	50671	1.36	10.313	14.211
5	delta 6- EE	34.952	13273	0.08	42776	1.17	11.779	15.717
6	Ethinyl Estradiol	36.872	1235104	7.00	53085	1.67	0.437	0.697
7	Drosperinone	43.848	16335383	92.58	16873	2.16	1.961	3.037
8	unknown_1	60.571	13765	0.08	375421	1.28	10.188	10.806

Figure 2: Chromatogram of Sample Spiked at 210nm



	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	Purity1 Angle	Purity1 Threshold
1	Drosperinone	44.550	718895	100.00	43447	1.68	0.145	0.341

Figure 3: Chromatogram of Diluted Std at 245nm



	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	Purity1 Angle	Purity1 Threshold
1	unknown_1	18.316	6745	0.01	26426	1.50	11.988	15.576
	unknown_2	33.890	4513	0.01	63646	1.08	10.456	13.796
3	unknown_3	34.947	5275	0.01	47826	1.77	12.772	16.825
4	Ethinyl Estradiol	36.864	13364	0.02	77052	1.03	0.379	0.649
5	unknown_4	41.275	3955	0.01	51817	0.99	14.286	18.760
6	unknown_5	42.341	1359	0.00	118681	1.35	26.082	36.065
7	Drosperinone	43.847	66834163	99.89	16749	2.18	1.959	3.040
8	unknown_6	46.286	12035	0.02	79959	1.79	4.387	5.482
9	unknown_7	48.584	8994	0.01	93840	1.01	16.898	20.474
10	unknown_8	49.491	8705	0.01	103930	1.79	17.292	19.452

Figure 4: Chromatogram of Sample Spiked at 245nm

Force Degradation: The peak purity of Drospirenone and Ethinyl Estradiol and their impurities in degraded samples passed. The forced degradation data of Drospirenone, Ethinyl Estradiol and their impurities is tabulated in Table 4-5.

Table 4: Forced degradation data of Drospirenone

Sr.No	Condition		% Degradation	Peak Purity	
				PA	PT
1	Control Sample		NA	0.145	0.341
2	Acid degradation	1.0 mL of 0.1M Methanolic HCl; sonication for 60 minutes	29.1	0.150	0.448
3	Alkali degradation	1.0 mL of 0.1M Methanolic NaOH; sonication for 60 minutes	21.9	0.160	0.507
4	Peroxide degradation	1.0 mL of 30% H_2O_2; kept on bench for 24hours	15.6	0.422	0.762
5	Photolytic	Exposed the sample for 1.2 million lux.	6.0	1.18	3.250
6	Thermal degradation	Exposed the sample at 102°C for 24 Hrs.	3.1	1.18	3.466
7	Humidity degradation	Exposed the sample at 40°C/75 % RH for 7 days.	0.0	1.18	3.510

Table 5: Forced degradation data of Ethinyl Estradiol

Sr. No.	Condition		% Degradation	Peak Purity	
				PA	PT
1	Control Sample		NA	23.867	34.588
2	Acid degradation	1.0 mL of 0.1M Methanolic HCl; sonication for 60 minutes.	11.1	0.215	0.513
4	Alkali degradation	1.0 mL of 0.1 M Methanolic NaOH; sonication for 60 minutes	11.0	0.240	0.549
5	Peroxide degradation	1.0 mL of 30% H_2O_2; kept on bench for 24	14.3	0.323	0.799
6	Photolytic degradation	Exposed the sample for 1.2 million lux.	5.42	0.423	0.931
7	Thermal degradation	Exposed the sample at 102°C for 24 Hrs.	6.72	0.441	0.930
8	Humidity degradation	Exposed the sample at 40°C/75 % RH for 7	0.00	0.507	1.045

Precision: % RSD (n=6) for peak area counts of Drospirenone and Ethinyl Estradiol and Ethinyl Estradiol Related compound B from six replicate injections of diluted standard solution was found within acceptance criteria.

Method precision (Repeatability): Six sample solutions of Drospirenone and Ethinyl Estradiol Tablets (2.5 mg/0.012 mg) were prepared by spiking the known impurities at the specification level and analysed as per methodology.

Intermediate Precision (Ruggedness):% RSD (n=6) for % of known impurities in six sample solutions and overall % RSD (n=12) for % of known impurities from Method Precision and Intermediate Precision results was found within acceptance criteria. Results are tabulated in table 6 to 8.

