

AN *In-vitro* STUDY OF A SIMULTANEOUS METHOD FOR DETERMINING RELUGOLIX, NORETHINDRONE ACETATE, AND ESTRADIOL BY UPLC

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ABSTRACT

Uterine Leiomyomata (commonly known as Fibroids in the uterus) are non-cancerous tumors, most commonly found in women at the premenopausal stage. They are estimated to be found at 33-77% during reproductive age and 70-80% during menopause. The current frontline treatments were ineffective and alternative pharmacological treatments were proposed. Thus, this study aimed at a therapeutic combination of Relugolix, Norethindrone acetate, and Estradiol is proposed. A precise, specific, simple, and selective stability indicating Ultra Performance Liquid Chromatography method for simultaneous determination of the combination has been developed and validated. The chromatographic separation was done by using an isocratic mobile phase of 50:50 v/v acetonitrile and buffer on a phenyl column (100 x 2.1 mm, 1.7 m) with 0.5 ml flow rate/minute, and 272 nm wavelength. The ability of the method to indicate stability was demonstrated by subjecting the Active Pharmaceutical Ingredients to various stressors using appropriate validity international standard methods. The current study found that the system acceptability (SA), reproducibility (R), detection limits (DL), quantitation limits (QL), linearity (L), accuracy (A), precision (P), and specificity (S) were all within acceptable limits.

Keywords: Uterine Fibroids, Menopause, UPLC-PDA Method, Relugolix, Norethindrone acetate, Estradiol, Stability Indicating, Stress Degradation.

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INTRODUCTION

Uterine Leiomyomata (commonly known as Fibroids in the uterus) are non-cancerous tumors, most commonly found in women at the premenopausal stage. They are estimated to be found at 33-77% during reproductive age and 70-80% during menopause.¹ The most common symptoms are abnormal uterine bleeding, abdominal protrusion, pelvic pressure, urinary urgency, frequency or incontinence; constipation, tenesmus, etc., and reproductive dysfunctions.²⁻⁴ The current frontline treatments include non – steroidal anti-inflammatory drugs, tranexamic acid, hormonal contraceptives, and hysterectomy. For the women with persistent symptoms or still worsening conditions, the current frontline treatments were ineffective, and alternative pharmacological treatments were proposed.⁵ The utilization of GnRH antagonists is found to be effective but has mitigated side effects. GnRH antagonists in combination with estrogen and progestin were found to have a statistically significant reduction in the manifestations. The first candidate Elagolix administered with norethindrone acetate and estradiol is approved for the treatment for 24 months. This treatment has long-term adverse side effects When combined with estradiol and norethindrone acetate, relugolix, an oral active non-peptide Gonadotropin-releasing hormone (GnRH) receptor antagonist, was found to be a better candidate for the pharmacological treatment of uterine fibroids.^{6,7} Relugolix is an orally administered non-peptide GnRH receptor antagonist. Its molecular formula is C₂₉H₂₇F₂N₇O₅S, and its molecular weight is 623.63g. It binds to pituitary GnRH receptors, limiting the release of LH and FSH, as well as the production of estrogen and progesterone.⁸

Norethindrone acetate belongs to the class of progestins. Its molecular formula is C₂₂H₂₈O₃ and its molecular weight is 340.5g. It is used to treat Endometriosis or women with abnormal periods or heavy menstrual bleeding.

