RESEARCH ARTICLE

Simultaneous Estimation of Orlistat and Clomiphene by Hplc: Stability-Indicating Method Development and Validation

Rubina Kauser[1*], Sunil Kumar Chaitanya Padavala[2], Venkatesan Palanivel[1]

[1]*Department of Pharmacy, Annamalai University, Annamalai Nagar, Chidambaram, Tamil Nadu, India.*
[2]*Department of Pharmaceutical Analysis, St. Pauls College of Pharmacy, Hyderabad, Telangana, India.*

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ABSTRACT

Aim: To quantify Clomiphene and Orlistat in pharmaceutical formulations simultaneously, this study is set out to create a sensitive, fast, and accurate stability-indicating RP-HPLC method.

Materials and Methods: The isocratic method was used to achieve the chromatographic separation of the Clomiphene and Orlistat mixture. A mobile phase was prepared using a 60:40% v/v ratio of acetonitrile to 0.01N Potassium dihydrogen orthophosphate (P^H 4.8). The Agilent C18 column, with dimensions of 150 x 4.6mm, a 5µm particle size, and a flow rate of 1.0 mL/min was used. With an injection volume of 10.0 mL, the detection system was observed at a maximum wavelength of 240 nm.

Results: The retention time for Orlistat was 2.12 minutes while that for Clomiphene was 2.88 minutes. Various factors, including heat, acidity, oxidation, photolysis, and alkalinity, were used to test the combined medication formulation of Clomiphene and Orlistat. To ensure that this method is accurate, precise, linear, and sensitive, it was validated according to the standards set out by the ICH.

Conclusion: The run time was reduced to 6.0 minutes, enhancing the method's precision and cost-effectiveness. Stability studies confirmed the technique's suitability for assessing the degradation of Clomiphene and Orlistat. The proposed method is well-suited for routine analysis in pharmaceutical quality control.

Keywords: Clomiphene, Orlistat, RP-HPLC, Stability, Validation.

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INTRODUCTION

Orlistat is a gastrointestinal lipase inhibitor that reduces the absorption of dietary fats, aiding in weight management and the treatment of obesity. It is marketed under the brand name Xenical, among others. Orlistat works by inhibiting pancreatic and gastric lipases, enzymes that break down triglycerides in the intestine.[1] This inhibition prevents the hydrolysis of triglycerides into absorbable free fatty acids, resulting in a reduction of caloric intake. Chemically, Orlistat is a white to off-white crystalline powder that is insoluble in water, but soluble in chloroform and methanol.[2]

Clomiphene, a Selective Estrogen Receptor Modulator (SERM), induces ovulation in infertile women, especially those with polycystic ovary syndrome. It functions by binding to estrogen receptors in the hypothalamus, leading to an increase in the release of gonadotropins (FSH and LH) from the pituitary gland, which in turn stimulates the growth and maturation of ovarian follicles.[3] Clomiphene is marketed under the brand name Clomid, among others. Clomiphene appears as a white to pale yellow, odourless, crystalline powder, which is freely soluble in methanol and slightly soluble in water.[4]

Orlistat and Clomiphene are two pharmacologically distinct agents widely used in therapeutic treatments. Recent studies have highlighted the potential benefits of using Clomiphene and Orlistat together. For instance, research by Jayagopal *et al*.[5] demonstrated that the addition of Orlistat to Clomiphene therapy significantly improved ovulation rates in obese women with PCOS compared to Clomiphene alone. This suggests that co-administration of these drugs could enhance treatment efficacy, addressing both infertility and obesity concurrently. Additionally, the combination approach aims to improve overall reproductive and metabolic outcomes, as indicated by another study that incorporated weight management strategies, including Orlistat, alongside Clomiphene for treating PCOS.[6]

