ORIGINAL ARTICLE

Developing and Validating a Stability-Indicating Ultra Performance Liquid Chromatography Method for the Concurrent Measurement of Estradiol and Dydrogesterone in Bulk and Tablet Doses

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Abstract

Purpose: A novel technique for quantitatively measuring estradiol and dydrogesterone (DYD) in pharmaceutical dose form is ultra-performance liquid chromatography which is easy, fast, accurate, sensitive, and repeatable. **Materials and Methods:** Using an acquity BEH (50 × 2.1 mm, 1.7 µm) column and a mobile phase comprising 45:55% v/v of NaH_2PO_4 and acetonitrile, chromatographic separation of estradiol and DYD was accomplished using a waters acquity high-performance liquid chromatographic system. At room temperature, a photodiode array detector was used for absorption at 260 nm to detect the 0.3 mL/min flow rate. **Results:** The results of a forced degradation investigation indicate that while less degradation was shown under hydrolysis (0.705%) and photolytic (0.927%) degradation conditions, considerable degradation was recorded under peroxide conditions (14.9%). The retention times for estradiol and testosterone were found to be 1.017 and 1.928 min, respectively. The linear calibration curves of (R_2 = 0.99985 and R^2 = 0.99972) were observed for the ranges of estradiol (2.5–15 g/mL) and testosterone (25–150 g/mL). The limits of detection and quantification were determined to be 0.03 µg/mL and 0.1 µg/mL, and 0.3 µg/mL and 1 µg/mL, respectively. It was discovered that all of the analytical validation parameters, including accuracy, linearity, and specificity, had percent relative standard deviation values <1%. The range of 101.0–99.2 was determined to be the recovery. When the purity angle is smaller than the purity threshold, the Empower software shows that the peak is homogeneous, as indicated by the purity flag "No." **Conclusion:** In compliance with International Council for Harmonisation principles, the suggested approach was verified. It was discovered that the technique was easy to use, affordable, precise, accurate, and robust for studying the stability of estradiol and DYD as well as for quantitative analysis.

Key words: Dydrogesterone, empower software, estradiol, purity flag and ultra-performance liquid chromatography

INTRODUCTION

Dydrogesterone (DYD) was created in the 1950s and became available for use in medicine in 196.[1] It is sold in Australia and other countries as well as being readily accessible throughout Europe, particularly the United Kingdom.[1,2]

DYD

DYD is a progestin drug that is marketed under several names, including Duphaston.[3] It is used for several conditions, such as endometriosis, dysmenorrhea, irregular cycles, premenstrual syndrome, dysfunctional bleeding, infertility resulting from luteal insufficiency, and recurrent or threatened miscarriages during pregnancy.[4] It is consumed orally.[4]

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Breast pain, headaches, nausea, and irregular menstruation are among the side effects of estrogen.[5,6] The biological target of progestogens, such as progesterone is the progesterone receptor, which is an agonist of Dydrogesterone as it is a progestin or synthetic progestogen.[4,7] Due to its unusual progestogen nature, the drug does not prevent ovulation.[4,8] There is no further significant hormonal action and only little antimineralocorticoid activity.[4,7]

Estradiol (E_2), the primary female sex hormone is estradiol (E_2), sometimes written as estradiol. It is an estrogen steroid hormone. Estrous and menstrual periods, two aspects of the female reproductive cycle that it regulates, are affected. The development of secondary sexual features in women, including breasts, hip enlargement, and a female pattern of fat distribution, is attributed to estradiol. In addition, during adolescence, maturity, and pregnancy, it plays a crucial role in the growth and maintenance of female reproductive tissues, including the uterus, vagina, and mammary glands.[9] In addition, it has significant effects on several other tissues, such as the skin, liver, bone, fat, and brain.[10]

Estradiol/DYD

Estradiol/DYD (E_2/DYD) [Figure 1] is a combination of estrogen (E_2) and progestin (DYD) that is marketed under the brand name Femoston, among other names. It is specifically used in menopausal hormone therapy to treat and prevent osteoporosis and hot flashes in post-menopausal women.[11-13] Each tablet, which is taken orally, includes 0.5, 1, or 2 mg E_2 and 2.5, 5, 10, or 20 mg DYD.[14-16] Worldwide, the drug is extensively marketed.[17] Neither Canada nor the United States may purchase it.[17] Combining two hormonal medications, estradiol/DYD, as part of hormone replacement therapy is known as estradiol + testosterone. By replenishing the body's declining estrogen levels, estradiol helps lessen the uncomfortable symptoms associated with menopause. However, it could impact the endometrium, the lining that lines the womb, and raise the risk of endometrial cancer. To counteract the detrimental effects of estradiol on the lining of the womb and lower the risk of cancer, DYD is given.

Literature review

A literature review using the reversed-phase high-performance liquid chromatography (HPLC) technique revealed findings about the quantification of estradiol valerate in pharmaceutical formulations and bulk.[10] However, there was no reporting of the combination medications (DYD and estradiol). The findings of the suggested approach show that the developed method may be applied to the measurement of bulk and pharmaceutical formulation estradiol. This standard procedure is appropriate for use in industry quality control testing.

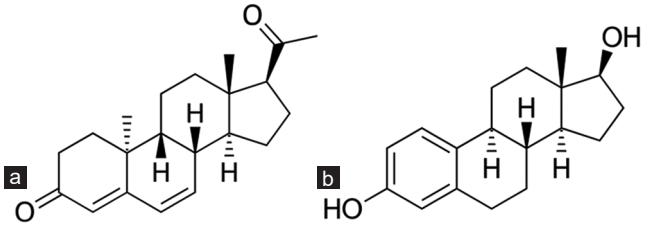

Figure 1: (a) Dydrogesterone and (b) estradiol

MATERIALS AND METHODS

Materials

Merck Chemicals, located in Mumbai, provided analytical grade chemicals and HPLC quality solvents. SJS Pharmaceuticals Lab in Hyderabad provided the standards for any possible contaminants in any of the chosen medications. HPLC analyzes and MilliQ water were utilized.

Instruments

Weighing and measuring the pH of the samples were done using digital balances (DENVER brand, SI234 model; Shimadzu AUX-220) and digital pH meters (Elico LI-120).

Ultra performance liquid chromatography (UPLC)

Estradiol and DYD were estimated using an Agilent1290 Infinity II LC System (Pump: Quaternary; Software: Empower 2.0) equipped with a photodiode array (PDA) detector.

Preparation of standard solution

Accurately weigh and transfer 10 mg of DYD and 5 mg of estradiol into a 10 mL dry volumetric flask. To completely dissolve the sample and adjust the volume, sonication, and diluent are utilized. After pipetting in 2 mL of the estradiol solution, fill a 10 mL volumetric flask with the diluent (stock solution).

One milliliter of the aforementioned stock solutions should be diluted with diluent to the proper amount in a 10-mL volumetric flask. Finally, repeat the procedure. (10 ppm of estradiol and 100 ppm of DYD).