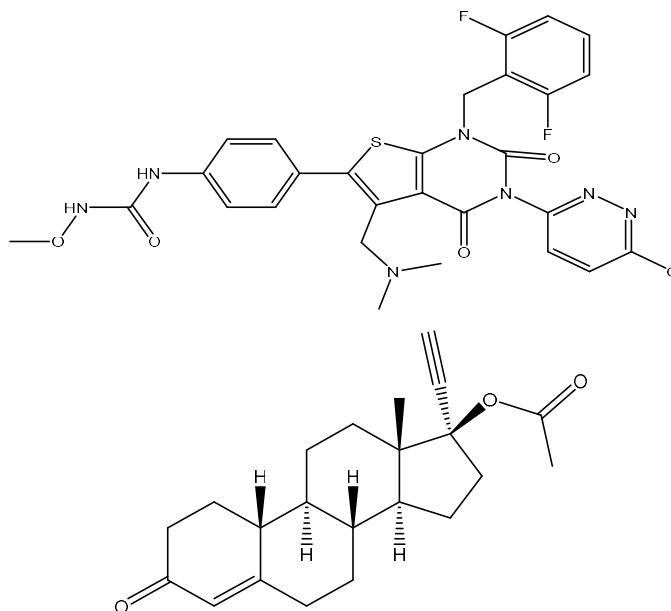


Fig.-1: Relugolix and Norethindrone Acetate

Estradiol is a synthetic form of estradiol, a steroid sex hormone important for female fertility and secondary sexual characteristics. The chemical formula is $C_{18}H_{24}O_2$ with a molecular weight of 272.4g. In combination with other drugs, it is used to treat menopause in women.

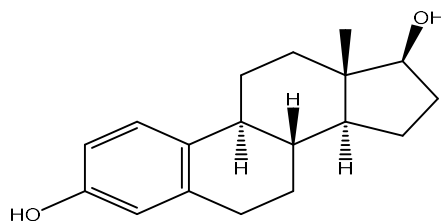


Fig.-2: Estradiol

Current studies utilized UPLC – MS / MS^{9,10}, HPLC¹¹, RP – HPLC^{12,13} separately and in combination to put out the determinations regarding RG, NA, and ED. A review of the literature reveals that few liquid chromatographic methods for individuals or combined with other molecules have been reported. A scant research method was reported for determining all three substances at once. Over the last decade, a concrete effort has been made to increase HPLC performance by means of smaller particle-packed columns. A revolutionary type of LC called ultra-performance LC (UPLC) that works on the same principles as HPLC, is governed by the Van - Demteer equation. Further, increased separation speed, sensitivity, and resolution were achieved with the utilization of sub - 2 μ m particle size column packing and high-pressure operation technologies. Present work aimed to develop a method of UPLC to measure RG, NA, and ED in samples collected during the procedure validation in manufacturing the medicine. This usage of the UPLC method improved the analysis time and solvent consumption. This process of extraction was simpler and faster.

EXPERIMENTAL

Materials Utilized

RG, NA & ED drug working values were procured from Pfizer, Mumbai. Merck, Mumbai, India, supplied HPLC-grade acetonitrile and formic acid. Milli - Q plus water purification system prepared high-purity water.

Instrumentation

A Waters Aquity UPLC instrument with a PDA detector, dual solvent manager, and autosampler system was used for chromatographic analysis. The signal was monitored and processed using Empower - 2 software. The prepared sample was sonicated prior to analysis.

Chromatographic Conditions

Acetonitrile and buffer (50:50 v/v), degassed and filtered through 0.22 μm , were used as the mobile phase. At room temperature, 5 μl of samples were injected into a phenyl column (100 x 2.1mm, 1.7 μm), and eluted components were measured at 272 nm.

Stock Solution Preparation

A 50:50 v/v diluent mixture of acetonitrile and buffer is used to prepare standard solutions. 40, 5, and 10 mg/l of RG, NA, and ED were weighed and transferred into a 100 ml volumetric flask. The stock concentrations after dilution were 40, 5, and 10 mg/l, respectively.

Working Standards Preparation

Diluting 5 ml of stock solution with the diluent in a 50 ml volumetric flask yielded working standards containing 40, 5, and 10 mg/l of RG, NA, and ED.