**Author for Correspondence: arkay0990@gmail.com*

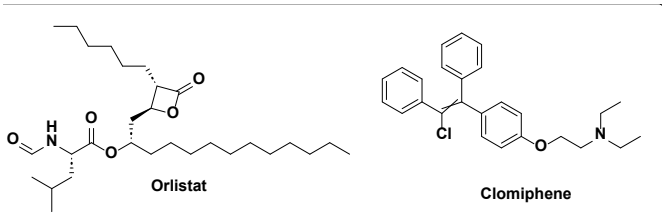

Figure 1: Structures of orlistat and clomiphene

Given these findings, there is a growing clinical interest in the concurrent use of Clomiphene and Orlistat. However, to ensure the safety and effectiveness of this combined therapy, reliable analytical methods are required to accurately quantify both drugs. The development of a simultaneous HPLC method involves optimizing chromatographic conditions to achieve adequate separation and quantification of Clomiphene and Orlistat. This includes selecting suitable mobile phases, columns, and detection wavelengths. The method must then be validated according to International Council for Harmonization (ICH) guidelines, evaluating parameters such as specificity, linearity, accuracy, precision, robustness, and detection limits.

MATERIALS AND METHODS

Chemicals and Reagents

Spectrum Pharma Research Solutions, Hyderabad, provided Clomiphene and Orlistat standard components as a gift. Locally purchased Mavyret 100 mg/40 mg film-coated tablets contained Clomiphene/Orlistat. A.B enterprises, Mumbai, India, supplied HPLC-grade acetonitrile and methanol. Ranchem, Mumbai, supplied orthophosphoric acid. Milli-Q Millipore water purification was utilized to process HPLC-grade water during method development.

Liquid Chromatography

Agilent column with dimensions of 150 x 4.6 mm and 5 m particle size, auto-sampler, and photodiode array detector are all components of the Waters HPLC system's chromatographic system. Using the water Empower-2.0 program, the output signal was integrated and monitored. The Agilent C18 (150 × 4.6 mm, 5.0m) column is employed to pump the isocratic mobile phase, which comprises 0.01N Potassium dihydrogen ortho phosphate (pH 4.8) and Acetonitrile in a 60:40% v/v ratio, at a constant flow rate of 1 mL/min. With a volume of 10.0 mL, the chromatograms were measured at detection wavelength of 240 nm.

Preparation of Buffer

About 1.36 grams of Potassium dihydrogen ortho phosphate was precisely weighed and placed in a 1000ml Volumetric flask. Approximately 900ml of milli-Q water was added and the mixture was degassed using sonication. The flask was then filled with water to reach the desired volume. Next, 1ml of Triethylamine was added and the pH was adjusted to 4.8 using a dilute Orthophosphoric acid solution.

Standard Stock Solutions Preparation

Orlistat stock solution

To prepare the Orlistat stock solution, 10 mg of Orlistat was accurately weighed and transferred to a 10 mL volumetric flask. The diluent, a 50:50 v/v mixture of water and acetonitrile, was added to the flask, dissolving the Orlistat completely. The volume was then adjusted to 10 mL with the diluent, resulting in a final stock concentration of 1 mg/mL.

Clomiphene stock solution

Clomiphene stock solution was made by properly weighing 10 mg and adding it to a 10 mL volumetric flask. The flask received 50:50 Water-Acetonitrile diluent. Clomiphene was thoroughly dissolved and diluted to 10 mL for a 1 mg/mL stock concentration.

Primary Standard Solution Preparation

Orlistat primary standard solution

To prepare the Orlistat primary standard solution, the stock solution was diluted to obtain a final concentration of 40 µg/mL. Specifically, 4 mL of the 1 mg/mL Orlistat stock solution was taken and diluted to 100 mL with the diluent.

Clomiphene primary standard solution

To prepare the Clomiphene primary standard solution, the stock solution was diluted to obtain a final concentration of 20 µg/mL. Specifically, 2 mL of the 1 mg/mL Clomiphene stock solution was taken and diluted to 100 mL with the diluent.

Working standard solution of orlistat and clomiphene

Each 5 mL of Orlistat and Clomiphene primary standard solutions were mixed in 10ml volumetric flask to achieve the working standard solution with the final concentration of 20 µg/mL for orlistat and 10 µg/mL for clomiphene.

Preparation of sample solution

Five tablets of Orlistat and Clomiphene were individually weighed and powdered. Equivalent to 100 mg of each drug, the powders were transferred separately into 100 ml volumetric flasks. To each flask, 50 ml of diluent was added, and the mixtures were sonicated until dissolved. The volume was then adjusted to 100 ml with diluent to achieve a concentration of 1 mg/ml.