Sample solution preparation

Following exact weighing and transfer of 7.075 mg of estradiol and DYD sample into a 10 mL dry volumetric flask, add diluent, sonicate for up to 30 min to completely dissolve it, and then centrifuge for a further 30 min using the same solvent to bring the volume up to par. It next goes through an injection filter (stock solution) with pore sizes of 0.45 µ. Then, pipette 1 mL of the previously specified stock solutions into a volumetric flask that holds 10-mL, and dilute

it to the necessary amount using diluent. (10 ppm of estradiol and 100 ppm of DYD).

Preparation of buffer (NaH_2PO_4) solution

1.19 g of sodium dihydrogen phosphate are dissolved in one liter of HPLC water. To filter, pass through a 0.45 µ nylon filter.

Preparation of mobile phase (MP)

The process for the preparation of the MP involved combining 45:55 ACN and NaH_2PO_4. To eliminate contaminants that might potentially affect the final chromatogram, it was passed through a 0.45 µ membrane filter.

Preparation accuracy sample 50%, 100% and 150% solutions

To prepare (0.5 mL stock solution dissolves into 10 mL volumetric flask) 50%, (1 mL stock solution dissolves into 10 mL volumetric flask) 100% and (1.5 mL stock solution dissolves into 10 mL volumetric flask) 150% solutions using the above stock solution. (5 ppm/10 ppm/15 ppm of estradiol, 50 ppm/100 ppm/150 ppm of DYD).

Preparation of degradation parameters

Degradation parameters preparation is given below in Table 1.

RESULTS AND DISCUSSION

Method development

A wavelength that is isobestic was employed to estimate two drugs at once. The wavelength at which two interconvertible substances have the same molar absorptivity is known as the isobestic point. To precisely estimate two drugs, this wavelength was employed in simultaneous estimation.

We used acetonitrile (ACN) and NaH_2PO_4 (55:45) as a blank, and a PDA detector scanned the wavelength of maximum absorption of the drug solution within the 200–400 nm wavelength range. 260 nm is the isobestic point on the absorption curve. The UPLC chromatographic technique chose 260 nm [Figure 2] as its detection wavelength.

In addition to other parameters (such as column, MP composition, buffers, pH, flow rate, and wavelength), the isocratic elution with various MP compositions was optimized until a well-defined separation of estradiol and DYD peaks along with suitable system suitability conditions were achieved. Conducted trails are given in below Table 2.

The optimized trial [Figure 3a-f] was conducted with an aquity BEH (50 × 2.1 mm, 1.7 µm), 0.3 mL/min flow rate, 5 min run time, 260 nm detection wavelength, and ACN: NaH_2PO_4 (55:45) as the MP conditions. Good response area, tailing factor, and resolution are provided by this path. DYD peaked at 1.922 min, with a peak area of 2431347, a tailing factor of 1.05, and a resolution of 3.26. Estradiol peaked at 1.013 min, with a peak area of 455159, and a tailing factor of 1.14. This trial was optimized conditions are given in Table 3.

System suitability and specificity

There was a system appropriateness test to make sure the existing approach was appropriate for the intended use. The optimized conditions for the current technique revealed system suitability characteristics that are within the acceptable range and are arranged in Table 4. System performance is supported by the system's good tabular values for system suitability parameters. A specificity analysis was conducted to confirm the interference from degradation products, blanks, and placebo [Figure 4a and b].

Table 1: Degradation parameters preparation

S. No.	Degradation	Conditions
1	Acid degradation	0.1 mL of 1N HCl and heated at 70°C for 1 h
2	Alkali degradation	1 mL of 1N NaOH and heated at 70°C for 1 h
3	Thermal degradation	Exposed at 80°C for at least 72 h
4	Peroxide degradation	0.5 mL of 30% H_2O_2 at 70°C for 1 h
5	Reduction degradation	1 mL of 10% sodium bisulfate and heated at 70°C for 1 h
6	Photolytic degradation	Exposed to 1.2 Million lux hours of light
7	Hydrolysis degradation	Heated at 70°C for 30 min

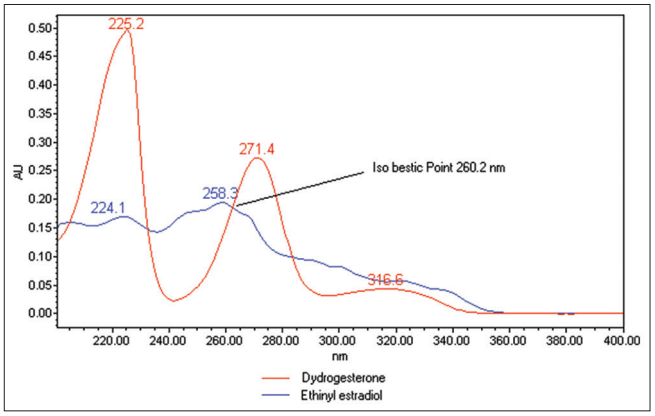

Figure 2: Photodiode array spectrum of estradiol and dydrogesterone

Table 2: Conducted trails and observation

S. No.	Column	Mobile phase and wavelength	Flow rate and run time	Observation
1	Acquity HPLC BEH C8 100×2.1 mm, 1.7 µm	Acetonitrile: 0.1% TFA 80:20 and 200–400 nm	0.3 mL/min and 5 min	Unknown peaks are observed
2	Acquity HPLC BEH C8 100×2.1 mm, 1.7 µm	Acetonitrile: 0.1% TFA 70:30 and 260 nm	0.3 mL/min and 10 min	Unknown peak and baseline drift are observed
3	Acquity HPLC BEH C8 100×2.1 mm, 1.7 µm	Acetonitrile: 0.1% TFA 60:40 and 260 nm	0.3 mL/min and 11 min	Resolution is very low
4	Aquity BEH (50×2.1 mm, 1.7 µm)	Acetonitrile: NaH_2PO_4 (60:40) and 260 nm	0.3 mL/min and 5 min	Unknown peak and baseline drift are observed
5	Aquity BEH (50×2.1 mm, 1.7 µm)	Acetonitrile: NaH_2PO_4 (70:30) and 260 nm	0.3 mL/min and 5 min	Resolution is <2000
6	Aquity BEH (50×2.1 mm, 1.7 µm)	Acetonitrile: NaH_2PO_4 (55:45) and 260 nm	0.3 mL/min and 5 min	Optimized condition

HPLC: high-performance liquid chromatography, TFA: Trifluoroacetic acid, ICH: International Council for Harmonisation

Table 3: Optimized chromatographic conditions

Parameters	Observation
Instrument used	Waters HPLC is equipped with a PDA detector
Injection volume	5 µL
Mobile phase/diluent	Acetonitrile and NaH_2PO_4 (55:45)
Column	Aquity BEH 50×2.1 mm, 1.7 µm
Detection wavelength	260 nm
Flow rate	0.3 mL/min
Runtime	5 min
Temperature	Ambient (25°C)
Mode of separation	Isocratic mode

HPLC: High-performance liquid chromatography,
ICH: International Council for Harmonisation, PDA: Photodiode array

Table 4: Estradiol and dydrogesterone system suitability parameters

S. No.	Parameter	Estradiol	Dydrogesterone
1	Retention time	1.017	1.928
2	USP plate count	163685	8730
3	USP tailing factor	1.18	1.09
4	USP resolution	--	3.22
5	Percent RSD	0.25	0.36
6	Purity angle	0.952	0.134
7	Purity threshold	3.331	1.228
8	Purity plag	No	No

RSD: Relative standard deviation

Neither diluent nor placebo has demonstrated interference at the estradiol and DYD retention time. The UPLC chromatogram indicates that the system is suitable [Figure 5] if the tailing factor value is <2 and the number of theoretical plates for the estradiol and DYD peaks is >2000.