Method Development:

Optimization of the Method

The chromatographic conditions were established after testing different ratios of the mobile phase in isocratic mode. The retention time and resolution were improved by changing the mobile phase composition. Better resolution is obtained using the phenyl stationary phase than C18. At optimized conditions of 0.5 ml/min mobile phase flow rate and 272 nm wavelength, retention times of 1.057, 1.754, and 2.863 and tailing factors of 1.10, 0.98, and 1.03 were obtained for RG, NA, and ED. Indicating the better elution, the theoretical plates of RG, NA, and ED were 3750, 5896, and 6585. The percent RSD for six replicates were reported as 0.102, 0.254, and 0.643. The anticipated method is precise, authenticated, and follows ICH guidelines.

RESULTS AND DISCUSSION

System Suitability

The system suitability study examines the performance and interdependence of tools, software, and analysis. The method's applicability is tested by injecting 40, 5, and 10 mg/l of RG, NA, and ED in six duplicates. The results are within ICH guidelines and are given below:

Table-1: System Suitability Parameters

Parameter	Acceptance Requirement	Name of the API		
		RG	NA	ED
PC*	</2000	3750	5896	6585
Tailing*	>/2.0	1.10	0.98	1.03
Resolution*	</2.0	--	3.15	4.94
RSD in percent	>/2.0	0.102	0.254	0.643
Retention time	</2.0	1.057	1.754	2.863

* - As per USP, PC- plate count, RT- Retention time

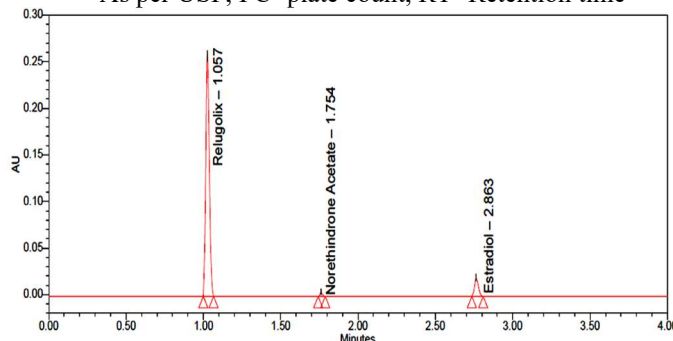


Fig.-3: Chromatogram of Standard

Specificity

Interferences from the placebo and blank were absent at the retention times of the RG, NA, and ED, demonstrating that the technique is more specific.

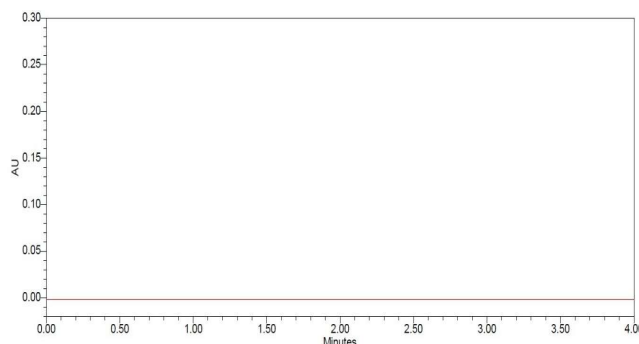


Fig.-4: Blank Solution Chromatogram

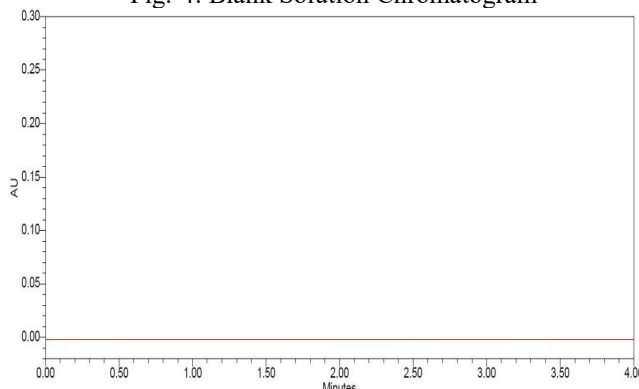


Fig.-5: Placebo Solution Chromatogram

Linearity

The range of techniques was determined by the process's linearity. The concentrations - peak area calibration curve plotting was utilized to determine the Linearity. This demonstrates that the curves of RG, NA, and ED were linear between 0 and 60, 0.75 and 1.5 $\mu\text{g} / \text{ml}$ respectively. The current study conclusions are depicted as follows:

Table-2: Show the Linearity Peak Values for RG, NA, and ED

Linearity	RG		NA		ED	
	Amount*	Peak Area	Amount*	Peak Area	Amount*	Peak Area
L - 1	0	0	0	0	0	0
L - 2	10.0	411715	0.13	12301	0.25	18337
L - 3	20.0	863987	0.25	23653	0.50	38045
L - 4	30.0	1277973	0.38	35299	0.75	57437
L - 5	40.0	1764341	0.50	47191	1.00	75224
L - 6	50.0	2157487	0.63	58539	1.25	94632
L - 7	60.0	2532197	0.75	70639	1.50	112632
Regression Equation	$Y = 42815.07 X + 2439.39$		$Y = 93695 X + 239.11$		$Y = 75380.71 X + 79.75$	
Slope	42815.07		93695		75380.71	
Intercept	2439.39		239.11		79.75	
Correlation coefficient	0.99955		0.99996		0.99992	

* - Amount in $\mu\text{g} / \text{ml}$, PA- Peak Area, L- Linearity

Accuracy

Accuracy determines the appropriateness of the procedure. Solutions containing RG (20, 40, and 60 mg/l), NA (0.25, 0.50, and 0.75 mg/l), and ED (0.5, 1.0, and 1.5 mg/l) were prepared, and three recovery studies were conducted at 50, 100 and 150 percent levels. When the test solution was spiked in triplicate, the percentage RSD values were less than or equal to 2 with 100 percent recovery rates. The recovered data was collected further expressed in percentages of recovery with mean, SD, and RSD's. The below table shows the conclusion of the findings.