Next, 4 ml of the Orlistat solution and 2 ml of the Clomiphene solution were transferred into separate 10 ml volumetric flasks and diluted with diluent. Subsequently, 5 ml of each diluted solution were combined in a 10 ml volumetric flask to prepare test concentrations of 20 µg/mL for Orlistat and 10 µg/mL for Clomiphene.

Forced degradation

In degradation studies, 1 mL of Clomiphene and Orlistat stock solutions was subjected to various conditions: acid (reflux with 2N hydrochloric acid), oxidation (20% hydrogen peroxide), alkali (2N sodium hydroxide), dry heat (105°C for 6 hours), photostability (UV light exposure), and neutral conditions (reflux in water). Each solution was diluted to 20 µg/mL

Clomiphene and 8 µg/mL Orlistat, and 10 µL was injected into the chromatographic system for analysis.

Analytical method validation

The developed method for Clomiphene and Orlistat was subjected for validation for the parameters like Limit of Detection (LOD), Limit of Quantification (LOQ), linearity, robustness, precision, system suitability and accuracy as per the guidelines of ICH.[7-14]

RESULTS

Method Development and Optimization of Chromatographic Conditions

The method development and optimization of chromatographic conditions involved systematic trials to determine the most suitable parameters for analysis. After evaluating various mobile phase compositions and other critical factors, the following conditions were selected for the chromatographic analysis:

The mobile phase comprised 0.01N KH_2PO_4 and acetonitrile in a 60:40v/v ratio. A flow rate of 1 mL/min was maintained to achieve optimal separation and elution of the compounds. Separation occurred on an Agilent C18 column (150 x 4.6 mm, 5 µm particle size), with detection at 235 nm, providing sufficient sensitivity for the target compounds.

To maintain reproducibility and accuracy, the column temperature was controlled at 30°C throughout the analysis. A sample injection volume of 10 µL was utilized to introduce the sample into the chromatographic system. The total run time for each analysis was 6 minutes, allowing sufficient time for elution and separation of peaks.

For preparation and dilution of samples, a diluent composed of water and acetonitrile in a ratio of 50:50 v/v was employed. This solvent mixture was chosen to ensure compatibility with the mobile phase and to optimize peak shapes and resolution.

These optimized chromatographic conditions were crucial in achieving reliable and precise results for the analysis of the target compounds under study.

Validation

System suitability

The findings of system suitability parameter were shown in Table 1 and related chromatograms were given in Figure 2 (C). System suitability is a critical aspect of chromatographic analysis, ensuring that the chromatographic system is functioning correctly and providing reliable and reproducible results. It involves evaluating various parameters such as peak area, retention time, resolution, plate count, and tailing factor, which are essential for assessing the performance of the HPLC system. Six replicates of the standard reference solution were processed and infused to perform the system suitability parameter and the resulting chromatograms peak area, retention time, resolution, plate count, and tailing were measured. The findings of system suitability parameter were shown in Table 1. These parameters confirm that the HPLC system is suitable for the analysis of Clomiphene and Orlistat, as all values fall within acceptable ranges for reliable and accurate measurements. The resolution between the peaks is adequate, ensuring that the compounds are well-separated and can be accurately quantified. The plate counts indicate efficient column performance, and the tailing factors are within acceptable limits, suggesting symmetrical peak shapes.

Specificity

Method selectivity refers to the capability of distinguishing and accurately assessing analyte components amidst potential interference from impurities, degradation products, excipients, and other compounds present. This parameter was evaluated by injecting and analyzing blank, placebo, standard, and sample solutions and their respective chromatograms. In the chromatograms of blank, placebo, and sample solutions, no peaks were observed at the retention times corresponding to Clomiphene and Orlistat. Figure 2 illustrates the chromatograms of Clomiphene and Orlistat in standards, blanks, formulations, and placebos.

Linearity

Aliquots of 0.25, 0.50, 0.75, 1.0, 1.25, and 1.50 mL were taken from standard stock solutions containing 10 µg/mL Clomiphene and 20 µg/mL Orlistat. Each aliquot was diluted to 10.0 mL with diluent, yielding Clomiphene and Orlistat solutions with concentrations of 2.5 to 15 µg/mL and 5 to 30 µg/mL, respectively. After analyzing these solutions with a chromatographic apparatus, a linearity graph was created by graphing peak area on the Y-axis against concentration on the X-axis. Figure 3-4 and Table 2 show the calibration graphs.