A specificity analysis was carried out to confirm whether any interference from degradation or other contaminants existed at the estradiol and DYD retention times. The present method's specificity is indicated by the lack of peak interference from blank at the retention times of 1.017 and 1.928 min for estradiol and DYD, respectively. When the purity angle (PA) is smaller than the purity threshold (TH), the empower software shows that the peak is homogeneous [Figure 6a and b], as indicated by the purity flag "No."

Degradation/stability indicating studies

To ascertain the specificity and stability-indicating characteristics of the suggested approach, forced degradation research was conducted. Chromatograms under various stress situations were acquired as part of degradation experiments [Figure 7a-h]. In every degradation condition, the estradiol and DYD peaks and all other degradant peaks are isolated from one another. Its degradation products did not cause any interference, thus. The suggested approach is a particular and stability-indicating strategy, it can be deduced from the data [Table 5]. Depicts how estradiol and DYD degrade in response to stress. Lower susceptibility to photolytic and hydrolysis degradation conditions is shown in estradiol and DYD; nonetheless, high degradation levels indicate sensitivity to acid, alkali, reduction, peroxide, degradation under light and ultraviolet radiation [Table 5]. Due to its shown selectivity, these results indicate that this technology is appropriate for regular quality control examinations. PA less than the Purity TH [Figure 8a-n] is determined by the empower software. Thus, this data indicates that the peak is homogenous.

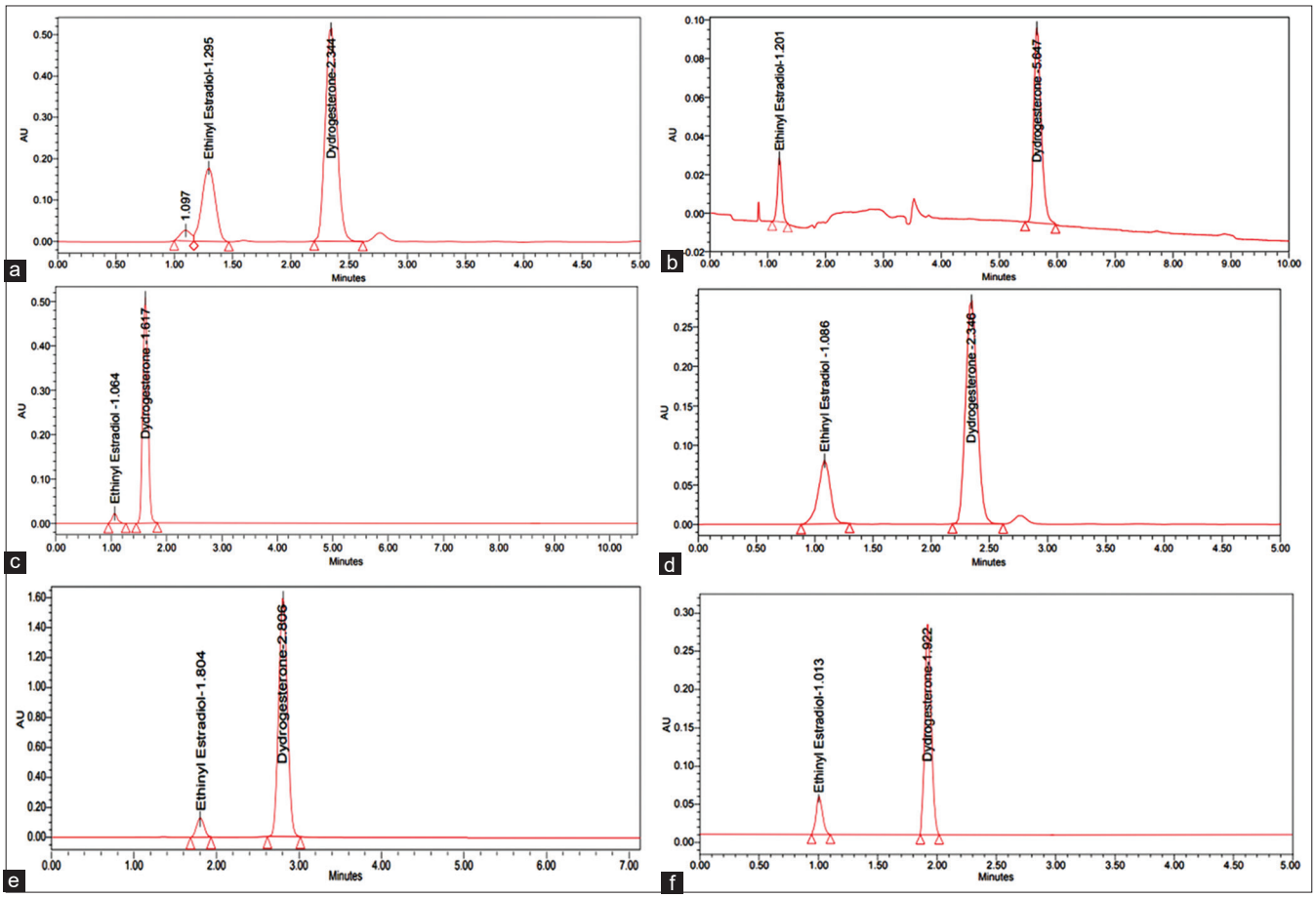

Figure 3: (a) Trail-1, (b) Trail-2, (c) Trail-3, (d) Trail-4, (e) Trail-5, (f) Optimized chromatograms