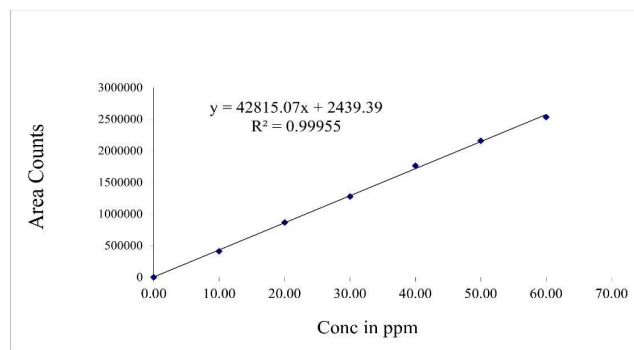


Fig.-6: Relugolix Linearity Plot

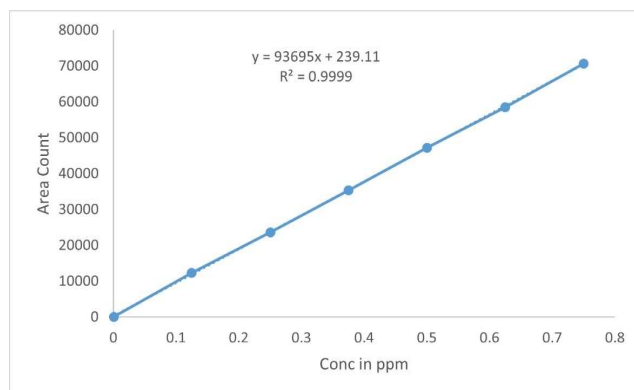


Fig.-7: Norethindrone Acetate Linearity Plot

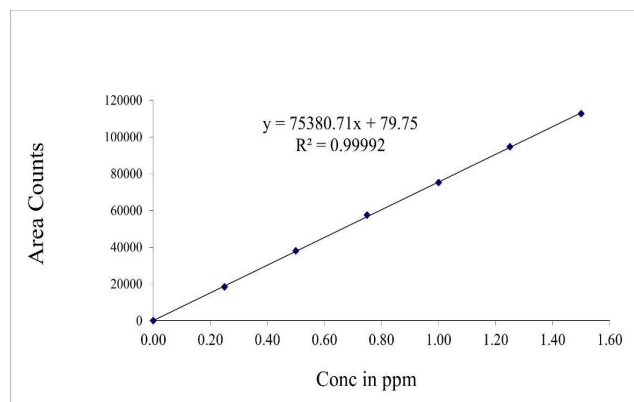


Fig.-8: Estradiol Linearity Plot

Table-3: Depicts the Percentages of Drugs Recovered which in Turn Relates to the Accuracy

S. No	% Level	% RG	%NA	%ED
		Recovered	Recovered	Recovered
1	50	101.3	99.6	100.3
2	100	100.6	99.2	100
3	150	100.4	98.4	99.3
Mean± SD		100.8±0.473	99.1±0.611	99.9±0.513
RSD (%)		0.47	0.62	0.51

R- recovered, SD- Standard Deviation

Method Precision

The closeness of homogeneous mixture sample readings during analysis was referred to as precision. Six injections of 40 g/ml RG, 5 g/ml NA, and 10 g/ml ED are used to determine the procedure's precision. The results are listed below.

Table-4: Intraday Precision Method Results

S. No	RG			NA			ED		
	Amount*	Area of Peak	Assay %	Amount*	Area of Peak	Assay %	Amount*	Area of Peak	Assay %
1	40	1819274	100.2	5	47579	99.8	10	75319	99.8
2		1799553	99.1		47432	99.5		75247	99.7
3		1794189	98.8		47387	99.4		75054	99.5
4		1785510	98.3		47485	99.6		75138	99.6
5		1804115	99.4		47066	98.7		75055	99.5
6		1813067	99.9		47263	99.1		75319	99.9
M	99.3			99.4			99.7		
SD	0.703			0.394			0.163		
RSD %	0.71			0.4			0.16		

* - Amount in mg / l, M- Mean, SD-Standard Deviation

Intermediate Precision

This was reported on separate days, where six replicates were examined by the researchers in the sample solution using various tools. Peak zones have been demarcated to assess RSD % averages. The below table depicts the results.

Table-5: Results for Inter-Day Precision

S. No	RG			NA			ED		
	Amount *	Area of Peak	Assay %	Amount *	Area of Peak	Assay %	Amount *	Area of Peak	Assay %
1	40	1791234	98.6	5	47786	100.1	10	75530	100.1
2		1785328	98.3		47367	99.2		75320	99.9
3		1781384	98.1		47542	99.5		75761	100.4
4		1793784	98.8		47143	98.7		75312	99.8
5		1793128	98.7		47357	99.2		75761	100.4
6		1813354	99.8		47687	99.9		75124	99.6
M± SD	98.7±0.591			99.4±0.513			100±0.327		
RSD %	0.6			0.52			0.33		

* - Amount in mg / l

Limits of Detection and Quantification

To detect and quantify the limits, calibration curves were used. UPLC was used to establish these detection and quantitation limits by injecting standard solutions in lower and higher quantities. The quantitation and detection limits for RG, NA, and ED, as well as their s/n values, are shown in the -6.

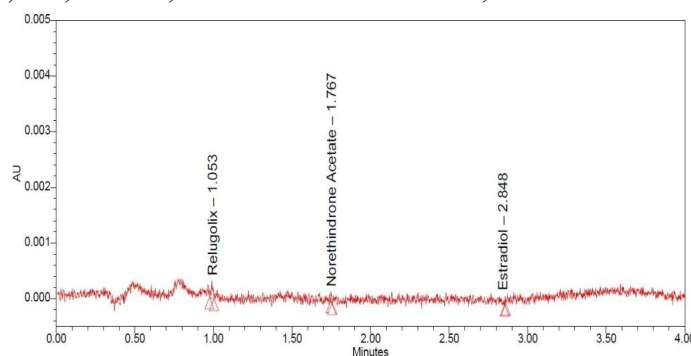


Fig.-9: Chromatogram for Detection Limit

Table-6: Show the RC, NA and ED, Quantification and Detection Limits

RG				NA				ED			
DL		QL		DL		QL		DL		QL	
A*	s/n	A*	s/n	A*	s/n	A*	s/n	A*	s/n	A*	s/n
40	3	40	10	5	3	5	10	10	3	10	10