The regression equations derived from the calibration curves were y = 140867x + 2919.9 for Clomiphene, and y = 94890x + 7410.8 for Orlistat, where y represents the peak area and x represents the concentration. The correlation coefficients (R^2) were 0.9998 for Clomiphene and 0.9995 for Orlistat, indicating excellent linearity for both compounds within the tested concentration ranges.

The high correlation coefficients confirm that the method provides a linear response over the specified concentration ranges, allowing for accurate and reliable quantification of Clomiphene and Orlistat in pharmaceutical formulations. This linearity validation is essential for ensuring that the method can be used effectively for quality control and stability testing of combined drug formulations.

Table 1: Clomiphene and Orlistat system suitability results.

S. No.	*Peak name*	*Peak area*	*Retention time*	*Plate count*	*Resolution*	*Tailing*
1.	Orlistat	768602	2.117	7725		1.13
2.	Clomiphene	2791171	2.871	9627	7.0	1.24

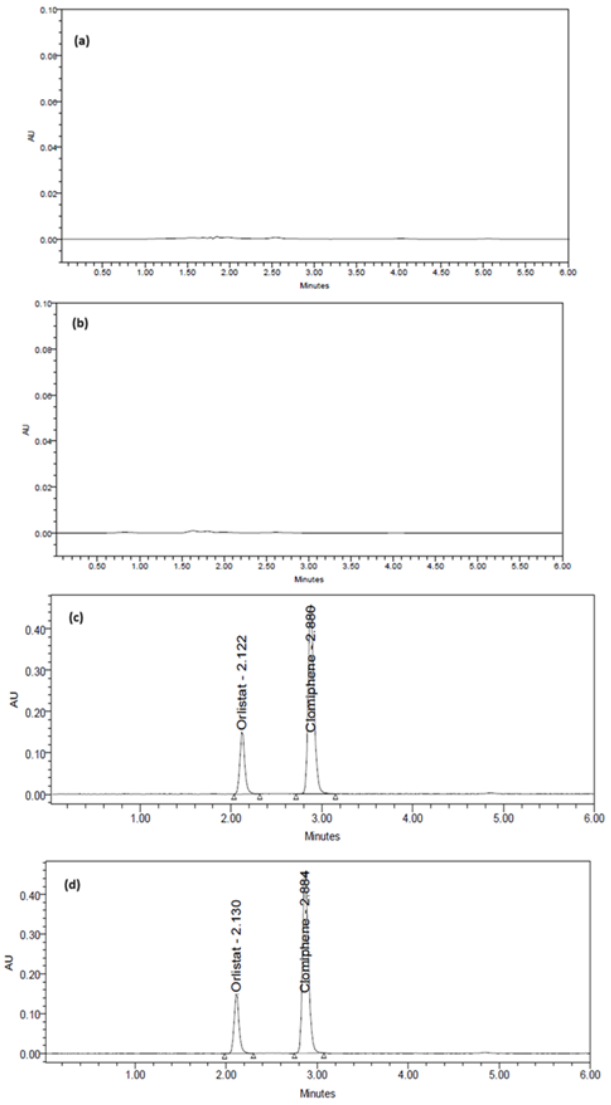

Figure 2: Chromatograms of A) Blank, B) Placebo, C) Standard and D) Formulation

Table 2: Linearity data of Clomiphene and Orlistat.

Clomiphene		*Orlistat*	
Concentration (µg/mL)	*Peak area*	*Concentration (µg/mL)*	*Peak area*
2.5	705085	5	196921
5	1418265	10	385675
7.5	2106886	15	585932
10	2811850	20	774240
12.5	3570247	25	964360
15.0	4199169	30	1130136
Regression equation			
y = 140867x + 2919.9		y = 94890x + 7410.8	
Correlation coefficient (R^2)			
0.9998		0.9995	