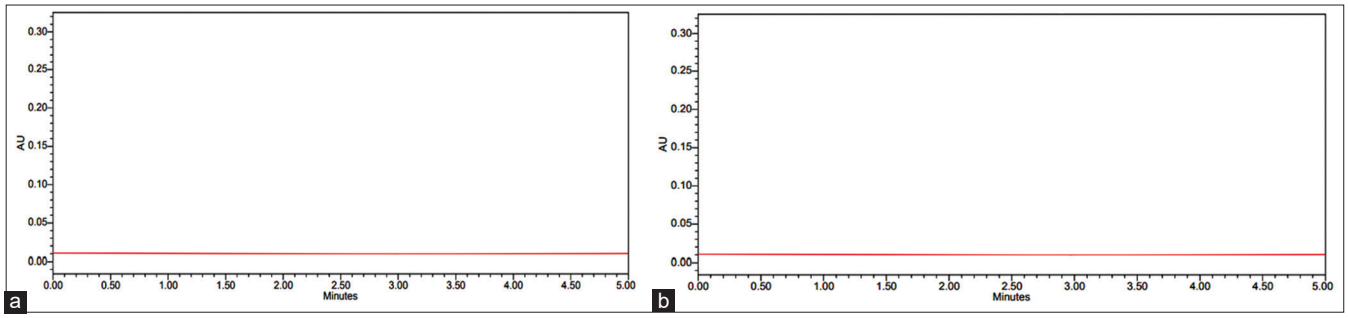

Figure 4: (a and b) chromatogram of blank and placebo

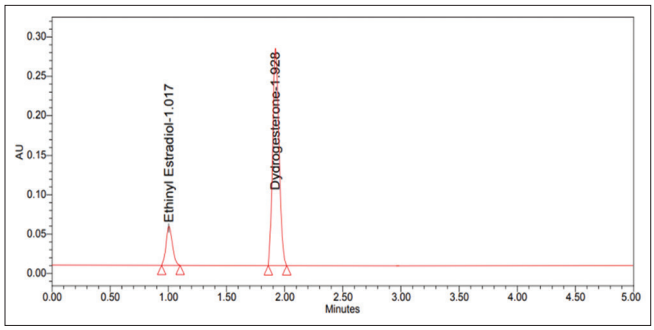

Figure 5: Standard chromatogram

Validation of method

To validate the above-mentioned optimized technique for the detection of estradiol and DYD, the current recommendations[18-20] were implemented.

Linearity

Assessed the linearity and observed linearity in the calibration curve [Figure 9a and b] that was generated by injecting varying amounts of estradiol and DYD (2.5–15 g/mL and 25–150) [Figure 10a-f]. Building the analytical

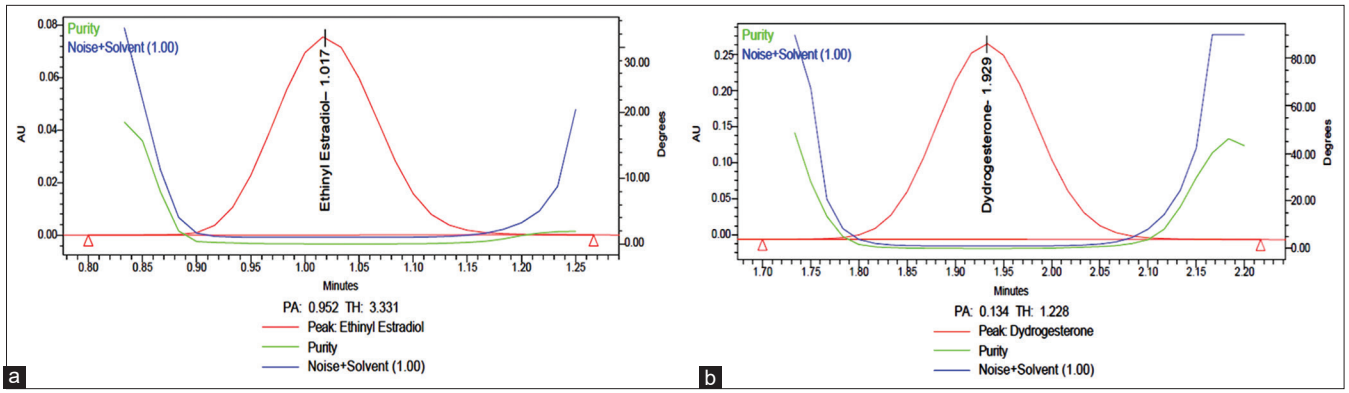

Figure 6: (a) Purity plot of ethinyl estradiol and (b) Purity plot of dydrogesterone

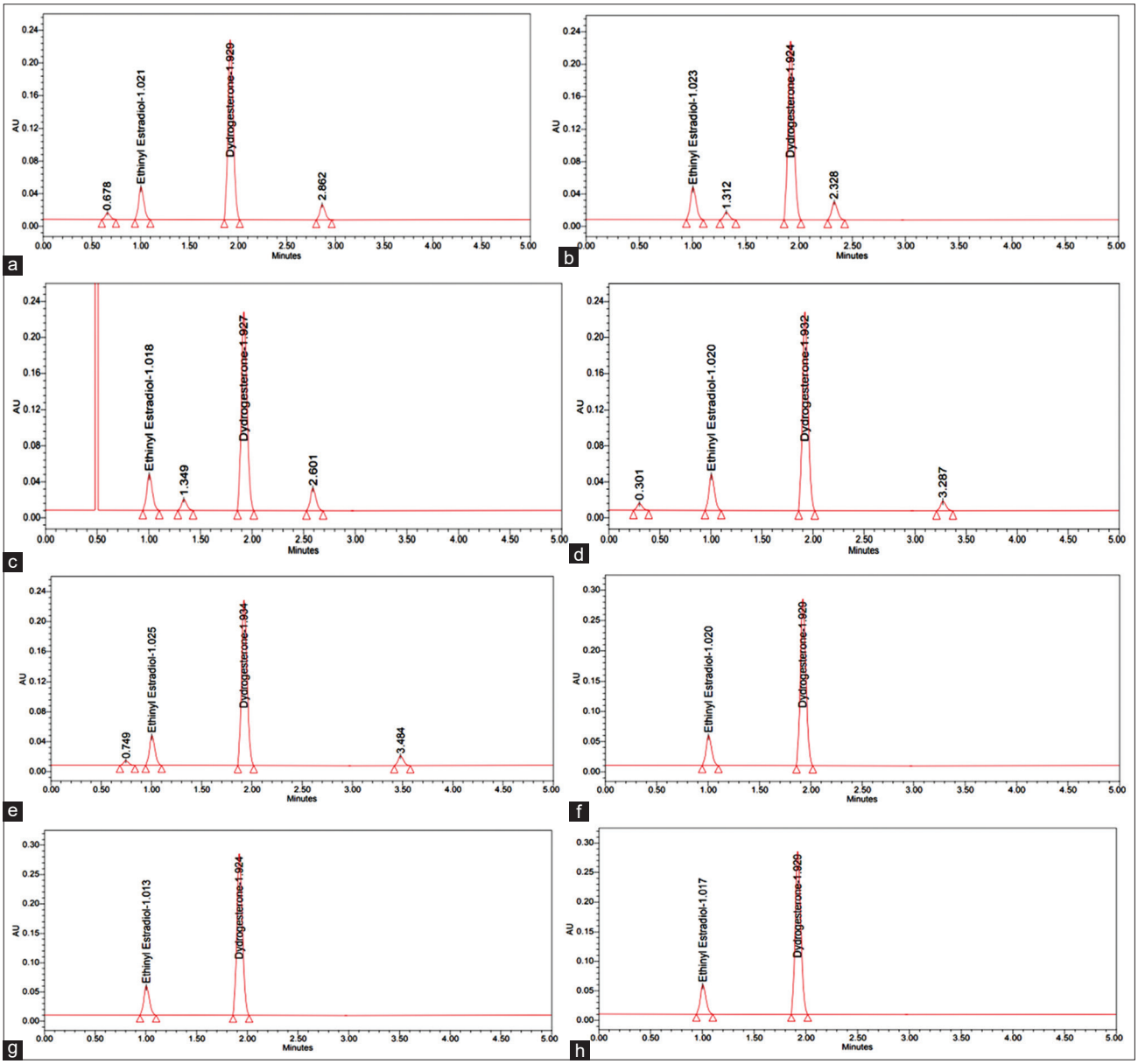

Figure 7: (a and b) Chromatograms of acid and alkali degradation. (c and d) Chromatograms of peroxide and reduction degradations. (e and f) Chromatogram of thermal and photolytic degradations. (g and h) Chromatogram of hydrolysis degradation and control degradation