Table 6: System Precision

Injection	Peak Area counts	
	Drospirenone	Ethinyl Estradiol
1	161669	12154
2	161606	12278
3	160473	12325
4	161443	12627
5	161510	12164
6	161213	12348
Mean	161319	12316
SD	443.50	172.40
% RSD	0.30	1.40

Table 7: Cumulative results of Method and Intermediate Precision (Dros & EE impurities)

	Single max Impurity	
	M.P.	I.P.
	(%w/w)	(%w/w)
1	0.152	0.153
2	0.149	0.149
3	0.152	0.153
4	0.152	0.149
5	0.149	0.153
6	0.152	0.149
Overall Mean (n=12)	0.151	
SD (n=12)	0.0017	
%RSD (n=12)	1.15	

Table 8: Cumulative results of Method and Intermediate Precision (Ethinyl Estradiol impurities w/w %)

	6α Hydroxy EE		6β Hydroxy EE		6 Keto EE		Δ9,11 EE		Δ6 EE	
	M.P.	I.P.	M.P.	I.P.	M.P.	I.P.	M.P.	I.P.	M.P.	I.P.
1	0.88	0.90	1.1	1.0	1.10	1.0	1.10	1.1	1.3	1.33
2	0.89	0.90	1.1	1.1	1.00	1.0	1.10	1.1	1.3	1.32
3	0.87	0.90	1.1	1.0	1.00	1.0	1.10	1.1	1.3	1.30
4	0.95	0.90	1.1	1.1	1.00	1.0	1.00	1.1	1.2	1.23
5	0.88	0.90	1.0	1.1	1.00	1.0	1.00	1.0	1.3	1.22
6	0.91	0.90	1.1	1.0	1.00	1.0	1.00	1.0	1.2	1.24
Overall Mean (n=12)	0.90		1.1		1.1		1.0		1.0	1.5
SD (n=12)	0.02		0.05		0.05		0.03		0.03	0.05
%RSD (n=12)	2.20		4.70		4.70		3.00		3.00	3.80

Limit of Detection (LOD) and Quantitation (LOQ): Limit of Quantitation (LOQ) was established by injecting six replicates of a solution containing Drospirenone, Ethinyl Estradiol and their known impurities having a concentration which is less than the reporting threshold (0.1%). Limit of Detection (LOD) was established by quantitatively diluting and injecting six replicates of the LOQ solution to obtain

the % RSD (n=6) not more than 30.0. % RSD (n=6) of peak area counts of Drospirenone, Ethinyl Estradiol and their known impurities in predicted LOD and LOQ solutions was found within acceptance criteria. The results are tabulated in Table 9-11.

Table 9: LOD & LOQ for Drospirenone

	Peak Area Counts	
	LOD	LOQ
	0.03% w/w	0.09% w/w
1	1736	5255
2	1726	5289
3	1722	5080
4	1713	5214
5	1744	5316
6	1728	5154
Mean	1728	5218
SD	10.80	88.50
%RSD	0.60	1.70

Table 10: Limit of Detection for Ethinyl Estradiol impurities

	Peak Area Counts (0.03% w/w each)					
	6α Hydroxy EE	6β Hydroxy EE	6 Keto EE	Δ9,11 EE	EE	Δ6 EE
1	383	394	393	402	413	428
2	382	373	415	391	429	406
3	388	374	415	359	428	422
4	369	379	409	364	402	419
5	381	382	415	364	419	405
6	354	393	388	398	436	420
Mean	376	383	406	380	421	417
SD	12.5	9.1	12.2	19.4	12.4	9.2
%RSD	3.30	2.40	3.00	5.10	2.90	2.20

Table 11: Limit of Quantitation for Ethinyl Estradiol impurities

	Peak Area Counts (0.09% w/w each)					
	6α Hydroxy EE	6β Hydroxy EE	6 Keto EE	Δ9,11 EE	Δ6 EE	EE
1	1114	1154	1239	1222	1230	1187
2	1151	1127	1262	1211	1233	1115
3	1139	1169	1241	1241	1241	1164
4	1186	1064	1267	1289	1260	1191
5	1168	1158	1279	1210	1254	1149
6	1143	1266	1200	1253	1262	1145
Mean	1150	1156	1248	1238	1247	1159
SD	24.8	65.7	28.1	30.3	13.9	28.5
%RSD	2.20	5.70	2.30	2.40	1.10	2.50