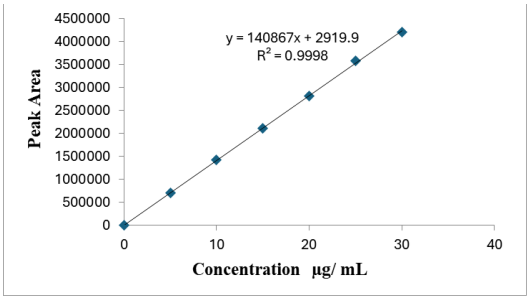

Figure 3: Linearity plot of Clomiphene

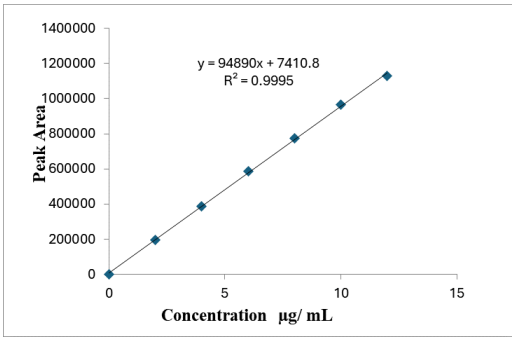

Figure 4: Linearity plot of Orlistat

Limit of Detection (LoD) and Limit of Quantification (LoQ)

The LoD and LoQ are crucial parameters in analytical method validation, determining the lowest concentrations of analytes that can be reliably detected and quantified by the method. The LOD and LOQ were assessed by measuring the S/N ratio at various concentrations of Clomiphene and Orlistat. The results were enumerated in Table 3.

For Clomiphene, the LOD was 0.15 µg/mL with an S/N ratio of 5.3, and for Orlistat, the LOD was 0.10 µg/mL with an S/N ratio of 7.5. These values represent the lowest concentrations at which the analytes can be detected with a reliable signal above the noise. For Clomiphene, the LOQ was 0.41 µg/mL with an S/N ratio of 24.9, and for Orlistat, the LOQ was 0.40 µg/mL with an S/N ratio of 36.8. These values denote the lowest concentrations at which the analytes can be quantified with acceptable precision and accuracy. These results confirm that the method has the necessary sensitivity for accurate detection and quantification of both compounds in pharmaceutical formulations, making it suitable for routine quality control and stability testing.

Precision

System precision

System precision involves evaluating the consistency of the chromatographic system when a standard preparation is repeatedly injected. For this study, a working standard solution of 10.0 µL was injected six times into the chromatographic system. The peak areas of Clomiphene and Orlistat were recorded, and the %RSD of these peak areas was calculated to assess the system's precision. The findings of system precision are shown in Table 4.

Table 3: Limit of detection and limit of quantification results

Parameter	*Measured concentration (µg/mL)*		*S/N Ratio*	
	Clomiphene	*Orlistat*	*Clomiphene*	*Orlistat*
LoD	0.15	0.10	5.3	7.5
LoQ	0.41	0.40	24.9	36.8

Table 4: Summary of System precision

S. No.	*Peak area response to drugs*	
	Clomiphene	*Orlistat*
1	2791171	768602
2	2824762	778670
3.	2788335	762737
4	2817550	779031
5	2789978	780274
6	2817388	774594
Average	2804864	775061
Standard Deviation	16709.4	6968.9
% RSD	0.6	0.9

Method Precision

To assess method precision, working sample solutions of 10 µL were injected six times into the chromatographic system, and the corresponding chromatograms were obtained. The %RSD of the assay results from these six preparations was calculated to evaluate the method's precision. The findings achieved for the assay were represented in Table 5.

Intermediate Precision

To evaluate intermediate precision, working standard preparations of 10 µL were injected six times into the chromatographic system. The peak areas were recorded, and the %RSD for these peak areas was calculated to determine the consistency of the method under varying conditions. The findings of intermediate precision study were represented in Table 6.

The low %RSD values for Clomiphene and Orlistat confirm the high system, method, and intermediate precision of the technique, demonstrating its reproducibility and robustness. This makes the method reliable for routine quality control and stability testing in pharmaceutical formulations.