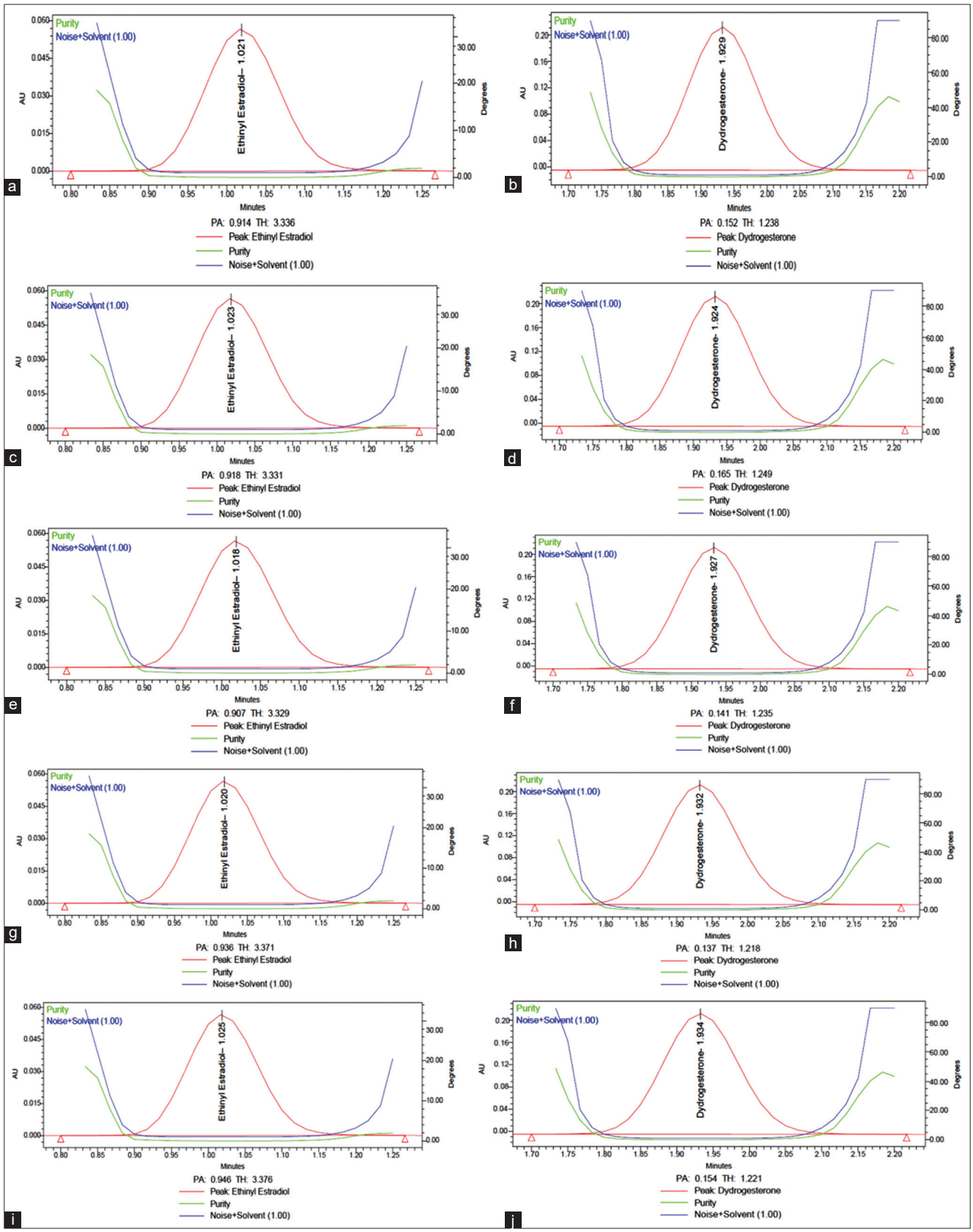

Figure 8: (a and b) Purity plot of estradiol and dydrogesterone (acid degradation). (c and d) purity plot of estradiol and dydrogesterone (alkali degradation) (e and f) Purity plot of estradiol and dydrogesterone (peroxide degradation). (g and h) Purity plot of estradiol and dydrogesterone (reduction degradation). (i and j) Purity plot of estradiol and dydrogesterone (thermal degradation). (k and l) Purity plot of estradiol and dydrogesterone (photolytic degradation). (m and n) Purity plot of estradiol and dydrogesterone (hydrolysis degradation). Purity plot of ethinyl estradiol and dydrogesterone (control)