Linearity: A series of solutions were prepared by quantitatively diluting the working standard stock solution of Drospirenone, Ethinyl Estradiol and their known impurities to obtain solutions at the level of about Limit of Quantitation (LOQ) to 200 % (i.e. at LOQ, 50 %, 80 %, 100 %, 120 %, 150 %, 200% and 250%) of the working concentration of Drospirenone and Ethinyl Estradiol in diluted standard and for

their known impurities with respect to specification limit. Each solution was injected in duplicate and mean area was calculated. The correlation coefficient of Drospirenone, Ethinyl Estradiol and their impurities was found within acceptance criteria. Linearity data reported in table 12-13 and Linearity graph reported in figure 5 to figure 10 .

Table 12: Linearity of Ethinyl Estradiol and their impurities

	6α Hydroxy EE		6β Hydroxy EE		6 keto EE	
Linearity Level	Concentration (µg/mL)	Mean Area	Concentration (µg/mL)	Mean Area	Concentration (µg/mL)	Mean Area
LOQ	0.0513	1133	0.0547	1141	0.0540	1251
50 %	0.2875	5855	0.3041	4677	0.3000	6567
80 %	0.4560	9130	0.4866	7468	0.4800	9520
100 %	0.5700	12267	0.6082	9297	0.6000	11319
150 %	0.8550	16554	0.9123	13916	0.9000	17686
200 %	1.1400	23756	1.2164	18051	1.2000	22180
Slope		20414.462	14689.033		18281.510	
Intercept		17.749	321.579		652.691	
RF		0.91	1.27		1.02	
Correlation Coefficient		0.9977	0.9998		0.9984	

Table 13: Linearity of Ethinyl Estradiol and their impurities

	Δ9,11 EE		Δ6 Ethinyl Estradiol		Ethinyl Estradiol	
Linearity Level	Concentration (µg/mL)	Mean Area	Concentration (µg/mL)	Mean Area	Concentration (µg/mL)	Mean Area
LOQ	0.0512	1217	0.0522	1232	0.0535	1151
50 %	0.2845	7503	0.2901	6959	0.2973	5671
80 %	0.4552	10285	0.4642	10060	0.4757	9728
100 %	0.5690	12480	0.5802	14085	0.5946	11425
150 %	0.8535	19525	0.8703	20968	0.8919	16460
200 %	1.1380	24592	1.1604	28086	1.1892	22564
Slope		21410.053	24365.680		18618.354	
Intercept		641.391	-312.879		298.967	
RF		0.87	0.76		1.00	
Correlation Coefficient		0.9978	0.9989		0.9990	