* - in mg / l, A- Amount, DL- Detection Limit. QL- Quantitation Limit

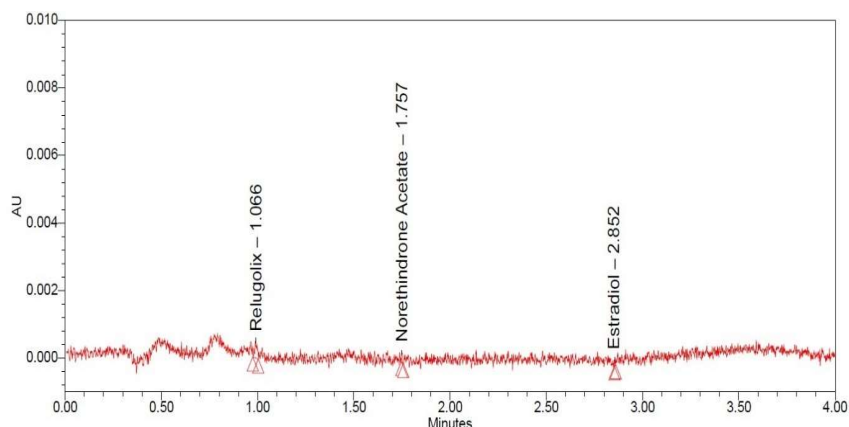


Fig.-10: Quantitation Limit of a Chromatogram

Robustness

The research was planned to test in the mobile phase regarding the robustness of changing flow rate and organic content.

Table-7: Robustness

Specification Name	% RSD		
	RG	NA	ED
RF (.8 ml/min)	0.72	0.71	0.21
RF (1.2 ml/min)	1.13	0.2	0.21
Org Plus (66:34)	0.36	0.27	0.21
Org Minus (54:46)	0.93	0.34	0.25

Studies on Stress Degradation

Degradation in Acid

In a volumetric flask of 50 ml, 5 ml of stock solution, a known volume of 0.1N HCl was taken, and kept aside for 15 minutes. Before dilution and analysis, using 0.1N NaOH, the above solution is neutralized. The same is repeated using 1N HCl and 1N NaOH.

Degradation in Alkali

In two trials, 5 ml of stock solution with known volumes of 0.1N NaOH and 1N NaOH were taken separately, set aside for fifteen minutes, and then analyzed after adding 0.1N HCl and 1N HCl.

Degradation in Peroxide

The test was carried out in two trials by adding 1 ml and 3 ml of H₂O₂ (10% and 30%) separately in two 50 ml volumetric flasks containing 5 ml of standard solution. This was then diluted up to the mark and analyzed.

Degradation in Reducing Agent

Sodium bisulphite solutions of 10% and 30% concentrations were used for analysis. For a 5 ml of standard solution taken in a 50 ml volumetric flask, 1 ml of reducing agent was added, kept aside for 15 minutes, and diluted before analysis.

Degradation in Heat

Analysis was performed by taking 5 ml of standard solution in two 50 ml volumetric flasks, diluted up to the mark, and heating them at 105°C for 3 hours and 6 hours respectively.

Degradation in Water

The analysis was performed after adding 1 ml and 3 ml of ultra-pure water in two 50 ml volumetric flasks containing of 5 ml of standard solution, kept aside for 15 minutes, and then diluted with solvent.