Table 5: Summary of Method precision

S. No.	*Assay result of six preparations*	
	Clomiphene	*Orlistat*
1	99.05	99.24
2	99.58	98.64
3.	100.07	99.65
4	100.12	99.53
5	99.39	99.98
6	100.78	99.80
Average	99.83	99.47
Standard deviation	0.62	0.480
% RSD	0.6	0.5

Table 6: Summary of Intermediate precision

S. No.	*Peak area response of drugs*	
	Clomiphene	*Orlistat*
1	272351	775632
2	275314	775464
3.	272658	762157
4	276324	778654
5	275874	765421
6	278954	778425
Average	275246	772626
Standard Deviation	2465.3	7050.8
% RSD	0.963	0.919

Accuracy

To determine the accuracy of the method, known amounts of Clomiphene and Orlistat were added to a pre-analyzed sample solution at three concentration levels: 50%, 100%, and 150%. These spiked samples were then injected into the chromatographic system in triplicate for each level. The percentage recovery was calculated by comparing the measured amount with the known added amount. The findings were presented in Table 7.

At the 50% spiked level, the mean percentage recovery for Clomiphene was 99.69%, and for Orlistat was 100.19%. At the 100% spiked level, Clomiphene showed a mean recovery of 99.85% and Orlistat 100.39%. At the 150% spiked level, the mean recovery for Clomiphene was 99.81%, and for Orlistat was 100.20%.

The high percentage recovery values at all spiked levels indicate that the method is accurate for both Clomiphene and Orlistat. These results demonstrate the method's capability to accurately measure the true number of analytes in the samples, confirming its reliability for routine quality control and stability testing.

Robustness

The robustness of the analytical method was evaluated by introducing variations in flow rate (±0.1 mL/min), mobile phase composition (±10% organic phase), and column temperature (±5°C). The %RSD values for peak area, retention time, plate count, and tailing factor remained within acceptable limits for both Clomiphene and Orlistat under these conditions. These results confirm the method's reliability and robustness, ensuring consistent and accurate measurements and making it suitable for routine quality control and stability testing.

Forced Degradation Studies

The results of the forced degradation studies were summarized in Table 8.

Clomiphene showed 96.11% recovery with 3.89% degradation under acid conditions. Orlistat had a recovery

Table 7: Summary of Percentage Recovery Experiments

	Clomiphene				*Orlistat*			
Spiked level	*spiked (µg/mL)*	*recovery (µg/ mL)*	*% recovery*	*Mean % recovery*	*spiked (µg/ mL)*	*recovery (µg/ mL)*	*% recovery*	*Mean % recovery*
50%	10	9.813157	98.13		4	3.975877	99.40	
	10	9.985383	99.85		4	4.009875	100.25	
	10	10.0236	100.24		4	4.012572	100.31	
100%	20	19.91689	99.58		8	8.049352	100.62	
	20	20.14974	100.75	99.69	8	8.040478	100.51	100.19
	20	19.84292	99.21		8	8.002708	100.03	
150%	30	30.0336	100.11		12	12.19996	101.67	
	30	30.08118	100.27		12	11.84334	98.69	
	30	29.71258	99.04		12	12.02866	100.24	

Table 8: Results of stress degradation study.

S. No	*Degradation condition*	*Clomiphene*		*Orlistat*	
		%recovery	*%Degraded*	*%recovery*	*%Degraded"*
1	Acid hydrolysis	96.11	3.89	93.09	6.91
2	Base hydrolysis	95.91	4.09	94.38	5.62
3	Peroxide	94.54	5.46	94.38	5.62
4	Dry heat	97.69	2.31	97.66	2.34
5	Photostability	98.71	1.29	97.70	2.30
6	Water sample	99.73	0.27	99.47	0.53

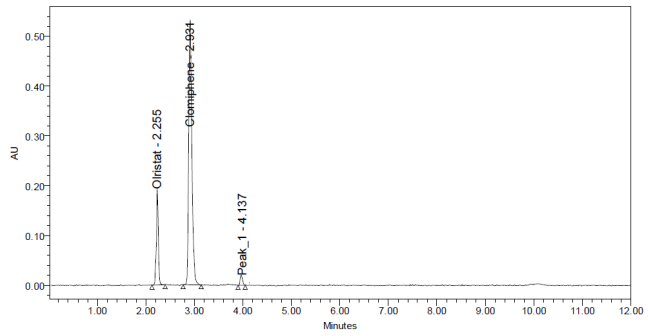

Figure 5: Chromatogram for acid degradation study.