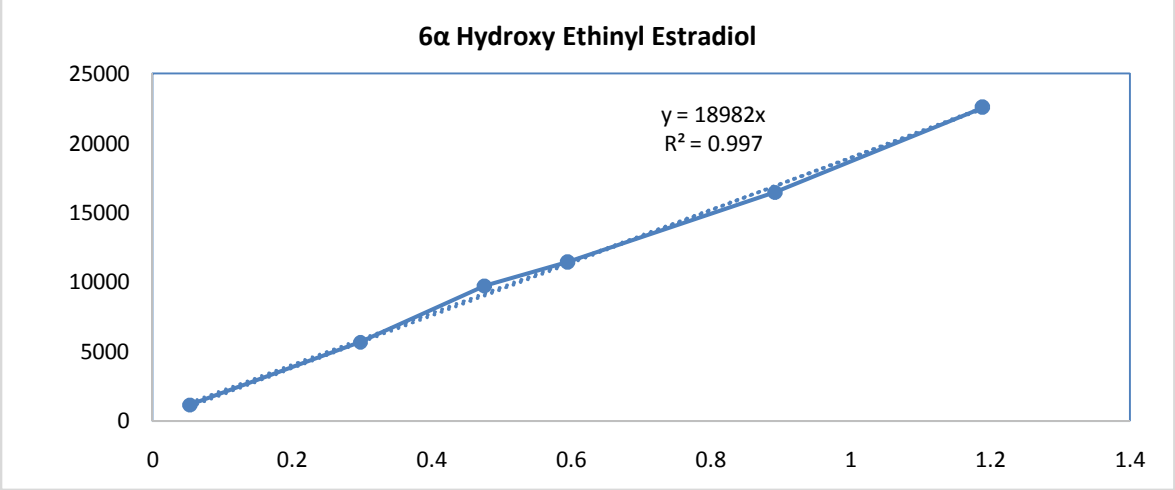


Figure 5: Linearity Graph of 6α Hydroxy Ethinyl Estradiol

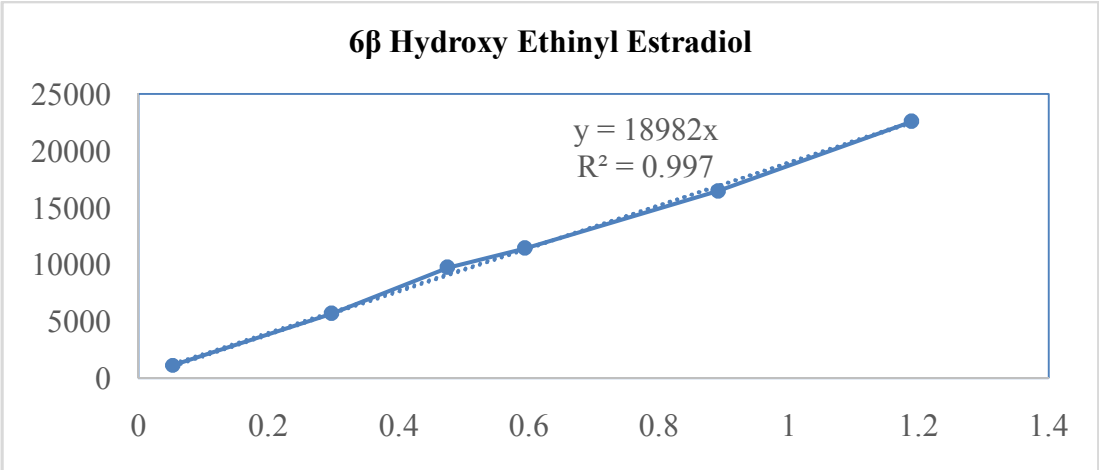


Figure 6: Linearity Graph of 6β Hydroxy Ethinyl Estradiol

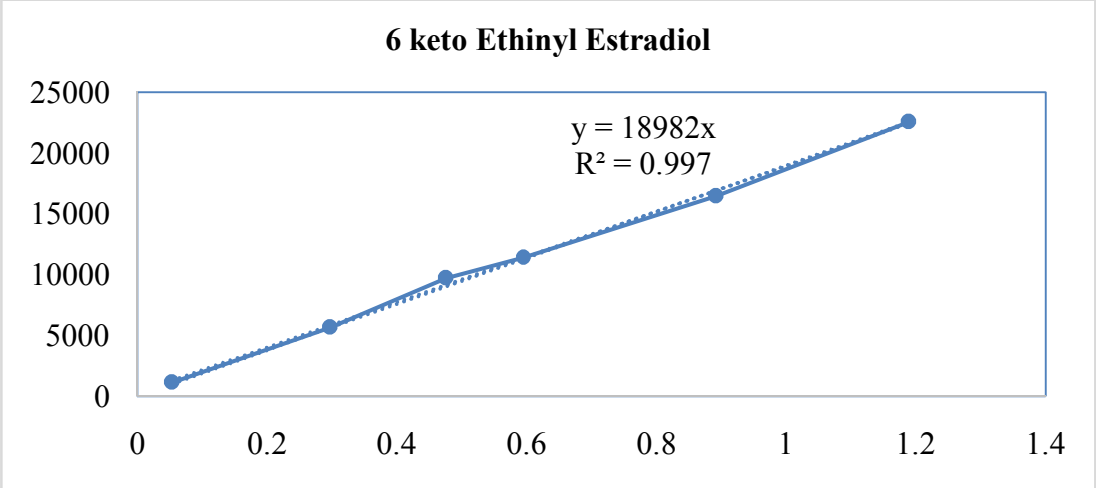


Figure 7: Linearity Graph of 6 keto Ethinyl Estradiol

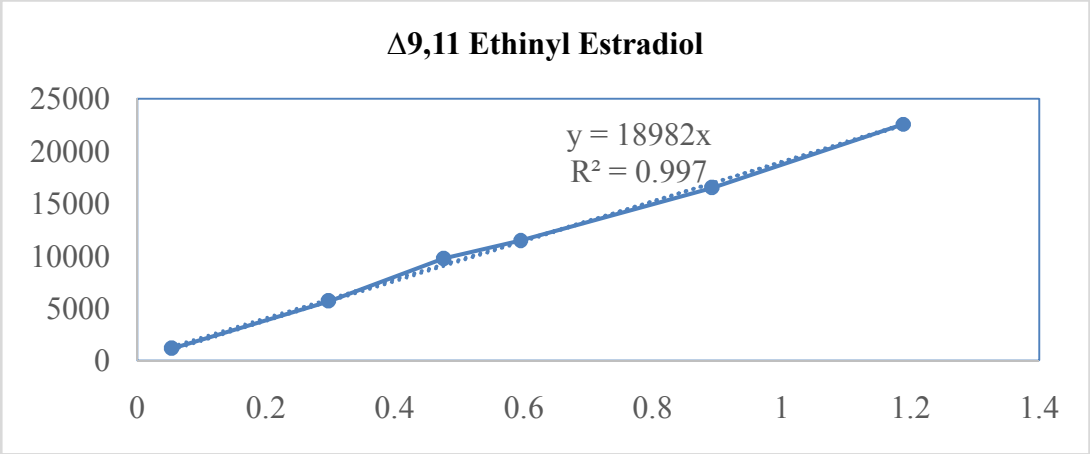


Figure 8: Linearity Graph of Δ9,11 Ethinyl Estradiol

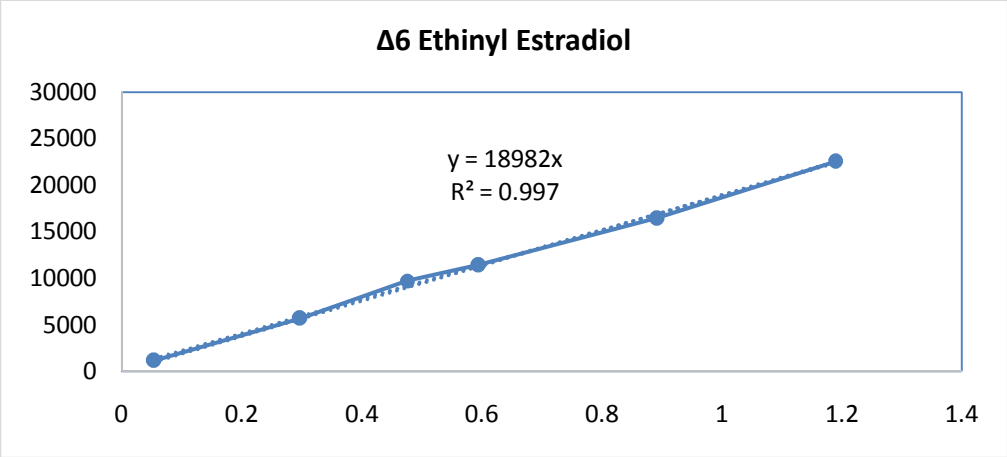


Figure 9: Linearity Graph of Δ6 Ethinyl Estradiol

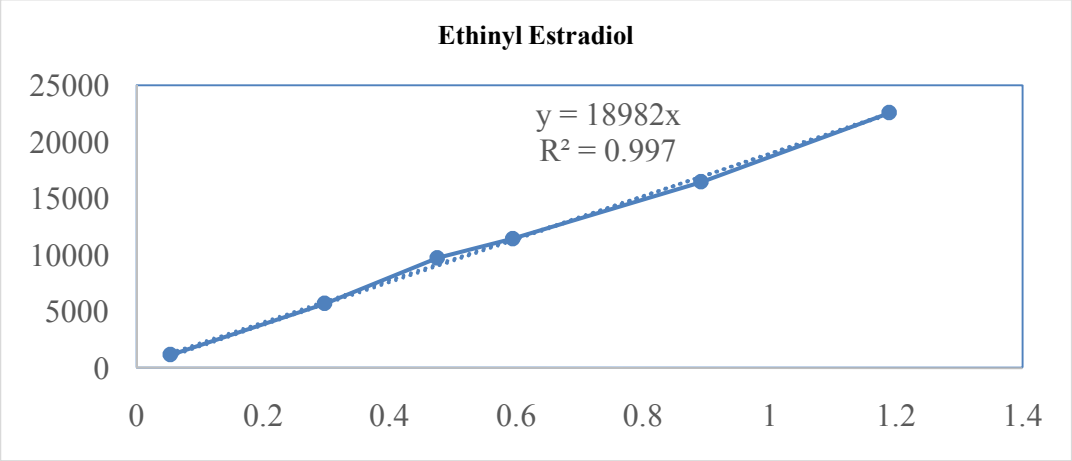


Figure 10: Linearity Graph of Ethinyl Estradiol

Accuracy: Accuracy stock solution of all known impurities of Drospirenone and Ethinyl Estradiol was prepared and spiked into sample solution (triplicate preparation) to achieve the concentration of impurities in the range of about LOQ, 50 %, 100 %, 150 % of the working concentration of Drospirenone and Ethinyl Estradiol in diluted standard and for its known impurities with respect to specification limit and injected into the chromatograph. For all levels individual and mean recovery was found within acceptance criteria. Overall % RSD (n=9) of % recovery at all levels (except LOQ) was found within acceptance criteria. Results are reported in table 14.

Table 14: % Recovery of Ethinyl estradiol Impurities

Level	6α Hydroxy EE	6β Hydroxy EE	6 keto EE	Δ9,11 EE	Δ6 EE
LOQ	118.8	112.1	106.5	108.2	109.8
	117.4	102.2	106.5	108.2	108.7
	116.3	105.5	101.1	108.2	108.7