Degradation in Sunlight

In two volumetric flasks of 50 ml, 5 ml of standard solution is taken, diluted up to the mark, and kept in sunlight for 3 and 6 hours and then analyzed.

Table-8: Depicts the Percentages Assay and Deviations of Forced Degradation for Three Drugs

Conditions for degradation		No. of deg. products	RG		NA		ED	
			% As	% D	% As	% D	% As	% D
Control		0	100.0	0	100.0	0	99.9	0.1
Acid degradation (HCl)	0.1N	0	96.3	3.7	96.2	3.8	96.9	3.1
	1N	3	86.6	13.4	86.3	13.7	87.3	12.7
Alkali degradation (NaOH)	0.1N	0	96.7	3.3	95.9	4.1	97.3	2.7
	1N	3	87.5	12.5	86.9	13.1	86.6	13.4
Peroxide degradation (H ₂ O ₂)	10%	1	95.9	4.1	95.4	4.6	96.2	3.8
	30%	4	85.2	14.8	85.9	14.1	86.1	13.9
Reduction degradation (Na ₂ SO ₄)	10%	0	96.8	3.2	97.9	2.1	97.6	2.4
	30%	3	91	9	89.1	10.9	90.0	10.0
Thermal degradation (at 105°C)	3 hrs	0	98.4	1.6	98.4	1.6	98.0	2.0
	6 hrs	0	96.6	3.4	96.1	3.9	98.4	1.6
Hydrolysis degradation (adding H ₂ O)	1 ml	0	98.3	1.7	96.7	3.3	98.1	1.9
	3 ml	0	96.7	3.3	96.9	3.1	99.1	0.9
PD	3 hrs	0	98.9	1.1	98.1	1.9	97.8	2.2
	6 hrs	0	97.8	2.2	94.4	5.6	98.3	1.7

DC- Degradation conditions, As- Assay, %- Percentages, D- Deviation, PD- Photo Degradation

Stability

The stability of the standard and formulated samples was investigated at RT and at 2°C to 8°C. The outcomes were charted below.

Table-9: Depict the Percentages of Assay and Deviations of Stability Studies for Standard of Three Drugs

S. No	Stability	RG		NA		ED	
	RT	% A	% D	% A	RT	% A	% D
1	Initial	99.0	0.00	100.0	0.00	99.9	0.00
2	6 hrs	98.5	-1.40	99.4	-0.60	99.7	-0.02
3	12 hrs	97.0	-2.90	99.2	-0.80	99.3	-0.06
4	18 hrs	96.5	-3.40	98.8	-1.2	98.5	-1.40
5	24 hrs	96.2	-3.70	98.7	-1.3	98.3	-1.60

Table-10: Stability Studies for Formulations

S. No	Stability	RG		NA		ED	
	2°C - 8°C	% A	% D	% A	% D	% A	% D
1	Initial	99.0	0.00	100.0	0.00	99.9	0.00
2	6 hrs	98.8	-1.10	99.6	-0.40	99.6	-0.30
3	12 hrs	97.9	-2.00	99.4	-0.60	99.5	-0.40
4	18 hrs	96.4	-3.50	99.2	-0.80	99.1	-0.80
5	24 hrs	95.9	-4.00	98.5	-1.5	98.7	-1.2

S- stability, A- Assay, D- Deviations

CONCLUSION

According to the ICH guidelines, a stability indicating UPLC method for simultaneous determination of Relugolix, Norethindrone acetate, and Estradiol was developed and validated. The chromatographic conditions were optimized at 272 nm and a flow rate of 0.5 ml/min. The analysis time is less than 3 minutes, with retention times of 1.057, 1.754, and 2.863, and tailing factors of 1.10, 0.98, and 1.03 for RG, NA, and ED, respectively. The theoretical plates were found to be 3750, 5896, and 6585, indicating effective drug elution, with percentage RSD values of 0.102, 0.254, and 0.643 for RG, NA, and ED, respectively. The proposed method is applicable to routine analysis in the pharmaceutical industry.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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