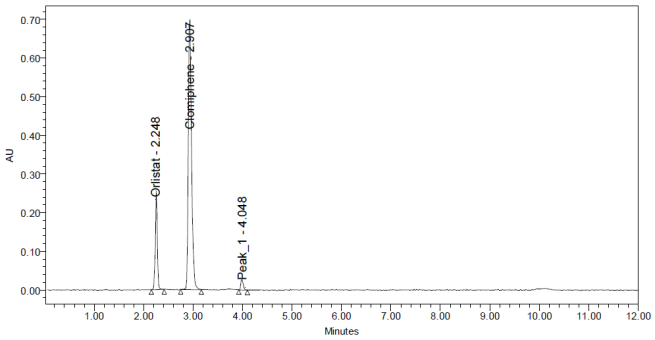

Figure 7: Chromatogram for alkali degradation study

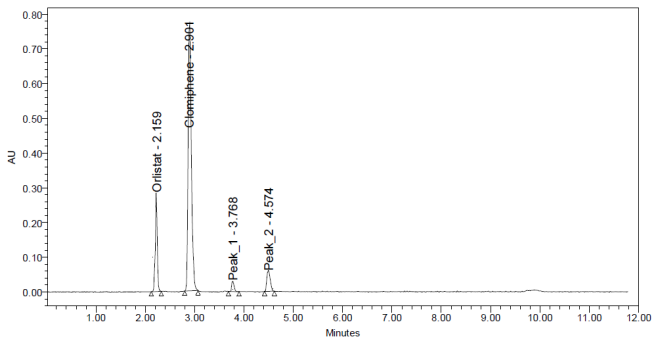

Figure 6: Chromatogram for oxidation degradation study

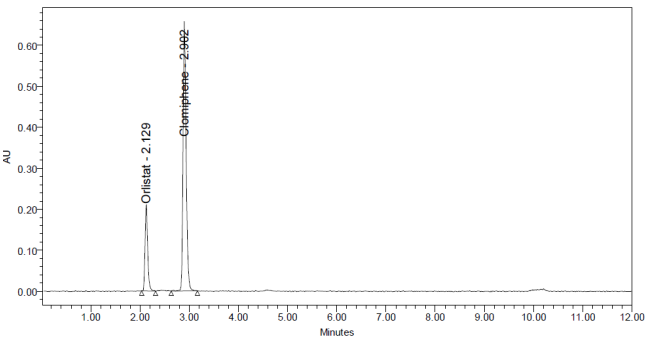

Figure 8: Chromatogram for dry heat degradation study

of 93.09%, with 6.91% degradation. This indicates that both compounds are relatively stable but do experience some degradation when exposed to acidic conditions. One stable degradation product was observed in the chromatogram (Figure 5) under acid stress conditions at RT 4.137.

Clomiphene has a recovery of 94.54%, with 5.46% oxidation degradation. Orlistat showed a recovery of 94.38%, with 5.62% degradation. This indicates that both compounds are susceptible to oxidation, with moderate degradation observed. Two stable degradation products were observed in the chromatogram (Figure 6) under oxidation stress conditions at RT 3.768 and 4.574.

Clomiphene showed a recovery of 95.91%, with 4.09% alkali degradation. Orlistat had a recovery of 94.38%, with 5.62% degradation. These results suggest that both compounds are slightly more stable under basic conditions than under acidic conditions, though they still undergo some degradation. One stable degradation product was observed in the chromatogram (Figure 7) under alkali stress condition at RT 4.048.

Clomiphene showed a recovery of 97.69%, with 2.31% dry heat degradation. Orlistat had a recovery of 97.66%, with 2.34% degradation. These results suggest that both compounds are quite stable under high-temperature conditions, with minimal degradation observed from the chromatogram (Figure 8)

Clomiphene having a recovery of 98.71%, with 1.29% photolytic degradation. Orlistat showed a recovery of 97.70%, with 2.30% degradation. These findings indicate that both compounds are stable under light exposure, with very little degradation observed from the chromatogram (Figure 9).