50 %	108.3	102.0	111.8	87.0	96.1
	110.4	103.9	109.6	87.0	98.0
	110.6	102.0	111.8	87.0	98.0
100 %	104.2	104.0	104.9	83.3	97.1
	107.4	105.0	104.9	83.3	98.0
	105.3	104.9	104.9	83.3	94.1
150 %	107.1	106.7	106.7	81.3	100.0
	107.1	106.7	106.7	81.3	100.0
	107.1	106.7	106.7	75.0	100.0
Overall Mean	**110.0**	**105.1**	**106.8**	**89.4**	**100.7**
Overall SD	**4.9**	**2.81**	**3.04**	**11.8**	**5.32**
Overall % RSD	**4.5**	**2.7**	**2.8**	**13.2**	**5.3**

Stability of Analytical Solution: One sample solution (unspiked) was prepared as per methodology and other by spiking known impurities at specification levels. Injected diluted standard solution, sample solution (unspiked) and spiked sample solution initially and at different time intervals upto 48 hours by storing the sample solutions at 25°C. For each sample solution % w/w of known and total impurities were calculated and compared with the initial results. Absolute value of % difference between % w/w of each known impurity in initial sample and sample injected at each time interval with respect to initial value should be NMT 20.0 (for impurities below 1.0%) and NMT 10.0 (for impurities above 1.0%). Spiked sample solution was found stable up to 48 hours at room temperature.

CONCLUSION