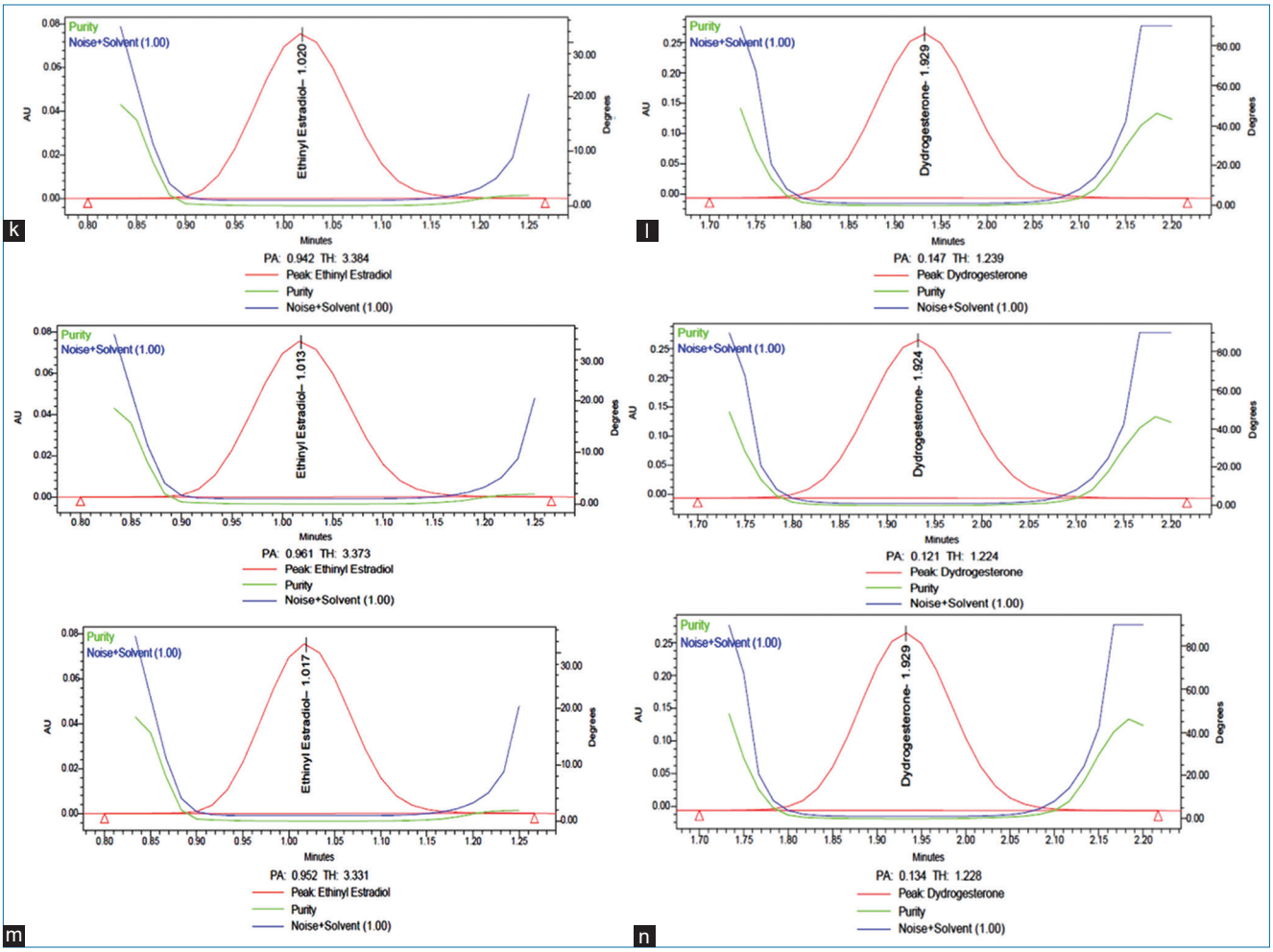

Figure 8: *(Continued)*

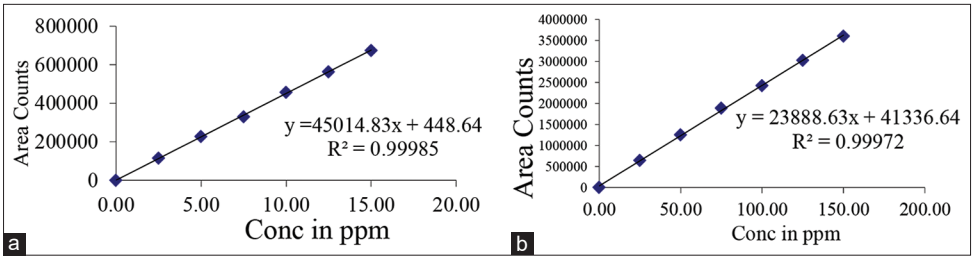

Figure 9: (a and b) Calibration curve for estradiol and dydrogesterone

curve involved plotting the area under the curve versus drug concentration. Y = 45014.83× + 448.64 and Y = 23888.63× + 41336.64 were determined to be the regression equations, with correlation coefficients of 0.99985 and 0.99972 [Table 6].

Accuracy

The technique's most important metric, accuracy, is represented by adding a specified amount of medication to the sample and then calculating the recovery percentage. Analytical technique accuracy is assessed by repeatability. Accuracy investigation was conducted using six replicate preparations at recovery levels of 50%, 100%, and 150% before being injected into a chromatograph [Figure 11a-c]. The results, which include the estimated percentage recovery from the chromatographic peak region at each recovery level, are displayed in Tables 5 and 6. Tables 7 and 8 show that the recovery percentages for estradiol and DYD were 99.6–99.2 and 101.0–100.6, respectively. Recovery values related to the analyte are within the limit since recovery findings are within the permitted limits. Thus, it suggests that the method for estimating estradiol and DYD that was suggested was accurate.

Table 5: Results of stress study data of estradiol and dydrogesterone

Parameter	Estradiol				Dydrogesterone			
	% Assay of degraded sample (A1)	% Degradation w.r.t. control sample (B1*)	PA	TH	% Assay of degraded sample (A2)	% Degradation w.r.t. control sample* (B2*)	PA	TH
Control	100.92	-----	0.952	3.331	100.29	------	0.134	1.228
Acid	88.12	12.68	0.914	3.336	86.99	13.25	0.152	1.238
Alkali	87.62	13.18	0.918	3.331	86.29	13.96	0.165	1.249
Peroxide	85.79	14.99	0.907	3.329	84.86	15.38	0.141	1.235
Reduction	90.26	10.56	0.936	3.371	89.15	11.10	0.137	1.121
Thermal	90.72	10.10	0.946	3.376	88.85	11.40	0.154	1.221
Photolytic	99.98	0.927	0.942	3.384	99.21	1.083	0.147	1.239
Hydrolysis	100.21	0.705	0.961	3.373	98.91	1.385	0.121	1.224

B1*=(100.92–A1)/100.92×100, B2*= (100.29–A2)/100.29×100

Table 6: Results of linearity for estradiol and dydrogesterone

S. No.	Estradiol		Dydrogesterone	
	Conc.(µg/mL)	Peak area	Conc.(µg/mL)	Peak area
1	2.50	114534	25.00	646501
2	5.00	226878	50.00	1253257
3	7.50	330326	75.00	1885746
4	10.00	456946	100.00	2421068
5	12.50	563167	125.00	3025715
6	15.00	674568	150.00	3598601
Regression equation	y=45014.83x+448.64		y=23888.63x+41336.64	
Slope	45014.83		23888.63	
Intercept	448.64		41336.64	
R^2	0.99985		0.99972	