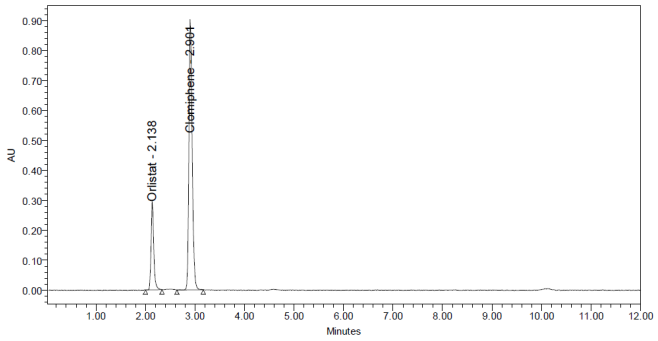

Figure 9: Chromatogram for photo stability study

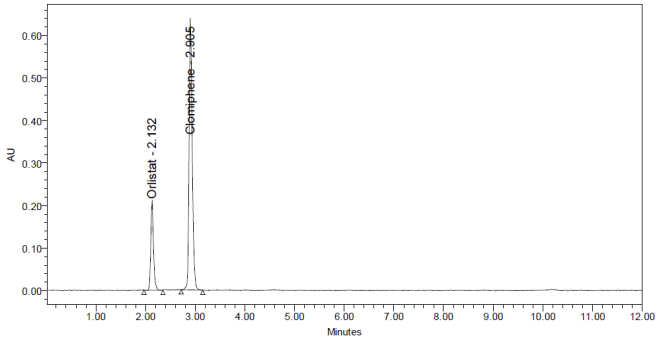

Figure 10: Chromatogram for neutral degradation study

Clomiphene showed a recovery of 99.73%, with only 0.27% neutral degradation. Orlistat had a recovery of 99.47%, with 0.53% degradation. These results demonstrate that both compounds are highly stable in neutral conditions, exhibiting minimal degradation observed from the chromatogram (Figure 10).

DISCUSSION

For the study, we selected HPLC to optimize the analysis duration and enhance overall efficiency. The method development process involved experimenting with various columns and mobile phases to improve separation efficiency. We refined the method by using a mobile phase consisting of a 0.01N solution of potassium dihydrogen orthophosphate (pH 4.8) combined with acetonitrile in a 60:40 (v/v) ratio. This optimized method was applied using an Agilent C18 column (150 × 4.6 mm, 5 µm particle size), with a flow rate set at 1.0 mL/min. This setup provided an effective balance between resolution and analysis time, ensuring accurate and efficient separation of the target compounds.

After optimizing the approach, a thorough validation was performed following the requirements outlined in ICH Q2R1. The evaluated parameters encompassed specificity, linearity, system appropriateness, LoD and LoQ precision, accuracy, and robustness. The created approach has successfully met all set acceptance criteria for validation, therefore demonstrating its dependability and precision.

Furthermore, the method that was created was subjected to forced deterioration experiments following the rules set by the International Council for Harmonisation (ICH). These studies included various conditions such as neutral degradation, photostability, dry heat degradation, alkali degradation, oxidation, and acid degradation. The degradation study's findings are extensively documented in the results section, offering valuable information on the stability and vulnerability of the investigated compounds when exposed to different stress conditions.

CONCLUSION

A sensitive, rapid, and accurate RP-HPLC method was developed and validated to simultaneously quantify Clomiphene and Orlistat in formulations, following ICH guidelines for stability-indicating methods. The retention times for Clomiphene and Orlistat were optimized to 2.88 min and 2.12 min, respectively. The mean percentage recovery for Clomiphene and Orlistat was determined to be 99.69 and 100.19%, respectively.

The LOD and LOQ were established using regression equations: 0.14 µg/mL and 0.41 µg/mL for Clomiphene, and 0.06 µg/mL and 0.19 µg/mL for Orlistat. The regression equations for Clomiphene and Orlistat were y = 140867x + 2919.9 and y = 94890x + 7410.8, respectively.

Stability studies demonstrated that the percentage degradation of the analytes ranged from 0.53% to 6.91%. The optimized method showed reduced retention and total run times for the analytes, enhancing efficiency. Therefore, this method

is both rapid and cost-effective, making it suitable for routine analysis in the quality control departments of pharmaceutical industries.

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