The related substance method of analysis for Drospirenone and Ethinyl Estradiol Tablets (3.0mg/0.03mg) was validated for Specificity, LOD/LOQ Precision, Intermediate Precision (Ruggedness), Linearity, Accuracy and Stability of Analytical Solution. The method meets the acceptance criteria for all parameters. The validated method is Specific, Linear, Accurate, Precise, and Rugged for determination of related substances of Drospirenone and Ethinyl Estradiol in Drospirenone and Ethinyl Estradiol Tablets 3.0 mg/0.03 mg. Hence, this method can be used for routine analysis for the determination of related substances Drospirenone and Ethinyl Estradiol in Drospirenone and Ethinyl Estradiol Tablets 3.0 mg/0.03 mg.

ACKNOWLEDGEMENT

Authors are thankful to department of science, Sri Satya Sai University of technology and medical science, sehore and Laurus laboratories Ltd for providing the necessary facilities & guidance to carry out this research.

LIST OF ABBREVIATIONS

% w/w	Percentage weight by weight
w.r.t	With respect to
EE	Ethinylestradiol
Dil Std	Diluted Standard
RT	Retention time
RRT	Relative Retention time
SD	Standard Deviation
RSD	Relative Standard Deviation
NLT	Not Less Than
NMT	Not More Than
PPM	parts per million
PA	Purity Angle
PT	Purity Threshold
MP	Method precision
IP	Intermediate precision

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CITATION OF THIS ARTICLE

A Dash, N Jain. Efficient, Cost Effective Analytical Method Validation for Simultaneous Estimation of Related Substances in Ethinyl Estradiol & Drospirenone By HPLC in Combined Dosage Form. Bull. Env. Pharmacol. Life Sci., Vol 9[9] August 2020 : 71-83