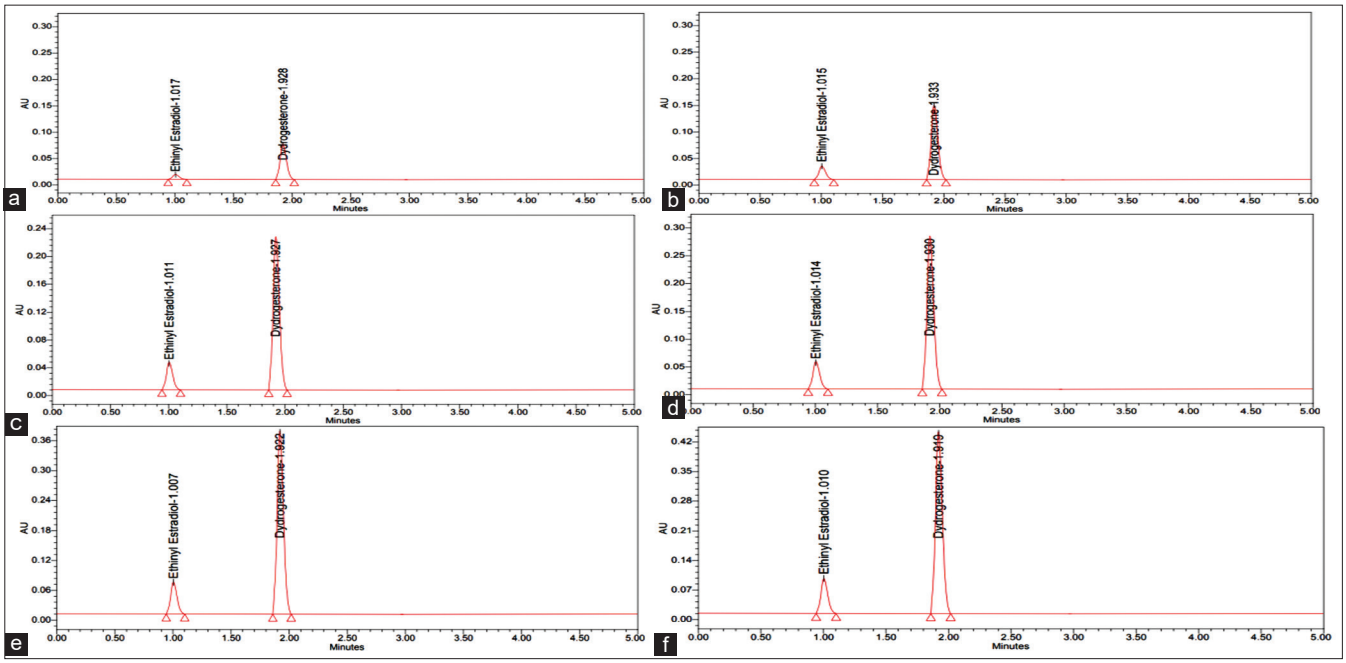

Figure 10: (a-f) Chromatogram of linearity-25%, 50%, 75%, 100%, 125% and 150%

Table 7: Accuracy results of estradiol by UPLC method

Recovery levels (%)	Peak response	Addition quantity (mg)	Recovery quantity (mg)	% Recovery	Average
50	225115	0.5	0.496	99.2	99.37
100	452458	1	0.996	99.6	
150	675478	1.5	1.49	99.3	

UPLC: Ultra-performance liquid chromatography

Table 8: The accuracy results for dydrogesterone by UPLC method

Recovery levels (%)	Peak response	Addition quantity (mg)	Recovery quantity (mg)	% Recovery	Average
50	1228746	5	5.04	100.8	100.80
100	2451068	10	10.06	100.6	
150	3692601	15	15.15	101.0	

UPLC: Ultra-performance liquid chromatography

Table 9: System precision table of estradiol and dydrogesterone

S. No.	Estradiol		Dydrogesterone	
	M.P	I.P	M.P	I.P
1	455884	455275	2468206	2458627
2	452327	452921	2450954	2461394
3	456309	456347	2434567	2444287
4	457567	453185	2454877	2439918
5	452256	454247	2448512	2452538
6	453368	452312	2425457	2447102
Average	454619	454048	2447096	2450644
Standard deviation	2260.489	1540.073	15154.454	8377.139
%RSD	0.50	0.34	0.62	0.34

Table 10: Robustness results of estradiol by UPLC

Changed parameters	Actual cond.	Altered cond.	RT (min)	Peak area	Resolution	Tailing	Plate count
Estradiol							
Control	--------	-------	1.017	455159	-----	1.18	163685
Flow rate change (mL/min)	0.3 mL	Less flow-0.27 mL	1.339	471222	----	1.13	166254
		More flow-0.33 mL	0.945	435300	----	1.21	160421
Organic Phase change	55:45	Less Org (49.5:50.5)	1.507	505365	-----	1.18	167632
		More Org (60.5:39.5)	0.717	416501	-----	1.10	168541
Dydrogesterone							
Control	--------	-------	1.928	2431347	3.22	1.09	8730
Flow rate change (mL/min)	0.3 mL	Less flow-0.27 mL	2.107	2567854	2.87	1.04	8860
		More flow-0.33 mL	1.629	2171320	2.79	1.03	8693
Organic phase change	55:45	Less org (49.5:50.5)	2.432	2751991	1.08	3.41	8860
		More Org (60.5:39.5)	1.324	1957482	2.58	1.04	8623

Precision

The precision measures the variability of the findings obtained from repeated analysis of the sample of estradiol and DYD under duplicate experimental conditions. Six replicates were employed to perform the investigations for both method and intermediate precisions (MP and IP) to validate the existing approach. Variations in the instrument, column, and analyst parameters were employed to achieve the IP. The computed percentage RSD values for MP and IP were 0.50 and 0.62, respectively, and 0.34, which falls within the International Council for Harmonisation

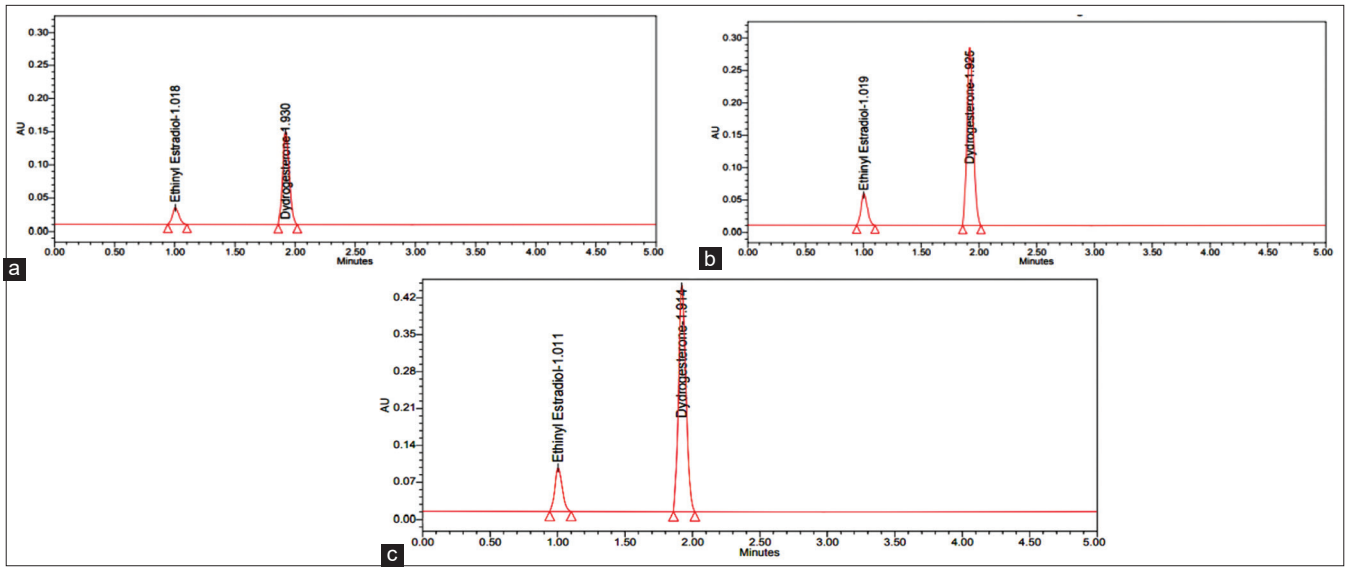

Figure 11: (a-c) Chromatogram of accuracy 50%, 100% and 150%

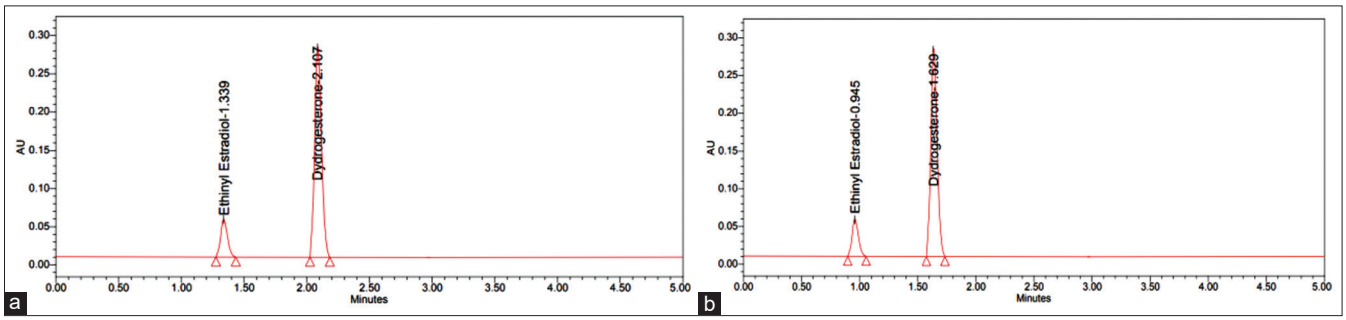

Figure 12: (a-b) Chromatogram for less flow rate (0.27 mL) and more flow rate (0.33 mL)

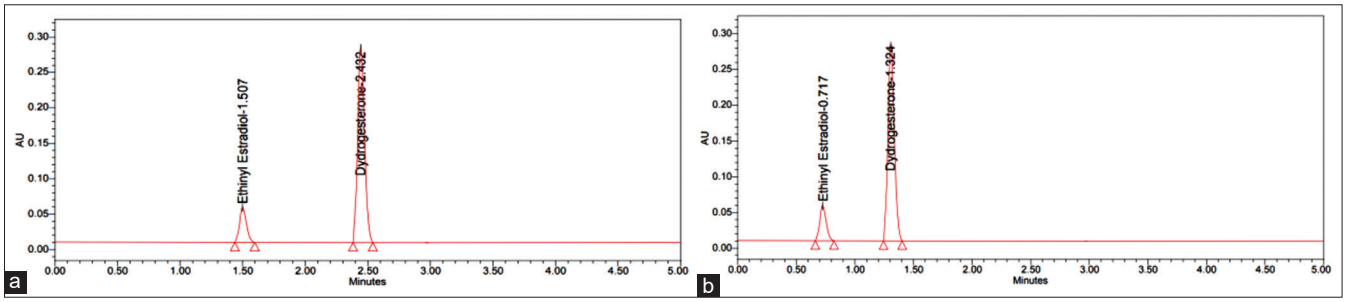

Figure 13: (a and b) Chromatogram for less organic phase (49.5:50.5) and more organic phase (60.5:39.5)

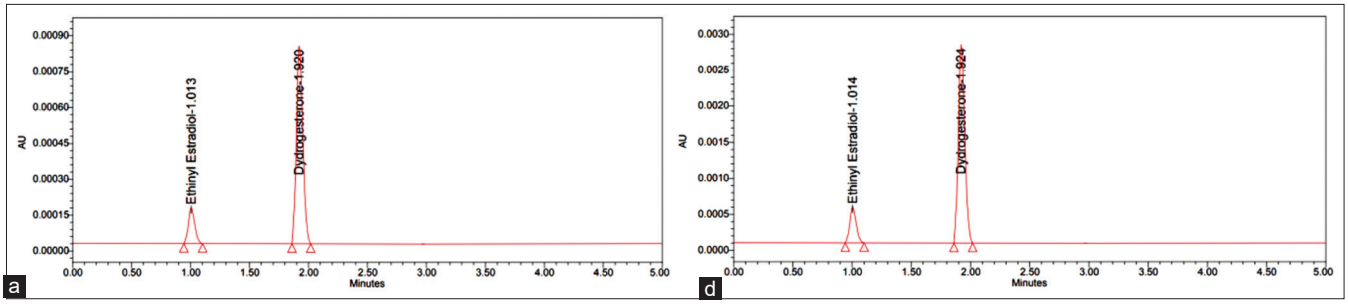

Figure 14: (a and b) Chromatogram for limit of detection and limit of quantification

Table 11: Pharmaceutical formulation assay

S No	Brand name	Dosage form	Dosage	Amount prepared (µg/mL)	Amount Found (µg/mL)	% Assay
1	Estrogen	Tablet	10 mg	1	0.996	99.6
2	Duphaston	Tablet	10 mg	10	10.06	100.6

(ICH) permissibility limitations [Table 9]. It shows a high degree of precision and a robust technique.

Robustness

The two main parameters flow rate [Figure 12a and b] and organic phase [Figure 13a and b] were carefully changed using a reference solution of and testosterone to evaluate the procedure's robustness. Peak area, tailing factor, retention length, and theoretical plates were all unaffected by the purposeful deviation of the experimental parameter values [Tables 10 and 11]. The content of organic solvent varied by approximately ±5%. Within bounds, that is, <2.0%, the observed variation in peak area demonstrates the dependability of the current methodology.

Limit of detection (LOD) and limit of quantification (LOQ)

It was discovered that the lower detection and quantification values for estradiol and testosterone were, respectively, 0.03 µg/mL and 0.1 µg/mL and 0.3 µg/mL and 1 µg/mL. Given that the LOD and LOQ [Figure 14a and b] values were discovered to be much below the designated limit, the approach is sensitive.[21-28]

Pharmaceutical formulation analysis

Determined the quantity of estradiol and DYD in the tablet formulation using the above approach, as the recovery values of both substances are good [Table 11]. These days, quality control laboratories in impoverished nations may employ the UPLC technique since, in addition to spectrophotometers, this equipment is reasonably priced. Consequently, by the current ICH standards, the present approach is used to determine the levels of estradiol and DYD in tablet formulations.

CONCLUSION

The study's conclusions indicate that the RP-UPLC technique's creation and validation are simple, and its run time is short. In addition, performs well in quality control labs for research into API and tablet dose formulation, stability signals, and quantitative detection of estradiol and dysgeresterone.

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